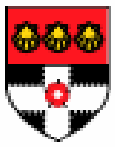

The University of Reading

Origin, biogeography, genetic diversity and taxonomic affinities of the cacao (*Theobroma cacao* L.) fungus *Moniliophthora roreri* (Cif.) Evans *et al.* as determined using molecular, phytopathological and morpho-physiological evidence

A thesis submitted by

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ABSTRACT

The origin, genetic diversity, biogeography and taxonomic affinities of the neotropical cacao pathogen *M. roreri* were studied through the application of various molecular, morpho-physiological and phytopathological methodologies to 96 isolates collected from throughout its geographic range.

AFLP, ISSR and ITS sequence analyses revealed that this fungus has a considerable genetic variation, although regions of comparative genetic uniformity were also identified in Central America, central Ecuador and Peru. The highest variability was observed in north-eastern Colombia indicating that this region represents the centre of diversity for the fungus. The pattern of declining variability outside this area also suggests that *M. roreri* originated in this country, possibly within or close to the Medium Magdalena. Five major subspecific groupings were identified, with each exhibiting a characteristic distribution: Bolívar and Co-West groups being widespread, whereas Co-Central and Co-East groups apparently endemic to Colombia and the Gileri group being restricted to Ecuador. ITS was useful to define two subspecific groups and to reinforce some of the AFLP/ISSR findings.

The morpho-physiological variation of 88 isolates and the effect of three *in vitro* temperature regimes were studied by evaluating eleven variables for which significant divergence was noted. Significant differences were observed between genetic groups for most variables, suggesting the variability may have a genetic basis.

The virulence of seven Colombian isolates representing four genetic groups was determined by artificially inoculating pods of five cacao clones in Colombia. Genetic variation detected between isolates was not matched by similar diversity in their virulence. There was apparently only limited variation in virulence between them.

Phylogenetic analysis of three different portions of the *M. roreri* genome (ITS regions, nLSU-rDNA, mtSSU-rDNA) confirmed that this fungus is a Basidiomycete and strongly suggest that it belongs to the order Agaricales, possibly belonging to the family Tricholomataceae. Sequences analysis also demonstrated that *M. roreri* is closely related to the witches' broom pathogen of cacao *Crinipellis perniciosa*.

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This thesis is dedicated to the memory of my brother Mario Ricardo and my nephews Miguel Angel and Edgar Alonso.

"Now to the King eternal, immortal, invisible, to God who alone is wise, be honour and glory forever and ever. Amen." 1 Timothy 1:17

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Moniliophtora roreri (Cif.) Evans *et al.* causes the moniliasis or frosty pod diseases, an extremely destructive pod rot of cacao that is confined to nine countries in South and Central America. The fungus only infects fruits of species belonging to the genera *Theobroma* and *Herrania* causing internal and external pod damage that results in the total loss of the pods, and more relevantly, the commercially important seeds that some of these species contain.

The devastating effects of this pathogen on cacao (*T. cacao*) have been dramatic and are well documented across different countries and epochs, including Ecuador in 1917 (Rorer, 1918; Rorer, 1926; Ampuero, 1967) and Costa Rica in 1978 (Enríquez *et al.*, 1982). Besides these countries, the fungus is present in Colombia, Venezuela, Peru, Panama, Nicaragua and Honduras, where it is invariably one of the main yield-limiting factors for cacao production in the affected areas. The fungus has continued with its invasive behaviour and was recently detected in Guatemala.

In the world context, current annual losses attributable to the pathogen are proportionally small, but the potential for extremely large-scale losses is substantial. The severity of the disease means that there is now increasing concern that it will disperse into other countries within America and thence to other continents. This situation is viewed as alarming, considering the pathogen's aggressiveness and the apparent susceptibility of most of the world's cultivated cacao genotypes.

Success in the development and dissemination of effective strategies for disease control depends on a sound the knowledge of specific aspects of the fungal biology, such as the level of genetic diversity and the sources of this variability. Zadocks (1997) analysed the world status of disease resistance in cacao and concluded that variability of the different

pathogens is often considerable but has not yet been satisfactorily explored. This author considered pathotype variability to be of utmost importance to cacao growers since, at any time, pathotype diversity may undermine limited inherent resistance in plantations and so threaten livelihoods by imposing a 'boom-and-bust' economic cycle. For *M. roreri*, information on variability is very scarce. So far, the few studies on the diversity of this species have been made using a few morpho-physiological traits characterised on a limited number of isolates (Herrera *et al.*, 1989; Ram, 1989).

Evans *et al.* (1978) used the fact that *M. roreri* has some traits that are distinctive of the Basidiomycetes group to argue that this fungus represents the asexual stage of an unknown Basidiomycete. However, there has been no subsequent research effort to test this hypothesis. A more detailed knowledge of the taxonomic affinities of *M. roreri* could benefit efforts to control the disease by enabling a rational transfer of genetic, pathological or biological information from a better studied but closely related fungal taxon (Berbee & Taylor, 1999).

It is concluded that in spite of the historical economic importance of *M. roreri* in different countries, several aspects of its basic biology remain unresolved. These include its origin, genetic diversity, biogeography and systematic position.

1.2 RESEARCH OBJECTIVES

1.2.1 GENERAL OBJECTIVES

There are two general objectives. The first is to study the origin, bio-geography and genetic diversity of the cacao pathogen *Moniliophthora roreri* in tropical America and to determine the possible sources of variation. The second is to study the phylogenetic affinity of *M. roreri* to the Basidiomycetes at the molecular level.

1.2.2 SPECIFIC OBJECTIVES

- To combine molecular, morphological and physiological information to determine the level of genetic diversity of *M. roreri* isolates in tropical America.

- To define the possible centres of origin and genetic diversity of the fungus.

- To study the geographical distribution of the genetic variants and infer the possible dispersal mechanisms involved.

- To determine the virulence of a representative set of isolates of *M. roreri* on different cacao clones.

- To explore the possibility of sexual reproduction in the species.

- To determine the taxonomic affinities of *M. roreri* within the Basidiomycetes with special reference to the cacao pathogen *Crinipellis perniciosa*.

1.3 GENERAL FEATURES OF THE FUNGUS AND THE DISEASE

General reviews on different aspects of the biology of *M. roreri* are summarised by Ampuero (1967), Thorold (1975), Evans (1981), Merchán (1981) and Evans (1986). Additional information of the pathogen and the disease can be also found in the following chapters.

1.3.1 HOST RANGE

M. roreri only attacks species belonging to the closely related genera *Theobroma* and *Herrania* (Whitlock & Baum, 1999), *T. cacao* being the most prominent species due to its commercial relevance and widespread cultivation. Other important species are *T. bicolor* (common names: *pataste, bacao, macambo, cacau do Peru*) an adulterant of cacao in Central America, and *T. grandiflorum* (*cupuaçu*) used in Brazil to prepare juice from the mucilage covering the beans (Wood & Lass, 1985).

The fungus has been recorded in the wild on natural populations of *T. bicolor* Humb. & Bonpl. and *Herrania balaensis* in Ecuador (Rorer, 1918) and on *T. gileri* Cuatr. in

Antioquia, Colombia (Baker *et al.*, 1954). In addition, *M. roreri* has also been observed on the following species in Ecuador (Evans, 1981) and Costa Rica; (Enríquez, 1981): *T. mammosum* Cuatr. & León; *T. grandiflorum*; *T. augustifolium* Moçino & Sessé; *T. simearum* Donn. Smith; *T. sylvestre* Mart.; *T. speciosum; H. nitida* (Poepp.) Schultes; *H. albiflora; H. purpurea; H. pulcherrima* Goudot and 4-5 unidentified *Herrania spp.* Evans (1981) suggested that the potential host range of *M. roreri* extends to all species of *Theobroma* and *Herrania*, which consists, respectively, of 22 and 17 species of trees growing in the understory of Neotropical lowland rainforests (Cuatrecasas, 1964).

T. cacao is traditionally divided into three "races" or groups: Criollo, Forastero and Trinitario. The "Criollo" types are believed to be the original domesticated cacao of Mexico and Central America (Cheesman, 1944) and these trees produce a superior chocolate flavour but have low fruit yields in most agricultural settings (Whitlock & Baum, 1999). The beans in this group tend to be rounded and are white in cross-section, producing cacao of a weak and special flavour (Wood & Lass, 1985). Criollos dominated the market until about the middle of the eighteenth century and accounted for most of the exports to Europe, however, this position was lost rapidly in the ensuing seventy years and today only a few, if indeed any, pure stands still exist (Wood & Lass, 1985).

The "Forastero" trees are believed to derive from a wild Amazonian lineage (Whitlock & Baum, 1999). The trees are hardy and vigorous which is why they now form the greater part of all cacao grown. Compared to Criollo the beans in this group are smaller and flatter and the cotyledons violet; the flavour derived from them is stronger and provides the basis for plain and milk chocolate (Wood & Lass, 1985).

The “Trinitario” group represents an intermediate race between the Criollos and Forasteros (Cheesman, 1944). The populations of this group are not found in the wild state but they are in cultivation mostly in the Caribbean area, in Cameroon and Papua New Guinea (Wood & Lass, 1985).

Schultes (1958) divided *Herrania* into two sections based on floral morphology. Members of section Herrania are confined to Central America and northern Colombia. Members of section Subcymbicalyx are found throughout Amazonia and in northern South America,

with only two species occurring in northern Colombia and southern Panama. In Colombia, 14 *Herrania* species are present, seven being endemic (Schultes, 1951).

1.3.2 THE FUNGUS

1.3.2.1 Etiology and taxonomy

There was much speculation over the identity of the causal agent of a new and very destructive disease appeared in Ecuador at the end of the 19th century and the beginning of the 20th century (Chapter 3). In 1892, Sodiro published the opuscule "Observations on the cacao disease called la mancha" in which explained that the disease originated from the lack of nitrogen in the soils (Sodiro, 1892). In an article published in a newspaper of Guayaquil (*El Grito del pueblo Ecuatoriano*), Dr. Dario Moral indicated that the causal agent of the disease was a parasitic organism (fidé Martínez, 1916). Elsewhere in the same newspaper, however, another article refuted this idea and attributed the disease to the *heladas* (i.e. sudden drop in temperature), as was commonly believed by the farmers at that time (fidé Martínez, 1916).

After examining pods from different farms, Martínez (1916) concluded that the new disease must be of cryptogamic order and caused by the fungus *Phytophthora cactorum*, although recognised that spores did not show the distinctive swollen papilla of this species. The same author presented a paper on cacao diseases in a conference held in Guayaquil in August 1916, which was later published together with reports from other authors including Campos and Holbert (Rorer, 1918). Here, Martínez made a distinction between the pod diseases called *helada, mancha* and *enfermedad de Quevedo,* and concluded that the first two diseases were caused by a species of *Phytophthora*, but the *enfermedad de Quevedo* had an unknown causal agent. Holbert insisted that the problem had a cryptogamic origin and Campos, after checking pods from Quevedo, concluded that *P. cactorum* was the causal agent of the disease (Rorer, 1918). It is interesting to note, however, as indicated by Jorgensen (1970), that the title of the latter paper mentions Monilia as the name of the disease.

Van Hall (1914) distinguished in Ecuador, two types of pod symptoms based on local names, *mancha* (lesion) with decay of the whole pod, probably caused by a fungus, and *helada* producing abnormal growth of the pods and beans. High losses due to the former disease were reported in south-west Ecuador in 1909. Evans (1981) speculated on the possibility that the pathogen was *C. perniciosa,* a fungus first observed in Ecuador in this same zone in 1922 (Rorer, 1926). Evans (1981) noted that the term *helada* is still used today by farmers in Ecuador and Colombia as a local name for the second pod rot and considered this an apt description of the external pod symptoms of moniliasis. Similarly, the first symptom of the disease on young pods is usually a gross swelling or abnormal growth. He concluded, therefore, that van Hall was unwittingly describing pod rot caused by *M. roreri* (Evans, 1981).

According to Rorer (1918), Mr. Ralph E. Smith of the University of California identified the fungus in 1917 from pod samples sent by Mr. Holbert. Smith remarked that the fungus seemed to belong to the Genus *Monilia* and is similar to *Monilia fructigena*, which is very common in the United States and other places, but not to *Phytophthora cactorum* as was mentioned on some reports received with the samples.

Ciferri & Parodi (1933) confirmed the provisional identification made by Smith and named the fungus *Monilia roreri* Ciferri in honour of J.B. Rorer who had pioneered research investigations into the disease. They classified the fungus as follows: Class: Deuteromycetes, Order: Hyphales, Genus: *Monilia*, species: *roreri*. Parodi (1936) complemented the fungal description with additional morphological data. It is perhaps not surprising that this taxonomic conclusion was never questioned because the fungus is superficially similar to several *Monilia* species both in macroscopic and microscopic appearance, and none of the mycological authorities had actually observed the fungus in the field but were working with dried specimens (Evans, 1986). There were no subsequent studies on the taxonomy of *M. roreri* until the decisive work by Evans *et al.* (1978).

Several characters were recognised by Evans *et al*. (1978) which did not conform to the contemporary concept of the genus *Monilia* or its relatives and which, when taken together with the behaviour of the pathogen in the host, suggested that a more highly evolved parasite was involved. Detailed analyses, using both scanning and transmission electron

microscopy, showed this to be the case since the asexual spores (conidia) are not formed in "monilioid" chains (with the youngest spore at the tip) but in exactly the reverse manner (Evans *et al.*, 1978; Evans, 1986). Further confirmation was obtained when the internal structure of the mycelium (fungal threads or hyphae) was found to be more typical of the mushroom group (basidiomycetes) rather than the Ascomycetes to which Monilia species belong (Evans *et al.*, 1978). Thus, the vegetative mycelium of *M. roreri* contains septa with typical dolipores as previously reported from Basidiomycete hyphae. The cacao pathogen was then identifiable as the asexual state of an unknown Basidiomycete fungus and a new genus had to be described in order to accommodate its novel method of spore formation. *Moniliophthora* was chosen as the generic epithet as it was felt that this name was not unlike the previous one and could be similarly abbreviated (Evans, 1986).

1.3.2.2 Dispersal of *M. roreri*

1.3.2.2.1 Sources of inoculum

Spores are the only infective propagules of *M. roreri,* and the pods of host trees being the only organs susceptible to be naturally infected by them (Evans, 1981). After a period of approximately three months, the infected pods become dry and mummified on the trees and remain attached to the trunk for long periods. These pods are a major source of inoculum responsible for new waves of infection of the disease over a considerable period of time (Ampuero, 1967; Castaño, 1952).

Spores are produced in great abundance on diseased pods. Sporulation density can reach 44 million spores cm^{-2} and a mature pod may produce over 7,000 million spores (Campuzano, 1981). The capability of infected pods to produce many crops of spores is considerable. Ram (1989) determined that as many as 20 periods of successive sporulation occur in four cycles over 80 days. Even old mummified pods collected either from the three or from the ground beneath cacao trees were capable of 13 to 16 sporulation periods and appeared capable of producing inoculum of *M. roreri* almost until they decomposed.

According to Wood & Lass (1985) spores are produced at high numbers for a few weeks after the onset of sporulation and then in reducing quantity for up to about ten weeks, after

which the number becomes insignificant. Evans (1981) reports that spores have been collected when air is blown over the sporulated pod surface more than one year after initial pod infection. Ampuero (1967) argued that spores from pods more than nine months after the onset of sporulation are probably not viable although retention of viability has been demonstrated in Ecuador for seven months (Wood & Lass, 1985), which is considered sufficient to provide an intercrop carry-over of inoculum into the next season.

After release, considerable numbers of infective spores become distributed over the canopy of the trees, the trunk, flower cushions and even the epiphytes common in the tropics from where they can infect the pods (Ram, 1989). Spores in the soil or on pod pieces in the soil survive no more than three months as they become invaded by micro-organisms (González, 1981).

M. roreri is a common component of the air-spora as determined in Ecuador (Evans, 1981), Colombia (Merchán, 1981) and Costa Rica (Schmitz, 1985). This means that infection could occur at any time if environmental conditions are suitable and susceptible tissues are available. Variations in the number of spores, however, have been identified between times of the day and seasons. For instance, in Pichilingue, Ecuador larger numbers of spores were trapped during the afternoons in the rainy season when winds tended to be stronger than at other times of the day and year (Evans, 1981).

1.3.2.2.2 Mechanisms of dispersal

Many aspects of the spread of the disease are still poorly understood (Wood & Lass, 1985). The dry powdery masses of spores formed on the pods are efficiently dispersed into the air by any physical contact with the infected pod (Evans, 1981). It is widely accepted that wind is the main mode of spore dissemination, although dispersal can also occur by water, insects and other animals (Naundorf, 1954).

1.3.2.2.2.1 Dispersal by wind

The sporulation density on the pod is such that by merely blowing on the surface, clouds of spores are liberated and freely carried by the wind or prevailing convection currents

(Naundorf, 1954). Evans (2002b) stated that there is ample evidence from the eastern Andes and Peru that the pathogen once free of natural barriers moves rapidly over considerable distances by airborne spores. However, although *M. roreri* is well-adapted to airborne dispersal having dry, powdery, thick-walled spores (Evans, 1986), apparently, spores are not able to travel for long distances due to their weight (Barros, 1981) and because they are susceptible to sun exposure, drought or excessive cold (Wellman, 1972). This aspect requires more investigation.

1.3.2.2.2.2 Dispersal by human beings

Wind dispersal fails to explain observed dispersal of the fungus over significant distances and geographical barriers. Such events are far easier to explain by human activities. In fact, human beings are considered as one of the most effective dispersal agents of moniliasis because the fungus is admirably suited to this indirect method of dissemination (Evans, 1986). The long period of pod colonisation prior to the manifestation of visible symptoms allows an apparently healthy, systematically infected pod to be selected and transported for use as a source of planting material or, more dangerously, for trade or demonstration purposes to friends or family where no selection operates (Evans, 1986). This is because, once these ripening pods are opened and found to be useless, sporulation on the cut surface is both rapid and abundant (Evans, 2002b).

The cryptic, latent period within the pod increases the chances of long-distance dissemination by man (Evans, 1986). Long-distance dispersal could occur in two ways: (a) in or on pods, although these are unlikely to be moved inter-continentally; or (b) on budwood or similar material. The powdery spores would readily adhere to such tissues and remain viable in this situation for many months. However, fungicide treatment would certainly reduce the inoculum and considerably limit the chances of an unwanted introduction (Evans, 1986). Since all such material is routinely passed through intermediate quarantine stations, then to date, this route to the Old World plantations has effectively been blocked (Evans, 2002a).

1.3.2.2.2.3 Dispersal by insects and other animals

Wood & Lass (1985) judged that the role of insects in moniliasis dispersal is probably minimal even though there have been no studies to test this hypothesis. What is clear is that the participation of insects is not necessary for penetration of the pathogen into the pods (Franco, 1958) because this process occurs naturally through direct entry of hyphae into the epidermis (Suárez, 1971).

Different species of animals such as monkeys, squirrels, rats, bats, deer, parrots and other kind of birds may have had an important role not only in the dispersal of *T. cacao* and its relatives in tropical America (Gumilla, 1791; Montaña, 1986; Patiño, 1963; van der Pijl, 1968; Young, 1994), but also in the spreading of *M. roreri*. Animals could favour the dispersal of the fungus in two ways: by directly carrying the spores on their bodies or within the digestive tracts or by transporting sick pods or parts of them from one place to another.

1.3.2.2.2.4 Dispersal by water

Water, as raindrops or drip, is effective in releasing small clouds of spores (Evans, 1981). Spores can be easily washed off the sporulating pods by rain and distributed to the canopy and particularly the trunk where it would be most likely to contact pods and so inoculate them (Ram, 1989). This author has demonstrated the importance of rain splash as a means of releasing spores from infected, sporulating pods on the ground, presumably by impact (Ram, 1989).

Besides human beings, water streams and strong winds such as hurricanes have been also suggested as possible dissemination agents of *M. roreri* over long distances (Porras & Enríquez, 1998), although their role has yet to be established.

1.3.3 THE DISEASE

1.3.3.1 Historical development and current economic importance

A detailed description of the historical development and current economic importance of *M. roreri* in tropical America is presented in Chapter 3.

1.3.3.2 Common names

The disease caused by *M. roreri* has been known with several names (Campuzano, 1980; Evans, 1981; Thorold, 1975). In some cases they referred to the external symptoms caused by the pathogen on the pods (*mancha* = lesion; *helada* = frost; *hielo* = ice; *pasmo* = wilt due to frost; *enfermedad* or *mal palúdico* = malaria); to the internal symptoms (*pudrición or podredumbre acuosa* = watery rot or *enfermedad acuosa* = watery disease) or to the signs of the pathogen (*ceniza* = ashes; *polvillo* = fine dust). The disease was also called *enfermedad de Quevedo* (Quevedo disease) as a reference of the place where apparently first appeared in Ecuador.

La mancha was apparently the earliest name used in Colombia (Chapter 3), although this name has been also recorded in Ecuador (van Hall, 1914; Rorer, 1918) and in Venezuela (Reyes & Pérez, 1964; León, 1976). The descriptive name "watery pod rot" was used because fungal invasion converted the pod contents into a wet and rotten mass. Ciferri & Parodi (1933) described affected pods as heavier and as more difficult to open than healthy ones of similar age. These features, and the white fungal "frosting" on the exterior, presumably account for the descriptive term *helada* (frost or frozen).

Monilia has been commonly used as part of the trivial name (Thorold, 1975). Currently, monilia or moniliasis are the most widely used common names of the disease used in Central and South America. These names originated from the generic epithet of the pathogen (*Monilia roreri*), and so Evans (1986) suggested it could be usefully changed to the more descriptive frosty pod rot. In spite of this, the use of monilia, moniliasis or monilla (in Ecuador) are becoming prevalent in Latin America. In the present work, the term moniliasis has been adopted due to its widely use in different countries.

1.3.3.3 Disease symptoms

Pods are the only part of the cacao plant susceptible to be naturally infected by *M. roreri* (Ampuero, 1967; Desrosiers & Suárez, 1974; Evans, 1986), although Evans (1981) was able to artificially induce infections of seedlings and flushes under laboratory and glasshouse conditions using abnormally high inoculum concentrations. However, the fungus failed to sporulate on such tissues and this was regarded by the author as purely artificial infection, not relevant to field conditions.

Pods are infected when they are young and become less susceptible to infection as they mature (Ampuero, 1967). Infection develops internally as the pod grows (Ampuero, 1967; Bejarano, 1961; Suárez, 1971) and some pods may be infected without any external symptom. The fungus typically produces the following symptoms (Plate 1.1).

External symptoms: The first indication of the disease is the appearance of small oily or water-soaked areas on pods 30-45 days after infection, which are usually difficult to be detected in the field until they increase in size to form a chocolate-coloured spot approximately 75 days after infection (Merchán, 1981). Other initial symptoms are the deformation or death of small pods (Campuzano, 1980). It is also frequent that some pods present a premature ripening, which is the appearance of areas of mature coloration on immature pods. Some 4-5 days after the appearance of the chocolate-coloured spots, pods become covered with a layer of white mycelium in which abundant spores are produced. This stage of the disease is the most characteristic symptom in the field (Ampuero, 1967; Campuzano, 1980). In subsequent weeks, the pod losses water and progressively becomes mummified (Suárez, 1971). Spores are produced in chains showing a basipetalous sporogenesis. Spores are easily separable, thick-walled, pale yellow, brown in masse, globose/subglobose to ellipsoid or cylindrical (Evans, 1981) (Plate 1.2).

Internal symptoms: When the infected pods are opened the husk, the central tissues, the pulp and the beans appear to form a compact, homogenous mass that is difficult to distinguish into component parts (Campuzano, 1980). These tissues are surrounded by a decayed watery substance as a result of tissue maceration (Rorer, 1918), which makes the pods weigh more than healthy ones (Campuzano, 1980). The beans may be partially or completely destroyed, depending on the stage of ripening when infection occurs (Ampuero, 1967).

Plate 1.1 Symptoms and signs of *Moniliophthra roreri* on cacao pods: 1. Small oily areas on pod surface; 2. Pod deformation; 3. Premature ripening; 4. Chocolate-coloured spots covered with the typical mycelium; 5. Release of spores from an infected pod; 6. and 7. Internal symptoms (Photos by A. Mora, CATIE).

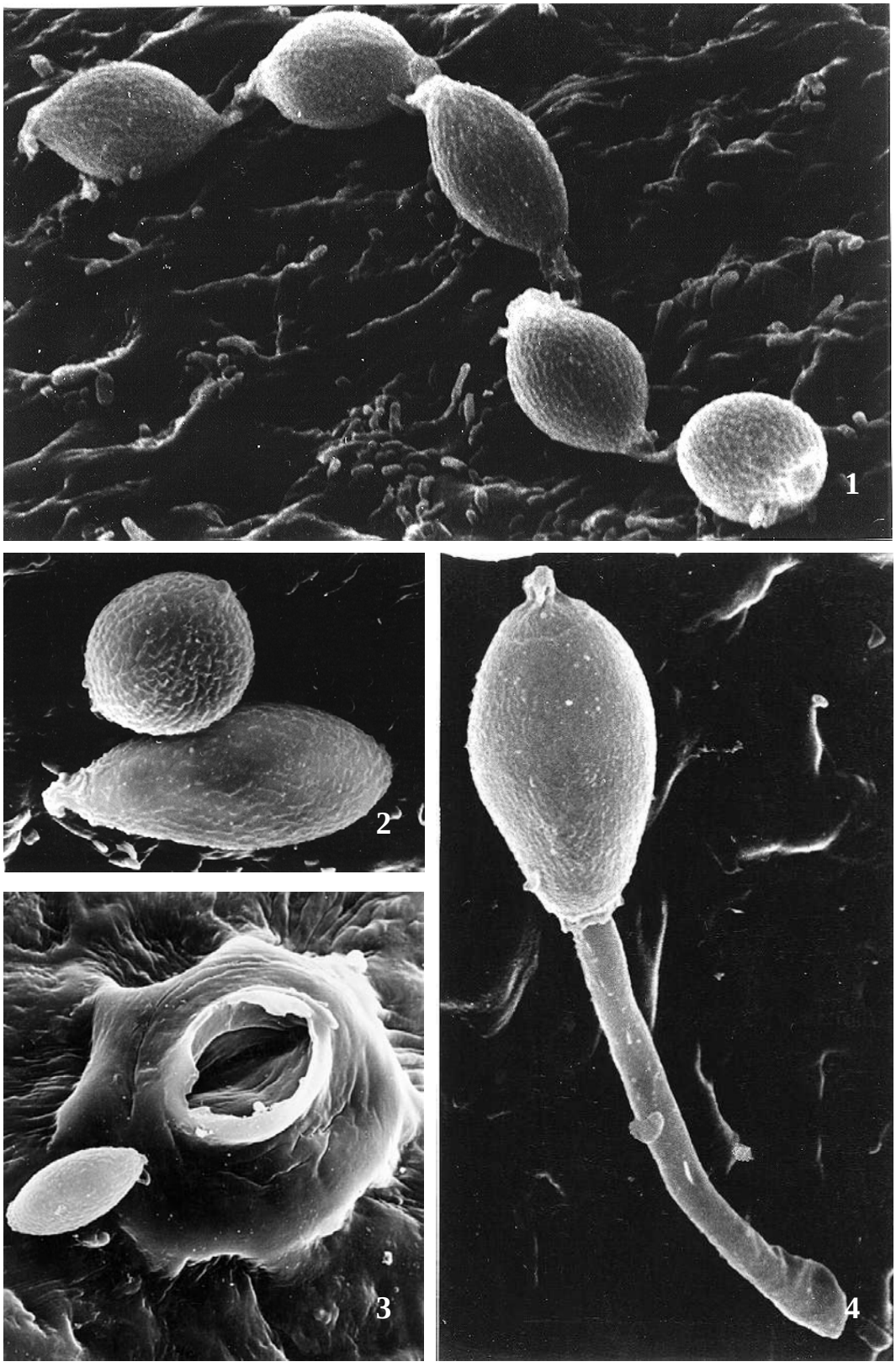

Plate 1.2 Scanning electron microscope (SEM) micrographs showing spores of *Moniliophthora roreri*: 1. Detached chain of mature spores; 2. Globose and ellipsoid spores; 3. Ellipsoid spore close to a stoma of *T. cacao*; 4. Germinated spore (Photos by D. Flores, Costa Rica).

Moniliasis has a long incubation period from initial penetration to the appearance of symptoms, and this can be modified to some extent by the age of the pod at inoculation and the genotype of the host (Evans, 1981). Suárez (1971) observed that spores of *M. roreri* germinate and penetrate the pod at all stages of development, directly through the epidermis or via stomata without the presence of wounds. Other results obtained by Suárez (1971) were summarised by Desrosiers & Suárez (1974) as follows:

- **Pod inoculated at 20, 40 and 60 days**: The entire process of penetration, intercellular and intracellular invasion (symptom appearance) takes place in 40 days. This results in a necrosis of the fruit that may easily be confused with physiological wilt (especially in pods inoculated at 20 and 40 days).

- **Pods inoculated at 80 days and some at 60 days**: The infective process, penetration to intracellular invasion, occurs at 60 days. These pods present symptoms of premature ripening, and deformation without externally visible necrosis. Some pods may appear healthy externally but may be totally destroyed within.

- **Pods inoculated at 120, 140 and 160 days**: In these pods infection develops more slowly. Small localised lesions may develop on the surface after 60 days. In many cases, the endocarp is not penetrated and the seed from infected pods may be salvaged.

1.4 FUNGAL DIVERSITY AND SYSTEMATICS

1.4.1 GENETIC DIVERSITY IN FUNGI

Fungi are characterised by possessing a greater complexity and diversity than other groups of microscopic pathogens (Talbot, 1998). Moreover, the genetic diversity of fungi allows them to evolve new pathogenic types quickly in response to enhanced resistance in crops brought about advances in crop breeding. It follows that an improved understanding of the mechanisms of pathogenicity and sources of genetic variation in plant pathogens is likely to be critical for the future control of fungal disease (Talbot, 1998).

Fungi possess a variety of mechanisms for introducing genetic variation in their life cycle, either during sexual reproduction or independently of it (Wang & Szmidt, 1998). The resulting variability is significant for a number of reasons. It can affect the pathogen's relationship with its host at many levels, and the genetic flexibility allows the fungi to adapt readily to changing environmental conditions, including the introduction of new host genotypes.

Sexual recombination is one of the most important sources of variability, but it is not universal among fungi. As many as one-third of all fungi may have both an asexual stage (anamorph) and a sexual stage (teleomorph) (Hawksworth *et al.,* 1996), with the latter often occurring infrequently. In many others, only asexual or parthenocarpic reproduction is known (Duncan *et al.*, 1998).

Many phytopathogenic fungi have no sexual stage or a sexual stage not yet found in nature (Brygoo *et al.,* 1998). At one extreme of the spectrum are fungi which are completely asexual (e.g. the deuteromycetes) but which nevertheless generate variation modifying genetic expression or adapting mitotic processes to produce recombinants or segregants as asexual propagules. At the other extreme are the diverse forms of sexuality, ranging from bipolar (unifactorial) incompatibility systems to (bifactorial) incompatibility systems, with mating types possibly switching in some organisms (Chiu & Moore, 1999).

Most fungi produce abundant asexual spores that are extremely effective in dispersing the organism. This being so begs the question over why so many fungi invest resources in the more complex process of sexual reproduction? Sex must have selective advantage if sexual stages are not to be replaced by asexual ones entirely (Chiu & Moore, 1999). Copious evidence exists to show that out-crossing certainly does promote variability, and that asexual lineages change little in time, in apparent support of the view that variability in the populations enables the organism to survive ecological and environmental challenges. Bernstein *et al.* (1985) suggested that repair of damaged DNA (caused by mutation or faulty replication) in one chromosome being repaired by recombination with the normal chromosome provided by the other parent is the underlying cause of the success of sexual reproduction. Out-crossing might also give rise to heterozygous advantage, where the heterozygous phenotype is more fit than either of its homozygous

parents. Evidence supporting this theory has frequently been presented in plants, animals and even in the yeast *Saccharomyces cerevisiae* (Chiu & Moore, 1999).

Brygoo *et al.* (1998) compared the genetic diversity, genotypic diversity and other parameters of populations from different fungal species (*Mycosphaerella fijiensis, Fusarium oxysporum, Magnaporthe grisea, Colletotrichum lindemuthianum, Botrytis cinerea* and *Erysiphe graminis*). Rather unsurprisingly, this study demonstrated that exclusively asexual populations tend to be genetically less diverse than sexual populations, in particular when considering overall genotypic diversity, as they are comprised of a small number of families composed of highly related clones (clonal lineages). Within such clonal lineages, a low level of polymorphism was detected, that is, in general, isolate specific. These case studies suggest that an analysis of population structures is simpler with asexual populations than with sexual species. Small samples and few molecular markers are sufficient to survey the structure of asexual populations. In particular, world-wide migration events can be traced easily when isolates from the same clonal lineages are detected in different parts of the world. Population dynamics in one specific region could also be analysed over time with confidence by measuring the fluctuation in frequency of the different resident clonal lineages **(**Brygoo *et al.,* 1998).

1.4.2 ANALYSIS OF FUNGAL GENETIC DIVERSITY

The analysis of genetic diversity and relatedness between or within taxa is a central task for many disciplines of biology. Historically, the phylogeny, taxonomy and characterisation of fungi have been inferred from various phenotypic characters, including morphological, physiological and developmental characters, and/or chemical components such as secondary metabolites (Mills *et al.,* 1998).

Morphological characters have long been used to identify species, families and genera (Bridge & Arora, 1998). Moreover, morphological as well as life history traits have been the subject of numerous studies in population genetics and agriculture, where fitness and yield are the biologically and evolutionary important factors. Levels of variability can be estimated for morphological characters, their response to selection and their genetic background can be determined, and genetic correlations and historical selection forces can

be inferred (Weissing *et al.*, 1995). This strategy has proved successful for a large number of fungi, although morphological characters have not always proved reliable source of information for identification purposes. Moreover, for fungi it is frequently difficult to find sufficient variable morphological characters to allow a comprehensive systematic resolution of all groups and many characters that are used can be strongly influenced by the environment (Weising *et al.*, 1995).

The introduction of molecular biological techniques has therefore been a major force in the areas of systematic and population biology of filamentous fungi (Bridge & Arora 1998). Application of a range of DNA-based techniques has revolutionised the discipline of fungal systematic and provided tools for both, discrimination of closely related fungi, at almost any required resolution, and also detection of fungal species at sensitivities, in some cases, far beyond those of traditional methods (Mills *et al.*, 1998; Tamakatsu, 1998).

The prerequisite of genetic analysis is to have tools that can discriminate between biological entities with different genetically-determined characters (Wang & Szmidt, 1998). A series of techniques and genetic markers have been developed to estimate genetic diversity but no single technique is universally viewed as ideal; each available technique exhibits both strengths and weaknesses. Therefore, the choice of technique is often a compromise that depends on the research question pursued and the genetic resolution needed, as well as on financial constraints and the technical expertise available (Mueller & Wolfenbarger, 1999).

1.4.3 FUNGAL PHYLOGENETICS

Phylogenetics is the study of genealogical relationships and of historical patterns of evolutionary change among organisms (Oliver & Schweizer, 1999). Phylogenetic studies contribute to intelligent transfer of information from the three or four very well known “model systems” fungal species to the hundreds of thousands of other less tractable, less well known fungi (Berbee & Taylor, 1999).

For hundreds of years, fungal phylogeny and taxonomy were based almost entirely on morphology. Morphological characters remain defining features for many fungal groups.

Useful characters can have one or more alternative character states (Berbee & Taylor, 1999). One of the underlying assumptions in phylogenetics is that patterns of change are recorded in the characters inherited by organisms from earlier generations. The record of change can be embedded in characters ranging from the morphological to genotypic features of DNA and protein sequences. Typically, phylogenetic relationships are diagrammed in the form of dichotomously branching trees generated, usually, by one of several computer algorithms. Most phylogenetics studies address practical concerns including classification of economically important fungi or detection of fungi of medical or environmental importance (Berbee & Taylor, 1999).

DNA sequence analysis has become the most widely used method for inferring phylogenetic relationships between organisms. The publication of sequences and their deposition in electronic databases (NCBI, GenBank, EMBL, DDBJ) allows the confirmation of results and their application to other taxa without the need to obtain strains or clones, or to repeat experiments (Takamatsu, 1998). There are several factors to consider in selecting a region for sequence analysis (Bruns *et al.*, 1991).

- The region should be evolving at an appropriate rate for the comparison of interest. Ideally this means that the region supplies enough consistent differences to separate the taxa into statistically supported monophyletic groups. Regions that are too conserved will provide too few changes. Regions that are too variable will contain too many inconsistent characters due to multiple substitutions at single positions, and alignment may be an additional problem.

- The region should present ideally as single copy or should at least evolve like a single copy region (e.g. rDNA, mtDNA). The danger of multicopy regions is that different copies might be compared in different species (i.e. paralogous comparisons)

- The region should have the same function in all taxa. Evolution of a new function changes selection pressures and therefore alters the rate and profile of sequence change.

- The effect of base composition and codon bias should be examined. Both factors can distort estimates of divergence.

1.5 MOLECULAR TECHNIQUES USED FOR GENETIC ANALYSIS OF FUNGI

1.5.1 AFLP ANALYSIS

1.5.1.1 General considerations

AFLP (Amplified Fragment Length Polymorphism) is a powerful DNA analysis originally described by Vos *et al.* (1995) that differs importantly from other random fingerprinting techniques by its robustness and reproducibility. The key feature of AFLP is its capability for the simultaneous screening of many different DNA regions distributed randomly throughout the genome (Mueller & Wolfenbarger, 1999). The analysis may be applied to DNA of any origin or complexity (Vos & Kuiper, 1998) producing large numbers of very clear bands of which as many as 10% may be polymorphic with reproductive patterns (Duncan *et al.*, 1998).

AFLP analysis has many of the characteristics of an ideal system for detecting genetic variation: variability is assessed at a large number of independent loci, AFLP markers are usually selectively "neutral" (i.e. not subject to natural selection) and extremely reproducible, variation is revealed over the entire genome and data are obtained very quickly (Majer *et al.*, 1996). Furthermore, the relative ease of implementation, large number of polymorphisms generated, small amount of genomic DNA required, and high reproducibility of band profiles generated render AFLP as an attractive method to study variation between genomes of closely related taxa (Innan *et al.*, 1999). Other advantages of the technique are mentioned by Mueller & Wolfenbarger (1999) as follows:

- **Taxonomic scope**: AFLP markers can be generated for any organism with DNA, and no prior knowledge about the genomic makeup of the organism is needed. Therefore, AFLPs have broad taxonomic applicability and have been used effectively in a variety of taxa, including bacteria, fungi, animals and plants.

- **Error levels**: AFLP amplifications are performed under conditions of high selectivity (a high stringency), thus eliminating the artifactual variation that is seen routinely in RAPD-PCR. Repeated AFLP amplifications show near perfect replicability and overall errors (including mispriming and scoring error) generally amount to less than 2%.

- **Quantity of tissue or DNA**: AFLP requires minimal amount of DNA and partially degraded samples can be used. Therefore, extremely small samples and very small organisms can be examined using this technique.

- **Time efficiency**: AFLPs can be generated at great speed.

- **Mendelian inheritance**: AFLP markers segregate in a mendelian fashion and so can be used for map-based applications.

- **Resolution**: because of the nearly unlimited number of markers that can be generated with AFLP using a series of different primer combinations, at least some AFLP markers will be located in variable regions and thus reveal even minor genetic differences within any given group of organisms.

The main disadvantage of AFLP is the difficulty in identifying homologous markers (alleles), rendering this method less useful for studies that require precise assignment of allelic states, such as heterozygosity analyses (Majer *et al.*, 1996; Mueller & Wolfenbarger, 1999). Although this technique is not a panacea for all molecular problems in ecology and evolution, it offers many advantages and therefore will probably replace several standard techniques (Mueller & Wolfenbarger, 1999). AFLPs are dominant markers but technical refinements to distinguish homozygous and heterozygous genotypes have recently been mooted (Vos & Kuiper, 1998).

1.5.1.2 Description of AFLP analysis

AFLP analysis is based on the selective PCR amplification of DNA restriction fragments under stringent PCR conditions (Kardolus *et al.*, 1998). DNA is prepared in an ingenious

but technically straightforward way that combines the reliability of restriction fragment analysis and the power of PCR (Mueller & Wolfenbarger, 1999). The technique comprises the following steps (Vos & Kuiper, 1998) which are summarised in Figure 1.1.

- **Restriction and ligation**: Restriction of the DNA using two different restriction enzymes, generally a rare-cutter (*Eco*R1) and a frequent cutter (*Mse*1), and the ligation of double-stranded adapters to the resultant ends of the restricted DNA fragments. The adapter and restriction site sequences serve as primer binding sites in the subsequent amplification steps.

- **Amplification of subsets of restriction fragments using selective AFLP primers**: Primers are used that correspond to the adapter and restriction site sequence and that have additional nucleotides at the 3' ends extending into the restriction fragments. These 3' extensions assure that only a subset of the restriction fragments are amplified, i.e. those fragments of which the sequence adjacent to the restriction sites match the 3' primer extensions. AFLP can be performed using up to two selective bases for each primer. One of the two AFLP primers used in the selective amplification reaction is usually labelled either using a radioisotope or a fluorochrome.

- **Analysis of the amplicon profile:** For this purpose, the labelled reaction products are separated by electrophoresis through denaturing polyacrilamide gels (PAGE) or in a capillary. After electrophoresis, gels are either silver stained or exposed to X-rays films to visualise the AFLP fingerprints. Single-nucleotide differences between AFLP fragments can be also resolved with the help of automated sequencer, but this high-resolution methods do require training and some laboratory set-up costs. It is also possible to analyse AFLP markers with technically simpler agarose gel electrophoresis, although simplicity is bought at the cost of lower resolution (Mueller & Wolfenbarger, 1999).

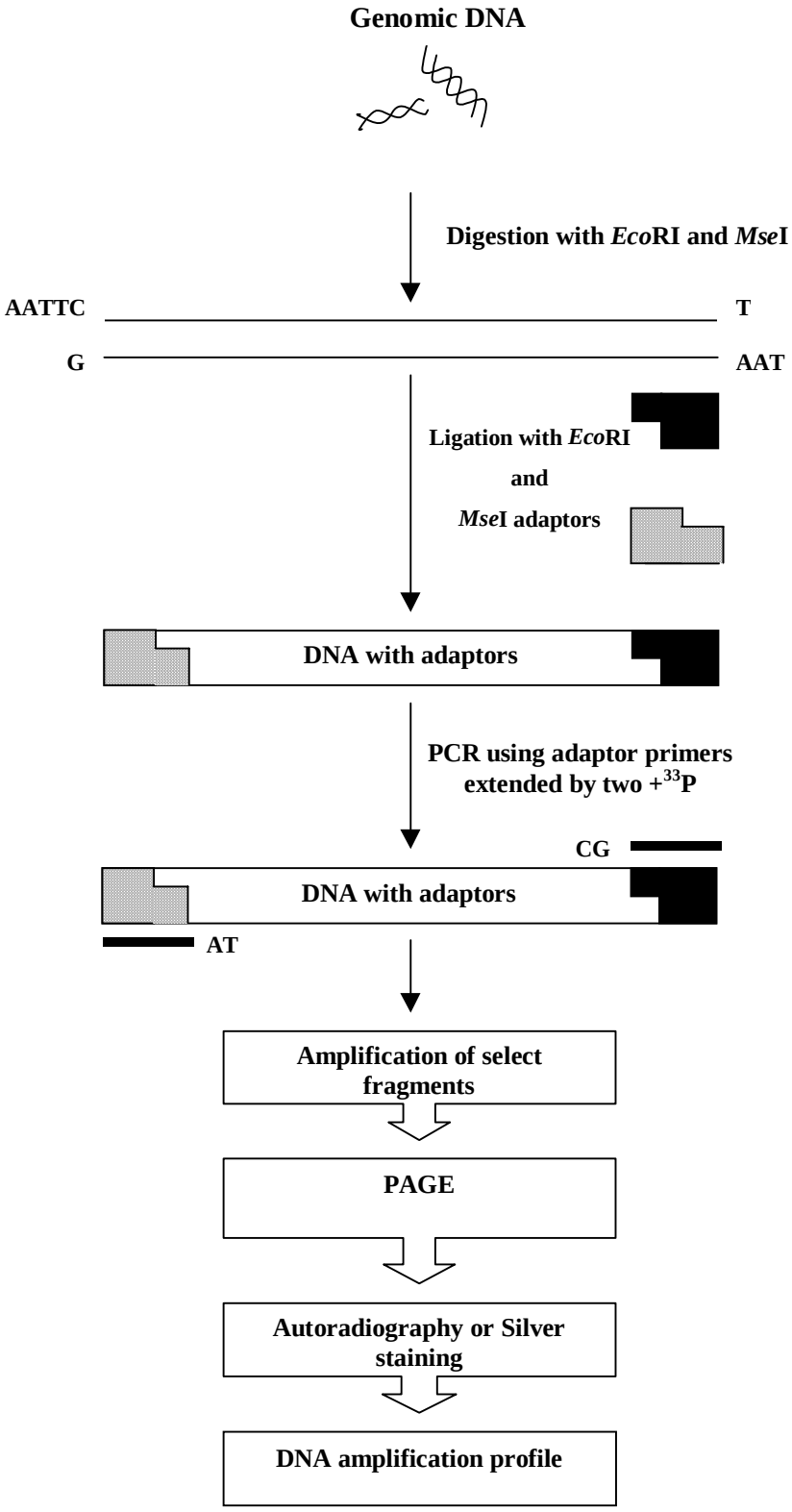

Figure 1.1 AFLP (Amplified Fragment Length Polymorphism) analysis.

1.5.1.3 AFLP applications in mycology

AFLP fingerprinting represents a relatively cheap and effective way to investigate several aspects of fungal molecular biology and genetic diversity (Majer *et al.*, 1996). The technique has proven to be a reliable and powerful analysis in a variety of applications including fingerprinting and identification of fungal isolates (Mueller *et al.*, 1996), studies of intraspecific genetic variation (Majer *et al.*, 1996) and even estimation of phylogenetic relationships (Tredway *et al.*, 1999).

The capacity of AFLP markers to resolve extremely small genetic differences has been demonstrated in several studies. For example, Mueller *et al.* (1996) found that AFLP was very efficient at detecting polymorphisms, even in species where very little variation could previously be found by RFLP analysis. Some general applications of this technique are summarised by Mueller & Wolfenbarger (1999) as follows:

- **Systematics, pathotyping and biodiversity surveys**: AFLP markers have been used to uncover cryptic genetic variation of strains or closely related species from a wide range of broad taxonomic groupings, including plants, fungi, animals and bacteria . AFLP markers have also been used to infer genetic relationships of closely related species based on measurements of genetic distance. At higher taxonomic levels, phylogenetic and genetic inferences based on similarities of AFLP profiles become problematic, largely because the high variability of AFLP markers.

- **Population and conservation genetics**: AFLP markers have found the widest application in analyses of genetic variation below the level of species and have proved particularly popular in investigations of population structure and differentiation. AFLP markers have been applied to evaluate gene flow and dispersal, outcrossing, introgression and cases of hybridisation. The high resolution of AFLP markers also enables testing for clonal identity between individuals (i.e. absence of recombination), and thus permits inferences about sexual versus asexual modes of reproduction.

- **AFLP fingerprinting and kinship**: AFLP markers have the potential to resolve genetic differences at the level of DNA fingerprinting for individual identification and parentage analysis. In the ideal case, a few primer combinations will suffice to generate an adequate number of polymorphic markers. In the worst case, many AFLP markers have to be generated with a series of primer combinations to reveal differences between closely related or inbred individuals, or to confirm a lack of differences for clonality. However, it is still unclear how many markers must be generated to ensure significant representation of hypervariable loci and to profile an individual at the level of a true DNA fingerprinting. This is crucial for unequivocal identification of clonally identical individuals, where an insufficient number of fragments might not uncover existing genetic differences, and thus lead to an incorrect conclusion of clonal identity and asexual reproduction.

1.5.2 ISSR ANALYSIS

1.5.2.1 General considerations

Inter Simple Sequence Repeat analysis (ISSR) is a quick, simple and reproducible PCR-based technique (Albani & Wilkinson, 1998; Prevost & Wilkinson, 1999, Gilbert *et al.*, 1999). The analysis has low running costs, requires only small quantities of template DNA and no prior DNA sequence information is required from the organism under study. ISSR analysis is highly informative generating sufficient polymorphisms to have potential for large-scale DNA fingerprinting purposes (Prevost & Wilkinson, 1999).

The technique is quicker and more straightforward than AFLPs and does not require the high development of conventional SSRs (McGregor *et al.*, 2000). Although the ISSR technique also yields dominant markers, it has been reported that a longer 5´-anchor can yield markers which are codominant (Fisher *et al.*, 1996). The ISSR-PCR protocol is nearly identical to that of randomly amplified polymorphic DNA (RAPDs) in that exponential amplification will occur from these single primer reactions only when the particular repeat used as a primer is represented in multiple copies, which are closely spaced (less 2-3 kb) and inversely oriented in the template DNA (Vogel & Scolnik, 1998). Typically, these reactions are multiplexed; multiple products are simultaneously co-

amplified, and therefore multiple loci can be detected from a genome using a single PCR reaction.

ISSR and RAPD analyses differ in that ISSR primer sequence are designed from microsatellite regions, the annealing temperatures used are higher than those used for RAPD markers (Wolfe, 2002), and the fragments are usually visualised on high-resolution polyacrilamyde gels by silver staining or a radioactivity source whereas the RAPD bands are resolved on low-resolution agarose gel using ethidium bromide staining. Since ISSR primers are longer, they allow more stringent annealing temperatures (Vogel & Scolnik, 1998; Wolfe & Liston, 1998). These considerations result in fewer problems with reproducibility, a complaint frequently levelled against the low-stringency RAPD assay with shorter primers (Nagaoka & Ogihara, 1997; Vogel & Scolnik, 1998; Wolfe *et al.*, 1998).

ISSR analysis can be completed within nine hours and produce complex band profiles that do not differ between replicates (Albani & Wilkinson, 1998; Prevost & Wilkinson, 1999). The protocol appears to be sufficiently reproducible for fingerprinting purposes. This is in concordance with previous studies where the reliability of the protocol was demonstrated between PCRs, DNA extractions and even laboratories when applied to oilseed rape (Charters *et al.*, 1996) and cacao (Charters *et al.*, 2000).

ISSR analysis exploits the abundance of genomic microsatellites (or simple sequence repeats, SSRs) to generate complex banding profiles that differ greatly both within (Salimath *et al.*, 1995; Albani & Wilkinson, 1998) and between species (Gupta *et al.*, 1994). Microsatellites are ubiquitously distributed repeats spread in genomes of very distant species. These sequence elements consist of tandemly repeat short sequence motifs such as penta-, tetra-, tri-, and dinucleotide repeats (Zietkiewicz *et al.*, 1994). They are abundant throughout the eukariotic genome (Tautz & Renz, 1984) and are highly polymorphic in length (Levinson & Gutman, 1987).

ISSR analysis uses primers that are complementary to microsatellites regions but which usually also contain one to three base oligonucleotide "anchor" at either the 3´ or 5´ end (Zietkiewics *et al.*, 1994). Base positions within the anchor may contain any nucleotide

other than that needed to continue the repeat sequence. The anchor serves to fix the annealing of the primer to a single position at each target site on the template, such that every new polymerisation event initiates at the same target position (Vogel & Scolnik, 1998). Thus, there is little or no chance for primer slippage on the template, and problems with priming out of register are drastically minimised. This modification was critical for enabling dinucleotide repeat primers to perform well and reduced product heterogeneity resulting from primer slippage (Vogel & Scolnik, 1998). The large numbers of amplicons generated consist largely of the region between neighbouring and inverted SSRs. Theoretically, polymorphisms should be easier to detect because variable regions in the genome are targeted (McGregor *et al*., 2000).

ISSR markers are presumably predominantly noncoding loci and therefore subject to fewer mutational constraints that are isozyme loci (Wolfe & Liston, 1998). Tsumura *et al.* (1996) found that most of the ISSR bands (96%) segregated according to Mendelian expectations and only three bands departed from mendelian expectations. The simple sequence repeats, which are the basis for the primer site of ISSR`s, are known to have a high rate of gaining and losing repeat units due to DNA slippage (Schlötterer, 1998). Chromosomal structural rearrangements have also been suggested as a source of ISSR variation (Wolfe & Liston, 1998).

1.5.2.2 Applications of ISSR-PCR.

The first studies employing ISSR markers were published in 1994 by Zietkiewics *et al.* (1994) and Gupta *et al.* (1994), who both demonstrated the hypervariable nature of ISSR markers. Several studies have applied the technique for a wide range of disciplines (Wolfe, 2002). Whilst there have been numerous such works on plants, there have been few studies that have used the technique to study fungal taxa, although some works did have direct relevance to plant pathology. For instance, Raman *et al.* (1999), used ISSR and AFLP to generate markers for bulk segregant analysis in order to locate a resistance gene in Barley against the fungus *Rhynchosporium secalis*. Camacho & Liston (2001) employed ISSR to determine the population structure and genetic diversity of *Botrychium pumicola*.

Indeed, to date, most ISSR analyses have been performed on cultivated plants such as maize, *Zea mays* L. (Kantely *et al.*, 1995); oilseed rape, *Brassica napus* L. ssp oleifera (Charters *et al.* 1996); wheat (Nagaoka and Ogihara, 1997); citrus (Fang & Roose, 1997); cacao, *Theobroma cacao* L. (Charters *et al.*, 2000); gooseberry, *Ribes grossularia* subgenus Grossularia (Lanham & Brennan, 1999); potato, *Solanum tuberosum* spp. andigena (Prevost & Wilkinson, 1999) and lupin (*Lupinus albus*) (Gilbert *et al.*, 1999). The technique has been used for testing of the genetic identity and purity of plant accessions held in germplasm collections (Gilbert *et al.*, 1999) and for studies of natural populations (Wolfe *et al.*, 1998). ISSR analysis have been also used to detect geneflow (Allainguillaume *et al.*, 1997), for mapping purposes (Kojima *et al.*, 1998) and in studies of somaclonal variation (Albani & Wilkinson, 1998). Wolff *et al.* (1995) used this technique to characterise genetic variability between cultivars, clonal variant families and sports derived from micropropagation in chrysanthemum.

ISSR-PCR products can be easily excised from the dried gel and cloned or re-amplified to be used as probes; this could provide an alternative strategy for identifying microsatellites in genomic libraries (Zietkiewicz *et al.*, 1994).

1.5.3 DNA SEQUENCE ANALYSIS OF THE NUCLEAR RIBOSOMAL DNA (rDNA)

1.5.3.1 General considerations

The genes encoding ribosomal RNA are a major group of genes that have been extensively studied and characterised in prokaryotic and eukaryotic organisms. In eukaryotic organisms, the ribosomal RNA (rRNA) gene cluster is found both in nuclei and plastid genomes and consists of a mix of highly conserved and variable regions (White *et al.*, 1990).

rDNA sequences are often used for taxonomic and phylogenetic studies because they are found universally in living cells in which they have an important function; thus, their evolution might reflect the evolution of the whole genome (Edel, 1998). Besides that, different regions of the mitochondrial and nuclear rRNA genes diverge at different rates;

therefore, if ones wishes to obtain the maximum amount of information from a minimum amount of sequencing, then Bruns *et al.* (1991) argued the question of which regions are most appropriate for a specific level of comparison is important. Historically, rDNA has therefore proved the most commonly used region for this purpose.

1.5.3.2 Structure of the rDNA locus

The fungal nuclear rRNA genes are arranged as tandem repeats with several hundreds copies per genome, whereas an additional single copy is located on the mitochondrial DNA (Bridge & Arora, 1998). Accordingly to Turner (1993), in filamentous fungi there are about 100-150 rRNA copies per haploid genome, although this amount may vary widely.

In the nuclear-encoded ribosomal RNA genes (rDNA) each copy or cluster contains the genes for the small subunit (SSU or 18S), the 5.8S, and the large subunit (LSU or 25-28S) rRNA (Takamatsu, 1998), which are transcribed as a single unit (Turner, 1993) (Figure 1.2). In each copy, the genes are separated by two internal transcribed spacers (ITS1 and ITS2), and two rDNA units are separated by the intergenic spacer (IGS) or nontranscribed spacer (NTS). A 5S rRNA gene may or may not be within the repeated unit, depending on the fungal taxon (Edel, 1998).

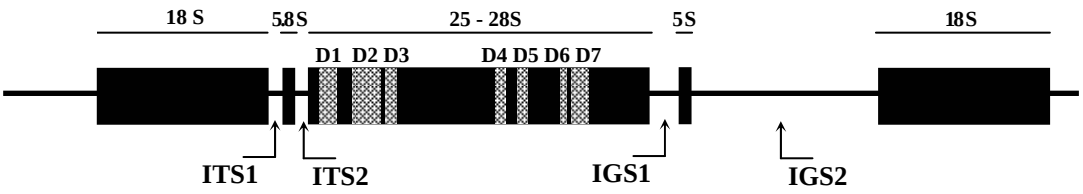

Figure 1.2 Structure of the nuclear-encoded ribosomal RNA gene (rDNA).

The rDNA locus contains both variable and conserved regions, allowing the comparison and discrimination of organisms at different taxonomic levels (Edel, 1998). The ribosomal coding regions are the best conserved, the internal transcribed spacers (ITS) display a

certain degree of variation, and the intergenic spacers (IGS) are the most variable regions (Lanfranco *et al.*, 1998). A more detailed description of the different elements in the rDNA is provided below.

1.5.3.3 Genes in the rDNA locus

The Small Sub Unit (SSU) evolves relatively slowly and is useful for comparing distantly related organisms (Edel, 1998), whereas LSU includes regions with different levels of variation (Figure 1.2). Most molecular studies only utilise the first 600-900 bases from the LSU gene, which includes three divergent domains (D1, D2, D3) that are among the most variable regions within the entire gene (much of the LSU is invariant even across widely divergent taxa) (Gutell & Fox, 1988; Hillis & Dixon, 1991). DNA sequences of the divergent domains are useful for determining phylogenies of relatively closely related organisms (Takamatsu, 1998). The D1-D3 region has been shown to contain most of the phylogenetically informative sites in the nuclear LSU gene (Hillis & Dixon 1991, Hopple & Vilgalys, 1999), D2 domain being particularly useful for studying relationships at or near the generic level (Seifert *et al.*, 1995). For example, Hopple & Vilgalys (1999) found that 97% of the average divergence among 47 species of *Coprinus* and 19 additional species from the families *Coprinaceae, Strophariaceae, Bolbitiaceae, Agaricaceae, Podaxaceae* and *Montagneaceae* was located within the divergent domains, with D2 and D8 being most divergent and domains D7 and D10 the least divergent.

The 5.8S is a small gene (160 nucleotides) and similar to LSU in terms of function and structure (Jacq, 1981). This 5.8S gene is rather highly conserved region with similar level of variability to the small subunit gene and can be used in evolutionary studies of taxa diverged before the Paleozoic (Fatehi, 2000). However, the shortness of this gene has limited its utilisation (Bruns *et al.*, 1991).

The 5S is a very small gene (about 120 nucleotides) found in prokaryotes and eukaryotes. In many fungal species this gene is not present in the cluster and copies are scattered around the genome (Metzenberg *et al.*, 1985), although in some yeasts and many filamentous fungi the gene is part of the cluster (Turner, 1993). This was the first gene to be sequenced extensively, but it is too small and evolves too rapidly to be suitable for the

study of most fungal phylogenetic relationships (Bruns *et al.,* 1991). Additionally, compensatory substitutions that maintain RNA secondary structure and the existence of multiple, independently evolving copies of the genes in filamentous Ascomycota, including *Neurospora* and *Aspergillus* present further problems.

1.5.3.4 Spacers in the rDNA locus

The spacer regions between the subunits, called the internal transcribed spacer (ITS), and between the gene clusters, called the intergenic spacers (IGS), are considerably more variable than the subunit sequences, and have been used widely in studies for comparing fungal species within a genus or strains within a species (Edel, 1998; Takamatsu, 1998).

1.5.3.4.1 Internal transcribed spacers (ITS)

The ITS consist of two non-coding variable regions that are located within the rDNA repeats between the highly conserved small subunit, the 5.8S subunit, and the large subunit rRNA genes (Figure 1.2). ITS1 and ITS2 lack a functional role, which is though to explain the high level of sequence variation within them. According to Bridge & Arora (1998), the ITS region is a particularly useful area for molecular characterisation studies in fungi for four main reasons:

- The ITS regions is relatively short (500-800 bp) and can be easily amplified by PCR using universal single primer pairs that are complementary to conserved regions within the rRNA subunit genes.

- The multicopy nature of the rDNA repeat makes the ITS region easy to amplify from small, dilute or highly degraded DNA samples.

- The ITS region may be highly variable among morphologically distinct species and so ITS-generated RFLP restriction data can be used to estimate genetic distances and provide characters for systematic and phylogenetic analysis.

- PCR-generated ITS species-specific probes can be produced quickly, without the need to produce a chromosomal library, and many researchers have selected sequences from the ITS region to develop species-specific probes because the sequences occur in multiple copies and tend to be similar within and variable between fungal species.

ITS is now the most commonly sequenced region of DNA within the fungal genome. Universal primers designed by White *et al.* (1990) have enabled the determination of many ITS sequences from divergent fungi and these have been used to investigate taxonomic and phylogenetic relationships between species within different genera, such as *Colletotrichum*, *Phytophthora* and *Penicilium* (Edel, 1998).

Differences in ITS sequences have been widely used to develop rapid procedures for the identification of fungal species and also for the detection of many phytopathogenic fungal species in host plants without previous isolation of the fungi (Bridge & Arora, 1998; Edel, 1998). The rapid evolution of ITS regions means that they have also proved useful for reconstructing phylogenies of closely related species in a wide variety of fungi, red algae and flowering plants (Berbee & Taylor 1999). Studies of ITS regions from rusts, *Colletotrichum* and *Alternaria* have shown sequence variation at the interspecific level but generally low levels of intraspecific variation within well defined species (Cooke & Duncan, 1997). ITS variation among *Laccaria* species is 1-3% (Gardes *et al.,* 1991). However, within *Armillaria* ITS sequence variation may be insufficient to resolve relationships within all species, a portion of the IGS appearing to be more useful (Bruns *et al.*, 1991).

1.5.3.4.2 Intergenic spacer (IGS)

The large intergenic spacer (IGS) is found between the 3´ end of the 25-28S and the 5´ end of the 18S genes (Takamatsu, 1998) (Figure 1.2). Two spacers (IGS1 and IGS2) exist when the 5S rRNA is present within the repeated unit.

It is often possible to differentiate between closely related fungal strains through comparisons of this sequence since it is generally a more variable region of DNA than is

ITS (Edel, 1998). Rather surprisingly given this observation, and in contrast to the ITS region, there have been relatively few studies that have utilised this region for phylogenetic reconstruction (Bridge & Arora, 1998), with most works considering other rDNA regions. There are nevertheless some important exceptions that have incorporated IGS information into larger data sets. For instance, Bunyard *et al.* (1996) used PCR-RFLP of the 26S, 5S and IGS for a phylogenetic study of *Agaricus* species and James *et al.* (2001) studied the dispersal and population structure of the common split-gilled mushroom *Schizophyllum commune* using both ITS and IGS1. The phylogeny of IGS1 sequences revealed strong geographic patterns and supported three evolutionary distinct lineages within the global population.

1.5.3.5 Variation between copies of rDNA within the same genome

As rDNA sequences are present in high copy number in the fungal genome, their use generally increases the sensitivity of a detection assay (Edel, 1998). The occurrence of differences between copies is widely believed to be prevented by a process known as concerted evolution, which seemingly maintains the homogeneity of the gene cluster and spacer regions (Hillis & Dixon, 1991; James *et al.*, 2001). Thus, Isikhuemhen *et al.* (2000) found that ITS sequences of *Pleurotus tuberregium* were typically highly uniform within populations and individual isolates from nature, and James *et al.* (2001) reported for the mushroom *Schizophyllum commune* that the large level of haplotype diversity detected in the ribosomal spacers appears to be entirely due to differences between individuals rather than differences among the tandem repeats within an array.

Despite the presumed dominance of concerted evolution and the consequent gene homogenisation, there is significant evidence that individual dikaryotic and monokaryotic strains can sometimes possess more than a single rDNA variant (Hibbett, 1992). An organism thus, could have multiple forms of a gene cluster of different sequences. Variation has been found within the rDNA repeating units, mostly within IGS and ITS (Stambrook, 1978, Yakura *et al.*, 1984; O´Donnell & Cigelnik, 1997; Fatehi & Bridge, 1998) or in ribosomal transcribed genes (González *et al.*, 1985; Carranza *et al.*, 1996) among different copies obtained from within the same individual. The significance of these events in the interpretation of data for fungal taxonomy has not yet been fully investigated, although some authors have indicated

that it could sometimes confound phylogenetic studies (O´Donnell & Cigelnik, 1997). In addition, the likelihood of detecting such occurrences, given the competitive nature and concentration dependency of the PCR reaction has not been widely considered (Bridge & Arora, 1998). Hong *et al*. (2002) recommended that phylogenies based on 18S or ITS rDNA should be verified by reference to other sources of data such as mt rDNA.

1.5.3.6 Application of rDNA sequence information

One of the first applications of PCR for mycological research was to enable the DNA sequences that encode ribosomal RNAs to be determined (White *et al.*, 1990). These data have since been widely utilised for both molecular systematic studies and population genetics (James *et al.*, 2001). For this purpose, “universal" primers sequences were designed for both nuclear and mitochondrial rRNA genes, as well as for the internal transcribed spacer (White *et al.*, 1990; Vilbalys & Hester, 1990).

There have been many ways in which the rRNA gene cluster can be used to study filamentous fungi at the species level, ranging from simple size comparisons of PCR amplicons through to extensive sequence analyses (Bridge & Arora, 1998). Indeed, a growing number of phylogenetic studies have used evidence from ribosomal RNA gene sequences to address the evolutionary relationships of mushrooms and their relatives (Bruns *et al.*, 1992, 1998; Moncalvo *et al.*, 1993; Hopple & Vilgalys, 1999; Johnson & Vilgalys, 1998).

The conserved nature of the coding regions in the large subunit (LSU) and small subunit (SSU) genes have been exploited to study the many relationships among distantly related fungi or for studies at the genus and interspecies level (Bridge & Arora, 1998). Thus, comparison of the sequences from two domains of the nuclear LSU gene was employed to assess the phylogenetic relationships within different species of *Fusarium* (Guadet *et al.*, 1989), and phylogenetic relationships were investigated in the mushroom genus *Coprinus* based on the same gene (Hopple & Vilgalys, 1999). Phylogenetic relationships of mushrooms and their relatives within the order Agaricales were addressed using nuclear LSU (Moncalvo *et al.*, 2000). Approximately 900 bases of the 5´end of the nucleus-encoded large subunit RNA gene were sequenced for 154 selected taxa representing most

families within the Agaricales. The resultant nuclear LSU sequences provided suitable resolution for identifying major lineages of agaric fungi, with good support for many terminal clades and many internal branches.

The spacer regions between the subunits (ITS, IGS) have been used widely at the specific, infraspecific and population levels (Edel, 1998; Takamatsu, 1998). For example, Lee & Taylor (1992) published the ITS1 and ITS2 sequences of the tropical *Phytophthora* species *P. palmivora, P. megakarya, P. capsici, P. citrophthora* and *P. cinnamoni,* and showed excellent resolution at the species level. They found that cacao isolates of *P. capsici* and *P. citrophthora* were closely related, that *P. palmivora* and *P. megakarya* shared a common lineage, and that *P. cinnamomi* was only distantly related to the other species. When Cooke & Duncan (1997) expanded the scope of this study to include other species of *Phytophthora*, they also identified polymorphisms within the ITS regions of individual isolates of some species. Crawford *et al.* (1996) also used rDNA sequences in an evolutionary analysis of 15 *Phytophthora* species. Analysis of papillate, semi-papillate and non-papillate species showed that sporangium papillation had phylogenetic significance, with the three morphological groups each forming separate clusters.

Hibbett *et al.* (1995) studied the phylogenetic relationships of the agarical shiitake mushroom (*Lentinus* spp.) on the basis of polymorphism in ITS sequences. They found four independent lineages in Asia-Australasia, and this provided some support for the morphologically based species concept. There was a strong correlation between the geographic origins of the isolates within each lineage and a biogeographic interpretation of the ITS tree suggesting that the centre of origin for shiitake in the Asia-Australasia region was located in the South Pacific.

Isikhuemhen *et al.* (2000) investigated genetic relationships among several populations of *Pleurotus tuberregium* using ITS sequence data. Nucleotide sequences of the ITS region of the rDNA gene were determined for 30 isolates and used to infer phylogenetic structure of populations. The resultant phylogenetic analysis showed that African and Australasian-Pacific isolates derived from at least two distinct evolutionary lineages.

Interpretation of ITS sequence data can sometimes be complicated by considerable non-informative variation (Bridge & Arora, 1998). On occasions, however, variation within species can prove informative. For instance, the analysis of the ITS region has been used to designate subspecific groups within *Fusarium oxysporum* (Bridge & Arora, 1998).

1.5.4 ANALYSIS OF THE SMALL SUBUNIT RIBOSOMAL MITOCHONDRIAL DNA (mtSSU rDNA)

1.5.4.1 General considerations

Fungi have extrachromosomal genetic elements, the most important of which are found in the mitochondria, which account for 1-20% of the DNA in fungal cells (Moore-Landecker, 1990). Mitochondrial genomes provide another source of genetic variability that is independent of sexual reproduction because mitochondria usually exhibit uniparental (maternal) inheritance (Taylor, 1986). Thus, in mammals the mitochondria derives from the female gamete and in *Neurospora crassa* from the protoperithecium. The complexity of fungal life cycles can confuse the apparent simplicity of plastid inheritance. For example in *Saccharomyces cerevisiae* there is no obvious differentiation between male and female strains and so both parents contribute mitochondria to the offspring (Carlile & Watkinson, 1994).

The mitochondrial genome of fungi differs in size between species, from an average of 18.9 kbp in *Torulopsis* to 176 kbp in *Agaricus bitorquis* (Fatehi, 2000). In a strain of *S. cerevisiae* genes account for 16% of the mtDNA, introns for 22% and intergenic regions for 62% (Carlile & Watkinson, 1994). Although the nuclear genome of fungi is several hundred times larger than the mitochondrial genome, mtDNA can nevertheless represent a considerable part of the total DNA in the cell, since cells contain many mitochondria, each perhaps with several mtDNA molecules.

A mitochondrial DNA (mtDNA) molecule normally consists of a circle of double-stranded DNA, but in a few organisms, including the yeast *Hansenula mrakii*, the molecule is linear (Carlile & Watkinson, 1994). Most of the mitochondrial genes coding for proteins give rise to enzymes that are involved in respiratory chain complexes including electron

transport and ATP synthesis. Other genes code for the transfer RNA (tRNA) and the small and large ribosomal RNA subunits (rRNA).

Mitochondrial DNA has proved to be a useful tool for taxonomic studies partly because of its relatively small size, making it possible to analyse the entire genome, uniparental inheritance, and its composition is not usually complicated by the recombination that occurs regularly in nuclei as a result of sexual reproduction (Taylor, 1986). The last point is not universally true, however, as such recombination does occur in some fungal species including *Aspergilus nidulans* (Earl *et al.*, 1981), *Coprinus cinereus* (Baptista-Ferreira *et al.*, 1983) and in the mushroom genus Armillaria (Saville *et al.*, 1998). Biparental inheritance has been also reported in *Didymium iridis* (Silliker, 1985).

1.5.4.2 Applications of mt DNA sequence information

Analysis of mtDNA variation has been used to discern subspecies, vegetative incompatibility groups and different populations (Gray 1989; Jacobs*on* & Gordon, 1990; Sm*ith et al.*, 1990). Also, it has been used in studies of fungal evolution (Moody & Tyler, 1990; Bruns *et al.*, 1991; Förster & Coffey, 1993). The many length mutations detected within the mitochondrial genome has limited the evolutionary value of mtRFLP profiles (Fatehi, 2000). To take advantage of the mitochondrial divergence in phylogenetic studies, it is necessary that the source of variability and the types of mutations, such as nucleotide substitutions, length mutations or rearrangements, be identified and compared independently (Taylor, 1986). One option to avoid this complexity is to consider only conserved regions of the mitochondrial genome such as rRNA genes (Fatehi, 2000).

1.5.4.2.1 rRNA gene cluster

There is a single copy rRNA gene cluster located on mtDNA of fungi, which has also been used to develop specific RFLP probes and PCR primers for the identification of fungal species (Bridge & Arora, 1998). Mitochondrial rDNA can be easily analysed among fungi after amplification using consensus primers (White *et al.*, 1990). As a single copy gene, this region of DNA would be free from the complications of different forms being present, and, as mitochondrial DNA is generally transmitted by uniparental inheritance, the gene

would be expected to be largely free of the effects of recombination (Bridge & Arora, 1998).

The rate of evolution of mitochondrial DNA is different from that of the nuclear genome. The mitochondrial rRNA genes evolve approximately 16 times faster than the nuclear rDNA (Bruns & Szaro, 1992), and so are useful for phylogenetic studies at an intermediate taxonomic level (Bruns *et al*., 1991; Simon *et al*., 1994). Thus, they are believed to have a potential to fill phylogenetic gaps at a family level between those available from 18S and ITS rDNAs (Hong *et al*., 2002). In fungi, the major source of variability in mitochondrial genome is length mutation (deletion and insertion). This form of genetic change occurs at high frequency within the mitochondrial genome (Fatehi, 2000).

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CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 EXPERIMENTAL MATERIAL

A set of 109-*M. roreri* isolates representing the global geographic distribution of the pathogen constituted the experimental material used in this study. The isolates were obtained from two sources: 97 isolates (89%) were collected by the author and the remaining samples kindly donated from other researchers listed below. Collectively, samples were taken from all of the most important cacao growing areas affected by the disease in all countries where the pathogen was present: Colombia (Co), Venezuela (V), Ecuador (E), Peru (P), Panama (Pa), Costa Rica (C), Nicaragua (N) and Honduras (H) (Table 2.1). Details on the origins of the isolates used are shown in the appendix (Table A2.1).

Table 2.1 Number of isolates of *M. roreri* obtained from eighth countries in Tropical America.

Country	Major political division	Number of isolates
Ecuador	Provinces of Guayas, Los Ríos, Manabí, Esmeraldas, Napo, Imbabura, Carchí and Pichincha	39
Costa Rica	Provinces of Cartago, Limón, Puntarenas, Heredia, San José and Alajuela	29
Colombia	Departments of Norte de Santander, Santander, Antioquia, Caldas and Huila	18
Panama	Provinces of Chiriquí, Bocas del Toro and Colón	7
Nicaragua	Región Atlántico Norte, Provinces of Río San Juan and Matagalpa	6
Peru	Departments of San Martín and Huánuco	5
Venezuela	States of Mérida, Zulia and Táchira	4
Honduras	Department of Gracias a Dios.	1
	TOTAL	**109**

Eleven collecting trips were performed by the author between February and December 1999 to collect isolates from seven countries (Colombia, Costa Rica, Ecuador, Honduras, Nicaragua, Panama and Venezuela). Collaboration from different persons and institutions was obtained to carry out the collections (Table A2.2). Isolates were obtained from a wide range of altitudes (0-1520 m.a.s.l.). A high percentage of the isolates were obtained in Ecuador, since at the time of collection this country was commonly regarded as the centre of origin of the pathogen (Rorer, 1918; Evans, 1981). A considerable representation of isolates were also obtained neighbouring from Colombia and Costa Rica in order to compare changes in genetic diversity of the fungus with distance from the presumed centre of origin. It was not possible to obtain samples from the Departments of Nariño, Meta, Chocó and Valle in Colombia, from the Province of Darién and Comarca of San Blás in Panama or from the Catatumbo river in Venezuela. Furthermore, it was only possible to obtain one Honduran isolate due to the very limited distribution of the pathogen in this country at the time of sampling.

Ninety-nine samples (90.8%) were isolated from fungal genotypes growing on *Theobroma cacao* trees, with the remaining 10 samples being taken from the following species of *Theobroma* and *Herrania* growing in the International Germplasm Collection of CATIE: *T. grandiflorum* (C14), *T. bicolor* (C20), *T. speciosum* (C22), *T. mammosum* (C25), *H. nitida* (C18) and *H. albiflora* (C23). In addition, one fungal isolate was collected from *T. bicolor* (E36), one from *Herrania* sp. (E38) and two from *T. gileri* (E42 and E43) trees growing in Ecuador.

All samples were taken from pods showing initial or intermediate stages of external necrosis and were mostly processed the same day of harvest. The pods were cleaned externally with water and then transversally sectioned using a sterilised knife. A small section of the shell (approx. 3 x 3 cm) was cut from the margins of tissue exhibiting fungal infection. Then, small pieces of internal tissues from the border of the necrotic area were obtained using a sterilised scalpel and aseptically transferred into Petri dishes containing 3% w/v agar-water. The fungus was allowed to grow for 3-5 d, and then a small piece of agar-mycelium was transferred to a Petri dish containing 20 ml of modified V8 medium (20% v/v V8 juice, Campbell Soup Company, Camden, NJ, USA, 0.1% w/v asparagine, 2% w/v maltose and 1.8% w/v agar). The dishes were sealed with Parafilm tape

(American National) and incubated in a Percival Boone 50036 model I-35LL incubator at 24 ºC under 12/12 alternate periods of light/darkness, with light provided by fluorescent lamps.

The isolates collected by the author were complemented with some isolates obtained by other investigators in Ecuador and Peru (Table A2.1). Five Peruvian isolates collected by U. Krauss and W. Soberanis in 1997 were obtained from the CABI´s collection at Ascot, UK in 2000. Seven isolates collected by H. Evans and collaborators in the Ecuadorian Provinces of Napo (E36, E37), Los Ríos (E38, E39, E40), Carchí (E42) and Imbabura (E43) were obtained from CABI, Ascot (Dis series) and from the *Centraalbureau voor Schimmelcultures Baar-Delft,* Netherlands (CBS series).

To study the phylogenetic relationship between *M. roreri* and the basidiomycetes, three *C. perniciosa* isolates were obtained from H. Evans, CABI, Ascot (Table 2.2).

Table 2.2 Isolates of *Crinipellis perniciosa* obtained from CABI, Ascot, UK to study the phylogenetic relationship between *M. roreri* and Basidiomycetes.

CABI I.D.	Collecting date	Place	Host
Dis 43	29/9/97	Yarinacocha, Pucallpa, Department of Ucayali, Peru	*Theobroma bicolor*
Dis 70	26/3/99	Yanayacu (Panacocha), Rio Napo, Napo Province, Ecuador	*Liana* sp.(?Bignoniaceae)
Dis 71	25/3/99	Río Anagucocha (Panacocha), Rio Napo, Napo Province, Ecuador	*Theobroma cacao*

2.2 MAINTENANCE AND PRESERVATION OF THE ISOLATES

Isolates were grown on modified V8 medium. The collection was maintained during the study by subculturing at four monthly intervals onto fresh stocks of he same medium. Two replicates of the collection (in slant tubes and in small Petri dishes) were maintained in the dark at 15 ºC and another two replicates were kept under laboratory conditions (21 ºC, natural daylight). *M. roreri* and *C. perniciosa* isolates were transferred into the CABI´s collection at Egham, UK for a more durable preservation using freeze-drying and cryopreservation techniques.

2.3 ISOLATION OF GENOMIC DNA

2.3.1 PRODUCTION OF MYCELIUM

Genomic DNA was extracted from dry mycelium obtained using the following procedure. Isolates were grown in Petri dishes (8.5 cm diam) containing modified V8 medium for 11-15 d. A conidia suspension was then prepared by adding, in three steps, 20 ml of a 0.01% Tween-80 solution to each Petri dish and gently removing the spores with a paintbrush. The suspension was transferred into a sterile beaker and gently agitated for 10 min to separate the spores. The solution was then aliquoted into a 250 ml Erlenmeyer flask containing 100 ml of mineral liquid medium (Table A2.3). The isolates were cultured at 25 °C, 12/12 alternate periods of light/darkness and subjected to continued shaking (150 rpm) for 8 d. Mycelia were harvested by vacuum filtration (Whatman No. 3), washed with sterile distilled water and dried in an oven at 35 °C for 48 h. Dried mycelia were ground in liquid nitrogen and transferred into 1.5-ml microcentrifuge tubes for storage at -20 °C.

Mycelial cultures of *C. perniciosa* were obtained by growing isolates in liquid Glucose Yeast medium (GYM, 1 lt. distilled water, 10 g glucose, 1g NH_4 H_2PO_4, 0.2 g KCl, 0.2 g $MgSO_4$ $7H_2O$, 5 g yeast extract, 1 ml 1% $ZnSO_4$ $7H_2O$, 1ml 0.5% $CuSO_4$ 5 H_2O) (Mugnai *et al*., 1989). Universal bottles containing 10 ml of GYM were inoculated with mycelia from cultures grown on modified V8 medium for 10 d. The inoculants were then incubated at 24 ºC for 48 h in an orbital shaker (Gallenkamp, Orbital Incubator) at 180 rpm. These cultures were transferred aseptically into a 500 ml Erlenmeyer flask

containing 100 ml GYM and incubated under the same conditions for a further 3 d. Cultures were harvested aseptically by vacuum filtration (Whatman No. 3), washed with sterile water and transferred into Petri dishes and lyophilized overnight using a freeze-drying machine (Eduard, Ltd.). The freeze dried mycelia were then powdered by grinding in liquid nitrogen and transferred into 1.5 ml microcentrifuge tubes for storage at –20 °C.

2.3.2 DNA EXTRACTION

DNA was extracted from the powdered mycelia (<100 mg) using a DNeasy Plant Mini Kit (Qiagen, UK) according to the manufacturer's instructions. Each sample was lysed in 400 µl of Buffer AP1 and 4 µl of RNAse was added to digest the RNA. The mixture was vortexed briefly and incubated at 65 ºC for 10 min. Following lysis, the sample was mixed with Buffer AP2 and incubated for 5 min on ice to remove detergent, proteins and polysaccharides. The clear lysate was then passed through a spin column and centrifuged at 13,000 rpm to remove the precipitates and cell debris. The flow-through fraction was transferred to a new tube and 0.5 volume of Buffer AP3 and 1 volume of 100% ethanol were added. This step is to aid the binding of the DNA to the column in the following step. The solution was passed through a DNeasy Mini spin column at 8,000 rpm to bind the DNA to the membrane. Two washes were performed with 500 µl of Buffer AW and the tubes spun at 8000 rpm to clean DNA. A final spin at 13,000 rpm dried the column and the DNA was then eluted into 200 µl of Buffer AE. The samples were stored at –20 ºC.

2.3.3 DNA QUANTIFICATION

The quantity and quality of DNA extracted was evaluated by reference to known standards fractionated on 1% (w/v) agarose gel stained with ethidium bromide (0.5 µg ml^{-1}) and visualised under UV. DNA samples were also quantified using a DyNA Quant fluorimeter (Höefer Scientific Instruments, USA) according to manufacturer's instructions.

2.4 AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP) ANALYSIS

Amplified Fragment Length Polymorphism (AFLP) reactions (Vos *et al*., 1995) were conducted using the Gibco BRL AFLP ™ Analysis System II kit (Life Technologies, UK). The manufacturer's instructions were followed except that both the reaction volumes and the amount of |y^{33}P|-ATP added were reduced by 50%. The procedure used is described below.

2.4.1 RESTRICTION DIGESTION OF GENOMIC DNA AND LIGATION OF ADAPTERS

DNA digestion was carried out using two different restriction enzymes (the frequent cutter *Mse*1 and the rare cutter *Eco*R1). Thin-walled microcentrifuge tubes containing 2.5 µl DNA (50 ng/µl), 1.0 µl *Eco*R1/*Mse*1, 2.5 µl 5x reaction buffer (50 mM Tris-HCl pH 7.5, 50 mM Mg-acetate, 250 mM K-acetate) and 6.5 µl of nanopure water were first incubated at 37 °C for three h, followed by heat deactivation at 70 °C for 10 min. Ligation of double-stranded adapters to the ends of the restriction fragments was performed immediately by adding 0.5 µl T4 DNA ligase and 12 µl adapter-ligation solution to the digestion tubes and incubating at 20 °C for 2 h. The ligation mixture was then diluted 1:10 in TE buffer.

2.4.2 PREAMPLIFICATION REACTIONS

PCR preamplifications were performed in 0.2-ml thin-walled microcentrifuge tubes containing 2.5 µl of 1:10 ligation mixture, 20 µl pre-amp primer Mix II, 2.5 µl 10x PCR buffer (Qiagen, UK), 0.1 µl *Taq* DNA Polymerase (5 units/µl, Qiagen, UK) and 0.5 µl of 0.2 mM each dATP, dCTP, dGTP and dTTP (Bioline, UK). The reaction mixture was subjected to 20 cycles of 94 °C for 30 seconds (denaturing), 56 °C for 1 min (annealing) and 72 °C for 1 min (extension) in a Phoenix thermocycler (Helena Biosciences). The PCR products were separated by electrophoresis on a 1% (w/v) agarose gel stained with ethidium bromide (0.5 µg ml^{-1}) and visualised under UV. Successful preamplification products were then diluted by 1:50 in TE buffer.

2.4.3 PRIMER LABELLING

*Eco*R1 primer were labelled by phosphorylation of the 5´ end using [γ-^{33}P]-ATP and T4 kinase. A Labelling Mix was prepared in a 1.5-ml microcentrifuge tube by adding the following ingredients (for 50 samples): 9 µl *Eco*R1 primer (27.8 ng/µl), 5 µl 5x kinase buffer (350 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 500 mM KCl, 5mM 2-mercaptoethanol), 1 µl T4 kinase (10 U/µl), 7.5 µl nanopure water and 2.5 µl [γ-^{33}P]-ATP (25 µ Ci) (Amersham Pharmacia Biotech, UK). The mixture was incubated at 37 °C for 1h. The reaction was stopped and the kinase denatured by heating to 70 °C for 10 min.

2.4.4 SELECTIVE AFLP AMPLIFICATION

A Primer Mix including 0.5 µl of Labelling Mix and 4.5 µl *Mse* 1 primer was prepared in a 1.5-ml microcentrifuge tube for each sample. PCR amplifications were then performed in a final volume of 20 µl comprising 5 µl Preamplification product, 2 µl 10x PCR buffer (Qiagen, UK), 0.1 µl *Taq* Polymerase (5 units/µl, Qiagen, UK), 7.9 µl nanopure water and 5 µl Primer Mix. The PCR conditions shown in Table 2.3 were applied using a thermal cycler (MJ Research, Watertown, MA).

Table 2.3 Thermal cycler programme used for selective AFLP amplifications.

File	Denaturing		Annealing		Extension		N°Cycles	Link to	Type
	Temp. (°C)	Time (sec)	Temp. (°C)	Time (sec)	Temp. (°C)	Time (sec)			
1	94	60	65	60	72	90	1	2	Step
2	idem		64	60	idem		1	3	Step
3	idem		63	60	idem		1	4	Step
4	idem		62	60	idem		1	5	Step
5	idem		61	60	idem		1	6	Step
6	idem		60	60	idem		1	7	Step
7	idem		59	60	idem		1	8	Step
8	idem		58	60	idem		1	9	Step
9	idem		57	60	idem		1	10	Step
10	idem		56	60	idem		1	11	Step
11	94	30	56	30	72	60	23		Step

2.4.5 SAMPLE PREPARATION

Selective amplification products (5 µl) were mixed with 5 µl of loading buffer (6.4 ml formamide 99%, 560 µl 10x TBE, 200 µl 50 mM EDTA, 0.01 gr 0.1% (w/v) xylene cyanol, 0.1 gr (w/v) Bromethanol blue and 2840 µl nanopure water).

2.4.6 PLATE PREPARATION

The glass plates were washed using a commercial wetting agent (Fairy, Procter and Gamble, UK), rinsed thoroughly in tap water and rinsed in RO water. The plates were dried with tissue paper and cleaned a further three times using 100% ethanol. A thin film of Gel Slick (Flowgen) was carefully applied to the internal side of the smaller glass plate and the excess removed by wiping the surface with 100% ethanol. When dried, two 0.4 mm plastic spacers (Life Technologies) were positioned along the sides of the larger glass plate. The smaller glass plate was then placed so that it rested on top of the spacers and the two plates were fixed together using tape (3M Sensitive Tape) and bull-dog clips.

2.4.7 PREPARATION AND POURING OF POLYACRILAMIDE GEL

Labelled AFLP amplification products were separated on denaturing 7% polyacrilamide gel prepared as follows. A gel solution of 80 ml SequaGel (National Diagnostics) was prepared by mixing 22.4 ml concentrate solution, 8 ml buffer, 49.6 ml diluent solution, 32 µl Temed and 500 µl fresh solution of amonium persulphate (0.1 mg ml^{-1}). The gel solution was poured between the two plates using a 50 ml syringe. Once the gel was poured, the flat edge of two 0.4 mm combs (Half Width Sharkstooth of 24 lanes each, Life Technologies) were successively inserted into the top of the gel to an approximately depth of 0.8 cm. The gel was allowed to polymerise for at least two hours. The outer edge of the glass plates were then washed under a running tap to eliminate excess polyacrilamide and the combs were carefully removed and washed.

2.4.8 ELECTROPHORESIS

The gel sandwich was clamped into a S2 Sequencer (Life Technologies). The upper and lower reservoirs were filled with 0.1x TBE electrophoresis buffer (National Diagnostics). The gel was pre-run for 1 hour at 40 W to pre-warm the plates and the combs prior to electrophoresis. The deposed urea was removed from the comb area by rinsing with running buffer and a small syringe. The combs were placed between the glass plates with the teeth down toward the gel and inserting them until they just made contact with the surface of the gel.

The samples were denatured at 90 ºC for 3 min and immediately placed onto ice until loading. Each sample (5 µl) was then dispensed into the respective lane using a P10 Gibson pipette. When all samples were loaded, the gel was run at 40 W for 2 h or until the slower dye (light blue) was two-thirds down the length of the gel.

2.4.9 PROCESSING OF THE GEL

After electrophoresis, the gel was transferred to a rigid support (Whatman 3MM), covered with cellophane (Bio-Rad) and dried on a GD40/50 dryer (Life Technologies) at 80 °C for 2 h. The gel was exposed to X-rays films (Kodak X-OMAT AR) to visualise the AFLP intensity of radiation as measured using a Geiger counter. Film was extracted from the cassette, developed and dried at room temperature. Restriction fragments were scored by visual examination and recorded as present or absent.

2.5 INTER SIMPLE SEQUENCE REPEAT (ISSR) ANALYSIS

The ISSR (Inter Simple Sequence Repeat) protocol was based on the protocol described by Charters *et al*. (1996), with minor modifications.

2.5.1 PCR AMPLIFICATIONS

PCR amplifications were performed in a final volume of 20 µl using the following ingredients: 1x *Taq* polymerase buffer (Bioline, UK), 2 mM $MgCl_2$, 0.375 µM primer, 0.2 mM DNTPs (Bioline, UK), 0.05 U/µl *Taq* polymerase (Bioline, UK) and 20 ng of total genomic DNA (Table 2.4).

The ISSR primers anchored at 3′ or 5′ end were obtained from Set 9 of UBC (the University of British Columbia, Nucleic Acid-Protein Unit, c/o Biotechnology Laboratory, Room 237-Wesbrook Building, 6174 University Boulevard, Vancouver, B. C. V6T 1Z3).

Table 2.4 Reaction mixture used for PCR reactions (ISSR).

	Stock concentration	Final concentration	1 sample
Buffer	10x	1x	2.0 µl
$MgCl_2$	50 mM	2 mM	0.8 µl
Primer	15 µM	0.375 µM	0.5 µl
DNTPs	10 mM	0.2 mM	0.4 µl
***Taq* polymerase**	5 U/µl	0.05 U/µl	0.2 µl
Nanopure water	---	---	12.1 µl
DNA solution	5 ng/µl	1 ng/µl	4.0 µl [1/]
Final Volume			**20.0 µl**

[1/] 4.0 µl of a 5 ng/µl DNA solution is equivalent to 20 ng/sample.

PCR reactions were performed in a Phoenix thermal cycler (Helena Biosciences). The programme consisted of 35 cycles of 1 min at 94 °C (denaturing), 2 min at 55 °C (annealing) and 30 seconds at 72 °C (extension), and a final cycle of 5 min at 72 °C. The efficiency of amplification was checked by fractionating the PCR products on a 1.5% w/v agarose gel stained with ethidium bromide (0.5 µg ml^{-1}) at 100 V for 30 min and visualising under UV. Failed reactions were repeated prior to fractionation of all samples by low temperature PAGE.

2.5.2 LOW TEMPERATURE POLYACRILAMIDE GEL ELECTROPHORESIS OF ISSR AMPLIFICATION PRODUCTS

A pre-cast 5/10% polyacrylamide gel (Cleangel 48s, Amersham Pharmacia Biotech., St. Albans, UK) was placed polyacrylamide side faced down into rehydration buffer (Table A2.4) and agitated gently for 1 h. Ten min before the gel was fully rehydrated, the cooling unit (Multitemp II, Amersham Pharmacia Biotech.) attached to a flatbed (Multiphor II, Amersham Pharmacia Biotech.) was set to 10 °C. The gel was removed from the rehydration buffer and wiped dry using Whatman No. 1 filter paper until the wiping action caused the gel to "squeak". Anode and cathode wicks (25 x 4 cm strips of Whatman No. 1 filter paper; five for each electrode) were moistened with 20 ml of electrode buffer (Table A2.4) for each wick. Approximately 2 ml of distilled water was placed onto the centre of the flatbed onto which the gel was carefully lowered (plastic side down). The gel was positioned so that the edge with the wells aligned with the 4th line on the flatbed. Care was taken to ensure that no air bubbles formed between the gel and the flatbed. The excess water was removed from the edge of the gel using filter paper. The moistened wicks were then placed onto the flatbed so that they overlay the gel by 4 mm at either end. Air bubbles were removed by gently flattening the wicks onto the surface of the gel. The gel was then loaded with a mix of 1 µl loading buffer (Table A2.4) and 5.5 µl amplification product. The electrodes were then placed onto the wicks about 4 mm from the outer edge of the wick. The following power programme was set: 20 min at 200 V max, 20 mA max and 10 W max; 50 min at 380 V max, 30 mA max and 20 W max; 60 min at 450 V max, 30 mA max and 20 W max. The programme was run until 5 min after the blue dye ran to the margin of the anode wick.

2.5.3 SILVER STAINING

After electrophoresis, the gel was placed with polyacrylamide side up into a pyrex casserole dish containing 250 ml of 10% acetic acid, and gently agitated for 30 min to allow fixation. The gel was then washed three times in fresh distilled water (2 min each), transferred into silvering solution (Table A2.4) and agitated gently for a further 30 min. The gel was then rinsed in 250 ml RO water for 20 seconds and placed into a pre-chilled dish containing developing solution (Table A2.4) and agitated slowly. Bands profiles

normally appeared after 5-10 min, after which the gel was transferred into stop solution (Table A2.4) for 10 min and then placed into a impregnation solution where it was agitate gently for a further 10 min. The gel was then removed and air-dried overnight at room temperature. Once the gel was dry, it was place onto a "photocopy acetate" and stored. Restriction fragments were scored by visual examination and bands at designated positions recorded as being present or absent. The resultant data set was assembled into a Microsoft Excel © spreadsheet to allow for subsequent analysis.

2.6 ANALYSIS OF THE INTERNAL TRANSCRIBED SPACERS (ITS REGIONS)

A segment of the ribosomal RNA genes in nuclear DNA was amplified using the primers ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) and ITS5 (5`-GGAAGTAAAAGTCGTAACAAGG-3`) (White *et al*., 1990). These primers amplify a segment comprising the following sections: 18S (partial), ITS1, 5.8S, ITS2, 25S (partial) (Figure 2.1).

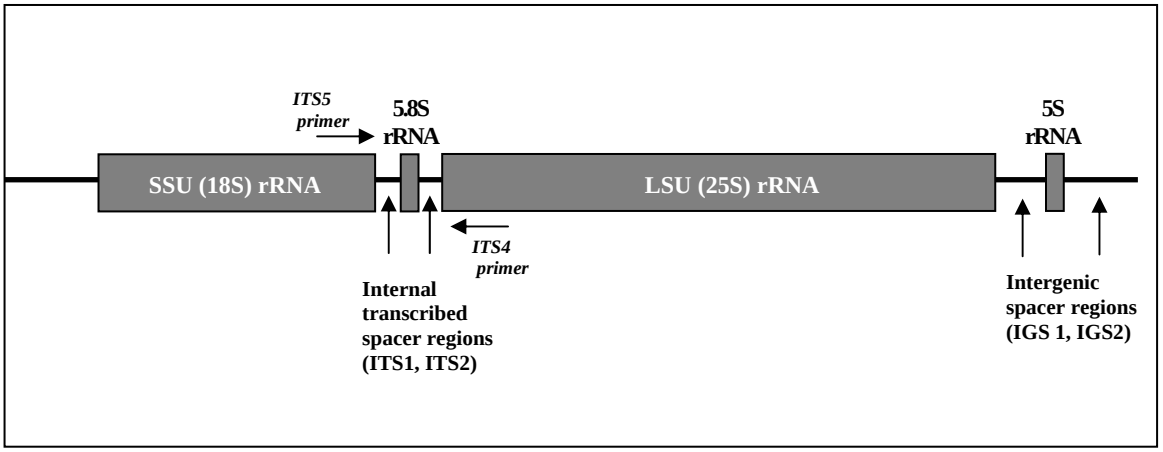

Figure 2.1 Location of the primers ITS5 and ITS4 on rDNA array

2.6.1 PCR AMPLIFICATION

PCR amplification were performed in a final volume of 25 µl consisting of 1x *Taq* polymerase buffer (Bioline, UK), 1.5 mM $MgCl_2$, 0.5 µM each primer, 0.2 mM DNTPs (Bioline, UK), 0.02 U/µl *Taq* polymerase (Bioline, UK) and 10 ng of total genomic DNA (Table 2.5).

Table 2.5 Reaction mixture used for PCR reactions (ITS).

	Stock concentration	Final concentration	1 sample
Buffer	10x	1x	2.50 µl
$MgCl_2$	50 mM	1.5 mM	0.75 µl
Primer ITS4	10 µM	0.5 µM	1.25 µl
Primer ITS5	10 µM	0.5 µM	1.25 µl
DNTPs	10 mM	0.2 mM	0.50 µl
***Taq* polymerase**	5 U/µl	0.02 U/µl	0.10 µl
Nanopure water	---	---	16.65 µl
DNA solution	5 ng/µl	0.4 ng/µl	2.0 µl [1/]
Final Volume	---	---	**25.0 µl**

[1/] 2.0 µl of a 5 ng/µl DNA solution is equivalent to 10 ng/sample.

PCR reactions were performed in a Phoenix thermal cycler (Helena Biosciences). Thermal cycling conditions consisted of an initial cycle at 94 °C for 1 min (denaturing); 30 cycles of 94 °C for 1 min (denaturing), 52 °C for 30 seconds (annealing) and 72 °C for 1 min (extension) and a final cycle at 72 °C for 7 min. Efficacy of PCR preamplification was checked by fractionation of products on 2% w/v agarose gels (1x TAE buffer) stained with ethidium bromide (0.5 µg ml^{-1}) and visualised under ultraviolet light. Size of the fragment was determined by comparison with 100 bp ladder (Gibco-BRL).

2.6.2 PURIFICATION OF PCR PRODUCTS.

After the PCR-amplification, the double-stranded PCR samples were purified of contaminating polymerase, salts and unincorporated nucleotides and primers using the NucleoSpin Extract Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. PCR products were mixed with four volumes of NT2 buffer and loaded into a column placed inside a collecting tube. The tubes were centrifuged for 1 min at 13,000 rpm. Flowthrough was discarded and then, 600 µl of buffer NT3 added into the column and the tubes centrifuged at 13 000 rpm. The flowthrough was again discarded and the process repeated using 200 µl of buffer NT3 and centrifugation for 2 min at 13,000 rpm. Finally, the column was placed into a clean tube and 30 µl of buffer NE were added. The tubes were centrifuged for 1 min at 13000 rpm and the eluted DNA collected in the tube. The purified products were checked by electrophoresis on 1.5% agarose gel stained with ethidium bromide (0.5 µg ml^{-1}) and visualised under UV.

2.6.3 SEQUENCING REACTIONS

The sequencing reactions were carried out using the ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit. Two single-stranded sequencing reactions were carried out per sample (one for each, forward and reverse primers). The reaction mixture (10 µl) containing 0.4 µl of primer (4 µM), 4 µl of Kit Mix and 5.6 µl of PCR-product was aliquoted into 0.2 ml microcentrifuge tubes.

The sequencing reactions were subjected to PCR in a thermal cycler using the following programme: 30 cycles of 10 seconds at 96 °C, 5 seconds at 50 °C and 4 min at 60 °C. The resulting products were cleaned using Edge Gel Filtration Cartridges (Edge BioSystems) following the manufacturer's instructions. The cartridges were centrifuged at 3000 rpm for 2 min and transferred to a clean tube. The sample was placed in the middle of the gel and the tube was centrifuged at 3000 rpm for 2 min. Finally, the samples were dried by placing the tubes in a Labcoco dryer at 60 ºC for 10 min.

2.6.4 SEQUENCING

The samples were subjected to cycle sequencing according to Sanger *et al*. (1977) using an automatic sequencer Perkin-Elmer ABI-373XL and the options: Power setting 35, Collection time 14.5 and Basecaller ABI50. The polyacrylamide concentration used was 5.25%. The sequences were then processed using Lasergene software (DNASTAR, Inc.).

2.7 REGION 25 S OF NUCLEAR RIBOSOMAL DNA (nLSU-rDNA)

Primers used for PCR amplification of the nLSU-rDNA were 5.8SR (5`-TCGATGAAGAACGCAGCG-3`), and LR7 (5`-TACTACCACCAAGATCT-3`), and sequencing primers were LROR (5`-ACCCGCTGAACTTAAGC-3`), LR3R (5`-GTCTTGAAACACGGACC-3`), LR5 (5`-TCCTGAGGGAAACTTCG-3`) and LR16 (5`-TTCCACCCAAACACTCG-3`) (Hopple & Vilgalys, 1999). These primers amplify a segment comprising the following sections: 5.8S, ITS2 and 25S (partial) (Figure 2.2).

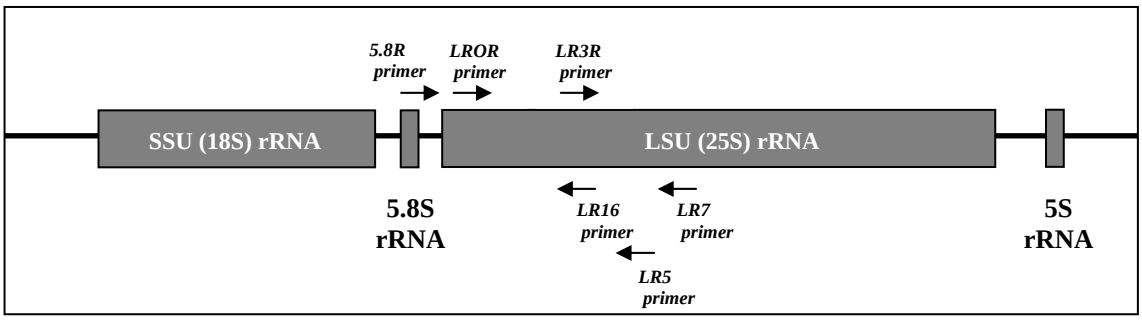

Figure 2.2 Location of the primers used to amplify a portion of the 25S gene on rDNA array.

The same procedure described for ITS analysis was used except for the primers and the PCR programme. The programme consisted of an initial cycle of 30 cycles of 94 °C for 1 min (denaturing), 50 °C for 45 seconds (annealing) and 72 °C for 1 min (extension) and a final cycle of 72 °C for 7 min.

2.8 SMALL MITOCHONDRIAL rDNA (mtSSU rDNA)

A section of the small subunit gene of the mitochondrial ribosomal RNA gene was targeted for PCR amplification using primers MS1 (5`-CAGCAGTCAAGAATATTAGTCAATG-3`) and MS2 (5`-GCGGATTATCGAATTAAATAAC-3`) (White *et al*., 1990) (Figure 2.3).

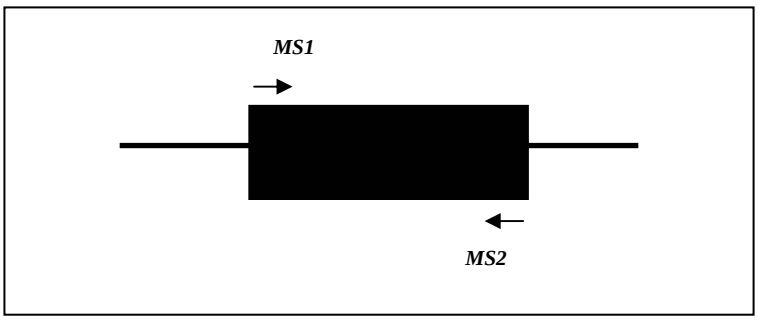

Figure 2.3 Location of the primers used to amplify the small subunit of the mitochondrial ribosomal RNA gene.

2.9 REFERENCES

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2.10 APPENDIX

Table A2.1 Geographic origin of 109 isolates of *M. roreri* collected in tropical America.

ID [1/]	Major political division [2/]	Place of collection [3/]	Altitude m.a.s.l.	Latitude	Longitude
C1	Prov. Cartago	CATIE, Cantón Turrialba (collected in 1987)	602	9º 53' N	83º 40' W
C2	Prov. Limón	La Lola Farm, Cantón Matina	40	10º 6' N	83º 25' W
C3	Prov. San José	10 km from San Isidro del General	703	9º 37' N	83º 45' W
C4	Prov. Puntarenas	La Cuesta, Cantón de Corredores	80	8º 6' N	83º 10' W
C5	Prov. Puntarenas	Km 7, La Purruja, Cantón Golfito	50	8º 38' N	83º 9' W
C6	Prov. Limón	Keköldi Indian Reservation, Cantón Talamanca	180	9º 38' N	82º 45' W
C7	Prov. Puntarenas	Buenos Aires, Cantón Osa	350	9º 24' N	83º 20' W
C8	Prov. Alajuela	Road Upala-Guatuso, Cantón Upala	70	10º 53' N	85º 0' W
C9	Prov. Alajuela	Upala, Cantón Upala	70	10º 53' N	85º 0' W
C10	Prov. Alajuela	Quebradón, Road Upala-La Cruz, Cantón Upala	70	10º 53' N	85º 0' W
C11	Prov. Limón	Earth, Cantón Pococí	250	10º 15' N	83º 40' W
C12	Prov. Cartago	Seis de Peralta, Santa Teresita, Cantón Turrialba	450	9º 53' N	83º 40' W
C13	Prov. Alajuela	Monterrey, La Fortuna, Cantón San Carlos	250	10º 29' N	84º 38' W
C14	Prov. Cartago	CATIE, Cantón Turrialba, *T. grandiflorum*	602	9º 38' N	83º 38' W
C15	Prov. Alajuela	Veracruz, Pital, Cantón San Carlos	156	10º 27' N	84º 27' W
C16	Prov. Heredia	Puerto Viejo, Cantón Sarapiquí	37	10º 30' N	84º 7' W
C17	Prov. Limón	Penshurt, Valle de la Estrella, Cantón Limón	18	9º 45' N	82º 54' W
C18	Prov. Cartago	CATIE, Cantón Turrialba. *H. nitida*	602	9º 38' N	83º 38' W
C19	Prov. Puntarenas	Punta Uvita, Distrito Bahía Ballena, Cantón Osa	10	9º 9' N	83º 55' W
C20	Prov. Cartago	CATIE, Cantón Turrialba. *T. bicolor*	602	9º 38' N	83º 38' W
C21	Prov. Cartago	CATIE, Cantón Turrialba. From APA-4 clone	602	9º 38' N	83º 38' W
C22	Prov. Cartago	CATIE, Cantón Turrialba. *T. speciosum*	602	9º 38' N	83º 38' W
C23	Prov. Cartago	CATIE, Cantón Turrialba. *H. albiflora*	602	9º 38' N	83º 38' W
C24	Prov. Cartago	CATIE, Cantón Turrialba. From SPA-11 clone	602	9º 38' N	83º 38' W
C25	Prov. Cartago	CATIE, Cantón Turrialba. *T. mammosum*	602	9º 38' N	83º 38' W
C26	Prov. Cartago	Guayabo, Cantón Turrialba.	602	9º 38' N	83º 38' W
C27	Prov. Limón	Playa Manzanillo, Cantón Talamanca	0	9º 38' N	82º 38' W
C28	Prov. Limón	Betania, Cantón Siquirres	70	10º 9' N	83º 29" W
C29	Prov. Limón	Cahuita, Cantón Talamanca	0	9º 32' N	82º 41' W

[1/] **ID** = Isolate identification indicating the country of origin as follows: Colombia (Co), Venezuela (V), Ecuador (E), Peru (P), Panama (Pa), Costa Rica (C), Nicaragua (N) and Honduras (H)

[2/] **Prov**. = Province, **Dep**. = Department, **Reg**.= Region.

[3/] **Munic.** = municipality.

Continuation Table A2.1.

ID	Major political division	Place of collection	Altitude m.a.s.l.	Latitude	Longitude
Co1	Dep.Norte Santander	Vereda Pedregales, Munic. Zulia	317	7º 44' N	72º 44' W
Co2	Dep.Norte Santander	Vereda Punta del Palo, Munic. Tibú	98	8º 37' N	72º 43' W
Co3	Dep. Santander	La Suiza CORPOICA´s Farm, Munic. Río Negro	500	7º 22' N	73º 10' W
Co4	Dep. Santander	La Suiza CORPOICA´s Farm, Munic. Río Negro	500	7º 22' N	73º 10' W
Co5	Dep. Santander	La Suiza CORPOICA´s Farm, Munic. Río Negro	500	7º 22' N	73º 10' W
Co6	Dep. Santander	Bijagual, Vereda Puente Piedra, Munic. Bucaramanga	800	7º 7' N	73º 1' W
Co7	Dep. Antíoquia	La Nacional Farm, Munic. Támesis	1190	5º 39' N	75º 43' W
Co8	Dep. Antioquia	Agricultural Institute, Leticia, Munic. San Jerónimo	703	6º 25' N	75º 42' W
Co9	Dep. Caldas	Lucker Farm, Corregimiento Arauca, Munic. Palestina	1120	5º 7' N	75º 35' W
Co10	Dep. Caldas	Vereda La Inquisición, Munic. Palestina	1520	5º 1' N	75º 36' W
Co11	Dep. Caldas	Vereda La Inquisición, Munic. Palestina	1350	5º 1' N	75º 36' W
Co12	Dep. Caldas	Vereda La Inquisición, Munic. Palestina	1200	5º 1' N	75º 36' W
Co13	Dep. Antioquia	Munic. Carepa, Urabá	132	7º 42' N	76º 39' W
Co14	Dep. Santander	Vereda Santa Inés, Munic. San Vicente de Chucurí	750	6º 52' N	73º 25' W
Co15	Dep. Santander	Vereda Barranco Amarillo, Munic. El Carmen Chucurí	820	6º 43' N	73º 29' W
Co16	Dep. Santander	Vereda Rancho Grande, Munic. El Carmen de Chucurí	460	6º 43' N	73º 29' W
Co17	Dep. Huila	Vereda El Guadual, Munic. Rivera	800	2º 51' N	75º 14' W
Co18	Dep. Caldas	Lucker Farm, Corregimiento Arauca, Munic. Palestina	1120	5º 7' N	75º 35' W
E1	Prov. Guayas	Munic. Naranjal	30	2º 41' S	79º 38' W
E2	Prov. Guayas	Recinto Rio Congo, Cantón Balzar	60	1º 7' S	79º 42' W
E3	Prov. Guayas	Recinto San Juan, Cantón El Empalme	120	1º 3' S	79º 38' W
E4	Prov. Los Rios	Recinto Cuatro Vientos, Cantón Mocache	120	1º 6' S	79º 29' W
E5	Prov. Los Rios	Recinto La Carmela, Cantón Mocache	120	1º 6' S	79º 29' W
E6	Prov. Los Rios	Recinto Agua Prieta, Cantón Mocache	120	1º 6' S	79º 29' W
E7	Prov. Los Rios	Recinto San Pedro, Cantón Mocache	120	1º 6' S	79º 29' W
E8	Prov. Los Rios	Recinto San Ignasio, Cantón Mocache	120	1º 6' S	79º 29' W
E9	Prov. Los Rios	Recinto Come Gallo, Cantón Quevedo	120	1º 6' S	79º 29' W
E10	Prov. Los Rios	Recinto Toquillal, Cantón Quevedo	120	1º 6' S	79º 29' W
E11	Prov. Los Rios	Pichilingue, Lote Caseta, Cantón Quevedo	120	1º 6' S	79º 29' W
E12	Prov. Los Rios	Km30 on the Quevedo-Babahoyo road	60	1º 14' S	79º 25' W
E13	Prov. Los Rios	Recinto Ventanilla Sur, Cantón Ventanas	60	1º 29' S	79º 25' W
E14	Prov. Los Rios	Recinto Juana de Oro	150	1º 40' S	79º 38' W
E15	Prov. Los Rios	Recinto Las Pampas, Cantón Vinces	41	1º 37' S	79º 43' W
E16	Prov. Los Rios	University of Guayaquil, Cantón Vinces	41	1º 35' S	79º 49' W
E17	Prov. Los Rios	Isla Bejucal, Cantón Baba	41	1º 46' S	79º 39' W
E18	Prov. Los Rios	Pichilingue, Lote Adentro, Cantón Quevedo	120	1º 6' S	79º 29' W
E19	Prov. Los Rios	Pichilingue, Nuevo Bambú, Cantón Quevedo	120	1º 6' S	79º 29' W
E20	Prov. Los Rios	Nestlé Farm, Recinto San Pablo	120	1º 6' S	79º 29' W
E21	Prov. Manabí	Parroquia Chone, Cantón Chone	69	0º 41' S	80º 6' W

Continuation Table A2.1.

ID	Major political division	Place of collection	Altitude m.a.s.l.	Longitude	Latitude
E22	Prov. Manabí	Parroquia Quiroga, Cantón Bolívar	200	0º 52' S	80º 5' W
E23	Prov. Manabí	Parroquia Calceta, Cantón Bolívar	180	0º 51' S	80º 10' W
E24	Prov.Esmeraldas	Recinto El Control, Cantón San Mateo	50	0º 57' N	73º 38' W
E25	Prov.Esmeraldas	Parroquia Chinca, Cantón Quinindé	95	0º 52' N	79º 33' W
E26	Prov.Esmeraldas	Recinto Viche, Cantón Quinindé	30	0º 38' N	79º 33' W
E27	Prov.Esmeraldas	Recinto El Consuelo, Cantón Quinindé	60	0º 5' N	79º 24' W
E28	Prov. Pichincha	Recinto Urbano, Cantón Santo Domingo	660	0º 18' S	79º 14' W
E29	Prov. Pichincha	Recinto Urbano, Cantón Santo Domingo	660	0º 16' S	79º 10' W
E30	Prov. Los Rios	La Variante, Cantón Buena Fe	120	0º 56' S	79º 34' W
E32	Prov. Guayas	Tenguel	15	2º 59' S	79º 44' W
E33	Prov. Guayas	Tenguel	15	2º 59' S	79º 44' W
E36	Prov. Napo	Nueva Primavera, Río Napo. Collected from pod of *T. bicolor* by H. Evans/S. Thomas in 1999 (Dis 67 f).	215	0º 25' S	76º 43' W
E37	Prov. Napo	Anangu, Rio Napo. Collected from pod of *T. cacao* by H. Evans in 1999 (Dis 122).	215	0º 30' S	76º 23' W
E38	Prov. Los Rios	Pichilingue, Cantón Quevedo. From an artificially inoculated pod of *Herrania sp*. by H. Evans in 1977 (CBS 199.77).	120	1º 6' S	79º 29' W
E39	Prov. Los Rios	Pichilingue, Cantón Quevedo. From an artificially inoculated pod of *T.cacao* by H. Evans in 1977 (CBS 201.77).	120	1º 6' S	79º 29' W
E40	Prov. Los Rios	Pichilingue, Cantón Quevedo. From an artificially inoculated seedling of *T. cacao* by H. Evans in 1977 (CBS 202.77).	120	1º 6' S	79º 29' W
E42	Prov. Carchi	El Rocio, Guadual. From a pod of *T. gileri* by H. Evans/R. Mack in 1999 (Dis 115i).	571	0º 43' N	78º 19' W
E43	Prov. Imbabura	Lita-Alto Tambo. From a pod of *T. gileri* by H. Evans/R. Mack in 1999 (Dis 116e).	571	0º 52' N	78º 19' W
H1	Dep. Gracias a Dios	Planyare, Wampusirpe, Mosquitia	10	15º 11' N	84º 36' W
N1	Reg. Atlántico Norte	Kosuly, Munic. Waslala	118	13º 14' N	85º 21' W
N2	Reg. Atlántico Norte	El Guabo, Munic. Waslala	118	13º 14' N	85º 21' W
N3	Dep.Río San Juan	Munic. San Carlos	40	11º 3' N	84º 15' W
N4	Reg. Atlántico Norte	Munic. Puerto Cabezas	28	14º 39' N	84º 45' W
N5	Dep. Matagalpa	Paiwas, Munic. Rio Blanco	118	12º 51' N	85º 12' W
N6	Dep. Matagalpa	La Ponzoña, Munic. Rio Blanco	118	12º 51' N	85º 12' W
P1	Dep. Huánuco	Tingo Maria, Province Leoncio Prado. By U.Krauss/W. Soberanis in 1997 (Dis160b)	669	9º 49' S	75º 57' W
P2	Dep. San Martín	Tocache , Province Mariscal Cáceres. By U.Krauss/W. Soberanis in 1997 (Dis160a)	669	8º 10' S	76º 31' W
P3	Dep. Huánuco	Marona, Province Leoncio Prado. By U.Krauss/W. Soberanis in 1997 (Dis160d)	666	9º 32' S	76º 2' W
P5	Dep. Huánuco	Castillo Papayal, Province Leoncio Prado. By U.Krauss/W.Soberanis in 1997 (Dis160c)	666	9º 10' S	76º 13' W

Continuation Table A2.1.

ID	Major political division	Place of collection	Altitude m.a.s.l.	Longitude	Latitude
P6	Dep. San Martín	Juanjui. Collected by U.Krauss/W. Soberanis in 1997 (Dis160e)	?	7º 10' S	76º 44' W
Pa1	Prov. Chiriquí	Paso Canoas, Panama-Costa Rica border	80	8º 6' N	83º 10' W
Pa2	Prov.Bocas Toro	Loma Brava, Almirante	10	9º 17' N	82º 25' W
Pa4	Prov.Bocas Toro	Loma Brava, Almirante	10	9º 17' N	82º 25' W
Pa5	Prov.Bocas Toro	Tibete, Almirante	10	9º 17' N	82º 25' W
Pa6	Prov. Colón	Coclecito	30	8º 49' N	80º 33' W
Pa7	Prov. Colón	Corregimiento Buena Vista	38	9º 15' N	79º 40' W
Pa8	Prov. Colón	Corregimiento Escobal	30	9º 14' N	79º 56' W
V2	State Mérida	La Rocolita, Munic. Carracciolo Parra y Olmedo	130	9º 1' N	71º 9' W
V3	State Zulia	Playa Grande, Munic. Heras	130	9º 1' N	71º 9' W
V4	State Zulia	Km 47 Santa Bárbara-El Vigía road, Munic. Colón	5	8º 37' N	71º 41' W
V5	State Táchira	Munic. Colón	105	8º 2' N	72º 15' W

Table A2.2 Collection of isolates of *M. roreri* and persons and institutions contacted.

Country	Dates	Persons and institutions contacted
Colombia	30 Oct.-8 Nov. and 29-30 November	Hernando Castro, CORPOICA, Cúcuta. Guillermo Jaimez, CORPOICA, Cúcuta. Isnardo Galvis Pinzón, Director Regional de Planeación, CORPOICA, Bucaramanga. Orlando Argüello, CORPOICA, Bucaramanga. Luis Mejía, CORPOICA, Bucaramanga. Jairo Alvarado Gomez, La Suiza Experimental Farm, CORPOICA, Rio Negro. Sergio Cadavid, Compañía Nacional de Chocolates, Medellín. Victor Merchán, Sanidad Vegetal, ICA, Manizales. Fabio Aranzazu, Corpoica, Manizales. Alberto Agudelo, Compañía Luker, Manizales. Gabriel Cubillos, Centro Nacional de Investigaciones Tulenapa, ICA, Urabá.
Costa Rica	1-3 February, 18 March, 26-28 Aug, 4 November, 9 December and 16-18 December	Marvin Hernández, CATIE. Fernando López, EARTH.
Ecuador	30 Nov.-5 Dec.	Sergio Cedeño, General Manager of Industrial y Agrícola Cañas S.A., Naranjal, Guayas. Esteban Quirola, Secadal Farm and the Machala Bank. Fernando Crespo, Association of Cacao Producers, Guayaquil. Carmen Suárez, cacao phytopathologist, INIAP, Pichilingue. Karina Solis, Pichilingue, Quevedo. Dennis Durango, Pichilingue, Quevedo. Grisnel Quijano, Pichilingue, Quevedo. Carlos Ruales, Nestlé R&D Center, Quito. Tatiana, Nestlé R&D Center, Guayaquil. Lorena Vásquez, Nestlé R&D Center, Quito. Julio Moreno Figueroa, Nestlé R&D Center, Quito. Hans Beltán, Nestlé R&D Center, Quito.
Honduras	16-20 October	Jesús Sánchez, FHIA, San Pedro Sula.
Nicaragua	20-24 September and 20-22 October	Ana Rosa Cruz and Rudolf Sollanek, Proderbo, Río Blanco. Hans Griebe, ProMundo Humano, Waslala. Xavier Escorcia, CATIE, Río San Juan. Gerardo Gutierrez, Centro Conades, Puerto Cabezas.
Panama	15-19 March and 7-11 December	Zuleyka Maynard, Smithsonian Institute. Luis Mejía, Smithsonian Institute. Enith Rojas, Smithsonian Institute. Gumercindo Añino, Coordinador Agrícola y Sanidad Vegetal, MIDA. Fermín Romero, Subdirector MIDA, Colón.
Venezuela	2-3 November	Alexander Vieto, Universidad de Los Andes, Mérida. Maria Marcano, Universidad de Los Andes, Mérida. Rosana Castro, Experimental Station Chamo, Corpozulia/FONAIAP, Zulia.

Table A2.3 Mineral liquid medium preparation for the *in vitro* growth of fungi [1/]

Liquid medium recipe

Ingredient	Amount
Dilution A	10 ml l^{-1}
Dilution B	10 ml l^{-1}
Dilution C	10 ml l^{-1}
Dilution D	1 ml l^{-1}
Glucose	30 g l^{-1}
Yeast extract	2 g l^{-1}
Peptone	2 g l^{-1}
pH = 6.0	

Preparation of the dilutions

DILUTION	INGREDIENTS
(A) Phosphate buffer	1.54 g l^{-1} KH_2PO_4
	0.26 g l^{-1} K_2HPO_4
(B) Mineral salts y microelements.	2.00 g l^{-1} NH4 NO3
	0.40 g l^{-1} Na_2SO_4
	0.15 g l^{-1} $MgSO_4$ $7H_2O$
	0.02 g l^{-1} $MnSO_4$ $4H_2O$
	0.02 g l^{-1} $CUSO_4$ $5H_2O$
	0.02 g l^{-1} $ZnSO_4$ $7H_2O$
(C) Iron dilution	0.15 g l^{-1} $Fe(NH_4)_2SO_4$
(D) Calcium dilution	1.10 g l^{-1} $CaCl_2$

[1/] **Weising *et al*. (1995).**

Table A2.4 Recipes for ISSR analysis

Rehydration buffer:

Chemical	Amount	Final Conc.	Source
Trisma acetate	13.5 g	75 mM	Sigma Cat. No. T1258
Nanopure water	1.0 l	---	---

Electrode buffer:

Chemical	Amount	Final Conc.	Source
Tris Base	24.2 g	0.2 M	Sigma Cat. No. T-8524
Tricine	35.8 g	0.2 M	Sigma Cat. No. T-0377
SDS	5.5 g	0.55 % w/v	Fisher Cat. No. S/5202/70
Adjust to 1 litter and pH 8			

Loading buffer:

Chemical	Amount	Final Conc.
Bromethanol Blue	0,01 g	0.02% w/v
Urea	15.1 g	5 M
Nanopure water	50 ml	---

Silver solution:

Chemical	Amount	Final Conc.	Source
$AgNo_3$	0.2 g	0.1% (w/v)	Sigma Cat. No. 56506
Nanopure water	200 ml	---	

Stop/de-silver solution:

Chemical	Amount	Final Conc.	Source
Glicine	5 g	2% (w/v)	Sigma Cat. No. G4392
EDTA-Na2	1.25 g	0.5% (w/v)	Fisher Cat. No. D/0650/50
Nanopure water	250 ml	---	---

Developing solution:

Chemical	Amount	Final Conc.	Source
Na_2Co_3	1.9 ml	2.5% (w/v)	Sigma Cat. No. S1641
Nanopure water	200 ml	---	

CHAPTER 3

Origin, dispersal and impact of the cacao fungus *Moniliophthora roreri* in tropical America: an historical review

3.1 INTRODUCTION

Moniliophthora roreri is the causal agent of moniliasis or frosty pod, a serious disease with the capability to infect pods of *Theobroma* spp. and *Herrania spp.* The disease has economic importance because it is particularly pathogenic against *T. cacao*, the most widely spread and commercially important plant species of the host group. The pathogen is restricted to tropical America but has progressively increased its range within the region over the past century. This is perhaps most simply reflected by successive new reports of the fungus in nine countries over this moment.

The dispersal and impact caused by *M. roreri* have been relatively well documented since the beginning of the 20th century, when the fungus caused an outbreak in Ecuador, the world's major cacao producer at that time. This outbreak was widely considered to be the first record of moniliasis in cacao and therefore established Ecuador as the probable place of origin for the disease. However, the large scale of this outbreak, coupled with a marked absence of appropriate documentation during previous decades means that there is considerable scope for uncertainty over the validity of the hypothesis.

There is a reasonable body of literature on the distribution of moniliasis since the Ecuadorian outbreak, although most of this has described incidence of the disease over very restricted geographic areas. In contrast, there is very little widely available information about the occurrence and spread of the disease during the period prior to the Ecuadorian outbreak. In order to improved the knowledge on the origin, dispersal and impact of *M. roreri* in tropical America, and to related this information with biological findings obtained in the following chapters, a bibliographical search comprising both periods (before and after the Ecuadorian outbreak) was carried out and the results summarised in this chapter.

3.2 MATERIALS AND METHODS

A bibliographical search was carried out in various libraries and academies of history (Table A3.1) in Colombia and, in a lesser level, in other countries, to collect information about the origin, dispersal and impact of the cacao fungal pathogen *M. roreri* in tropical America. Over 300 related references were collected during the period 2001 and early 2002. References were summarised and organised using the Endnote® Software. Illustrative maps were generated using the *Atlas Mundial* Microsoft® Encarta® 2001.

3.3 LITERATURE REVIEW

3.3.1 ORIGIN AND FIRST REPORTS OF THE APPEARANCE OF MONILIASIS

Various authors such as Rorer (1918), Holliday (1957, 1971), Thorold (1975) and Evans (1981, 2002) have speculated on the possible origin of *M. roreri* mainly based on the presence of infected wild host. Thus, Rorer (1918) concluded that moniliasis was a new fungal pathogen indigenous to Ecuador, because the same disease was found in the forest on pods of wild cacao species, such as *T. bicolor* and *T. baloensis* (possibly *Herrania balaensis*). This belief was widely adopted by many authors, for example Briton-Jones (1934); Erneholm (1948); Ampuero (1967); Barros (1977) and Delgado & Suárez (1993).

Holliday (1957, 1971) speculated that *T. gileri* was the original wild host of *M. roreri* in north-west Colombia because the fungus was observed on this species in a primary forest near Villa Arteaga (Department Antioquia). Correspondingly, Thorold (1975) suggested that *M. roreri* may have evolved in the forests that cover the slopes of the western Andean cordillera of Colombia; a zone rich in *Herrania* and *Theobroma* species.

Evans (1981) extended the area of origin by arguing that the presence of the fungus on wild *Herrania* and *Theobroma* species in Ecuador and Colombia indicates that *M. roreri* is indigenous to the north-western part of South America. He believed that the introduction of susceptible continuous-cropping Forastero cacao selections into these countries may have brought the pathogen into prominence, as both the Nacional trees of Ecuador and the

Criollo cacao types (native) of Colombia have been noted as possessing some resistance to the disease (Evans, 1981).

Recently, Evans (2002) concluded that it would seem probable that *T. gileri* does represent the coevolutionary host of *M. roreri*, with its endemic range extending from western Ecuador to north-west Colombia. This author also suggested that almost certainly, *M. roreri* invaded the burgeoning cacao plantations of coastal Ecuador from infected forest loci, on the lower, western slopes of the Andean cordillera, sometime during the 19th century.

Thus, Ecuador has been usually regarded as the centre of origin of the pathogen since the time of Rorer's work due largely to the very noticeable effects of the epidemic caused by the pathogen in one of the most prominent cacao producing countries. However, there are also reports suggesting that Colombia could be the place where the pathogen first originated. Bibliographical evidence supporting both hypotheses is provided below.

3.3.1.1 First reports of moniliasis in Ecuador

3.3.1.1.1 Moniliasis in Central Ecuador, Provinces Los Ríos and Guayas

The first authenticated report of the appearance of moniliasis in Ecuador was written by J.B. Rorer in 1918 (Rorer, 1918), although the disease had apparently been noticed some years earlier (Rorer, 1926a). Moniliasis became so serious in Ecuador that Rorer, a mycologist of the Agricultural Society in Trinidad and Tobago at that time, and later recognised as the pioneer of research into the disease, was invited to advise over what could be done to eradicate the problem.

During his first visit to Ecuador from late 1917 to early 1918, Rorer observed that the main focus of moniliasis was concentrated in the area of Quevedo, Province Los Ríos, about 200 km north of Guayaquil (Figure 3.1) (Rorer, 1918). He concluded that moniliasis was a new fungal pathogen indigenous to Ecuador, because of the presence of infected host in the wild.

Figure 3.1 Map of Ecuador showing places indicated in the text.

Rorer travelled down the Quevedo river and noted that the further he moved away from the humid climate of Quevedo, the fewer diseased pods he encountered. When he finally arrived at the town of Vinces, he could not find a single pod with moniliasis, although as he travelled towards the foot hill of the Andes, the disease once again became the more serious. Rorer (1918) also failed to observe the disease in the area of Machala, located in the southern Department El Oro.

According to Rorer (1918), some previous reports of pod losses in Ecuador attributed to different abiotic or biotic causes such as lack of nitrogen in the soil (Sodiro, 1892), *heladas* (i.e. sudden drop in temperature) and *Phytophthora cactorum* (Martínez, 1916) were caused by moniliasis. Since the etiology of the new disease had not been properly clarified at that time, and the symptoms were in some cases similar to those caused by *Phytophthora*, much confusion existed during this period in relation to the identity of the organism. In fact, different names were used for the same disease for example *enfermedad acuosa* (watery rot), monilia, *enfermedad de Quevedo* (Quevedo disease), *helada* (frost) and sometimes *mancha* (lesion).

Various bibliographical sources support the thesis of Rorer (1918) concerning an earlier appearance of moniliasis in Ecuador (van Hall, 1914; Martínez, 1916; Jorgensen, 1970; Garcés, 1939; Crawford, 1980). Jorgensen (1970) indicates that a private diary entry mentioned that moniliasis or some very similar fungal disease appeared in 1895 in Hacienda Maravilla (Figure 3.1) in the neighbourhood of Hacienda Clementina, Province Guayas. The document describes symptoms that match closely with those of moniliasis: "most of the pods become white while maturing on the trees, and on opening the inside is watery and the beans are rotten and useless". In 1896 the diary reports that "happily the dreaded disease of last year has disappeared". This is an abnormal behaviour for moniliasis, disease that usually becomes prevalent and is very difficult to eradicate once it has appeared in a new region (Pereira, 1996), there are circumstances associated with this case, however, that could account for the apparent disappearance of the disease. For example, the very young age of the plantation at the time of the epidemic and the peculiar environmental conditions in this area, are both unfavourable for pathogen development (Wood, 1959; Evans, 1981). Moreover, the pathogen may struggle to survive in this area during the dry months, particularly in view of the lack of both host material and favourable

conditions for infection (Evans, 1981).

Van Hall (1914) reported that high pod losses were caused by a disease in south-west Ecuador in 1909. The disease produced decay of the whole pod and was locally called *mancha*, which was one of the names used for *M. roreri* (Rorer, 1918*)*. Evans (1981) suggested the possibility that the pathogen was *C. perniciosa*, however this fungus was first observed in the same zone until 1922 (Rorer, 1926).

3.3.1.1.2 Moniliasis in northern Ecuador, Provinces Esmeraldas and Carchí

Crawford (1980) quotes other references possibly related to moniliasis including a mention in 1911, that a consular official of the United States in the Province of Esmeraldas had requested information about a "disease or insect that has been attacking cacao trees during some years". Other reference mentions the presence of an important disease attacking cacao plantations near Esmeraldas. According to Garcés (1939), a letter written on 24 May 1912 indicates that two diseases called *hielo* and *mancha* were abundant in north Ecuador between Hato Viejo (Province Carchí) and Esmeraldas (Province Esmeraldas) (Figure 3.1), causing the destruction of the cacao plantations.

3.3.1.2 First reports of moniliasis in Colombia

3.3.1.2.1 Moniliasis in western Colombia, Department Antioquia

Various authors (Holliday, 1953; Baker *et al.*, 1954; Thorold, 1975; Evans, 1981) have suggested that moniliasis first appeared in Colombia for as long ago as 1851. These references are partially based on an article quoted by Parsons (1949) but originally appeared in the local newspaper "*Los Ecos del Ruiz*" from the city of Manizales on 5 December 1880. The article mentions that large-scale plantings were made around the towns of Antioquia and Sopetrán (Figure 3.2) in the lower Cauca Valley after 1830 (see section 3.3.2.3.2 for details), only to be devastated by a fungus disease which had brought ruin to the growers (Parsons, 1949).

Figure 3.2 Map of Colombia showing places indicated in the text.

A disease called *la mancha* was first recorded at the end of 1851 in the plantations east of the Cauca River and north of Sopetrán, Department of Antioquia. It spread rapidly to all of the cacao plantings along the Cauca, causing total loss of the 1852 crop. The newspaper article described the disease as "a virulent velvety fungus growth developing to an impalpable dust..... and attacking the fruit only". This description matches moniliasis very well (Holliday, 1953; Evans, 1981). In a more recent version of his book, Parsons (1968) attributed the disease to *C. perniciosa*, although this seems unlikely since there was no other report of this pathogen in Colombia until 1929 (Mejía & Gaviria, 1937; Garcés, 1940). The case for the epidemic having been moniliasis is also supported by the presence of a susceptible wild host (*T. gileri*) in the lower Cauca Valley that may have acted as a native host from which moniliasis spread into cultivation (Holliday, 1953).

Other references mention the presence of an important disease in the same area during this period. Aguirre (1881) reported that a disease called *la mancha* invaded the cacao plantations of Anzá, western flank of the Cauca river, Department Antioquia (Figure 3.2). Much of the cacao in the Cauca Valley was cultivated near Antioquia and this disease devastated these plantations at some time during the middle of the 19th century (van Hall, 1932).

Hettner (1976) visited the Central Andean area of Colombia between 1882-1884 and reported that cacao was being imported into Antioquia from the region of Pereira and Cartago to replace the cacao lost due to *la mancha* since the 1850´s. Brew (1977) mentioned that a very pathogenic disease appeared in mid Cauca in 1851 and quoted a reference from 1863 (José María Martínez Pardo. "La enfermedad del Cacao", Diario Oficial 43. November 13, 1863) indicating that many wealthy families in Antioquia had been ruined because of this problem. Barros (1981) indicated that due to the significant effects of la mancha since 1850´s, the Assembly of Antioquia eliminated the cacao taxes in 1869 and promulgated new measurements to favour the reduction of the problem in 1877. Finally, an article in El Agricultor newspaper (Series II, 1880) mentioned that incidence of the disease was so severe that it was necessary to abandon the cacao plantations in some districts of Antioquia and to establish new plantations in the margins of the River Cauca. Sadly, the new plantations were also attacked (Carrasquilla, 1916).

In other cases described elsewhere in the region, the symptoms described as *la mancha* undoubtedly corresponded to diseases other than moniliasis. For example, Ospina quoted (1887) described a destructive disease that provoked the appearance of spots (*manchas*) in new leaves and related this problem with soil exhaustion. Other authors described diseases whose description match very well with the trunk canker (Anon, 1822; Martínez, 1879; Palau, 1889) or with the black pod disease (Preuss, 1901; Carrasquilla, 1916) caused by *Phytophthora*. Garcés (1939) suggested that some of the trunk necrosis could have been provoked by *Fusarium*.

3.3.1.2.2 Moniliasis in eastern Colombia, Departments Norte de Sandander and Santander

A group of largely overlooked references found during the bibliography search strongly suggest that an outbreak caused by *M. roreri* occurred north-eastern Colombia in the Department Norte de Santander (Figure 3.2) as early as 1817 (Anon, 1832b; Anon, 1850, Ancizar, 1956; Arenas, 1993). The earliest of these references originally published in the newspaper *El Cultivador Cundinamarqués o Periódico de la Industria Agrícola y de la Economía Doméstica* in 1832 (Anon, 1832b) and republished in the *Gazeta Oficial* of Colombia in 1850 (Anon, 1850), describes disease symptoms that correspond very well to those of moniliasis and so possibly represent the oldest known record of the disease, occurring some 100 years before from the outbreak reported by Rorer (1918) in Ecuador. A summary is presented below.

Although only scattered cacao plantations existed in Cúcuta (also called San José de Cúcuta) in 1623 (Patiño, 1963), the cacao production of the region became one of the most important cacao producing areas of the Viceroyalty of Nueva Granada during the 18th century and the first decades of the 19th century (see section 3.3.2.3.2 for details) (Anon, 1832a; Ancizar, 1956). By 1817 and particularly by 1821 this situation dramatically changed due to the appearance of two problems: *la mancha* (the spot) and *el gusano* (a pod perforator worm), both of which seriously affected the cacao trees and finally destroyed the growing activity in this area (Anon, 1832b). In the words of the latter author: "*la mancha* was recognised by the farmers because the pods acquired a *cenizoso* (ash-grey) colour and stopped growing since the first small spot appeared. No other external symptoms were

observed, but if the pods were infected when tender, the seeds appeared watery, stuck together and became lost" (Anon, 1832b). This description closely matches with the symptoms caused by moniliasis. According to the farmers, la mancha spread rapidly and could destroy the entire yield in one week. The report indicates that from the experience of some farmers in Neiva (department not specified but possibly Huila), it was recommended to externally apply fire to the cacao trunks as a good preventive of the disease (Anon, 1832b). This last comment is very relevant as it suggests that moniliasis was known even before this outbreak in at least one other place.

In 1831, the government reduced the taxes imposed to the cacao producers of Cúcuta considering the effects since 1812 of both, the War of Independence and losses caused by the pod pests. In spite of this measure, however, cacao activity was not able to survive due to continued losses caused by the pests (Arenas, 1993). Ancizar (1956) reported that by 1850, the cacao plantations in the circuit of Cúcuta had disappeared due to both the soil exhaustion and *la mancha*. *La mancha* continuously destroyed cacao yields in San José de Cúcuta and Rosario (Department Norte de Santander), causing the paralysis of the commercial activities and the impoverishment of the previously rich towns (Ancizar, 1956).

The problem subsequently also appeared in San Juan de Girón, in the current Department of Santander (Carrasquilla, 1916; Garcés, 1939; Galán, 1947; Arenas, 1993) (Figure 3.2). Daniel Martínez mentioned in a letter published in *El Agricultor* in 1882 that a fungus or *mancha* was destroying the cacao yields appeared in Zapatoca, Department Santander in 1878 (Carrasquilla, 1916; Garcés, 1939): "The disease become evident during the develop of the pod which externally acquired a dry, woody and black in colour appearance with a grey mould on it. Internally the disease arrests the development of the pod so that the beans rot." Garcés (1939) argued that this description probably refers to moniliasis since the description of symptoms closely matches those of this disease.

There is a quotation that chronologically relates the two mentioned outbreaks occurred in Colombia. Thus, Eustasio de la Torre wrote in *El Agricultor* (Series II, 1879) as follows "remembering the destruction of the cacao plantations in the Cúcuta Valley in 1834 and

later in Antioquia...” (Carrasquilla, 1916). This clearly established that a cacao outbreak occurred first in Cúcuta, Eastern Colombia and after that in Antioquia, Western Colombia.

Whilst it is not possible to confirm with absolute certainty that these reports refer to moniliasis, the consistency that the described symptoms correspond to this disease is strongly suggestive that they do. This finding has significance since the centre of origin of the disease was presumed to be Ecuador based largely on this country holding the first historic record of its occurrence.

3.3.2 DISPERSAL AND ECONOMIC IMPORTANCE OF MONILIASIS IN TROPICAL AMERICA

3.3.2.1 Current geographical distribution of moniliasis and historical dispersal

Moniliasis is restricted to tropical America where it has been successively reported in nine countries over a period nearly 200 years: Colombia in 1817 (Anon, 1832b), Ecuador in 1895 (Jorgensen, 1970), Venezuela in 1941 (Muller, 1941), Panama in 1956 (Orellana, 1956), Costa Rica in 1978 (Enríquez & Suárez, 1978), Nicaragua in 1980 (López & Enríquez, 1980), Peru in 1988 (Hernández *et al*., 1990), Honduras in 1997 (Porras & Enríquez, 1998) and Guatemala in 2002 (J. Sánchez, FHIA, Honduras, personal communication).

The talcum powder-like qualities of the spores, combined with their longevity, have made *M. roreri* a formidable invader once it escaped the relatively narrow confines of its origin; if there are no extensive geographic barriers then there is little that can be done to prevent the entry of this pathogen into new territories (Evans, 2002). Holliday (1971) stated that in view of the frequent movement of cacao material in the Americas tropics it was surprising that moniliasis is not more widespread, specially since it seems to occur over both wide altitudinal and rainfall ranges.

Until 1950´s moniliasis was geographically isolated, being confined to north-west South America, and therefore of interest only to countries of that region. However, its appearance in Panama in 1956 and Costa Rica in 1978, increased the geographic confines

of the pathogen and changed the perception of the disease (Evans, 1986).

M. roreri is still on a invasive front, with recent reports of its arrival in Honduras (1997) and Guatemala (2002), and it now threatens not only Mexico to the north but also Bolivia in the south, as well as the much more extensive and economically important plantations in the Brazilian Amazon and Bahia (Evans, 2002).

During recent years, a more active dispersal pattern of the pathogen is being observed, possibly attributable to increasing human activity in the region. Thus, in South America, *M. roreri* spread approximately 1,100 km within Peru in just 10 years. The pathogen has moved rapidly southwards along the eastern slopes of the Andes to reach northern Peru in 1989 and, by stepwise movements through the isolated cacao-growing valleys of the Huallaga, Apurimac and Ene rivers, it arrived in the Cuzco region in 1996 (Evans *et al*., 1998). If it moves across the Sierra Madre de Dios, the pathogen then poses a direct threat to the Brazilian plantations in Rondonia, as well as to Bolivian cacao (Evans, 2002). This situation has obviously increased the fears over the possibility of further dispersal into Brazil and Bolivia.

As Evans (1986) pointed out, the natural progress of the pathogen movement into the Brazilian Amazon may be slowed, since wild and planted cacao have a scattered distribution along the headwaters of the Amazon, but Indian farmers or traders could hasten the process in the quest for better cacao material or new markets. Evans *et al.* (1998) estimated that it took 5-7 years to reach north-east Peru from the Napo region of Ecuador, a distance of some 500-600 km. It is not too dissimilar a distance to the Rondonian plantations from south-east Peru, and, seemingly, it is be only a matter of time before it invades plantations in the south-east of Mexico from Guatemala (Evans, 2002).

Evans (2002) concludes that movement of *M. roreri* from its possible western Colombia forest habitats was much earlier; firstly, eastwards to Zulia State of western Venezuela, probably in the 1940s, and northwards to eastern Panama in the 1950s and Costa Rica in the 1970s. He believes that dissemination may have been by airborne spores although recognises that the pathogen was probably introduced accidentally by man into Panama.

M. roreri recently moved from the Nicaraguan-Honduran border to the Honduran-Guatemalan border (approximately 370 km) in five years, so that, it is expected that it may arrive soon to Belize and to the southern Mexican states of Tabasco and Chiapas, the most important cacao-producing areas in Mexico.

3.3.2.2 Economic importance of the disease

M. roreri causes an extremely destructive pod rot of cacao and represents one of the principal yield-limiting factors in many countries. Within its geographic range, estimates of disease incidence during an outbreak are highly variable, ranging from 10% (Katip, 1994) to 100% (i.e. Rorer 1926a, 1926b; Katip, 1994) depending on factors such as local geography, period of the year and weather conditions. Most reports mention pod losses over 30%, but can exceed 90% in some circumstances, leading to the total abandonment of cacao cultivation as have occurred in parts of Ecuador (Rorer, 1918, 1926b; Delgado & Suárez, 1993), Colombia (Barros, 1977), Venezuela (Reyes & Pérez, 1964), Costa Rica (Enriquez *et al*., 1981) and Peru (Katip, 1994).

Moniliasis is a potential threat to new cacao-growing areas in these countries as well as to the established industries in those countries in tropical America and elsewhere into which the disease has yet to spread (Evans, 1981). In the world context, the current annual loss from moniliasis is small but the potential danger presented by the disease is enormous (Gregory, 1978). Moreover, *M. roreri* ranks with any of the other major pod pathogens in terms of its economic impact during an epidemic (Evans, 1986). Moniliasis has been reported to be twice as destructive as *Phytophthora* disease (Desrosiers & Diaz 1957) and more dangerous (Orellana, 1954; Wellman, 1956) and difficult to control (Aranzazu, 2000) than witches´ broom. In Colombia, where moniliasis and witches´ broom are widely distributed, Aranzazu (2000) noted that moniliasis continues to be the most limiting of the two diseases for cacao production.

There follows a detailed description of the appearance, dispersal and economical impact of the disease on a country by country basis.

3.3.2.3 Colombia

3.3.2.3.1 General physiography of the country and main cacao regions

Colombia covers an area roughly equal in size to that of France, Spain and Portugal combined. It occupies the north-western part of South America and is the only country in this region with coasts on both the Pacific and the Caribbean. Colombia claims to have the highest number of species of plants and animals per unit area of any country in the world (Dydynski, 1995). This abundance reflects Colombia's equatorial location and its immense topographical and climatological variety.

The outstanding feature of Colombia's topography is the Andean mountain system (Figure 3.2) whose three massive ranges traverse the country for one thousand miles, and in the north spread out like three great fingers known as the Western Cordillera, Central Cordillera and Eastern Cordillera (Whitbeck, 1926). The Western Cordillera is the lowest of these ranges; it runs parallel to the Pacific coast for about 1,100 km before petering out in northern Antioquia. Westwards, the mountains give way to the hot and very wet, forested and narrow plain of the Pacific coast (Holliday, 1953). The Central Cordillera, which is considered an extension of the main Andean range, and is the highest and most volcanic (Dydynski, 1995). The Eastern Cordillera is the longest and widest massif. It spreads across the middle of the country, bordered by the vast plains of Los Llanos to the east. The range culminates in the snowy summits of the Sierra Nevada del Cocuy, after which is heads north-eastwards into Venezuela. Over 50% of the territory east of the Andes is a vast lowland which can be broadly divided into two regions; Los Llanos to the north and the Amazon to the south. Los Llanos, roughly 250,000 sq. km in area, is a huge open savannah lying in the basin of the Río Orinoco.

Two valleys, the Valle del Cauca and Valle del Magdalena, are sandwiched between the three cordilleras. Their rivers flow northwards, more or less parallel, until the Cauca River (1,350 km long) and ultimately join the Magdalena (1,538 km) which the flows into the Caribbean near Barranquilla (Dydynski, 1995).

A national census carried out by the National Federation of Cacao Producers in 1998

determined that 82,300 ha of cacao are grown in Colombia, involving 19,194 farms and 17,857 owners (Rojas, 2000). Cacao cultivation is widespread with twenty-two departments producing the crop, the most important being in decreasing order: Santander, Nariño, Norte de Santander, Huila, Arauca, Tolima, Antioquia, Caldas, Risaralda, Cundinamarca, Valle, Cauca, Cesar and Magdalena.

3.3.2.3.2 Historical development of cacao

According to Wood & Lass (1985), Colombia did not become a large producer of cacao until the 20th century. The historical role of this country as a cacao producer, however, whilst not comparable with that of Ecuador, has been possibly underestimated due to the fact that most of the production was consumed locally or exported to Europe and North America through other countries. The lofty mountain barriers, which stretch the entire length of the country, made interior communication extremely difficult which limited the exportation of the product. Plantings have been, and still are, concentrated in the interior of the country and the enormous costs of transport to the ports or to the great centres of consumption impede competitiveness on the world market.

Erneholm (1948) indicates that the first time cacao is mentioned in Colombia is in a work of León de Pinelo published in 1636, who wrote that this plant was discovered "*en los montes de Zaragoza de Antioquia*". Erneholm also indicates that cacao cultivation possibly begun in the eastern parts of Colombia on both sides of the Cordillera from where it latter spread south and west.

In a comprehensive review of the history of cacao in Colombia, Patiño (1963) quoted references of regular plantations since the 16th century with records becoming far more abundant from the 17th century. The first cacao plantings were apparently established near the city of Cartago after the Conquest Period, from where the crop spread later northwards following the River Cauca (Patiño, 1963) (Figure 3.2). During the 17th century, cacao plantations existed in several departments, notably including Cauca, Valle del Cauca, Boyacá, Magdalena, Norte de Santander, Santander, Huila, Caquetá and in the regions of Los Llanos, Orinoquia, and the flanks of the Rivers Cauca and Magdalena (Patiño, 1963; Montaña, 1986; Arenas, 1993).

Regular cacao plantations existed in the middle of the 18th century on the banks of the river Magdalena and yielded a product of high quality that demanded a higher market price than beans from Caracas and Guayaquil (Erneholm, 1948). By the end of that century, the cacao from Magdalena became the second most important exportation item of the Viceroyalty of Nueva Granada (Posada & Ibañez, 1910). By 1782, cacao plantations were so abundant in the valley of Timaná (Upper Magdalena, Department Huila) that 830,000 trees were destroyed by the earthquake of Gigante in 1827 and 104,000 by a flooding of the River Suaza one year later (Charry, 1922).

In the Department Nariño, it is possible that there existed a cacao district during the early colonial age along the west coast near the present Tumaco, which possibly formed a part of the cultivated district in Northern Ecuador at the time of the Spanish conquest (Erneholm, 1948).

Large quantities of cacao were produced in the Circuit of Cúcuta (Provinces of Pamplona and Maracaibo) from 1700´s to the first decades of 1800, with some of the farms accounting more than 30,000 trees each (Guerrero *et al*., 1998). The product was mostly exported via Maracaibo using the rivers Zulia, Catatumbo and Pamplonita, but cacao also travelled through Ocaña to the River Magdalena or into the interior of Colombia for local consumption. During this period, cacao was the base of the economy and commerce of the valleys of Cúcuta, Zulia and San Faustino and responsible for the foundation of various towns including the currently important cities of San José de Cúcuta (Cúcuta) in Colombia and San Antonio del Táchira in Venezuela (Anon, 1832b; Angel, 1990; González, 1996; Guerrero *et al*., 1998; Pabón, 2001). As mentioned earlier, at the beginning of the 19th century cacao yields in this area were devastated by persistent '*la mancha'* epidemics (probably moniliasis), causing the paralysis of the commercial activities and the impoverishment of the previously rich towns (Anon, 1832b; Ancizar, 1956).

Galán (1947) indicated that as the cacao production decreased in Cúcuta, its importance increased in the current Department of Santander, where the crop had been exploited since the Colonial Period. The cacao produced in San Juan de Girón was famous due to its high quality. It supplied the necessities of the Department during part of the 19th century until

diseases caused significant yield reductions and the abandonment of many plantations (Galán, 1947). Interestingly, this author mentioned that wild *T. cacao* and *Herrania umbratica* were present in this area at that time, specifically in the flanks of the rivers Chucurí and La Colorada.

Cacao plantation were regularly established in Antioquia at the end of 18th century as a consequence of various political and economic driving forces, the most important being the exhaustion of the mines (main activity since the Colonial period), the change of the capital of the State from Santa Fe de Antioquia (now Antioquia) to Medellín and the construction of a new road from Medellín to access the River Magdalena (Poveda, 1988; Londoño, 1995). José Antonio Mon y Velarde, Governor of Antioquia during 1785-88 stimulated the establishment of cacao plantations in Antioquia but the activity did not become important until the beginning of the 19th century. New plantations were established along the River Cauca in Santa Fe de Antioquia, Arisa, Sopetrán, Santa Bárbara, Arauca and San Jerónimo (Montaña, 1986). The region of the central valley of the River Cauca became the most important cacao area and cacao production the most important economical activity in western Antioquia (Brew, 1977). As mentioned above, the boom ended when *la mancha* destroyed yields.

By 1823, cacao was the most important commercial commodity of Colombia after salt (Mollien, 1944) and the country become self-supporting of this product. Since the beginning of the 1920´s the country has regularly imported cacao to cover domestic consumption (Erneholm, 1948). By the third decade of the 20th century, the cultivation of cacao was only conducted on a moderate scale in Colombia; but "it was of no importance from a commercial point of view, as almost all the cacao was consumed locally and very little was exported" (van Hall, 1932). At that time, cacao plantations were distributed throughout the country, especially along the Magdalena River, above Honda, in the whole Cauca Valley, in the Sinú Valley, south of Santa Marta and Ciénaga, in the departments of Bolivar, Magdalena, near Santander and in the Tolima district (van Hall, 1932).

Currently, there are 82,400 ha of cacao in Colombia with an estimated production of 40,000 Mt produced in 22 departments, the following being the most important in percentage terms: Santander 47%, Norte de Santander 10%, Huila 10%, Arauca 6%,

Antioquia 6%, Tolima 6%, Nariño 4% and Caldas 3% (Mantilla *et al*., 2000).

Originally Criollo cacao was grown in all the areas except the eastern plains, but Forastero or Trinitario types were introduced at various times since 1890 and a range of hybrids now also exist (Wood, 1959; Wood & Lass, 1985). Some Forastero pods were introduced from Trinidad and planted into Antioquia in 1901 by Carlos Pattin (Rivera, 1952). The variety was called Pajarito and spread from Antioquia to many other places in Colombia.

3.3.2.3.3 Appearance, dispersal and current geographic distribution of moniliasis

The early reports of moniliasis in Colombia (rough dates) occurred in the current departments of Norte de Santander, Santander and Antioquia (see section 3.3.1.2). New records of moniliasis in other departments appeared much later. Chardon (1929) observed the disease in the Department of Cauca in 1929, which Merchán (1981) almost certainly erroneously considered it to be the first report of moniliasis in this country. Garcés (1940), Llano-Gómez (1947) and Ciferri (1948) subsequently noted that all cacao plantations in Colombia were infected by moniliasis, in particular those plantations in the Departments of Cauca and Valle, the major cacao producing departments at that time. By 1941, all cacao plantations in the Department of Caldas were also infected as indicated by Henao (1941), however, higher levels of infection were observed in the banks of La Miel river at altitudes lower than 500 m.a.s.l. Ciferri (1948) suggested that the presence of the disease in the Department of Santander opened the possibility that moniliasis could be also present in the State of Zulia, the zones of El Amparo-Guasdualito and in the valleys of the rivers Arauca and Sarere, all within Venezuelan territory, but this possibility was not confirmed.

An Anglo-Colombian expedition carried out in Colombia during the period 1952-1953 (Baker *et al*., 1954) found the disease in all three main Colombian producing areas (the valley of the upper Magdalena, the lower Cauca Valley (Department Antioquia) and the upper Cauca Valley (Departments Valle and Cauca) (Holliday, 1953; Baker *et al*., 1954). In western Colombia, the disease was also found in the Pacific coastal plain. Interestingly, it was also observed on a wild species of *Theobroma* (*T. gileri* Cuatrecasas.) in a primary forest near Villa Arteaga (a rubber station), which is approximately half-way between San

Jerónimo and the Colombian-Panamanian border (Figure 3.2) (Holliday, 1953; Baker *et al*., 1954).

Detailed local reports contradicted the common presumption by Garcés (1940), Llano-Gómez (1947) and Ciferri (1948) of a general presence of moniliasis in all cacao plantations in Colombia before 1950. For instance, the Anglo-Colombian expedition did not find the disease in the south eastern forest areas of the Departments of Putumayo, Caquetá, Amazonas and Vaupés (Holliday, 1953; Baker *et al*., 1954) where the pathogen has not been recorded, except in the Department of Caquetá. Also, Mejía & Gaviria (1937) did not mention the disease in the region of Tumaco, Department Nariño when they made an inventory of the cacao diseases there. Unexpectedly, the Anglo-Colombian expedition did not see the disease in the Departments of Magdalena and Norte de Santander (Holliday, 1953; Baker *et al*., 1954) where the pathogen had been apparently present for a long time (section 3.3.2.2). Hardy (1957) considered that the moniliasis has spread into the Department of Huila in 1954-5, although Holliday (1953) had recorded the disease as established in this department in 1953.

Currently, moniliasis is present in all major cacao producing areas of Colombia (Mantilla *et al*., 2000), which comprises the Departments of Santander, Nariño, Norte de Santander, Huila, Arauca, Tolima, Antioquia, Caldas, Risaralda, Cundinamarca, Valle, Cauca, Cesar, Magdalena, Meta, Chocó, Quindio and Caquetá (Barros, 1970; Cubillos, 1970; Rojas, 2000). As far as the author of this thesis has been able to investigate, moniliasis has not been reported yet in the eastern Colombian Departments of Putumayo, Amazonas, Vaupes, Guaviare, Guainia, Vichada and Casanare. The Andes therefore seem to have been an effective barrier to the dispersal of moniliasis to the eastern side of the Andean cordillera (Evans, 1981). Recent reports of the fungus in at least three departments in this area, (Meta, Caquetá and Arauca) however, suggest that this substantial physical barrier has now been traversed.

In the first part of the 20th Century, the other important cacao pathogen in Colombia, *C. perniciosa*, was restricted to the eastern side of the Andes until it appeared for the first time in the region of Tumaco, Department Nariño in 1929 (Pound, 1943; Mejía & Gaviria, 1937; Garcés, 1940). From here, the pathogen dispersed slowly by colonising the

Colombian Pacific coast, the wettest zone of the country (Ciferri, 1948). Then, the pathogen was recorded sequentially in Gigante, Huila in 1963; Urabá, Antioquia 1969; Arauca 1972; Marsella, Risaralda 1973; Palmira, Valle 1976; Victoria, Caldas 1988 and Santander Department in 1989 (Aranzazu, 2000). The area with the highest incidence in Santander Department is Landázuri, where all plantings are infected (Mantilla *et al*., 2000). Other affected municipalities in Santander are El Carmen de Chucurí (81%), San Vicente de Chucurí (14%), Rionegro (2%) and Lebrija (2.5%).

3.3.2.3.4 Historical and current economic impact of moniliasis

In spite of the increasing presence of *C. perniciosa* in most cacao-producing areas of Colombia, moniliasis has been historically, and continues to be the most important and extended cacao disease in this country (Garcés, 1940; García & Naundorf, 1952; Barros, 1981; Aranzazu, 2000). Pod losses are put at 30-40% (García & Naundorf, 1952; Barros, 1981; Anon, 1986; Arguello, 1996), however, in some areas such as San Vicente del Caguán (Department of Caquetá) (Barros, 1970; 1981) and in the municipalities of Baudó, Lloró, Bojayá, Bagadó and Quibdó (Department of Chocó) (Martínez, 1970), the pathogen has caused over 80% of pods losses and led to the abandonment of many plantations.

Garcés (1940) pointed out that moniliasis increased alarmingly its presence in most cacao areas of Colombia, causing serious yield losses that could easily reach 90-100%. In relatively dry areas such as the North of the Department of Cauca and the Departments of Valle and Huila, infections are higher than 25% (Barros, 1970; 1977; 1981), but can reach 90% during years that favour the disease (Llano-Gómez 1947; Anon, 1986). In Santander, the major cacao department in Colombia, yield losses of around 40% pod mean the loss of approximately 15 000 Mt dry cacao a year (Arguello, 1996; Mantilla *et al*., 2000). At current world market value, this amounts to a shortfall of 33 millions US$ in the local economy.

Thorold (1975) mentions that it is uncertain why the economic impact of moniliasis disease was apparently less severe in Colombia than in Ecuador. He explains that the difference may have been due to the generally lower rainfall and humidity in Colombia, but it is worth noting that cacao areas were isolated and smaller, and that plantations are in

general placed at higher altitudes in Colombia than in Ecuador.

3.3.2.4 Ecuador

3.3.2.4.1 General physiography of the country and main cacao regions

The Andes Mountains, traverse Ecuador from north to south and divide the Republic into three distinct geographical regions: the Littoral or Coastal, the Sierra and the Oriente or Amazonian as seen in Figure 3.1 (Fowler & López, 1949). Cacao grows in both the Littoral and Amazonian regions. The Amazonian region was, until recently, a remote area east of the Andes and colonised during the 1970s (Evans, 1986). The Littoral region is the traditional cacao-growing area. It is west of the Andes and extends from the Pacific ocean to the western foothills of the Andes, comprising from north to south the Provinces of Esmeraldas, Manabí, Los Rios, Guayas and El Oro. The Littoral region is low, relatively flat and well drained for the most part, with the exception of a few disconnected scattered ranges of low hills near the coast (Fowler & López, 1949).

3.3.2.4.2 Historical development of cacao

There is no firm evidence that cacao was cultivated in Ecuador at the time of the Spanish conquest (Wood & Lass, 1985). However, it is said that, when Pizarro reached Bahia de Mateus in the present-day province of Esmeraldas on his first expedition along the west coast of South America 1526-27, he found cultivated areas of maize and sweet potatoes, and on lower levels flourishing cacao plantations (Erneholm, 1948). In any case, cacao cultivation only started to expand significantly in the 17th century. In 1619, cacao became an important product in Guayaquil from where it was exported to Peru and Spain (Patiño, 1963).

A first cacao boom occurred in Ecuador from the 1750´s to the 1820´s (Arosemena, 1991; Contreras, 1994) as a direct result of the Bourbon monarchs´ gradual relaxation of intra-imperial commercial restrictions (Lipscomb, 1949). There was a second boom between 1870 and 1925 in a response to a significant increase in world consumption of the product.

During most of the 19th century and the early part of the 20th century, Ecuador became established as the world's leading cacao producer, famed for its high quality Arriba cacao (cacao Nacional produced in the Province of Los Rios). Importance of the crop to the Ecuadorian economy was such that much of the agricultural landscape became a cacao monoculture (Erneholm, 1948; Greenhill, 1972; Guerrero, 1980; Crawford, 1980; Chiriboga, 1980; 1988; Rodríguez, 1985; 1992; Arosemena, 1991; Pineo, 1994; Henderson, 1997). Even with increased foreign competition dislodging Ecuador from its top position in the early years of the 20th century, the volume of cacao exports nevertheless more than doubled between 1900 and 1920 (Henderson, 1997).

The period from about 1890 to the beginning of the 1920´s is characterised by the introduction of new foreign genotypes of cacao and by a very marked expansion of cacao production (Erneholm, 1948). During this time, the country passed through a period of economic prosperity without parallel in its history. Prior to 1890 only one variety of cacao, known as "Cacao Nacional" was grown in Ecuador, except in the Province of Esmeraldas, from which the exports were very small (Rorer, 1926a). Esmeraldas cacao was finer and nobler than the others and sometimes much resembled the Venezuelan Criollo, especially when the beans were white, as sometimes happens (van Hall, 1932). The variety was tried in the other provinces of Ecuador, but without success, the trees dying from no apparent reason when seven or eight years old (van Hall, 1932).

About 1890 an Ecuadorian plantation owner returning from Europe brought to Ecuador a few cacao pods that he had collected in Trinidad (Rorer, 1926a; Evans, 1977). It was found that this variety, now know throughout the country as "Venezuela" (according to Rorer, this cacao is a typical Forastero of the Amelonado type, which is grown throughout Trinidad and the greater part of Venezuela, and is not to be confused with the very fine Venezuelan Criollo), bore abundantly, was not so particular in regard to soil as the "Nacional" types, and gave good yields on lands on which the latter variety would be an utter failure (van Hall, 1932). Cacao pods for planting were also imported from Venezuela and Colombia (Erneholm, 1948). The result was a great impetus in cacao planting, and so from 1890, hundreds of thousands of "Venezuela" trees were planted. The result of the large planting of Venezuela cacao can be easily seen in the great increase in exports during the three decades from 1891 to 1920. At the same time, however, there were many

complains from end-users (chocolate manufacturers) that the quality of Ecuador cacao was gradually deteriorating (Rorer, 1926a). Ecuadorian exports therefore declined after reaching a peak in 1914, when 47,210 tons were exported, such that by 1930, Ecuador had fallen to N°5 in the list of cacao exporters, with an annual export of no more than about 20,000 tons.

Other introductions of planting material were carried out in Ecuador in the first half of the 20th century, mostly to address the increasing problem of yield losses through fungal diseases. During the 1920´s, the United Fruit Co. made several introductions of cacao clones from Trinidad into the Hacienda Tenguel. Miguel Aspiazu similarly imported material for his states during the same period (Arosemena, 1991). The families Seminario and Aspiazu also introduced clones from Trinidad and Venezuela during the period 1945-46. In 1949, the government of Ecuador initiated a programme to renovate the cacao plantations with clonal materials by creating an operation centre in Babahoyo and propagation facilities in Naranjal, Rocafuerte, Vinces and Milagro-Yaguachi, which used vegetative materials from Pichilingue and from private producers (Arosemena, 1991).

3.3.2.4.3 Appearance, dispersal and current geographic distribution of moniliasis

Appearance of moniliasis in Ecuador was discussed in section 3.3.1.1. The first well documented outbreak occurred at the beginning of the 20th century when moniliasis invaded some of the most important cacao-producing areas of the country causing devastating loss of yield (Rorer, 1918).

Moniliasis appeared with great effect particularly in the Quevedo area, Province Los Rios about 200 km north of Guayaquil (Figure 3.1) (Jorgensen, 1970). The disease spread rapidly from Quevedo, so that, all cacao plantations in Ecuador were infected before 1924, by which time witches´ broom disease had become a new menace in the cacao producing areas after being reported in the Balao region in 1921 (Fowler & López, 1949).

By 1949, pod losses attributed to moniliasis across all growing regions were estimated at 40%, with the heaviest infections being reported from haciendas in the more humid parts

of the Arriba and Machala Regions and the lowest in the Arriba Region along the Puebloviejo and Caracol Rivers (Fowler & López, 1949). By 1954, the disease was very widely dispersed and destructive, particularly in the wetter areas (Orellana, 1954).

There were some places in Ecuador that apparently remained free of moniliasis during much of this time. For instance, Erneholm (1948) did not find moniliasis nor witches´ broom in the irrigated plantations in the Province of Manabí (Figure 3.1). Furthermore, cacao-collecting expeditions in the eastern Amazonian region of Ecuador never recorded the presence of *M. roreri* on cacao until the 1980s when the introduction of "improved" cacao hybrids via pods from the other side of the Andes was quickly followed by the subsequent arrival of *M. roreri* (Evans, 1986). The Andes had been an effective barrier to airborne dispersal of *M. roreri* to this region, although, with increasing exploitation and colonisation of Napo, the risk of chance introduction through infected pod material increased (Evans, 1981). As a result, by 1982, moniliasis had invaded all cacao regions of Ecuador (Suárez, 1982).

3.3.2.4.4 Historical and current economic impact of moniliasis

Data provided by Rorer (1926b) shows the dramatic effect of the appearance of moniliasis on a representative cacao plantation in Ecuador at the beginning of the 20th century. The plantation yielded about 33 tonnes of dry cacao in 1917 when the disease was absent, 10 tonnes in 1918 when the disease appeared, and 1.5 tonnes in 1919 once the disease had reached epidemic levels. The plantation was consequently abandoned in 1920. This pattern of decline was reflected in the exports of cacao from Ecuador with production falling from over 46,000 tons in 1917 to about 37,700 in 1918, a drop which must be ascribed principally to the ravages of moniliasis (Erneholm, 1948). Peak production in Ecuador of nearly 50,000 tons in 1915-16 declined rapidly to about 30,000 tons in 1922-3 when moniliasis became widespread, and fell further to about 20,000 tons in 1925 with the onset of witches´ broom disease (Thorold, 1975). The result of the rapid spread of moniliasis was that plantations containing several millions of Venezuelan trees were completely abandoned since 1919, while others in districts where the rainfall is light yielded enough to pay for their upkeep and in some years even to produce modest profits (van Hall, 1932).

Successive appearance of moniliasis and witches´ broom diseases together with the effects of the First World War and the unfavourable prices associated with competitive production in West Africa and Brazil, had serious consequences on the cacao production of Ecuador at the beginning of the 20th century (Crawford, 1980). The economic depression in Ecuador began with the appearance of moniliasis disease and many farmers were forced, as a last resource, to sell their cattle to solve some of their immediate financial difficulties. Thus, decline in cacao led to a corresponding reduction in cattle production over the same time period (Jorgensen, 1970).

In 1982, moniliasis was present in all cacao areas in Ecuador (Suárez, 1982). Now, the disease is widely dispersed in most cacao areas on both the Coastal and the Amazonian regions, and together with witches´ broom disease accounts for an average of 60% pod losses in Ecuador (Suárez-Capello, 1999).

3.3.2.5 Venezuela

3.3.2.5.1 General physiography of the country and main cacao regions

Venezuela is situated on the northern coast of South America, north of Brazil and between Colombia and Guyana (Figure 3.3). The Southeast is dominated by the Guyana Highlands, and the vast grassland takes up a further 30% of the country plain of the central Llanos, which is drained by the Río Orinoco. The continent's largest inland lake, Lake Maracaibo, lies in the north-west, surrounded by marshy, fertile lowlands. South of the lake rise the northern end of the Andes.

The Eastern and Barlovento regions are the main cacao-producing areas in Venezuela. The Central and Western regions produce comparatively low quantities of high quality cacao. In particular, a much sought after form of Criollo cacao called Porcelana, is only grown in the southern area of the Maracaibo lake (Portillo *et al*., 1995).

Figure 3.3 Map of Venezuela showing some places indicated in the text.

Figure 3.4 Map of Peru showing some places indicated in the text.

3.3.2.5.2 Historical development of cacao

The first reference to cacao cultivation in Venezuela dates from 1579 and refers to the presence of cacao trees in the Maracaibo basin, although the record does not indicate whether these trees had been deliberately planted (Erneholm, 1948; Patiño, 1973; Alden, 1976). After the conquest of Mexico, cacao cultivation spread to Caribbean islands and parts of South America, Venezuela being one of the first countries where the growing of cacao was started in the 16th century (Wood & Lass, 1985). It was the Maracaibo basin that first appeared as an exporter of cacao (Erneholm, 1948). This area was the first district to become a leading cacao exporter no only in Venezuela, but also in the whole continent. Cultivation subsequently spread to eastern Venezuela and then to other areas of production.

During the 18th century, Venezuelan cacaos came to be esteemed as being of superior quality to all others grown in the New World and so Venezuela became the Europe's dominant supplier during the 1790´s (Alden, 1976). By the end of the decade, however, the industry was in distress because access to Peninsular markets had been interrupted by a renewal of Anglo-Spanish maritime conflict. In 1810, the long series of bitter campaigns for independence began, and apart from the effects of rival royalist and patriot coastal blockades, the cacao industry suffered particularly because of destructive campaigns waged in cacao-producing districts and the dispersal of its slave labour force (Alden, 1976). The leading position of Venezuela was thereby ceded to Ecuador because unstable political conditions led to a great decline in production (Erneholm, 1948).

3.3.2.5.3 Appearance, dispersal and current geographic distribution of moniliasis

In 1901 Venezuelan cacao did not apparently suffer from a significant disease problem, with only one sporadic report of a disease (Preuss, 1901) whose symptoms match most closely to those of black pod disease (*Phytophthora* spp). By 1937, however, Arostegui (1937) mentioned that other diseases were attacking cacao in Venezuela but moniliasis was not listed among them. Indeed, it was not until 1941 that moniliasis was first reported in this country in the area of the Catatumbo river, State of Zulia, Western region (Figure 3.3)

(Muller, 1941). The fungus apparently remained isolated for some time in this area (Erneholm, 1948), although according to Ciferri (1948), the presence of the disease in the Department of Santander in Colombia opened the possibility that moniliasis could be also present in the State of Zulia and in the zones of El Amparo-Guasdualito and the valleys of the rivers Arauca and Sarere in the State of Apure, all within Venezuelan territory. This speculation was not corroborated. By 1949, the disease was present in some cacao plantations in the States of Zulia, Trujillo, Mérida and Táchira, all of them within the Western region (Ciferri, 1949), but it was not reported in the State of Apure.

Although moniliasis was recorded in the south of the Maracaibo Lake since 1949, observations made by the author in this area at the end of 1999 indicated that *M. roreri* was still in an invasive phase. Thus, some old plantations have been recently infected and other remained free of the disease in spite of the proximity of infected plantations (for instance the cacao plantations of the "Chamo" Experimental Station in the Municipality of Colón, State of Zulia).

Monilasis is currently restricted to western Venezuela, with the most important producing areas in the central and eastern part of the country remaining free of the pathogen. The only affected area is physically separated from the coastal region of Colombia by the narrowest part of the Andean cordillera. Evans (1981) therefore postulated that it is possible, that the present distribution of the disease is the result of unchecked airborne spore movements along the western side of the Andes.

C. perniciosa was first reported in the area of the Orinoco river and in the Peninsula and Gulf of Paria in 1937 (Figure 3.3), but Palma (1949) surmised it had probably spread into the region considerably earlier this because of the high level of damage observed. The fungus remained in the Eastern region (States of Sucre and Delta Amacuro) for a long time. Ciferri (1949) hypothesised that the mixing of geographic distributions of both pathogens, *M. roreri and C. perniciosa*, was prevented by the "diaphragm" of cacao culture under irrigation of the State of Aragua, at the Central region. It could be argued however, that the geographical barriers between the Western and Central regions and the unfavourable environment for the development of the pathogen in the Central region are possibly the main reasons that *M. roreri* has not yet invaded other regions in Venezuela.

3.3.2.5.4 Historical and current economical impact of moniliasis

In south Maracaibo Lake, the disease causes important pod losses mostly in the very desirable Porcelana cacao type, in which losses of up 90% has been estimated by Reyes & Pérez (1964) or 40-50% by González (1965). Moniliasis was considered as responsible for the loss of Criollo cacao in the States of Mérida and Táchira (Reyes & Pérez, 1964).

3.3.2.6 Peru

3.3.2.6.1 Cacao in Peru

Evans *et al*. (1998) reviewed the history and distribution of cacao in Peru. North-eastern Peru has been recognised as a centre of origin and diversity of the crop, with commercial cultivation in the tropical valleys of eastern Peru along the rivers Amazonas, Apurímac, Marañon, Huallaga, Pachitea, Ucayali and Urubamba below 800 m above sea level, distributed unevenly between Departments (Figure 3.4). There were nearly 50,000 ha of cacao in Peru in 1995 with an average yield of 593 kg ha^{-1} (Adriazola, 1998). The main producing departments were Cuzco (24.6%), Ayacucho (23%) and Junín (18.6%), although cacao plantings are present in various regions and departments in this country.

3.3.2.6.2 Appearance, dispersal and current geographic distribution of moniliasis

Many authors (Desrosiers & Díaz, 1957; Ampuero, 1967; Thorold, 1975; Gregory, 1978) have mentioned the existence of *M. roreri* in Peru. It seems likely that many of these records are based on an earlier report by McLaughlin (1950), who erroneously recorded moniliasis in Jaén (Cajamarca Department), Tingo María (Huánuco Department) and Convención (Cuzco Department). These records remained controversial until Hernández *et al*. (1988, 1990) reported the appearance of the fungus in the Department of Amazonas in 1988. This later report is regarded by Evans *et al*. (1998) as the first validated record of the disease in Peru.

In September 1988, *M. roreri* was detected in Bagua Grande, Province Utcubamba,

Department Amazonas (Figure 3.4) (Hernández *et al*., 1990; Arévalo & Hernández, 1990). One month later, it was observed in the sector of La Victoria (440 m.a.s.l.), six kilometres away from Bagua Grande in the same Department (Arévalo & Hernández, 1990). Spread of the fungus was later traced in the Provinces of Jaen and Cutervo (Cajamarca Department) and Condorcanqui (Amazonas Department) in February 1989 (Arévalo, 1989).

In March 1989, the distribution and incidence of moniliasis in Peru was as follows (Hernández *et al*., 1990): In north-east the Amazonas Department the disease was only present in Chapiza with an incidence of 73.6% , but it was not found in Chinganaza, Yutupís, San Juan, Santa Rosa, Chingamar and Seasmi. In the central area of this department, *M. roreri* was detected in Huampami 61.4%, Mamayaque 69.5%, Wawain 39.7%, Chipe 64.7%, Uut 88.8%, Duship 51.2%, Yamayakat 47.5%, Shushunga 51.8% and Wawas 64% but not in Pagki and Tutino. The fungus was found in Bagua 65.8%, Bagua Grande 65.8% and Chamaya 37.2% in northen Amazonas Department and in the near localities of Bellavista 35% and Jaén 95.4% in the Department of Cajamarca, but it was not detected in la Peca (Amazonas Dep.) and San Ignacio (Cajamarca Dep.). Thus, by 1989, the only affected Departments were Amazonas and Cajamarca and the highest concentration of infected plantations were in Jaén and Bagua.

The pathogen reached San Martín Department in the Upper Huallaga Valley in 1992 (Ríos *et al*., 1993), the Apurimac Valley in 1995 and the Ene Valley in 1996 (Arévalo & Ram, 1997) (Figure 3.4). Only the cacao-producing areas of San Alejandro in the Ucayalli Valley, Quillabamba in the Urubamba Valley and Madre de Dios remained free of disease by 1998 (Evans *et al*., 1998). In August 1998 the disease was recorded in San Iriato and Kepiarato, Urubamba Valley, Department Cuzco (Arévalo-Gardini, 1999). In 1999, the fungus was present in the Regions and/or Sub Regions of Jaén, Chachapoyas, San Martín, Huánuco y Ayacucho (Valley of the river Apurimac and Ene) (Ministerio de Agricultura de Perú, 1999). Arévalo-Gardini (1999) indicated that in 1999, nearly all (99%) cacao plantings in Peru were infected by moniliasis and that no geographical barriers existed at that moment for the dispersal of the fungus into the Department of Madre de Dios and afterwards into Brazil.

Arévalo (1989) and Hernández *et al*. (1990) suggested that the pathogen was introduced from Ecuador into Peru via San Ignacio Province (Cajamarca Department) by human-mediated transport throughout the river Marañón, and that it may also spread into Brazil via the same river. This is possible, since prior to the border conflict, Ecuadorians and Peruvians exchange products in agricultural fairs in this region. Alternatively, *M. roreri* may have entered naturally along the Rio Santiago because the disease was first reported near the border of Chapiza after several negative surveys in the area. However, it is more probable that the fungus dispersed southwards from infested areas in Amazonian Ecuador, notably in the Napo region (Evans *et al.*, 1998).

Up until the arrival of *M. roreri* in the late 1980´s, *C. perniciosa* was the major cacao pathogen in Peru (Evans *et al*., 1998). This fungus is indigenous to eastern Peru (Pound, 1943). The initial symptoms of pod infection cannot be distinguished from those caused by *M. roreri*, and there can now be little doubt that the erroneous records of frosty pod rot in northern Peru in the 1950´s and 1960´s. were, in fact *C. perniciosa* pod infection (Evans *et al*., 1998).

3.3.2.6.3 Economic impact of moniliasis

Diseases are the major constraint to cacao production in Peru, moniliasis being the paramount problem and the main focus of national germplasm improvement programmes (Evans *et al*., 1998). Until 1990, the major cacao disease in Peru was witches´ broom disease, with a complex of *Phytophthora* diseases a poor second. The situation changed rapidly and dramatically when the moniliasis appeared causing an overall fall in production of 40-50%, and even total crop loss in some areas, leading to the abandonment of farms (Evans *et al*., 1998). In affected areas, losses due to moniliasis have ranged from 10 to 100% (Katip, 1994). For example, after the arrival of moniliasis in 1991, production in the Huallaga Valley, the main cacao-producing area of the Huánuco Department, fell from 1,276 Mt to 745 Mt in 1992 and 427 Mt in 1993 (Evans *et al.*, 1998).

By 1988 it was estimated that 88% of Peruvian cacao farms were affected by the disease (Evans *et al*., 1998). The magnitude of the losses and the lack of effective control measures led to neglect and abandonment of many cacao fields (Katip, 1994; Krauss &

Soberanis, 2001). Over 50% of the national area under cacao, equivalent to 16,500 ha was abandoned mostly as a result of moniliasis, causing Peru to become a net importer of this product (Krauss & Soberanis, 2001).

3.3.2.7 Panama

3.3.2.7.1 Cacao in Panama

Commercial cacao production was largely a development of the United Fruit Company (Bergmann, 1957). The Almirante Division in the Province of Bocas del Toro was planted to bananas in 1903, and became a major banana district of this company before the destructive infestation of Panama disease (*Fusarium oxysporum*). Cacao plantations were then established in the period 1913 to 1919. Seed was selected from trees of the local Indians and settlers, and planted on abandoned banana lands (Bergmann, 1957). By 1957, the Almirante Division produced nearly all of Panama's cacao and owned all the cacao farms in Costa Rica's Sixaola District. Also on the Caribbean littoral small isolated plantings of Indian and Negro farmers were found on the mainland and island shores bordering Chiriqui Lagoon. Elsewhere in Panama, cacao was gathered locally from widely scattered groups of a few trees. An estimated 1,000 trees were found along the rivers of San Blas Province, the fruit gathered and utilised by the local Indians. In the province of Darién occasional plantings of ten to 20 trees may be vestiges of larger plantings. Cacao production is currently not significant in Panama, aside from the farms in the Province of Bocas del Toro in north-west Panama.

3.3.2.7.2 Appearance, dispersal and geographic distribution of moniliasis

Orellana (1956) recorded moniliasis for the first time in Panama in April 1956. The disease was found in Ailigandi, *Comarca* of San Blas in the eastern extreme of the Atlantic coast and in Paya, Province of Darién 15 km from the Colombian border (Figure 3.5). Wellman (1956) enlarged this list by adding the following places as invaded by the fungus: Achutuppu and Puerto Obaldía in San Blas and Tuira in Darién. He also confirmed the diagnosis made by Orellana (1956) by analysing specimens from the Province of Colón, on the Atlantic side of Panama.

Figure 3.5 Map of Central America showing some places indicated in the text.

Consequently, by 1956 the moniliasis has been described in two Departments (Darién and Colón) and in the *Comarca* (San Blas) in Panama.

Orellana (1956) suggested a recent introduction of *M. roreri* into Panama and, based on the analogies of the cacao Amelonado observed in both areas he also suggested that a possible spread of the disease into this country occurred from infected pods brought from the region of San Jerónimo-San Vicente area, Department of Antioquia, Colombia (Figure 3.5). Conversely, Holliday (1957, 1971) suggested that wild trees of *T. gileri* may be responsible for the natural spread of the disease from Colombia into Panama, which may have been present but undetected in the latter country for a long time.

In support of this theory, Baker *et al*. (1954) reported that the disease occurred on a wild species (*T. gileri* Cuatrecasas) as well on cacao itself in north-west Colombia and was found in primary forest on this wild host near Villa Arteaga (a rubber station), which is approximately half-way between San Jerónimo and the Colombian-Panamanian border. However, here is important to take in account that the mountain range of Darién constitutes a considerable barrier for the natural dispersal of the fungus from Colombia into Panamá.

A new introduction of the pathogen apparently occurred from Costa Rica into the western Province of Bocas del Toro in 1979 (Enríquez *et al*., 1981). The disease is now widely dispersed there. Moniliasis is also present in the Province of Chiriquí but is apparently restricted to the sector of Paso Canoas on both sides of the Costa Rican-Panamanian border as observed by the author of this thesis in 1999. Similarly, inspections of some of the very scarce cacao plantations along the Inter-American road in the Province of Chiriquí failed to detect the disease. By 1999, the disease appeared to be widely dispersed in the Province of Colón. Indeed, when plantations in Coclesito, Alta Divisa and Escobal were visited by the author in 1999, all were found to be severely infected by *M. roreri*.

Panama is the only country in Central America that has been invaded by *C. perniciosa*. In fact, Panama is the northern limit of the geographic distribution of this pathogen where the pathogen is restricted to the Province of Colón.

3.3.2.7.3 Economic impact of moniliasis

Pod losses due to moniliasis in Panama are very high even in distant areas such as the provinces of Darién, Colón and Bocas del Toro, where most of the cacao plantations of this country are concentrated.

3.3.2.8 Costa Rica

3.3.2.8.1 Cacao in Costa Rica

Cacao is a traditional crop in Costa Rica, and since colonial days, had constituted one of the principal farming activities and an important source of foreign exchange, as well as providing employment for the country's rural population (Patiño, 1963; Enríquez *et al*., 1981). The crop was early established by Spaniards almost three centuries ago (Bergmann, 1957). An inventory made in 1682 recorded 78,000 cacao trees planted on 55 farms in the Matina Valley. Production continued and even expanded over the following century despite major handicaps, including frequent raids by Miskito Indians, Zambos and English pirates, difficult transportation to the Central Highland, and Indian labour which was both scarce and lethargic (Bergmann, 1957).

By the beginning of the 19th century, these difficulties, heightened by inadequate government protection, sufficed to reduce the industry to less than half its former stature. Following independence from Spain, Costa Rica's cacao industry remained in a state of decay until the beginning of 20th century when the United Fruit Company began replacing much of its abandoned banana plantation owing to the uncontrollable infestation of Panama disease (Keithan, 1940). Modern planting of cacao began around 1905 and continued intermittently until 1923, after which the Company planted no more cacao until 1950. Production increased steadily until 1930 as the orchards reached maturity. However, the depressed prices of the thirties were insufficient to pay even for harvesting the crop. The orchards, which could not be cleaned, pruned or sprayed, fell into a state of semi-abandonment, and remained thus until the Second World War (Bergmann, 1957).

By 1973, there were 20,213 ha of cacao under cultivation divided into a total of 2,823

farms of various sizes, mainly concentrated in Province Limón, in the Atlantic region (Enríquez *et al*., 1981) (Figure 3.5). Since 1973, the area planted to cacao increased by approximately 750 ha yr^{-1}. However, the arrival of *M. roreri* in 1978 arrested this progress. Today, the crop is scattered cultivated in different areas mainly along the Atlantic Coast, the Northern and Southern regions and the Central Pacific area.

3.3.2.8.2 Appearance, dispersal and current geographic distribution of moniliasis

Moniliasis was reported for the first time in Cahuita, Province of Limón in 1978 (Enríquez & Suárez, 1978) (Figure 3.5), where it possible arrived few years before since farmers in the area have noticed its presence in previous harvest seasons but had attributed the damage to *Phytophthora palmivora*, the prevalent pathogen at that time in Costa Rica (Enríquez *et al*., 1979). Accordingly to Evans (1986), the arrival to Costa Rica may have been by airborne inoculum from Panama, although it is more likely to have been introduced accidentally by man. The huge distance that separate the cacao plantations in Costa Rica from the infected plantations in the Province of Colón in Panama, over 300 km away, seemingly render the first hypothesis as very improbable.

The spread of the pathogen within Costa Rica was very fast. In two years, the fungus had moved from the Atlantic to the Pacific coastlines (approximately 130 km east to westwards) and from the Panamanian to the Nicaraguan border (approximately 300 km south to northwards) (Figure 3.5). The Central Pacific region remained free of the disease during this phase. The discontinuity and distance that separate the main cacao growing regions in Costa Rica and the presence of important geographical barriers, the most important being the mountainous system that cross longitudinally the country north to south, infer that the intervention of human beings was decisive in the spread of the fungus within Costa Rica. Conversely, Evans (1986) suggested that this spread was due to the ability of the spores of *M. roreri* to be airborne transported, which could explain some of the dispersal events but not all.

Inspections made in 1979 to detect the geographic distribution of moniliasis in the Atlantic coast of Costa Rica showed that the disease was present in some 900 ha of cacao located in

a triangle formed by the drainage of La Estrella river and the communities of Cahuita y Pandora (Enríquez *et al*., 1981). One year later, the disease had spread to the whole southern part of the Limón Province and to numerous scattered plantations along the highway line, totalling some 7,150 ha of cacao affected. In June 1979, the disease was discovered attacking trees of different species (*T. mammosum, T. grandiflora, T. augustifolia* and *Herrania purpurea*) in La Lola farm in Matina (Enríquez, 1981).

Moniliasis was recorded in Golfito on the Pacific coast in 1980 (Brenes, 1980a) and in Santa Clara and San José, Canton de Upala close to the Nicaraguan border in October 1980 (Brenes, 1980b) (Figure 3.5). It seems plausible that the incidence of the disease in the region of Upala may have originated from cacao plantations in Nicaragua rather than from more distant plantations within Costa Rica, leading to the suggestion, that the disease appeared first in Nicaragua and then was dispersed into that area (Enríquez *et al*., 1981).

3.3.2.8.3 Economic impact of moniliasis

Damage to the cacao industry of Costa Rica has been substantial. Nine hundred hectares were initially affected in the Atlantic Zone in 1978, but a year later, the disease spread throughout the country, affecting 7,150 hectares (Enríquez *et al.*, 1981). Between 1978 and 1983 cacao production in Costa Rica declined by 72% and dry cacao bean exports decreased by 96%. Many farms abandoned cacao or turned to other crops, especially bananas. Since then, Costa Rican cacao production has not recovered. The disease impact has provoked that, currently, this country is not able to fulfil its own domestic market, and so has moved from a cacao exporter to a net cacao importer in only a few years.

3.3.2.9 Nicaragua

3.3.2.9.1 Cacao in Nicaragua

Cacao has been a traditional crop in Nicaragua since Indian times. Although Nicaragua never became a major international supplier of cacao (Bergmann, 1957), the Nicaraguan Criollo cacao was very appreciated during the Colony period due to its fine quality and large seed size. To give an idea of the importance of cacao during this period, the Valley

of Rivas had 400 rich cacao states in 1736 (Thienhaus, 1992). The industry reached its climax towards the end of the 18th century when problems of expensive transportation to Mexico, insect plagues, competition from cheaper though inferior cacao from Guayaquil, and finally the conditions of anarchy during the revolution against Spain, resulted in the decline of cacao as an important product in Nicaragua (Bergmann, 1957).

Now cacao is grown by smallholders in different localities of Nicaragua mostly located in the Department of Matagalpa (Figure 3.5). In the North Atlantic coast there are different ethnic groups such as Sumus and Misquitos for which cacao is an important crop for local consumption. Although 350,000 ha have been identified as potential areas, only 6,155 ha were planted with this crop in 1992 (Thienhaus, 1992).

Most cacao plantations are located lower than 400 m.a.s.l. and under precipitation regimes between 2,000 and 4,000 mm $year^{-1}$. The most important cacao producer is the Department of Matagalpa with 4515 ha (73% of the total area) distributed in the Municipalities of Waslala, Río Blanco, La Dalia, Rancho Grande and Matiguás (Ministerio de Agricultura y Ganadería de Nicaragua, 1992). Other important areas in order of importance are the Región Autónoma Atlántico Sur, the Región Autónoma Atlántico Norte and the areas of Río San Juan and Rivas/Granada.

3.3.2.9.2 Appearance, dispersal and current geographic distribution of moniliasis

M. roreri moved into southern Nicaragua (Department of Río San Juan) from Costa Rica in 1980 (López & Enríquez, 1980). Some areas in this department, such as La Esperanza and Palo de Arco, remained free of the disease in 1992. In March 1991 the pathogen was detected in the centre of the country in the locality of La Patriota, Department Matagalpa, where most of the cacao plantations are concentrated (Ministerio de Agricultura y Ganadería de Nicaragua, 1992) (Figure 3.5). By 1992, the pathogen has dispersed from this focus into other localities such as Rancho Grande and Waslala, although the Municipalities of Río Blanco and Nueva Guinea still remained free of moniliasis (Ministerio de Agricultura y Ganadería de Nicaragua, 1992). From La Patriota, the pathogen continued spreading in a north-easterly direction as that is the region where cacao

is cultivated (Porras & Enríquez, 1998). In March 1997, an expedition found the fungus in northern Nicaragua in the rivers Atapalito, Bocay and Wina which are all tributaries of the Coco river (Porras & Díaz, 1997). In April 1997 *M. roreri* was identified along the Coco River itself (Porras & Enríquez, 1998). This river serves as border between Nicaragua and Honduras. An expedition in the same area between the communities of Raiti in the upper reaches of the Coco River and Waspam near the Caribbean coast of Nicaragua was carried out in March-April 1997. It was found that the disease was present in all cacao plantations inspected (Porras & Enríquez, 1998). Apparently, the fungus had spread to Waspam over a year earlier and, according to farmers, the phenomenon had been hastened by the swelling of the rivers due to hurricane Cesar in September 1996 (Porras & Enríquez, 1998).

Moniliasis is currently affecting most cacao plantations in the Departments of Matagalpa and Río San Juan and in the Región Autónoma Atlántico Norte. Possibly, the only areas that still remain free of the pathogen are the Región Autónoma Atlántico Sur and the Department of Rivas/Granada (Figure 3.5). Dispersal of *M. roreri* in Nicaragua has been related to the fact that a high percentage of farmers use local seeds for new plantings, so that, it is frequent the movement of pods from one place to another (Ministerio de Agricultura y Ganadería de Nicaragua, 1992). Also, the presence of wild trees of *T. angustifolium* and *T. bicolor* particularly in the Departments of Rio San Juan and Matagalpa possibly has contributed with this dispersal.

3.3.2.9.3 Economic impact of moniliasis

Before the appearance of moniliasis, black pod disease (*Phytophthora palmivora*) was the most important cacao disease in Nicaragua (Thienhaus, 1992). Moniliasis has surpassed this fungus in infected areas to become the most important limiting factor for cacao production.

3.3.2.10 Honduras

3.3.2.10.1 Cacao in Honduras

As in other Central American countries, cacao is a traditional crop in Honduras. A few tons of cacao were produced in the Ulua Valley where in early colonial times it was cultivated by the Indians (Bergmann, 1957). By 2001, Honduras had become the major cacao producer in Central America with 6,000 ha distributed along the Atlantic Coast and a yearly production of 5,000 Mt dry cacao (Anon, 2001a).

3.3.2.10.2 Appearance, dispersal and current geographic distribution of moniliasis

Several expeditions were carried out along the rivers Patuca and Coco (or Segovia) in the Honduran Mosquitia (Department Gracias a Dios) between 1993 and 1996 (Porras & Cruz, 1993; 1996) but all failed to find the fungus. The pathogen was first detected in Wampusirpi, half -way up the Patuca river in the Honduran Mosquitia (Gracias a Dios Department) in March-April 1997 (Figure 3.5). In an inspection carried out in May 1997 along the Patuca river in the communities of Bilalmuk and Ahuas, and Kurpa and Tukrun, down and upstream Wampusirpi, it was determine that *M. roreri* was restricted to Wampusirpi (Porras & Enríquez, 1998). Some farmers there believed that the fungus had been spread along the Coco river to this community by travellers, as there is a direct route over the mountains that connect both sectors.

Until October 1999, *M. roreri* was restricted to a few localities in the Mosquitia (Department Gracias a Dios) including Wampusirpi and Wawina. Other places in this area such as Ahuas remained free of the disease. However, in March 2000, the fungus was detected in Guaymás, Yoró Department some 270 km from this area, and five months later in Cuyamel, Cortés Department near to the Guatemalan border (Jesús Sánchez, FHIA, personal communication). The spread of the fungus from the Mosquitia to other areas was possibly fostered by the regular movement of dry cacao from there to the other cacao areas in Honduras.

Porras & Enríquez (1998) concluded that *M. roreri* reached Honduras as a result of hurricanes and floods, and possibly through exchanges of agricultural products or dried cacao. They also concluded the proximity of the cacao plantations along the coast lead to the expectation that the fungus will continue its spread along the Caribbean coast of Central America into reach the rest of Honduras, then Guatemala, Belize and Mexico.

Currently, the fungus is present in all major cacao areas in Honduras, specifically in the Departments of Gracias a Dios, Atlántida, Yoró and Cortés (Figure 3.5) (Jesús Sánchez, FHIA, personal communication).

3.3.2.10.3 Economic impact of moniliasis

Moniliasis has had a dramatic impact on the cacao industry in Honduras. For instance in Guaymás, Yoró Department, 500 smallholders were seriously affected since the appearance of the disease in year 2000 due to the significant and progressive reduction of cacao yield that in some cases was as high as 93% of the pods (Anon, 2001b). Currently, pod losses due to moniliasis are estimated in 80% in Honduras (Jesús Sánchez, FHIA, personal communication).

3.3.2.11 Guatemala

At the time of the conquest, the Pacific coastal plain of western Guatemala and adjacent Mexico was one of the main source areas of cacao for the courts of Aztec emperors in the Valley of Mexico. This cacao was the exceptionally fine, white-bean Criollo type (Bergmann, 1957). The crop lost its importance in this country some time ago, and by 1990 only 1,500 Mt of dry cacao were produced in Guatemala.

M. roreri was detected in one cacao plantation in the Department of Izabal, Guatemala near to the border with Honduras in 2002 (J. Sánchez, FHIA, Honduras, personal communication), however, losses have not been estimated yet due to the recent and limited presence of the pathogen in this country.

3.4 CONCLUSIONS

The information presented here suggests that *M. roreri* has attacked cacao plantations in tropical America for nearly 200 years causing, invariably, dramatic effects. The first outbreaks probably occurred in Colombia and some time later in Ecuador. This finding contradicts the previously accepted dogma that the disease had originated in Ecuador, first postulated by Rorer (1918), but commonly reproduced by other authors.

Historic records indicate that *M. roreri* first appeared in north-eastern Colombia and after that in the north-western area of this country, which open the possibility of an origin of the pathogen in the former area. From Colombia, the pathogen has been successively dispersed into other countries in South and Central America. During recent years, a more active dispersal pattern is being observed, possibly attributable to the increasing efficacy of human-mediated dispersal brought about by improvements in transport infrastructure in the region.

The first reports of the appearance of the disease in Colombia and Ecuador were associated with outstanding booms in cacao cultivation, which presumably stimulated the mobilisation of infected planting material either from the wild or from other producing areas. Apparently, human activities have been the major force responsible for the dispersal of moniliasis into new areas and countries, although the relative importance of this to other dissemination agents has yet to be properly addressed.

In all countries where the disease has arrived, significant pod losses have been reported. In some cases the disease has been responsible for 100% yield loss and the total abandonment of the plantations or the change of activity. In countries such as Ecuador and Costa Rica *M. roreri* has damage of such a scale to inflict macroeconomic consequences. It can be reasonably anticipated that the spread of the disease will continue unless dramatic steps are taken to arrest its progress.

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3.6 APPENDIX

Table A3.1 List of libraries and other institutions visited in Colombia and in other countries to collect information about the origin and dispersal of *M. roreri* in tropical America

Country	Library or institution
Colombia	▪ Library of CORPOICA, Santa Fé de Bogotá. ▪ Hemeroteca Nacional, Santa Fé de Bogotá. ▪ Library Luis Angel Arango, Santa Fé de Bogotá ▪ Library Universidad Nacional, Santa Fé de Bogotá. ▪ Colombian National Library, Santa Fé de Bogotá. ▪ Faculty of History, Universidad Industrial de Santander, Bucaramanga. ▪ Academy of History of the Santander State, Bucaramanga. ▪ Library Universidad Industrial de Santander, Bucaramanga. ▪ Public Library, Cúcuta. ▪ Registro de Instrumentos Públicos (Lic. Rafael Angel Mogollo), Cúcuta. ▪ Academy of History of the State of Norte de Santander, Cúcuta. ▪ Library of the Universidad de Santander, Cúcuta. ▪ Library of the Universidad Nacional, Medellín.
Costa Rica	▪ Orton Commemorative Library, CATIE, Turrialba. ▪ Rodrigo Facio Library, University of Costa Rica, San José.
England	▪ Library of the University of Reading. ▪ Library of Cadbury Schweppes, Bournville, Birmingham. ▪ Library of CABI, Egham.
Mexico	▪ Centro de Estudios Avanzados, Universidad de Chiapas ▪ Library Municipal, Chiapas State.
United States	▪ Library of the International University of Miami, Miami.

CHAPTER 4

Origin, dispersal and molecular variation of the cacao (*Theobroma cacao* L.) pathogen *Moniliophthora roreri* (Cif.) Evans *et al*. in tropical America determined through AFLP, ISSR and ITS analyses.

4.1 ABSTRACT

AFLP, ISSR and ITS sequence analyses were performed to determine the origin, biogeography and molecular variation of *M. roreri* in tropical America. Ninety-six isolates representing the global range of the fungus were included in the study. AFLP and ISSR produced congruent trees (Mantel test, 0.92 of correlation, $P < 0.001$) both of which indicated the highest levels of genetic diversity occur in the Middle Magdalena area of Colombia. The declining pattern of variation outside this region is also suggestive that this region also represents the centre of origin for the fungus.

Five genetic groups of *M. roreri* were identified. Two groups have a wide geographic range: the Bolívar group extends from the Colombian department of North of Santander, through Ecuador, Venezuela and Peru in the south; the second (Co-West group) encompasses western Colombia, central Ecuador and four countries in Central America (Panama, Costa Rica, Nicaragua and Honduras). The other groups are all apparently endemic to Colombia (Co-Eastern group restricted to the east and Co-Central group to the central regions) or north-western Ecuador (Gileri group). Considered collectively, there are four genetic groups in Colombia, three in Ecuador and only one in Venezuela, Peru and throughout Central America. The analysis of ITS regions provided much less information on the variability of the fungus but this technique was useful to define two subspecific groups (Eastern and Western) and to reinforce some of the AFLP/ISSR findings.

Keywords: AFLP, Colombia, frosty pod disease, genetic diversity, ITS, ISSR, moniliasis, *Moniliophthora roreri*.

4.2 INTRODUCTION

Fungi characteristically possess a greater genetic diversity than other groups of microscopic pathogens. This variation can affect all aspects of the biology of these organisms and allows them to evolve new pathogenic types quickly in response to enhanced resistance in crops brought about advances in crop breeding (Carlile & Watkinson, 1994; Talbot, 1998).

Although levels of variability can be estimated using morphological characters, this source of information has not proved reliable for identification and discrimination of many fungi due to the difficulty detecting sufficient numbers of characters to enable a comprehensive resolution. Moreover, many of the morphological characters that are used for identification purposes can be strongly influenced by the environment (Weising *et al*., 1995).

Application of a range of DNA-based techniques has provided tools for the discrimination of closely related fungi at almost any required resolution and also for the detection of fungal species at sensitivities, in some cases, far beyond those of traditional methods (Mills *et al.*, 1998; Tamakatsu, 1998). The need for such genetic analysis is to develop tools that can discriminate between biological entities with different genetically-determined characters (Wang & Szmidt, 1998). A series of techniques and genetic markers are now available to measure genetic affinities of taxa although no single technique is universally viewed as ideal; each exhibits its own strengths and weaknesses. Therefore, the choice of technique is often a compromise that depends on the research question posed and the genetic resolution needed, as well as on financial constraints and the technical expertise available (Mueller & Wolfenbarger, 1999).

The Amplified Fragment Length Polymorphism analysis or AFLP (Vos *et al.*, 1995) is a powerful DNA analysis with the capability for the simultaneous screening of many different DNA regions distributed randomly throughout the genome (Mueller & Wolfenbarger, 1999).

AFLP fingerprinting represents a relatively cheap and effective way to investigate several aspects of fungal molecular biology and genetic diversity (Majer *et al.*, 1996). The technique has proven to be a reliable and powerful analysis in a variety of applications including fingerprinting and identification of fungal isolates (Mueller *et al.*, 1996), studies of intraspecific genetic variation (Majer *et al.*, 1996) and estimation of phylogenetic relationships (Tredway *et al.*, 1999).

Inter Simple Sequence Repeat analysis or ISSR (Zietkiewics *et al.*, 1994) is a quick, simple and reproducible PCR-based technique (Albani & Wilkinson, 1998; Gilbert *et al.*, 1999). The profiles generated are highly informative and generate sufficient polymorphisms to have potential for large-scale DNA fingerprinting purposes (Prevost & Wilkinson, 1999). The ISSR protocol is nearly identical to that used for the notoriously less robust Randomly Amplified Polymorphic DNA analysis (RAPD analysis) but crucially differs in the design of the primers used. ISSR primers target neighbouring and inverted SSRs and are considerably longer (18-21 bases) than the random oligomers used for RAPD analysis (typically 10 bases). Increased length of ISSR primers allows for far more stringent annealing temperatures to be applied during the PCR and this improves reproducibility (Vogel & Scolnik, 1998; Wolfe & Liston, 1998).

Several studies have demonstrated the utility of ISSR in a wide range of applications and plant families including cacao (Charters & Wilkinson, 2000), although the technique has rarely been applied to fungal groups or to study the basis of plant resistance to infection. Hantula *et al*. (1996) first suggested the use of this technique (which they termed Randomly Amplified Microsatellite Regions, RAMS) after applying it to characterise genetic variation among a diverse range of distantly related fungi comprising *Armillaria cepistipes*, *Gremmeniella abietina*, *Heterobasidion annosum*, *Phytophthora cactorum*, *Phlebiopsis gigantea* and *Stereum sanguinolentum*. Raman *et al*. (1999) used ISSR with AFLP data to perform bulked segregant analysis of resistant and susceptible barley plants to locate a resistance gene against the fungus *Rhynchosporium secalis*.

The internal transcribed spacer (ITS) consists of two non-coding variable regions that are located within the rDNA repeats between the highly conserved small subunit, the 5.8S subunit and the large subunit rRNA genes. ITS1 and ITS2 lack a functional role, which is

thought to explain the high level of sequence variation within them. ITS is now perhaps the most widely sequenced region of DNA in the fungal genome and has been widely exploited to infer phylogenetic affinities of related groups. For example, Lee & Taylor (1992) studied the genetic relatedness of various *Phytophthora* species that attack cacao by using sequence from both ITS1 and ITS2.

The cacao tree *(Theobroma cacao)* is susceptible to various fungal pathogens, many of which are still poorly studied. The variability of these organisms is considerable but not yet satisfactorily explored (Zadocks, 1997). Molecular diversity studies have been carried out for cacao fungal pathogens such as *Phytophthora* spp (Förster *et al*., 1990; Lee & Taylor, 1992; Nyassé *et al*., 1999) and *Crinipellis perniciosa* (Andebrhan & Furtek, 1994 a, b; Griffith *et al*., 1994; Andebrhan *et al*., 1999). As yet, however, there have been no molecular studies for the commercially important fungus, *Moniliophthora roreri* (moniliasis or frosty pod disease). Indeed, estimates of genetic diversity and relatedness have thus far been based entirely on the study of morpho-physiological characters on a limited number of isolates (Herrera, 1988; Ram, 1989). Other aspects relating to the origin and dispersal mechanisms of this pathogen remain obscure in spite of the dramatic economic impact that *M. roreri* has had on the economies of many tropical American countries for nearly 200 years (Anon, 1832; Rorer, 1918; Enríquez *et al*., 1981; Evans, 1981) and the permanent threats that its further dispersal could provoke in other countries in America and other continents.

The objectives of the present research are to determine the level of molecular diversity of the cacao pathogen *M. roreri* in its entire geographic range, and to use this information to make inferences about the possible origin and biogeography of the pathogen. The study was carried out using three different molecular techniques: AFLP, ISSR and ITS.

4.3 MATERIALS AND METHODS

4.3.1 LOCATION OF THE EXPERIMENT

In vitro growth of the fungal isolates and DNA extractions were carried out in the Biotechnology and Phytopathology laboratories of the Tropical Agricultural Research and Higher Educational Centre (CATIE), Turrialba, Costa Rica from November to December 1999, and in the laboratories of CABI, Egham, UK from January to February 2000. The molecular studies of the isolates were performed in the Laboratories of the School of Plant Sciences, University of Reading from March to December 2000 and from April to May 2001.

4.3.2 EXPERIMENTAL MATERIAL

A set of 96 isolates of *M. roreri* was used in the molecular studies. The author collected most of these in 1999. The isolates and details of their geographic origin are described in Chapter 2 (Table A2.1). The following isolates included in this list were not used in the molecular studies: C4, C8, C10, Co3, Co18, E19, E33, E39, N2, N6, P6, Pa2 and Pa4.

All countries and most of the major areas affected by the disease are represented in the study (Figure 4.1). Sixteen isolates were obtained in Colombia, 36 in Ecuador, four in Venezuela, four in Peru, five in Panama, 26 in Costa Rica, four in Nicaragua and one in Honduras.

A high percentage of the isolates were obtained in Ecuador, because this country has been widely regarded as the centre of origin of the pathogen (Evans, 1981; Rorer, 1918). A considerable representation of isolates from Colombia and Costa Rica were also analysed to allow comparisons between the genetic diversity of the fungus in Ecuador and that encountered in presumed sites of secondary spread with a good historical account of the outbreak of the disease (See Chapter 3).

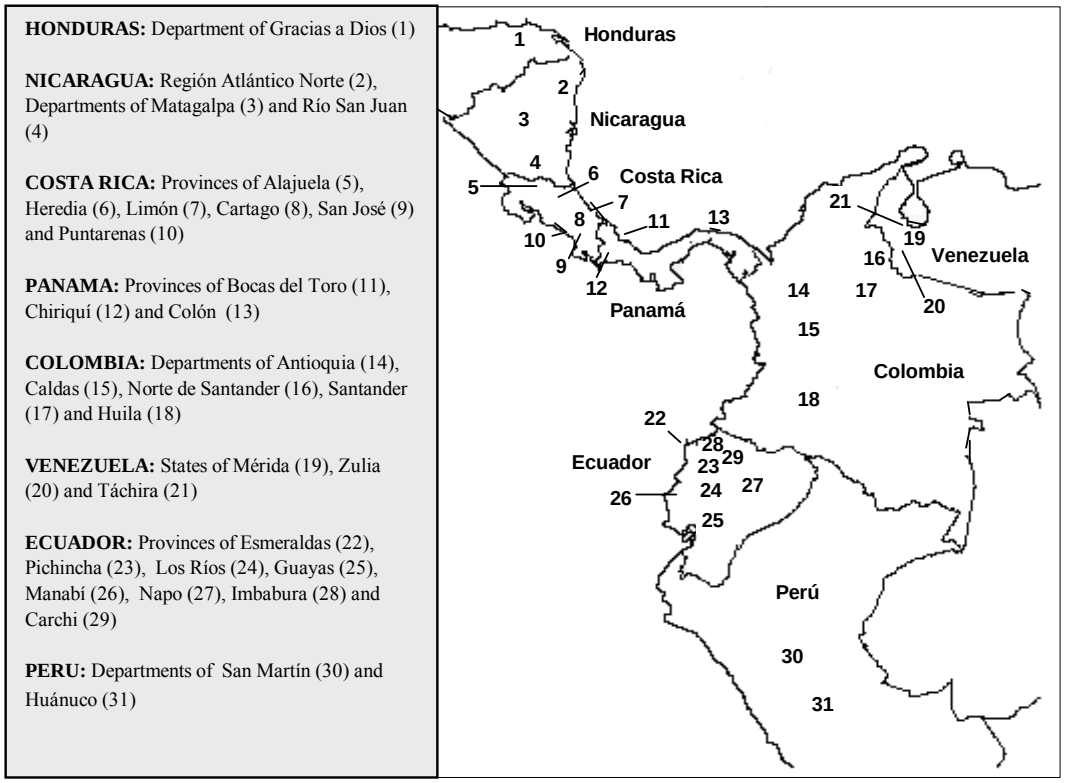

Figure 4.1 Countries and major areas of origin (indicated by numbers on the map) of 96 isolates used in the molecular survey of *M. roreri.*

4.3.3 METHODOLOGY

4.3.3.1 Extraction of the total genomic DNA

Genomic DNA was extracted from dry mycelium using a DNeasy Plant Mini Kit (Qiagen, UK) according to the manufacturer's instructions as described in Chapter 2. Mycelium of *M. roreri* was obtained by growing the isolates in mineral liquid medium (Weising *et al*., 1995) for eight days. DNA was quantified by reference to known standards on 1% (w/v) agarose gels and also using a DyNA Quant fluorimeter (Höefer) following the manufacturer's protocol.

Three different molecular techniques were performed to assess the genetic diversity of *M. roreri* in tropical America. Ninety-four isolates were analysed using AFLP, 96 using ISSR and 95 using ITS. General procedures for each technique are described as follows and detailed protocols are described in Chapter 2.

4.3.3.2 Analysis of Amplified Fragment Length Polymorphism (AFLP)

Ninety-four isolates were analysed using AFLP which comprise all isolates enlisted in Table A2.1 except those mentioned in section 4.3.2 and the Peruvian isolates P3 and P5. The samples were processed using the GIBCO-BRL/AFLP Analysis System II kit (Life Technologies, UK) as detailed in Chapter 2. Both the reaction volumes and the amount of $|\gamma^{33}P|$-ATP used were reduced to 50% of that recommended to maximise the utilisation of the kit. Twenty primer combinations were screened for the production of robust, polymorphic profiles (Table 4.1) using three-*M. roreri* isolates (Co-2, E-6 and N4), which represented extreme points in the geographical distribution of the pathogen.

Table 4.1 Selective primer combinations used for AFLP analysis

M-CAA x E-AA [1/]	M-CAC x E-AA
M-CAA x E-AT	M-CAG x E-AA
M-CAA x E-AG	M-CAG x E-AT
M-CAA x E-TA	M-CAG x E-TA
M-CAA x E-TC	M-CAG x E-TC
M-CAA x E-TG	M-CAG x E-TG
M-CAA x E-TT	M-CAG x E-TT
M-CAC x E-AG	M-CAC x E-TC
M-CAC x E-AT	M-CAC x E-TG
M-CAC x E-TA	M-CAC x E-TT

[1/] **M** = primer complimentary to *Mse*1 adapter sequence; **E** = Primer complimentary to *Eco*R1 adapter sequence; **A, C, G and T** = Selective nucleotides added to 3′ end of primers.

Digestion of genomic DNA was performed at 37 °C for 3 h using the restriction enzymes *Mse*1 and *Eco*R1 followed immediately by ligation of double-stranded adapters to the ends of the restriction fragments at 20 °C for 2 h. PCR preamplifications were then carried out in microcentrifuge tubes containing 2.5 µl of 1:10 ligation dilution, 20 µl pre-amp primer Mix II, 2.5 µl 10x PCR buffer (Qiagen, UK), 0.1 µl *Taq* DNA Polymerase (5 units/µl,

Qiagen, UK) and 0.5 µl of 0.2 mM each dATP, dCTP, dGTP and dTTP (Bioline, UK). Pre amplification PCR comprised 20 cycles at: 94 °C for 30 sec (denaturing), 56 °C for 1 min (annealing) and 72 °C for 1 min (extension) was used.

PCR amplifications were performed for each of the selected primer combinations. The primer was labelled by phosphorylating the 5′ end of the *Eco*R1 primers with [γ-^{33}P]-ATP. PCR reactions were applied to microcentrifuge tubes containing 5 µl Preamplification product, 2 µl 10x PCR buffer (Qiagen, UK), 0.1 µl *Taq* Polymerase (5 units/µl, Qiagen, UK), 7.9 µl nanopure water and 5 µl Primer Mix). The PCR products were separated on denaturing 7% polyacrilamide gel. After electrophoresis, the gel was transferred onto a rigid support, covered with cellophane and dried. The gel was exposed to X-rays films (Kodak X-OMAT AR) to visualise the AFLP fingerprints. Film was developed and the restriction fragments scored by visual examination and recorded as present or absent.

4.3.3.3 Analysis of Inter Simple Sequence Repeat (ISSR)

ISSR analyses were conducted for 96-*M. roreri* isolates enlisted in Table A2.1 except those mentioned in section 4.3.2. The methods reported by Charters *et al*. (1996) were used with minor modifications as described in Chapter 2. PCR amplifications were performed in a final volume of 20 µl (1x Taq polymerase buffer (Bioline, UK), 2 mM $MgCl_2$, 0.375 µM primer, 0.2 mM DNTPs (Bioline, UK), 0.05 U/µl Taq polymerase (Bioline, UK) and 1 ng/µl of total genomic DNA).

The following seven primers from Set #9 of the University of British Columbia were used: #823 (5′-TCTCTCTCTCTCTCTCC-3′), #874 (5′-CCCTCCCTCCCTCCCT-3′), #880 (5′-GGAGACGACAGGAGA-3′), #885 (5′-BHBGAGAGAGAGAGAGA-3′), #890 (5′-VHVGTGTGTGTGTGTGT-3′), #891 (5′-HVHTGTGTGTGTGTGTG-3′) and #816 (5′-CACACACACACACACAT-3′), where B = C, G or T; H = A, C or T and V = A, C or G. These were selected on the basis of reliable amplification following a pre-screen of 20 primers against a representative group of four fungal isolates (Co-16, E-6, Co-17 and N3) when fractionated on agarose and visualised by ethidium bromide staining.

All ISSR PCR reactions were performed in a Techne thermal cycler. The programme consisted of 35 cycles of 1 min at 94 °C (denaturing), 2 min at 55 °C (annealing) and 30 sec at 72 °C (extension) and a final cycle of 5 min at 72 °C. The PCR products were separated in 1.5% agarose gel stained with ethidium bromide (0.5 µg ml^{-1}) at 100 V for 30 min and visualised under UV. Failed reactions were repeated prior to fractionation of all samples by low temperature polyacrilamide gels electrophoresis.

The PCR products were loaded on a precast 5/10% polyacrilamide gel (Cleangels 48s, Amersham Pharmacia Biotech) and then run on a flatbed (Multitemp II and Multiphor II, Amersham) for approximately 3 h. Gels were silver stained and dried at room temperature in the dark overnight. Restriction fragments were scored by visual examination and recorded as present or absent.

4.3.3.4 Analysis of the Internal Transcribed Spacers (ITS)

A segment of the ribosomal RNA genes in nuclear DNA comprising the sections 18S (partial), ITS1, 5.8S, ITS2, 25S (partial) was amplified using the primers ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) and ITS5 (5`-GGAAGTAAAAGTCGTAACAAGG-3`) (White *et al*., 1990). Ninety-five *M. roreri* isolates were analysed for this segment which comprise all isolates enlisted in Table A2.1 except those mentioned in section 4.3.2 and isolate E-40.

PCR reaction mixtures (25 µl) consisted of 1x Taq polymerase buffer (Bioline, UK), 1.5 mM $MgCl_2$, 0.5 µM each primer, 0.2 mM DNTPs (Bioline, UK), 0.02 U/µl Taq polymerase (Bioline, UK) and 0.4 ng/µl of total genomic DNA. Thermal cycling conditions involved an initial cycle at 94 °C for 1 min (denaturing); 30 cycles of 94 °C for 1 min (denaturing), 52 °C for 30 sec (annealing) and 72 °C for 1 min (extension) and a final cycle at 72 °C for 7 min. Samples of the PCR products were checked for amplification by separation by electrophoresis on 2% agarose gels in 1x TAE buffer stained with ethidium bromide (0.5 µg ml^{-1}) and visualised under ultraviolet light. Size of the fragment was determined by comparison with 100 bp ladder (Gibco-BRL). Successful PCR amplification resulted in a single band of approximately 760 bp.

The PCR products were purified using the NucleoSpin Extract Kit (Macherey-Nagel, Germany) according to the manufacture's instructions. The sequencing reactions were carried out using the ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction. Two single-stranded sequencing reactions were carried out per sample (one for forward and one for reverse reaction). The reaction mixture was prepared with 0.4 µl of primer (4 µM), 4 µl of Kit Mix and 5.6 µl of PCR-product. The reactions were run in a thermal cycler using the following programme: 30 cycles of 10 sec at 96 °C, 5 sec at 50 °C and 4 min at 60 °C. The resulting samples were cleaned using Edge Gel Filtration Cartridges (Edge BioSystems) and dried by placing the tubes in a Labcoco dryer at 60 ºC for 10 min. The samples were subjected to cycle sequencing according to Sanger *et al.* (1977) using an automatic sequencer Perkin-Elmer ABI-373XL and the following options: Power setting 35, Collection time 14.5 and Basecaller ABI50. The polyacrilamide concentration used was 5.25%. The sequences were processed using the Lasergene software (DNAStar Inc.).

4.3.4 DATA ANALYSIS

Patterns of AFLP and ISSR bands were analysed both separately and jointly as a combined data set using the MVSP software (Multi-Variate Statistical Package) version 3-12d (Kovach Computing: Aglesea). The 94 isolates in common were included in the jointly analysis. Similarity matrices were compiled using Jaccard coefficient and subjected to the Unweighted Pair-Group Method, Arithmetic Mean (UPGMA) to generate dendograms. In order to estimate of the reliability of the various groupings in the dendogram, bootstrap values (Felsenstein, 1985) were determined using WinBoot software (Yap & Nelson, 1996) based on 1000 replications.

Cluster analyses were also separately performed for the isolates from Colombia, Ecuador and Central America using Jaccard coefficient and UPGMA. Bootstrap values were determined based on 1000 replications. Shannon's Diversity Index (Shannon & Weaver, 1949) and the percentage of polymorphic loci were calculated for each group identified using Popgene software (Yeh *et al*., 1997).

The software InfoGen Versión 1.0 (Grupo InfoStat: Universidad Nacional de Córdoba, Argentina) was employed to carry out a Principal Coordinates Analysis (PCO) using Jaccard distances and the 181 AFLP/ISSR bands. A Minimum Spanning Tree (MST) was obtained with the same software and superimposed on the PCO ordinations. The MST is the minimum-distance tree that connects all vertices in a graph, vertices are thus connected to one another by the smallest possible distance between them.

The Mantel test (Mantel, 1967) was used to test for correlation between the dissimilarity matrices generated using either the AFLP or the ISSR band profiles obtained for 94 isolates. The software Genetix (Laboratoire Génome et Populations, Université de Montpellier II, France) and 1000 permutations were used for calculations. The same procedure was performed to compare the dissimilarity matrix obtained through the combined analysis of the AFLP and ISSR band profiles and the dissimilarity matrix generated using the geographic distances between the places of collection of the isolates. This comparison was done using either the set of 94 isolates or a subset of them comprising only the 16 Colombian isolates and two Ecuadorian isolates (E42 and E43).

The ITS sequences were processed using the Lasergene System Software (DNAStar Inc.). Thus, sequences were assembled and checked for inaccurate base calling using SeqMan II. Consensus sequences were aligned manually using MegAlign and regions of ambiguous alignment and incomplete data at the beginning and end of sequences were excluded. Indels were determined and the isolates compared accordingly.

4.4 RESULTS

4.4.1 DNA EXTRACTION

DNeasy Plant Mini Kit produced a good DNA quality whose concentration varied from 9 ng/µl (E-2 isolate) to 290 ng/µl (Co-13 isolate). There was no relationship between DNA yield and origin of the isolates.

4.4.2 AFLP ANALYSIS

The following four combinations of primers were selected for analysing the complete set of isolates, since they showed the highest number of polymorphic bands (9-10 bands each) when were challenged against three representative genotypes of *M. roreri*: "M-CAA x EAT", "M-CAA x ETG", "M-CAG x ETG" and "M-CAG x EAT".

One hundred and sixty-three bands with an average of 40.8 bands per combination were obtained after evaluating the set of 94 isolates using these combinations (Table 4.2). Sixty-five percent of the bands (106 bands) were polymorphic and 20.2% (33 bands) were deemed as 'rare alleles' (present or absent only in one or two genotypes). Rare alleles were present only in the following isolates: E42 and E43 with 10 rare alleles, Pa8 with seven, C19 with 6, Co14 with 3, Co6 with 2 and Co4 with 2 and the following isolates with only one: Co5, Co12, Co13, Co15, Co16, C16, V2, V3 (Table 4.2).

Table 4.2 Summary statistics of bands obtained for each AFLP primer combination when applied to 94 isolates of *M. roreri.*

		PRIMER COMBINATION				Total	Avg
		MCAG x EAT	MCAA x ETG	MCAA x EAT	MCAG x ETG		
Total bands		37	41	44	41	**163**	**40.8**
Polymorphic bands [1/]		26 (70.3%)	26 (63.4%)	25 (56.8%)	29 (70.7%)	**106 (65%)**	**26.5**
Rare alleles [2/]		7 (18.9%)	10 (24.4%)	6 (13.6%)	10 (29.3%)	**33 (20.2%)**	**8.2**
Number of rare alleles	**Allele absent**	1 in C16 1 in E42/E43	1 in Co14/Co5	1 in E42/E43 1 in V2/V4	1 in Co14/Co6		
	Allele present	2 in C19 1 in Co13 1 in E42/E43 1 in Pa8	4 in Pa8 1 in E42/E43 1 in C19 1 in Co12 1 in Co4 1 in Co4/Co6	2 in E42/E43 1 in C19 1 in Co14	4 in E42/E43 2 in C19 2 in Pa8 1 in Co15/Co16		

[1/] Number and percentage of polymorphic bands in relation to the total bands.

[2/] Number and percentage of rare alleles (band present or absent only in one or two genotypes) in relation to the total bands.

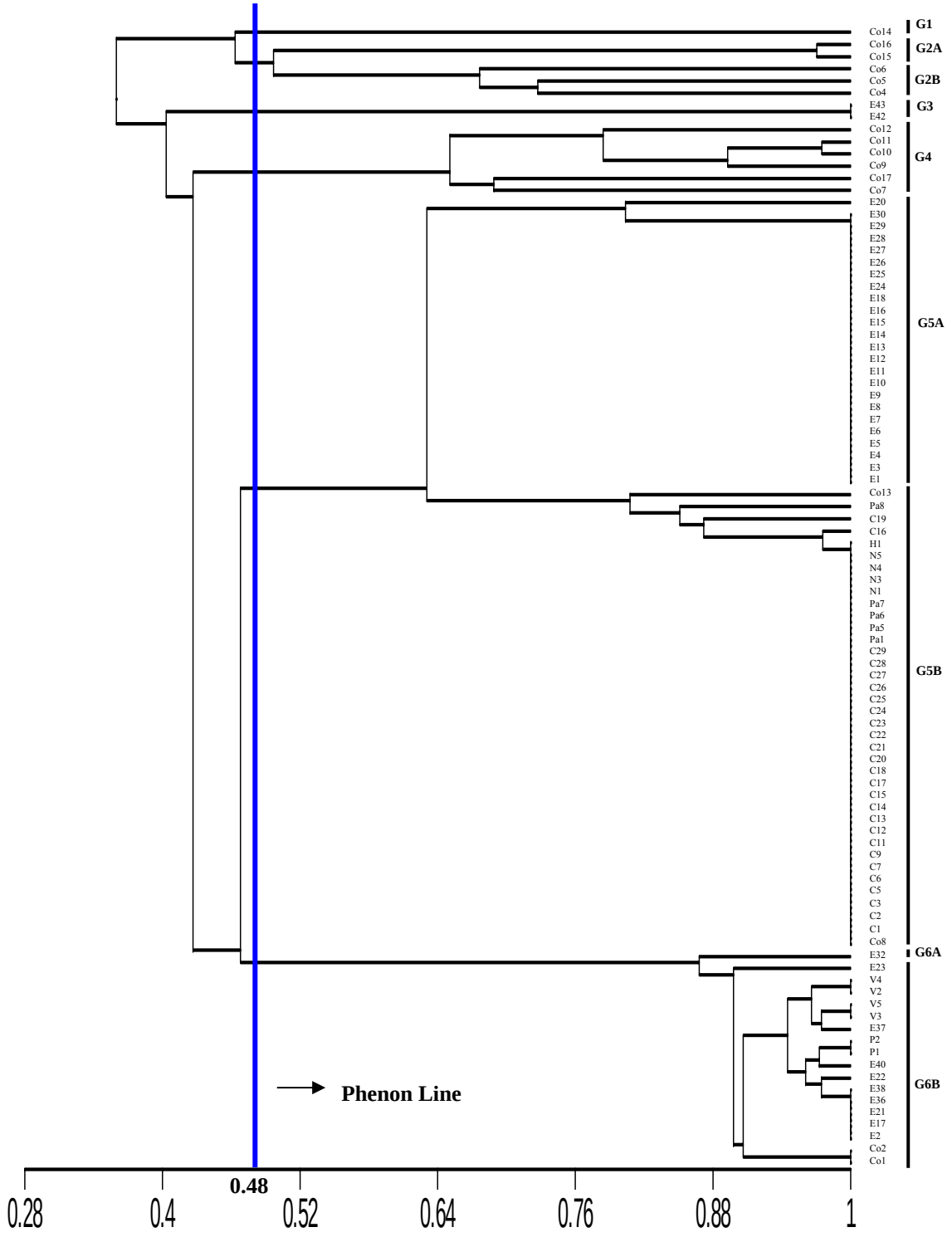

Figure 4.2 UPGMA dendogram comparing 94 isolates of *M. roreri* on the basis of banding profiles generated using primers AFLP.

UPGMA cluster analysis using Jaccard`s coefficient revealed several distinct clusters. These were resolved into groups using two approaches. First, imposition of a phenon line to the dendrogram at the 0.48 similarity level resulted in the designation of 6 groups (denoted G1 – G6) (Figure 4.2).

A second system for allocation of groupings was based on identification of the largest clusters with >95% bootstrap support. When this approach was used, six groups were identified (denoted I – VI), with two assemblages comprising two isolates and the remaining five groups comprising three or more genotypes (Figure 4.3).

4.4.2.1 Description of groupings identified by the phenon line

Group 1 (G1) only containing isolate Co14 from San Vicente de Chucurí with a similarity coefficient of 0.46 with respect to the most closely related (Figure 4.2).

Group 2 (G2) which includes isolates Co15 and Co16 from El Carmen de Chucurí (**G2A**) and isolates Co6, Co5, Co4 from Río Negro (**G2B)**.

Group 3 (G3) embraces two isolates (E42, E43) collected from *T. gileri* in north-western Ecuador, which show a similarity coefficient of 0.41 in relation with the next group.

Group 4 (G4) comprises isolates from the Departments of Antioquia, Caldas and Huila in Central Colombia (Co12, Co11, Co10, Co9, Co17, Co7) with a similarity coefficient of 0.43 and significant levels of differentiation between them.

Group 5 (G5) is the largest assemblage and includes two subgroups with a similarity coefficient of 0,63. **G5A** comprises isolates mostly from Central Ecuador with E-20 being the isolate that registered the highest differentiation in this subgroup. Interestingly, 44% of the polymorphisms observed in E20 (10 bands) were not detected in the remaining isolates of G5A, but they were present in all isolates belonging to Group 6. **G5B** comprises all Central American isolates and two Western Colombian materials from Antioquia (Co13 from Urabá and Co-8 from San Jerónimo).

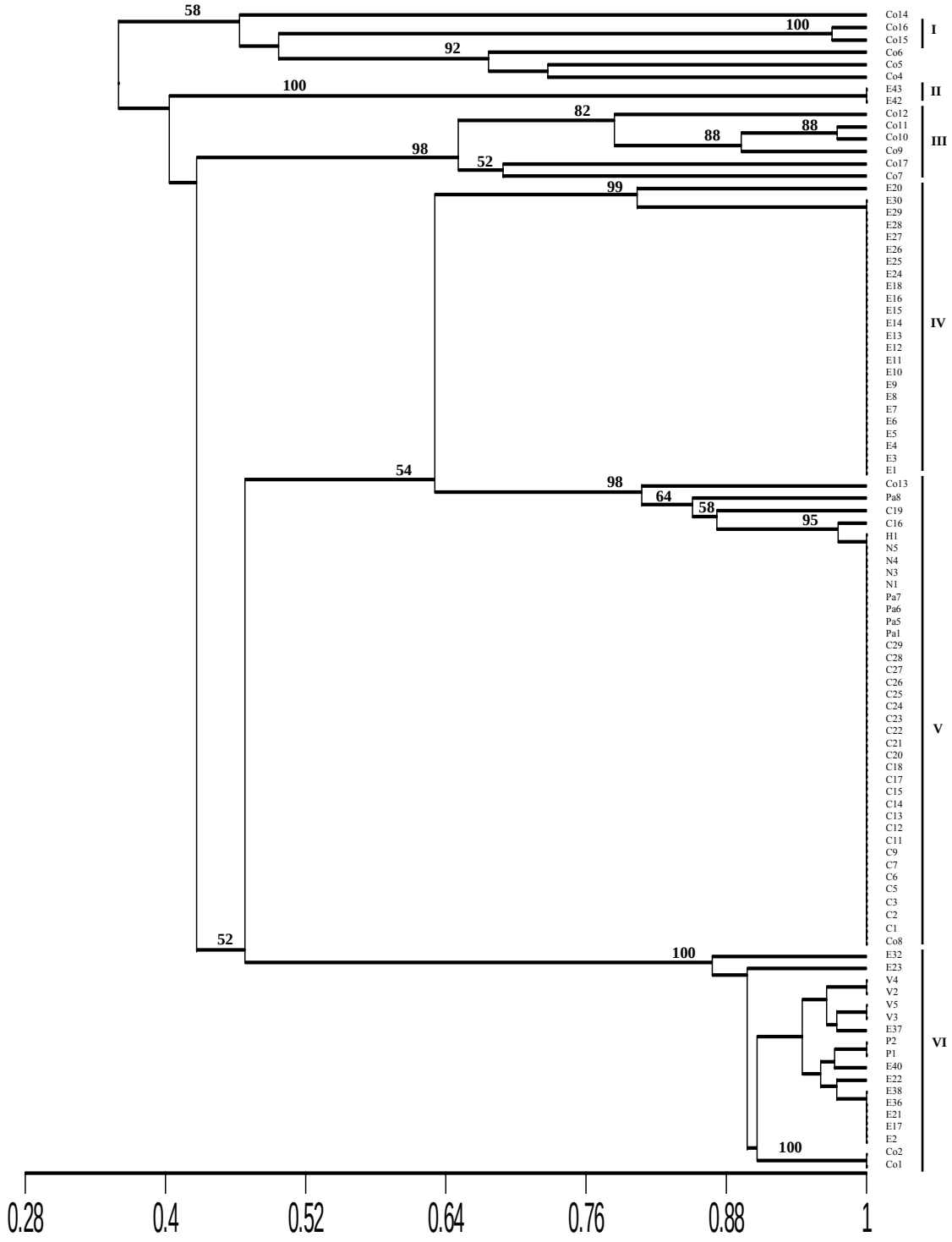

Figure 4.3 UPGMA dendogram comparing 94 isolates of *M. roreri* on the basis of banding profiles generated using primers AFLP. Bootstrap values above 50% (based on 1000 bootstrap re-samples) are given above branches.

Group 6B includes isolates from the Colombian Department of Norte de Santander (Co1, Co2), all Peruvian (P1, P2) and Venezuelan (V2, V3, V4, V5) isolates and 9 genotypes (E23, E37, E40, E22, E38, E36, E21, E17, E2) from different areas in Ecuador mostly located in the periphery of this country such as Manabí and Napo.

4.4.2.2 Description of groupings supported by bootstrap values above 95%

Group I comprises isolates Co16 and Co15 from El Carmen de Chucurí (Figure 4.3).

Group II includes the two isolates from *T. gileri* collected in Ecuador (E42 and E43).

Group III contains isolates from Central Colombia: Co12, Co11, Co10 and Co9 from the Department of Caldas and Co7 and Co17 from the departments of Antioquia and Huila, respectively.

Group IV comprises isolates from Central Ecuador including E20.

Group V is composed by two isolates from western Antioquia in Colombia and all Central American isolates. This group contains a subgroup with a high bootstrap support which includes the same isolates as Group V except for isolates Co13, Pa8 and C19.

Group VI includes genotypes from the Department Norte de Santander in Colombia, all the Peruvian and Venezuelan isolates, and isolates from peripheric areas of Ecuador including E32 from Tenguel. A subgroup comprising isolates Co2 and Co1 from the Norte de Santander Department in Colombia showed a high bootstrap support.

4.4.3 ISSR ANALYSIS

Several bands from every profile were not sufficiently distinct to allow the total number of bands to be determined each primer. Only very clear and distinct bands were included in the analyses. In this way, only seventy five polymorphic bands were scored overall from the seven primers used, with an average of 10.7 bands being produced per primer (Table 4.3). Twenty-four of these bands (32%) were present or absent in only one or two isolates

and so deemed as rare alleles. These rare alleles were most frequently observed in E42/E43 with 13 and Co5/Co6 with 3. The following isolates yielded only one rare allele: Co14, Co14/Co5, Co15, Co15/Co16, E32 and Pa8.

Table 4.3 Summary statistics of bands obtained for each ISSR primer when applied to 96 isolates of *M. roreri*.

Primers	UBC-823	UBC-874	UBC-880	UBC-885	UBC-890	UBC-891	UBC-816	TOTAL	AVG
Polymorphic bands	5	20	5	15	12	9	9	**75**	**10.7**
Number of rare alleles	3	8	1	1	3	5	3	**24**	**3.4**
% Rare alleles [1/]	60.0	40.0	20.0	6.7	25.0	55.6	33.3	**32%**	

[1/] Rare alleles = band present or absent only in one or two genotypes.

UPGMA cluster analysis generated using Jaccard`s coefficient yielded clear evidence clustering between isolates. Two strategies were again adopted for the designation of groupings from the dendrogram. First, a phenon line drawn at 0.29 generated six separate groups of isolates (G1 – G6). Second, five groups (I – V) were defined on the basis of the largest assemblage of isolates supported by bootstrap values >95%. This approach defined five groups of isolates.

4.4.3.1 Description of groupings defined by phenon line

Group 1 (G1) comprises only isolate Co14 from San Vicente de Chucurí with a similarity coefficient of 0.23 (Figure 4.4).

Group 2 (G2) consists of four isolates from the Department of Santander in Eastern Colombia with a similarity coefficient of 0.27. In this group, isolates Co15 and Co16 from El Carmen de Chucurí (**G2A**) present a significant separation in relation to isolates Co5 and Co6 from Río Negro (**G2B**).

Figure 4.4 UPGMA dendogram comparing 96 isolates of *M. roreri* on the basis of banding profiles generated using primers ISSR.

Group 3 (G3) contains the isolates from the Departments of Antioquia, Caldas and Huila in Central Colombia (Co17, Co12, Co11, Co10, Co9, Co7) and the isolate Co4 from the Department of Santander. This group shows a similarity coefficient of 0.51.

Group 4 (G4) is composed of two *T. gileri* isolates (E42, E43) from north-western Ecuador which show a similarity coefficient of 0.17 with respect to the remaining isolates.

Group 5 (G5) includes three subgroups of isolates with a similarity coefficient of 0.53: **G5A** which only comprises isolate Co13 from Urabá, Colombia; **G5B** consists of 24 isolates from Central Ecuador and **G5C** is composed of all Central American isolates and one Colombian isolate from San Jerónimo, Antioquia (Co-8). E20 showed the highest differentiation within G5B. Interestingly, one of the 21 polymorphisms recorded in this genotype was otherwise exclusively observed in Group 6.

Group 6 (G6) has a similarity coefficient of 0.24 and is composed by two subgroups: **G6A** comprising only isolate E32 from Tenguel, Ecuador. This isolate showed the highest level of differentiation within this group. Again, it is noteworthy that six of the 20 polymorphisms recorded in this genotype were typically observed in Group 5B. **G6B** embraces the two isolates from the Colombian Department of Norte de Santander (Co1, Co2), all Peruvian (P5, P3, P2, P1) and Venezuelan (V5, V4, V3, V2) isolates and 9 genotypes (E17, E37, E40, E38, E36, E23, E22, E21, E2) from different Ecuadorian areas mostly located in the periphery of the country including the Departments of Manabí and Napo.

4.4.3.2 Description of groupings supported by bootstrap values above 95%

Group I comprises isolates Co16 and Co15 from El Carmen de Chucurí in the Colombian Department of Santander (Figure 4.5).

Group II is composed of two *T. gileri* isolates (E42, E43) from Ecuador.

Group III contains isolates from Central Ecuador including E20.

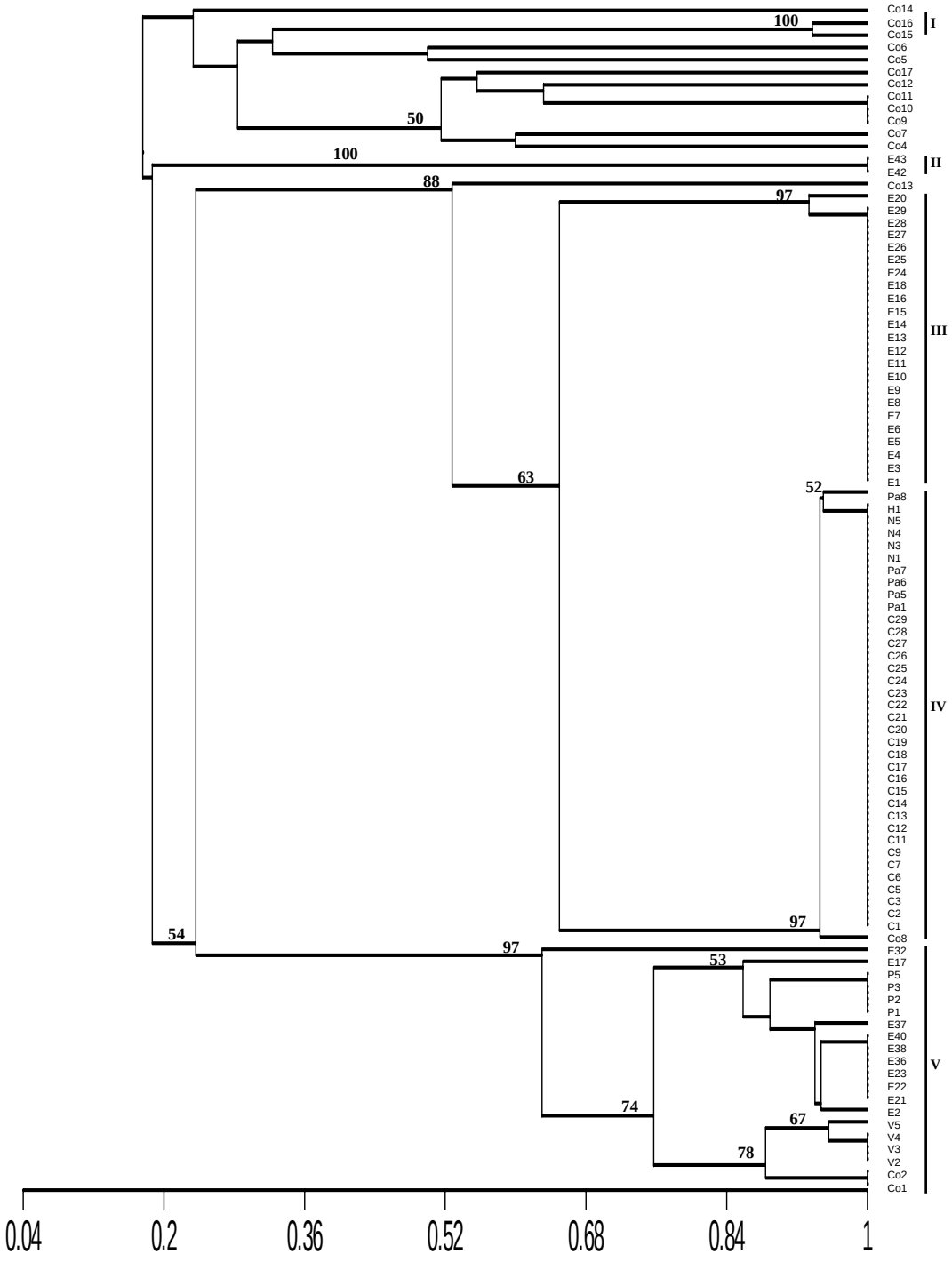

Figure 4.5 UPGMA dendogram comparing 96 isolates of *M. roreri* on the basis of banding profiles generated using primers ISSR. Bootstrap values above 50% (based on 1000 bootstrap re-samples) are given above branches.

Group IV is composed by one isolate (Co8) from western Antioquia in Colombia and all Central American isolates.

Group V includes genotypes from the Department Norte de Santander in Colombia, some peripheric areas of Ecuador and all Peruvian and Venezuelan isolates. Isolates Co1 and Co2 from the Norte de Santander Department in Colombia, which belong to this group, showed a high bootstrap support.

4.4.4 COMBINED ISSR AND AFLP DATA SETS

4.4.4.1 Comparison between AFLP and ISSR dendograms based on the phenon line.

Dendograms obtained using AFLP and ISSR band profiles compiled with Jaccard coefficient and subjected to UPGMA appear very similar (Figures 4.2 and 4.4). Both dendograms show basically the same clusters, although the following differences can be highlighted:

- Isolates from central Colombia (Departments of Antioquia, Caldas and Huila) and those from the Department of Santander grouped in two separate major clusters using AFLP analysis, but in the same major cluster when the ISSR band profiles were considered.

- Using AFLP, Co13 from Urabá, Colombia grouped in the subgroup including Co8 and the Central American isolates, but it formed an independent subgroup for ISSR.

4.4.4.2 Comparison between AFLP and ISSR dendograms based on bootstrap values.

When the groups were defined based on bootstrap values, the five groups detected using ISSR were also defined using AFLP (Figures 4.3 and 4.5). These groups comprise the following isolates: Co16/Co15; E42/E43; the Central Ecuadorian isolates; the Western Antioquia/Central American genotypes, and finally, a combination of isolates from the Department of Norte de Santander in Colombia, some peripheric areas of Ecuador and all

Peruvian and Venezuelan isolates. The only difference observed here between AFLP and ISSR was the exclusion of Co13 in the Western Antioquia/Central America group in ISSR.

One group was additionally supported by AFLP (bootstrap > 95%): the Central Colombian isolates (Co12, Co11, Co10, Co9, Co17, Co7).

4.4.4.3 Comparison between AFLP and ISSR dissimilarity matrices through the Mantel test

When the Mantel test was used to test for correlations between the dissimilarity matrices obtained with AFLP and ISSR analysis, a Pearson correlation coefficient of r =0.92 with a probability < 0.001 of independence between the matrices was found.

4.4.4.4 Combined analysis of AFLP and ISSR band profiles

Given the close similarity of dendrograms yielded by AFLP and the ISSR band profiles as determined by the Mantel test, a subsequent analysis was carried out using both sets of data (181 band profiles) across 94 isolates.

UPGMA cluster analysis generated using Jaccard`s coefficient yielded clear evidence of clustering between isolates. Two strategies were again adopted for the designation of groupings from the dendrogram. First, a phenon line drawn at 0.39 generated five separate groups of isolates (G1 –G5). Second, five groups (I – V) were defined on the basis of the largest assemblage of isolates supported by bootstrap values >95%.

4.4.4.4.1 Description of groupings defined by phenon line

Two major clusters are distinguished in the dendogram, which show a coefficient of similarity of 0.31 (Figure 4.6). The first main cluster shows the highest levels of genetic diversity and includes all Colombian isolates except Co8 and Co13 from Western Colombia and Co1 and Co2 from the Department of Norte de Santander. Two groups can be identified within this cluster:

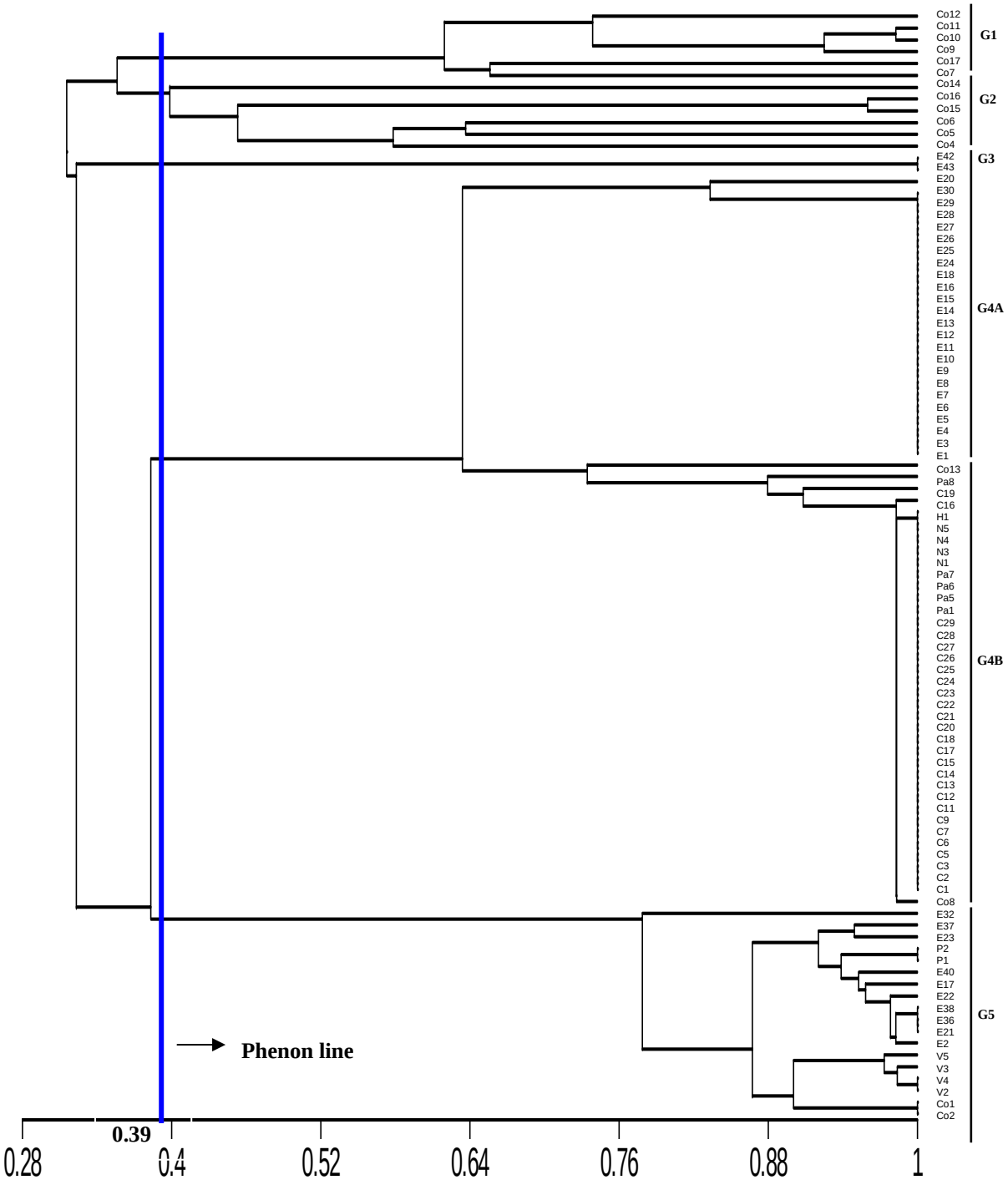

Figure 4.6 UPGMA dendogram jointly generated from AFLP and ISSR band profiles of 94 isolates of *M. roreri*.

Group 1 (Co-Central): This group includes isolates from three different departments in Central Colombia: all isolates from Department of Caldas (Co12, Co11, Co10, Co9); the only isolate from the Department of Huila (Co17) and one isolate from the municipality of Támesis in the Department of Antioquia (Co7).

Group 2 (Co-East): Consists of all isolates from the Department of Santander in Eastern Colombia: Co14 from San Vicente de Chucurí; isolates Co15 and Co16 from El Carmen de Chucurí and isolates Co6, Co5 and Co4 from Río Negro or near to this Municipality.

The second main cluster embraces isolates from all countries under study. However, three groups of isolates can be distinguished based on the molecular results:

Group 3 (Gileri): It is composed only of two *T. gileri* isolates (E42, E43) from north-western Ecuador.

Group 4 (Co-West): It includes two subgroups: **Group 4 A** consists of 24 isolates from Central Ecuador with E20 showing the lowest similarity coefficient. **Group 4 B** is composed of all Central American isolates and two Western Colombian materials from west Antioquia (Co13 from Urabá and Co-8 from San Jerónimo), Co13 being the isolate that registered the highest dissimilarity in this subgroup. Very little variation was observed within both the Central Ecuadorian and Central American isolates. In Central America, the most distinctive isolates were the Panamanian isolate Pa8 from Colón and C19 from the Pacific Coast of Costa Rica.

Group 5 (Bolívar): This group embraces the two isolates from Norte de Santander Department in Colombia (Co1, Co2), all Peruvian and Venezuelan isolates and 10 isolates mostly collected in the periphery of Ecuador: E32 from Tenguel in southern Ecuador which showed the highest differentiation in relation to the remaining isolates; isolates E21, E22, E23 from the Province of Manabí; isolates E36 and E37 from the Province of Napo; isolates E38 and E40 from Pichilingue (Province of Los Ríos) and finally isolates E17 and E2 from the localities of Baba and Río Congo, in the Provinces of Los Ríos and Guayas, respectively.

In the previous paragraphs, the name assigned by the author to each group it is indicated within parentheses. The names arise from the geographic origin of the isolates or from the host where the respective isolates were collected (Gileri). The name Bolívar was assigned to the group comprising isolates from Venezuela, Colombia, Ecuador and Peru as reference to Simon Bolívar who led the independence processes in these South American countries.

4.4.4.4.2 Description of groupings supported by bootstrap values above 95%

Group I contains isolates from Central Colombia: Co12, Co11, Co10, Co9 from the Department of Caldas and Co7 and Co17 from the Departments of Antioquia and Huila, respectively (Figure 4.7). A subgroup comprising all genotypes from the Department of Caldas (Co11, Co10 and Co9) except Co12 was supported by a bootstrap value of 100%.

Group II includes isolates Co 16 and Co15 from El Carmen de Chucurí in the Colombian Department of Santander.

Group III is composed of two *T. gileri* isolates (E42, E43) from Ecuador.

Group IV comprises isolates from Central Ecuador, two isolates from western Antioquia (Co13 and Co8) and all Central American isolates. Two subgroups are supported by high bootstrap values: one embracing isolates from Central Ecuador and the other comprising the isolates from western Antioquia and Central America. Also, high bootstrap support was obtained for the subgroups containing the Western Antioquia and Central American isolates except Co13.

Group V includes genotypes from the Department Norte de Santander in Colombia, some peripheric areas of Ecuador and all Peruvian and Venezuelan isolates. A subgroup including the two isolates from the Colombian Department of Norte de Santander (Co1 and Co2) was supported by a 100% bootstrap value.

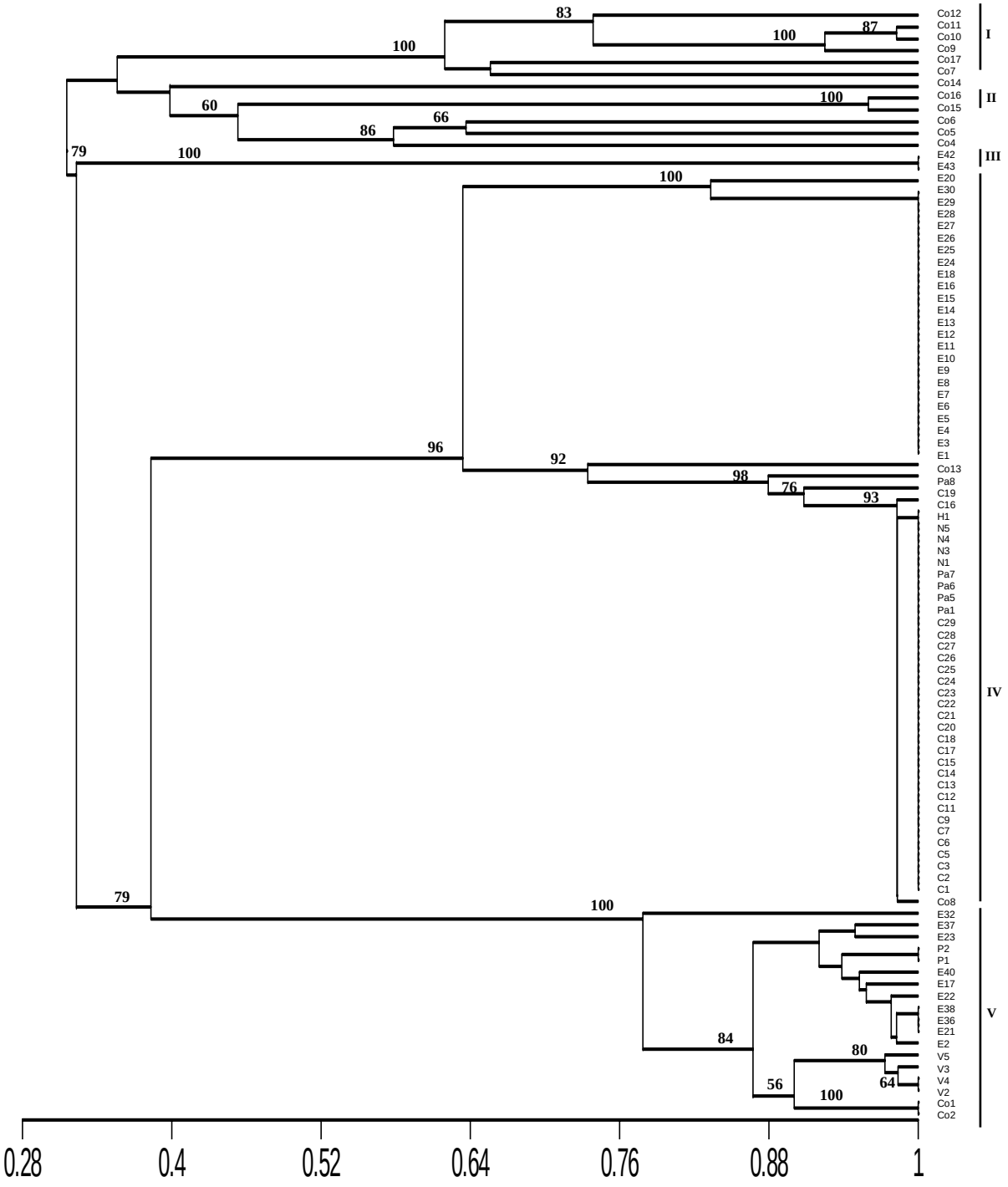

Figure 4.7 UPGMA dendogram jointly generated from AFLP and ISSR band profiles of 94 isolates of *M. roreri*. Bootstrap values above 50% (based on 1000 bootstrap re-samples) are given above branches.

4.4.4.5 Genetic diversity in Colombia, Ecuador and Central America

Separate UPGMA dendograms (Jaccard coefficient) were produced for isolates from Colombia, Ecuador and Central America using combined data sets from AFLP and ISSR band profiles (Figure 4.8). These images provide a clear visual indication of the level of genetic diversity of *M. roreri* represented in the three populations that correlates well with historical records on the disease.

Reference to Figure 4.8 reveals that the group of Colombian isolates registered the highest levels of genetic diversity and a coefficient of similarity ranging from 0.32 to 1 with an intermediate value at 0.66. Different groups of genotypes were supported by bootstrap values above 95% such as: isolates Co12, Co11, Co10, C09, Co17 and Co7 from Central Colombia (Co-Central); isolates Co16 and Co15 from El Carmen de Chucurí (Department of Santander) (Co-Eastern); isolates Co8 and Co13 from western Antioquia (Co-Western) and isolates Co2 and Co1 from the Department of Norte de Santander (Bolívar).

The Ecuadorian isolates showed an intermediate level of genetic diversity and a similarity coefficient ranging from 0.29 to 1 with an intermediate value at 0.65 (Figure 4.8). Three clusters were supported by bootstrap values of 100%, which involve three different genetic groups showing a specific geographic distribution: two wild isolates from *T. gileri* (Gileri) from north-western Ecuador; ten isolates mostly from peripheral areas of the country such as Tenguel, Manabí and Napo (Bolívar), and 24 isolates from Central Ecuador collected along an imaginary line from Esmeraldas in the north to Naranjal in the south (Co-West). Gileri isolates registered the lowest coefficient of similarity in relation to the remaining isolates.

The Central American isolates showed the lowest level of genetic diversity. They registered a coefficient of similarity ranging from 0.88 to 1 with an intermediate value at 0.94. The isolates belong only to one genetic group (Co-West).

In accordance with this, Colombia registered the highest levels of genetic diversity using Shannon's index (0.30) and on the basis of the percentage of polymorphic loci (69.1%) (Table 4.4).

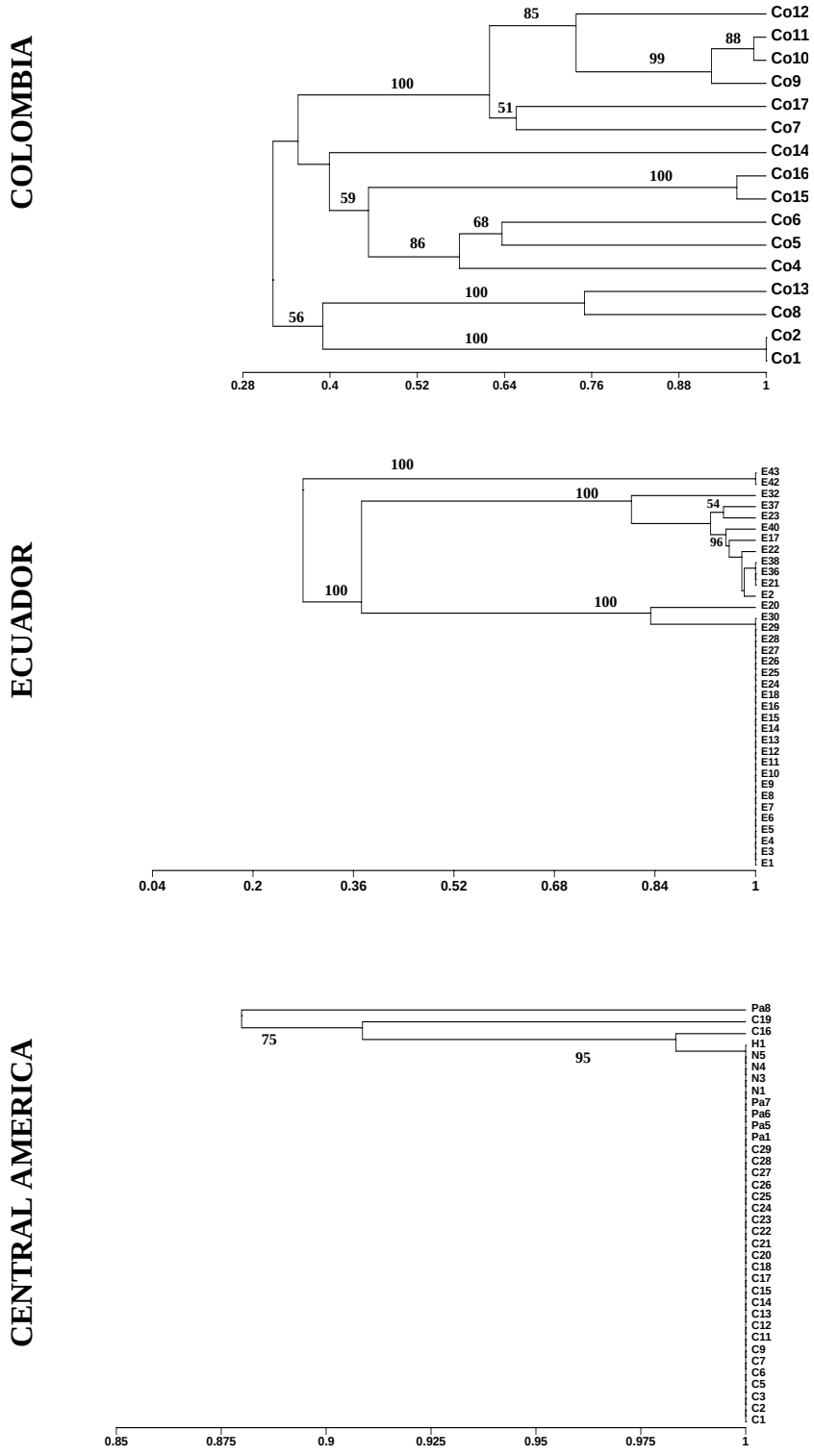

Figure 4.8 UPGMA dendograms of AFLP + ISSR band profile for isolates of *M. roreri* from Colombia, Ecuador and Central America. Bootstrap values above 50% (based on 1000 bootstrap re-samples) are given.

Table 4.4 Shannon index and percentage of polymorphic loci found in three populations of *M. roreri* when analysed using AFLP and ISSR.

Country (s)	Shannon Index	% of Polymorphic loci
Colombia	0.30±0.06 [1/]	69.1
Ecuador		
with E42/E43	0.23±0.04	56.4
without E42/E43	0.17±0.05	31.5
Central America	0.01±0.006	8.3

[1/] **Value ± standard error, (standard error = St. Dev. $n^{-½}$)**

Ecuador showed intermediate values with 0.23 and 56.4%, respectively, although a considerable proportion of this variation originated from the two isolates from Gileri group (E42 and E43). As mentioned above, these isolates registered the highest presence of rare alleles for both AFLP and ISSR analyses.

The lowest values of the Shannon index and the percentage of polymorphic loci were observed in Central American (0.01 and 8.3%, respectively), where the pathogen apparently arrived in recent history.

4.4.5 PRINCIPAL COORDINATES ANALYSIS (PCO).

The first three axes in the PCO plot accounted for a large proportion of the variation with 44%, 18% and 17%, respectively. The accumulative variation obtained for the first two axes was therefore 62%. Overall, the PCO identifies four groups (Figure 4.9).

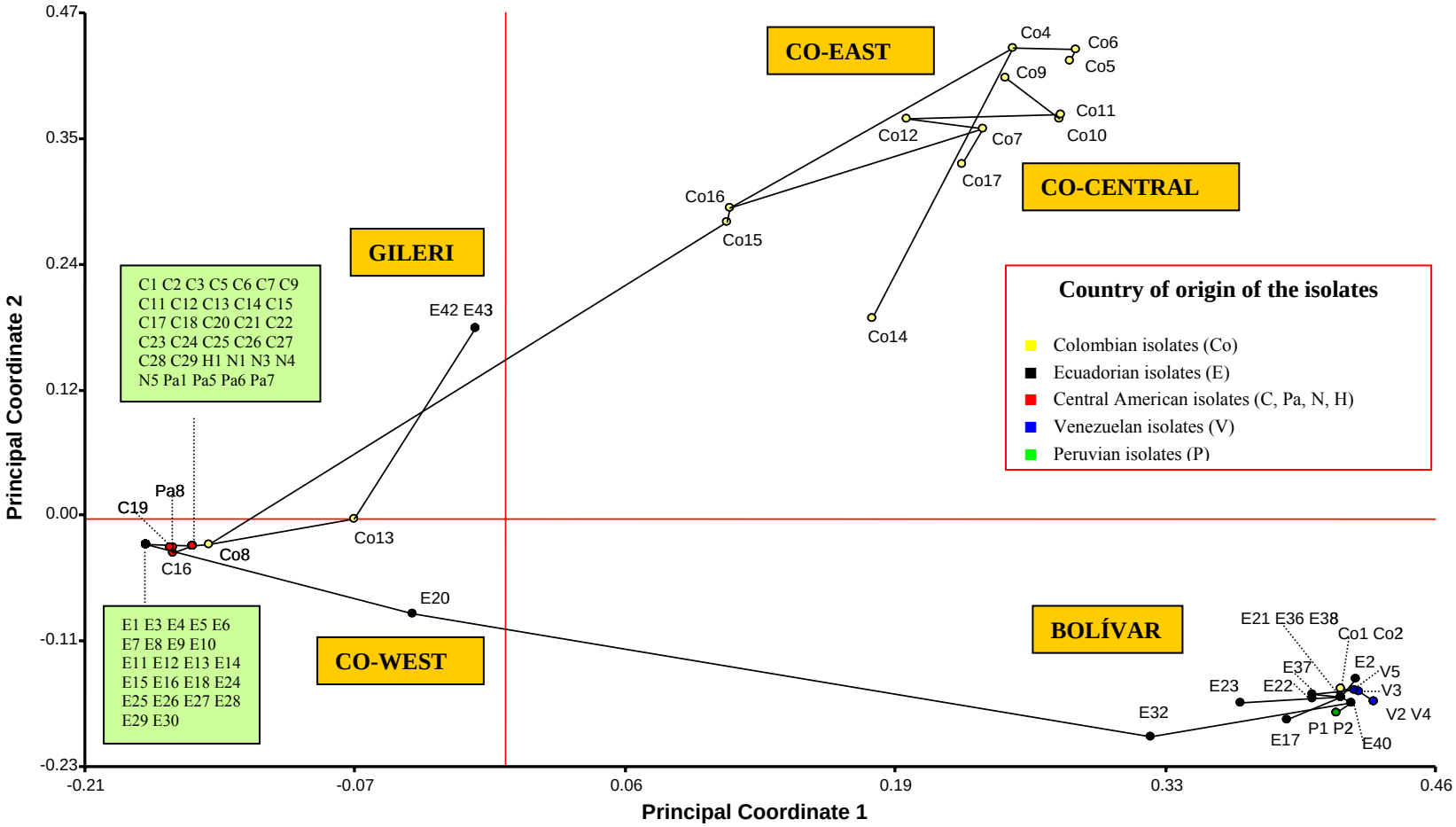

Figure 4.9 Principal coordinates analysis (PCO) and minimum spanning tree (MST) of Jaccard matrix of distances using 181 AFLP/ISSR bands of 94 isolates of *M. roreri*.

The main axis (Principal Coordinate axis 1) separates the isolates in two distinct groups: **Group A**: Isolates belonging to Gileri and Co-West genetic groups and **Group B**: Isolates belonging to Co-East, Co-Central and Bolívar genetic groups.

Principal Coordinate axis 2 separates the isolates into the following groups: **Group C**: Isolates belonging to Co-East, Co-Central and Gileri groups and **Group D**: Isolates from Co-West and Bolívar genetic groups.

Consequently, the whole set of isolates divided in four segments comprising one (Gileri, Co-West or Bolívar) or two (Co-Central/Co-East) genetic groups (Figure 4.9). Co-East and Co-Central isolates associated close together on the plot but retained some level of separation in particular Co14, Co15 and Co16. Isolates E20, E32 and Co13 showed an intermediate position between genetic groups: E20 and E32 between Co-West and Bolívar and Co13 between Co-West and Co-East/Co-Central.

It was observed that distribution of the genetic groups in Figure 4.9 closely coincided with their possibly geographical origin. This could be appreciated by reference of this figure to the map showing the involved areas (Figure 4.10).

Figure 4.10 Possibly geographic origin of five genetic groups of *M. roreri* determined using AFLP and ISSR analyses.

4.4.6 MANTEL TEST TO COMPARE THE MOLECULAR (AFLP+ISSR) AND GEOGRAPHIC DISSIMILARITY MATRICES

A Pearson correlation coefficient of r = 0.40 with a probability of 0.001 of independence between the matrices was found between the matrix of similarity based on the geographic distances between isolates and the matrix of similarity obtained through the combined analysis of the AFLP and ISSR bands when the set of 94 isolates was used. A higher coefficient (r = 0.62, probability = 0.001) was found when the set of 16 Colombian isolates and two Ecuadorian isolates from *T. gileri* (E42, E43) were analysed.

4.4.7 ANALYSIS OF THE INTERNAL TRANSCRIBED SPACERS (ITS REGIONS)

Sequences of approximately 760 bp in size were obtained for 95 *M. roreri* isolates, although this was reduced to 741 bp after editing consensus sequences to remove anomalies.

Ten mutations were detected among the isolates evaluated (Table 4.5). This means an estimated variation for the segment analysed of 1.4%. Ten point mutations involving single base pair changes were detected.

Table 4.5 Mutations for the ITS region obtained for 95 *Moniliophtora roreri* isolates.

Indel	Position in base pairs	Kind of mutation	Isolate (s) showing the indel
1	66	Transition G to A	Co13
2	133	Deletion of G	Western Group [1/] - Co13
3	134	Insertion of G	Co5, Co6
4	432	Deletion of A	Western Group
5	440	Insertion of T	E42, E43
6	441	Transition C to T	E42, E43
7	464	Insertion of G	Co5, Co6
8	483	Insertion of T	Western Group - E16
9	563	Transition T to C	Western Group
10	638	Insertion of G	Western Group

[1/] **Isolates comprising Western and Eastern Groups are indicated in Table 4.6**

Five rare mutations were observed in only one or two isolates: two rare mutations in Co5 and Co6 (Río Negro, Santander Department, Colombia), two in E42 and E43 isolates (*T. gileri*, Ecuador) and one in Co-13 (Urabá, Antioquia Department, Colombia) (Table 4.5).

The remaining mutations separated the set of 95 isolates into two distinct groups (Table 4.6). Group 1 named the Eastern Group comprises all Bolívar, Co-Central and Co-East isolates except Co15 and Co16 from El Carmen de Chucurí, Santander Department, Colombia. Consequently, all Peruvian, Venezuelan and peripheral Ecuadorian isolates and most of the Colombian isolates were contained in this group. The second group named the Western Group involves all Co-West and Gileri isolates, one Bolívar isolate from Tenguel, Ecuador (E-32) and the two Co-East isolates mentioned above (Co15 and Co16). Thus, four Colombian isolates, all Central American and most Central Ecuadorian isolates belong to this group.

Table 4.6 Isolates of *M. roreri* belonging to the Eastern and Western groups as determined using ITS analysis

Genetic Group	Eastern Group	Western Group
Co-East	**Colombia**: Co4, Co5, Co6, Co14	**Colombia**: Co15, Co16,
Co-Central	**Colombia**: Co7, Co9, Co10, Co11, Co12, Co17	
Bolívar	**Colombia**: Co1, Co2 **Ecuador**: E2, E17, E21, E22, E23, E36, E37, E38 **Peru**: P1, P2, P3, P5 **Venezuela**: V2, V3, V4, V5	**Ecuador**: E32
Co-West		**Colombia**: Co-8, Co13 **Ecuador**: E1, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, E18, E20, E24, E25, E26, E27, E28, E29, E30 **Central America:** C1, C2, C3, C5, C6, C7, C9, C11, C12, C13, C14, C15, C16, C17, C18, C19, C20, C21, C22, C23, C24, C25, C26, C27, C28, C29, H1, N1, N3, N4, N5, Pa1, Pa5, Pa6, Pa7, Pa8
Gileri		**Ecuador**: E42, E43

4.5 DISCUSSION

In spite of the outstanding historical and current importance of moniliasis in tropical America, there is still very little information available on the origin, genetic diversity and biogeography of its causal agent, *M. roreri.* This shortfall has been addressed in the present study, where isolates from the global range of *M. roreri* have been examined using AFLP, ISSR and ITS sequence data.

4.5.1 COMPARISON OF MOLECULAR TECHNIQUES

AFLP and ISSR produced extremely similar genetic affinities within moniliasis even though the two techniques target different random portions of the genome according to quite dissimilar principles. The similarity obtained is perhaps most evident when the respective UPGMA dendograms are compared visually. This apparent congruence was corroborated using the Mantel test for which a very high coefficient of correlation (0.92, $P < 0.001$) was revealed between the two dissimilarity matrices. This result suggests that any of the two techniques could have been independently used to study the genetic diversity of *M. roreri*. However, it was also manifest that the combined analysis of both sets of data improved the resolution of the clusters and increased the degree of support of the groups in accordance to the bootstrap support obtained for the groupings identified. This agrees with the opinion of Lanham & Brennan (1999) about the convenience to use a combination of marker types. Since the basis for detecting the markers is sometimes different; it would be expected that the data each generates would confirm or complement that generated by the others (Lanham & Brennan, 1999).

Results of this study thereby appear to suggest that AFLP and ISSR produced complementary information. Although AFLP and ISSR markers are randomly distributed throughout the genome (Zietkiewics *et al*., 1994; Majer *et al*., 1996; Maheswaran *et al*., 1997) and segregate in a Mendelian fashion (Tsumura *et al*., 1996; Mueller & Wolfenbarger, 1999), they have a different basis for detecting the markers. AFLP markers are based on the selective PCR amplification of DNA restriction fragments under stringent PCR conditions (Kardolus *et al.*, 1998). The basis of the observed polymorphisms in terms of nucleotide variation may be mutation in restriction site sequences or in sequences

complementary to the adaptor and selector nucleotides (Matthes *et al*., 1998). ISSR markers are also based on PCR under stringent conditions, but use primers that are complementary to the abundant and ubiquitously distributed microsatellites regions (Tautz & Renz, 1984). These primers usually also contain a one to three base oligonucleotide "anchor" at either the 3´ or 5´ end (Zietkiewics *et al*., 1994). The large numbers of amplicons generated by ISSR consist largely of the region between neighbouring and inverted SSRs (McGregor *et al*., 2000). Polymorphisms arise from nucleotide differences at the binding site corresponding to the 3' end of the primer, to absence of a primer binding site and, in the case where (as here) low temperature PAGE is used for fractionation, to small differences in internal sequence (Albani, 2002).

As Lanham & Brennan (1999) concluded, each marker type has its strengths and weaknesses. In the present research for instance, it was observed that AFLP produced a larger number of clearer and reproducible bands than ISSR, of which, as many as 65% were polymorphic. However, the AFLP analysis using autoradiography was more laborious and time-consuming methodology than ISSR, which could be an important consideration in future studies (Weising *et al*., 1995). The ISSR methodology employed was quicker and more straightforward than that used for AFLPs but generated fewer bands and presented more difficulties for profile scoring. The smaller size of the polyacrilamide gels used for ISSR (12.5 cm) in comparison with those used for AFLP (42.5 cm) caused some ISSR bands to separate poorly such that some bands became indistinguishable. Furthermore, the acute sensitivity of the silver staining detection protocol used meant that ISSR bands were easily either over- or under-exposed thereby increasing problems associated with the scoring of some bands.

4.5.2 PLACE OF ORIGIN OF *M. roreri*

The place of origin of *M. roreri* has been the subject of speculation since the first major appearance of the disease in Ecuador almost 100 years ago. Various authors (Rorer, 1918; Holliday, 1957; 1971; Thorold, 1975; Evans, 1981, 2002) have speculated about the country of origin of the fungus since that time, based primarily on historical accounts and observations of the presence of *M. roreri* on wild trees of *Theobroma* and/or *Herrania,* the only two genera susceptible to be infected by the fungus.

In this sense, the finding here that Colombia possesses the highest genetic diversity of *M. roreri* of all sampled countries establishes this country as the centre of diversity and so represents something of a surprise. This observation is also consistent with moniliasis having originated in Colombia and subsequently dispersed to the other countries in its range. This finding contradicts the most accepted version that the disease originated in Ecuador at the beginning of the 20th century, first postulated by Rorer (1918), but commonly reproduced by other authors (Briton-Jones, 1934; Erneholm, 1948; Ampuero, 1967; Barros, 1977).

The Colombian origin of *M. roreri* is apparently supported by different references mentioning the appearance and devastating effects of a cacao disease in the lower Cauca Valley, western Colombia during the second half of the 19th century (Aguirre, 1881; Carrasquilla, 1916; van Hall, 1932; Parsons, 1949; Hettner, 1976; Brew 1977; Barros, 1981). The effects of the disease first became evident in this area of Colombia in 1851, some fifty years before the much celebrated Ecuadorian outbreak. Based on this record and on the presence of wild hosts infected with moniliasis in this area, Holliday (1957, 1971) and Thorold (1975) postulated that western Colombia represents the possible place of origin of the pathogen. In accordance with this position, Evans (1981, 2002) concluded that *M. roreri* is indigenous to the north-western part of South America. More recently, this author suggested that it would seem that *T. gileri* probably represents the coevolutionary host of *M. roreri*, with its endemic range extending from western Ecuador to north-west Colombia (Evans, 2002).

In contradiction to the generalised beliefs that western Ecuador and/or north-west Colombia are the places of origin of *M. roreri*, the discovery here that the highest levels of genetic diversity are found in north-east Colombia revealed using both AFLP and ISSR data sets, are more consistent with this being the site of origin for the fungus. This thesis is supported by the decline in diversity with distance from this region. In this sense, a high correlation coefficient was obtained between the geographic and genetic matrices of distance through the Mantel test. Interestingly, the Principal Coordinates Analysis plot (Figure 4.9) shows a distribution of the Colombian isolates and the two Gileri isolates from Ecuador closely coinciding with their geographical distribution, suggesting divergence has arisen from isolation rather than recent spread of the disease.

The presence of seemingly ancient populations of wild species of *Theobroma* and *Herrania* in the Middle Magdalena area in Colombia (Galán, 1947) agrees with a possible origin of *M. roreri* in north-east part of the country since stable presence of the host is essential for the survival of such an obligate pathogen. Furthermore, various different historical references (Anon, 1832; Anon., 1850; Ancízar, 1956; Arenas, 1993) have mentioned the devastating effects caused in eastern Colombia (Department of Norte de Santander and later in the Department of Santander) by a pod disease matching the symptoms of moniliasis as early as 1817. These apparently represent the earliest references to the disease and date from 34 to 100 years before the referred outbreaks in the lower Cauca Valley and in Ecuador, respectively (see Chapter 3 for more details).

4.5.3 DISPERSAL

Dispersal of *M. roreri* from eastern Colombia to other areas in Colombia and later to other countries have possibly occurred over a long period of time, hypothetically starting with an ancient and perhaps natural spread of the pathogen throughout *Theobroma* and *Herrania* wild hosts as suggested by Holliday (1957, 1971), Thorold (1975) and Evans (1981). Records of the presence of *M. roreri* in the wild on *T. bicolor* and *Herrania balaensis* in Ecuador (Rorer, 1918) and on *T. gileri* in Antioquia, Colombia (Baker *et al.*, 1954) apparently support this hypothesis. However, the intervention of human activity in this very early dispersal process is also very probable considering both the large distances involved and the huge physical barriers separating some of the relevant areas of Colombia. Most notably, the Andean Cordillera splits into the three chains in Colombia and these represent the most notorious barriers to natural dispersal in the country. Isolates belonging to the Gileri group (the only genetic grouping found outside Colombia) could be a remnant of this earlier dispersal event (s) since they were collected both in Ecuador from wild trees of *T. gileri.*

After the first hypothetical dispersal event, it is plausible that *M. roreri* possibly remained in the wild in different places in Colombia, Ecuador and perhaps in Venezuela until more recent historical times when an explosion of cacao cultivation occurred in South America, most notably since the 18th century. This phenomenon increased the movement of planting material and provided new opportunities for dispersal of the disease from isolated wild

populations into semi-continuous stands of cultivated cacao. It can be speculated that infected pods from wild sources possibly initiated outbreaks of the disease in the Colombian Departments of Norte de Santander and Santander starting as far back as 1817 (Anon., 1832; Arenas, 1993), in the Colombian lower Cauca Valley starting in 1851 (Parsons, 1949) and in Ecuador around 1900 (Rorer, 1918).

Conversely, other outbreaks registered more recently in Central America (Enríquez *et al*., 1981; Anon., 2001) and Peru (Evans *et al*., 1998) are almost certainly the product of the invasion of the pathogen from infected cacao areas into cultivated stands of the crop in countries previously free of the disease. First time reports of the appearance of *M. roreri* in Venezuela, Panama, Costa Rica, Nicaragua, Honduras and Peru are all very recent (from Venezuela in 1941 to Honduras in 1998). The low genetic diversity of *M. roreri* within and between isolates from these countries is consistent with a rapid, recent spread of the fungus from a small founder population.

The very low levels of variation found between Co8 and the Central American isolates support a relatively recent introduction of *M. roreri* into Panama from infected pods brought from the region of San Jerónimo-San Vicente area, as suggested by Orellana (1956) based on the analogies of the cacao observed in both areas. Conversely, results contradict the thesis of Holliday (1957, 1971) who suggested that wild trees of *T. gileri* may be responsible for the natural spreading of the disease from Colombia into Panama, which may have been undetected in the latter country for a long time.

Results indicated that one or, alternatively, very few introductions of *M. roreri* may have occurred so far in Central America. The isolates from this area were very uniform in terms of AFLP and ISSR bands profiles, suggesting that the fungus has been mostly dispersed as a clone there.

4.5.4 GENETIC DIVERSITY OF *M. RORERI* AND DEFINITION OF GENETIC GROUPS

The existence of genetic groups of *M. roreri* was determined when the AFLP and ISSR bands profiles were analysed both separately and jointly. Since the combined analysis

increased the resolution and degree of support of the groups, the following comments refer to this analysis only.

Four main genetic groups of *M. roreri* (Co-Central, Co-West, Bolívar and Gileri) were consistently identified from the respective UPMGA dendogram using two approaches, by the imposition of a phenon line to the dendogram and based on the identification of the largest clusters with >95% bootstrap support.

A fifth genetic group (Co-Eastern) was identified under the phenon line approach, however it was not supported by a significant bootstrap values. Since the two subgroups comprising this group showed a bootstrap P value of 100% (Co15 and Co16) and 86% (Co4, Co5 and Co6) and the remaining isolate (Co14) was not bootstrap supported, it seems that these represent three different groups from the hypothetical centre of diversity of the disease. Considering that these groups are represented by a small number of samples in the present study and in order to simply the analysis and interpretation of the information, they will be treated as only one group here and in the remaining chapters.

In support to the Colombian origin of *M. roreri*, four of the genetic groups were found in Colombia, Co-East and Co-Central being apparently endemic in this country. Gileri is the unique group that, so far, has not been found in Colombia and is seemingly exclusively in Ecuador. Since the isolates of this group were collected very close to the Colombian/Ecuadorian border, however, it is entirely plausible that representatives of the group could be eventually identified within Colombia, particularly in the Department of Nariño.

Co-East and Co-Central isolates, although highly variable, clustered together when the Principal Coordinate Analysis was carried out, in contrast to the isolates from the other groups that clustered separately. This result suggests that Co-Central and Co-East are very genetically related. Since Co-East exhibited the highest genetic diversity observed for *M. roreri* and the area of collection of this group is adjacent to Central Colombia, it is possible that Co-Central is a subset of Co-East, adapted to the particular environmental conditions of Central Colombia.

It can be postulated that the adaptation of *M. roreri* to new environments occurred as consequence of historical dispersal events and this was the main cause for the differentiation of the fungus into genetic groupings. Under the working hypothesis that genetic groups arose within or close the areas where the isolates were collected in Colombia, it can be concluded that prevalent conditions in these places, mainly temperature and precipitation, may have been the key factors in the process of differentiation of the groups. This inference arises after taking in account the contrasting weather conditions in the places of collection of the isolates.

Thus, Co-East, the hypothetical original group, possibly evolved in the Middle Magdalena area at intermediate altitudes (approximately 500-800 m.a.s.l.) and under favourable environmental conditions in terms of relatively moderate temperature and precipitation for both the fungus and the host. Conversely, Co-Central possibly acquired their features in Central Colombia where is frequent to find cacao at altitudes over 1100 m.a.s.l., under adequate regimens of precipitation but at temperatures near to the minimum established for the host and the pathogen. The distinctive characters of Bolívar group possibly originated when *M. roreri* dispersed into the north-eastern flanks of the Cordillera Oriental of Colombia colonising habitats characterised by low altitudes, high temperatures, moderate to low precipitation and a marked drought period of 3-4 consecutive months (<100 mm precipitation $month^{-1}$) in some areas. Finally, Co-West and Gileri groups possibly differentiated, respectively, in western Colombia and north-western Ecuador. The predominating conditions in these two regions are low to medium altitudes, high temperatures, very high precipitation and the absence of a marked dry season.

Co-East comprised three subgroups that showed an outstanding level of differentiation. The different groups may represent ecotypes that arose from processes of local adaptation to specific environmental conditions in areas such as Rio Negro, San Vicente de Chucurí and El Carmen de Chucurí. The phenomenon probably also occurred in other places in the Middle Magdalena area but samples were not represented in the present study. This area represents the Centre of diversity and possibly also the centre of origin for the pathogen and so further collection expeditions to the region is strongly recommended to determine the existence of infected wild hosts, to collect new isolates and most importantly, to collect possible cacao sources of resistance to the pathogen.

The Co-Central isolates Co10, Co11 and Co12 were collected at three different altitudes (1200-1520 m.a.s.l) in a small farm from the Department of Caldas. However, they apparently have two different origin being Co10 and Co11 more related to Co9 isolate (Palestina, Department of Caldas) and Co12 to Co7 isolate (Támesis, Department of Antioquia). A similar situation occurred with Co 4 and Co5 (Co-East), which although were collected in a 32.6 hectare experimental farm in the Department of Santander, showed a significant level of genetic differentiation suggesting that at least two different introduction have occurred in this farm. This result is consistent with the information that different isolates of *M. roreri* were introduced in the mentioned farm in recent times.

Bolivar is a very widely spread group and is currently present in Colombia, Venezuela, Ecuador and Peru. It is possible that this group originated in the Colombian Department of Norte de Santander or perhaps in the area of the Rio Catatumbo in western Venezuela, where *M. roreri* was recorded for the first time in Venezuela in 1941 (Muller, 1941), but where the fungus had probably also existed for a long time in the wild. The River Catatumbo was a very important exportation route for cacao produced in the Valleys of Cúcuta during the 18th and 19th centuries (Angel, 1990; González, 1996). It is open to speculation that infected pods moved from this wild area into the outstanding cacao plantations of the Cúcuta Valley were the cause of the outbreak occurred here at the beginning of the 19th century (Anon, 1832; Anon., 1850; Ancízar, 1956; Arenas, 1993).

Isolates E40 and E38 were obtained from the *Centraalbureau voor Schimmelcultures Baar-Delft,* Netherlands, where they were reported as being collected in the Pichilingue Farm, central Ecuador. However, this is possibly a mistake since these isolates belong to the Bolívar group and no evidence of the current presence of this group was found in this farm in the present research.

The particular distribution of Bolivar group, being present in areas north east (in Colombia and Venezuela) and north west (in Ecuador and Peru) of South America is most consistent with movement of infected material via the intervention of human activity, who probably dispersed the group from its hypothetical place of origin within an area between Colombia/Venezuela into western Ecuador, afterwards into the Ecuadorian department of

Napo (eastern Ecuador) and from here into Peru. In this case and in other similar events, the participation of human beings spreading the disease into new areas and countries offers the most believable explanation for the presence of isolates of a specific group in areas far away of the centre of differentiation of that group. Thus, the introduction of Bolívar isolates into Ecuador may have occurred through infected pods from Venezuela, since different references indicate that the importation of this kind of material occurred from Venezuela/Trinidad into Ecuador starting in 1875 (Preuss, 1901; Rorer, 1918; van Hall, 1932; Arosemena, 1991). Conversely, Bolívar isolates arrived to the Napo region (Ecuador) and to Peru very recently, where the pathogen was detected after the 1980s in Napo and in 1989 in Peru (Evans *et al*., 1998). The fact that isolates from both areas belong to the same genetic group and were similar genetically, support the hypothesis of Hernández *et al*. (1990) and Evans *et al*. (1998) that isolates from the Province of Napo spread into Peru.

The Manabí area in Ecuador, where cacao has been cultivated along the rivers Chone and Calceta for a long time, is a good candidate for the first introduction of the Bolívar group in Ecuador. The increasing demand for cacao in the world market made the cultivation of cacao developed greatly in the Chone district during the last two decades of the 19th century (Erneholm, 1948). Since almost the whole crop was exported direct via the close Bahia de Caráquez or the neighbouring port of Manta (van Hall, 1932; Erneholm, 1948), it is very feasible as occurred in other places in Ecuador during this time (Preuss, 1901; Rorer, 1918; van Hall, 1932; Crawford, 1980; Arosemena, 1991), that introduction of cacao planting material from Venezuela and Colombia also occurred in Manabí, particularly in Chone, the most accessible area to the mentioned ports. On this respect, it is relevant to notice that although isolates from Manabí are genetically related, they show a significant differentiation suggesting that the fungus has been in this area for a reasonable number of years.

The previous hypothesis is supported by the molecular results since E21 isolate from Chone, not only shows connections in the minimum spanning tree with the other isolates from Manabí, but also with E2 and E17 from central Ecuador and with E36 and E37 from the Napo province, suggesting that dispersal of *M. roreri* to these places occurred from the Chone district. Also E21 is related to E38 and E40, isolates supposedly collected in the

Pichilingue farm but more probably originated in this area. Dispersal of *M. roreri* from Manabí into the Napo Province is probable since the development of a trans-Andean oil pipeline in the 1970s opened up the previously remote eastern region of Ecuador to colonists from different places in Ecuador (including Manabi), and cacao cultivars were imported from western Ecuador (Evans, 2002).

Co-West showed two distinctive subgroups. One subgroup comprises all isolates from Central American and two Colombian isolates (Co8 from San Jerónimo and Co13 from Urabá), and the other subgroup isolates from central Ecuador. Isolates from central Ecuador were mostly collected in places where *M. roreri* caused the first well documented outbreak around 1900 (Rorer, 1918). The relatively low levels of genetic diversity observed within this subgroup are more consistent with the disease being originated from an external source than from wild local sources where the fungus have supposedly remained for a long time. Since there are reports indicating that a similar disease to moniliasis appeared in the area of Esmeraldas and Hato Viejo before the outbreak in Central Ecuador (Crawford, 1980; Garcés, 1939), and that movements of pods for new plantings occurred from this area into central Ecuador before 1900 (Preuss, 1901), it is most plausible that moniliasis dispersed from north-western Ecuador into the centre of the country to cause these epidemics. However, the evident molecular differences found between isolates belonging to the Gileri and Co-West groups, clearly indicates that isolates of the former group were not the source of this dispersal.

4.5.5 SOURCE OF VARIATION

The present study provides a few strands of evidence supporting the enticing suggestion that *M. roreri* is capable of sexual reproduction. Evans *et al*. (2002) recently proposed a cytological mechanism that enables the fungus to undergo sexual reproduction. The apparent scarcity of evidence for recombination presented here perhaps indicates that sexual reproduction probably only occurs under very particular conditions. There is nevertheless some data that are at least consistent with recombination. Moreover, E20 and E32 showed some AFLP and ISSR bands typically present in other group and so these isolates may represent recombinants between two groups which were visualised in intermediate positions in the PCO plot (Figure 4.9). A cross between Co-West and Bolívar

is certainly feasible in the case of E20, since introductions of isolates for experimentation apparently occurred from Manabí into the area of San Pablo (Central Ecuador) where E20 was collected.

The few polymorphims observed in areas where *M. roreri* recently dispersed such as Central America, possibly originated in mutations since they are rare bands present only in one isolate. In this sense, Kohn (1994) and Anderson & Kohn (1995) have indicated that clonality produces a clear pattern in populations. Thus, for a series of loci known to be polymorphic, repeated recovery of the same multilocus genotype, especially over long distances (e.g. Central America) or periods of time, can be taken as a strong indication of clonal reproduction.

It is possible that mutations have had an important role generating genetic variability in *M. roreri*. In fact, gene mutation is the ultimate source of genetic variability occurring in 1% of the cells in each fungal generation as estimated by Carlile & Watkinson (1994). According to these authors, most of the cells in a fungal colony are capable of giving rise to further vegetative cells or to spores, so the number of cells in a fungal population that can undergo mutation and leave progeny can be very large. Furthermore, most fungi are haploid for a large part of their life cycle, so mutations will commonly be expressed, an if beneficial spread through a population by natural selection (Carlile & Watkinson, 1994). Conversely, it could be argued that the appearance of rare bands in one isolate is also consistent with fresh invasion or with recombination with another, as yet unidentified strain. More research is clearly required to resolve this problem.

4.5.6 GENETIC DIVERSITY REVEALED BY ITS

A relatively low level of intraspecific variation (1.4%) was obtained when ITS sequence data was analysed for 95-*M. roreri* isolates. This value is apparently significant considering both, the high frequency of ITS analysis that have produced non-informative variation in fungi (Bruns *et al*., 1991), and the very low levels of variation obtained within genera such as some rusts, *Colletotrichum* and *Alternaria* (Cooke & Duncan, 1997). For example, Gardes *et al*. (1991) estimated an ITS variation among *Laccaria* species and within *L. bicolor* of 3-5% and 1-2%, respectively.

In common with *M. roreri*, intraspecific informative variation has also been reported for some fungal species such as *Fusarium oxysporum* (Bridge & Arora, 1998) and *Pleurotus tuberregium* (Isikhuemhen *et al*., 2000).

Ten point mutations were found within *M. roreri* isolates, which agrees with the opinion expressed by Tredway *et al*. (1999) that non-point mutations rarely occur on ITS regions because these regions are tightly linked to rRNA genes with essential functions.

In spite of the few mutations found, however, the analysis of the ITS regions proved to be informative for *M. roreri* and allowed the designation of two subspecific groups, hereafter named 'Eastern' and 'Western' Groups. Fifty percent of the ten mutations detected made a clear separation between these two groups, the others five being rare mutations present only in one or two isolates.

Although the definition of the genetic groups was based on results from AFLP and ISSR, which, as suggested by Tredway *et al.* (1999) reveal a different forms of genetic variation than ITS, some correspondence was observed between both sets of results. Moreover, the isolates from the genetic groups originated in eastern Colombia (Co-East, Co-Central and Bolívar) also comprised the ITS Eastern Group, whereas isolates from the genetic groups originated in western Colombia (Co-West and Gileri) comprised the ITS Western Group. In support of this, the Principal Coordinate 1 in the respective plot, also clearly separated both groups of isolates (Figure 4.9).

The congruence between AFLP/ISSR and ITS data sets has significance and supports the division of *M. roreri* into two major groupings although whether the separation is sufficiently distinct to warrant recognition at some subspecific rank remains to be resolved. It is plausible that the two diverged after isolates of *M. roreri* dispersed from their place of origin in Eastern Colombia into Western Colombia, possibly involving several successive events, considering the huge geographical barriers separating both areas. Interestingly, two isolates from eastern Colombia (Co15 and Co16) possessed the typical ITS pattern of the Western Group. This establishes a link between the two groups, and suggests that the set of ITS mutations characterising the Western Group, possibly

originated in eastern Colombia and afterwards moved to Western Colombia. The presence of one isolate (E32) belonging to Bolivar but also showing the typical ITS pattern of the Western Group, suggests that the transfer of this set of mutations possibly also occurred from Eastern Colombia to isolates belonging to other genetic groups, or possibly that E32 is a recombinant between the groups.

It is concluded that *M. roreri* registered a considerable genetic variation in its global dispersal range, although genetic uniformity was also identified in some geographical areas such as Central America and Peru. The highest levels of variability found in Colombia suggest that *M. roreri* originated in this country, probably within or close to the Middle Magdalena area. From here the pathogen appears to have dispersed to other places where, by adaptation to the new environmental conditions, acquired particular molecular features that finally originated genetic groups. The five more prominent groups have a particular geographical distribution, Bolívar and Co-West being widely spread, and Co-Central, Co-East and Gileri apparently endemic to Colombia the first two and perhaps to Ecuador the later.

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4.7 APPENDIX

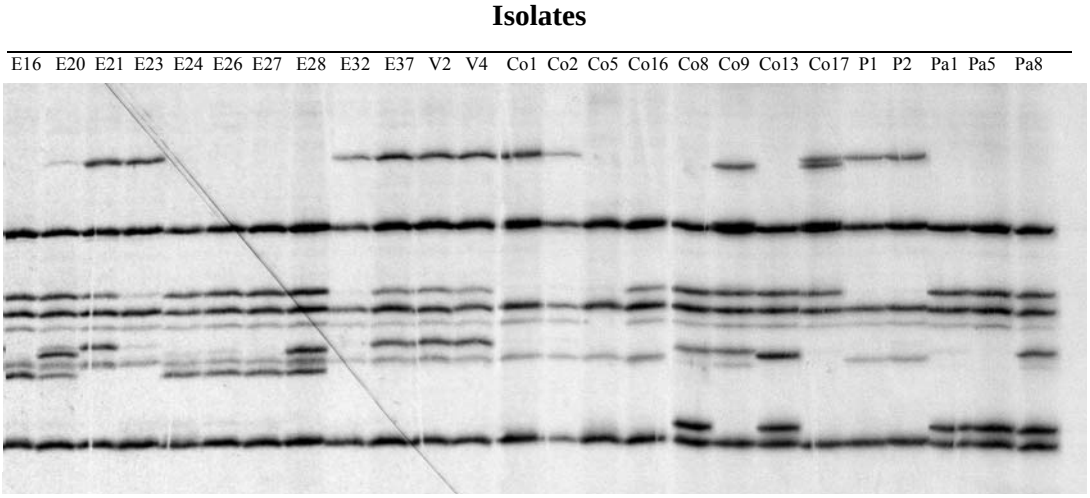

Plate A4.1 Part of an AFLP band profile generated using the primer combination "MCAG x EAT" on different isolates of *Moniliophthora roreri.*

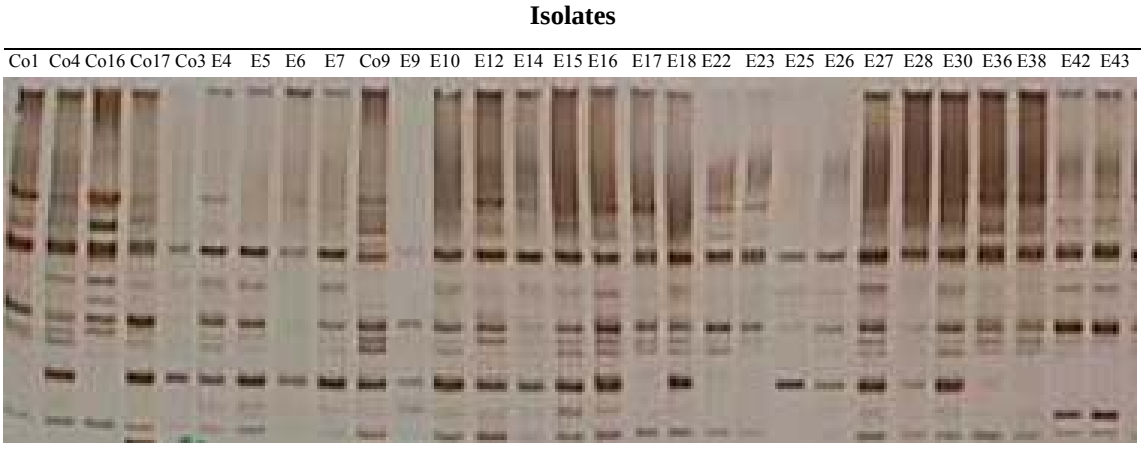

Plate A4.2 Part of an ISSR band profile generated using the primer UBC 880 on different isolates of *Moniliophthora roreri.*

CHAPTER 5

Genetic relationship between the cacao fungus *Moniliophthora roreri* (Cif.) Evans *et al.* and Basidiomycetes

5.1 ABSTRACT

In order to determine the taxonomic affinities of *M. roreri* with the Basidiomycetes, different portions of its genome were amplified by PCR, sequenced and compared with sequences from other basidiomycetes and in particular from the witches' broom pathogen of cacao, *Crinipellis perniciosa* (Agaricales, Tricholomataceae). Two segments of the nuclear ribosomal RNA genes were amplified using locus-specific primers: the internal transcribed spacer (ITS) regions (partial 18S, ITS1, 5.8S, ITS2, partial 25S) and part of the 25S ribosomal RNA gene (nLSU rDNA). The mitochondrial small ribosomal subunit RNA gene (mtSSU rDNA) was also targeted for analysis. Sequences from *M. roreri* and *C. perniciosa* were compared with those of related fungi deposited in the NCBI (National Centre for Biotechnology Information) database to infer taxonomic affinities. The results generated confirm that *M. roreri* is a Basidiomycete and strongly suggest that it belongs to the order Agaricales and possibly to the family Tricholomataceae. Sequence comparisons also indicates that this fungus is closely related to *C. perniciosa*, with only minor sequence anomalies being detected between them.

Keywords: Basidiomycetes, cacao, cocoa, *Crinipellis perniciosa*, frosty pod disease, moniliasis, *Moniliophthora roreri*, rDNA, *Theobroma cacao*.

5.2 INTRODUCTION

Moniliasis or frosty pod rot disease of cacao is a devastating disease currently present in nine countries of tropical America. Efforts to classify the causal agent of the disease with other fungi started in Ecuador at the beginning of the 20th century. Martínez (1916) first named the fungus responsible for moniliasis as *Phytophthora cactorum*, but R. E. Smith of the University of California soon reassigned the pathogen as belonging to the genus

Monilia, and described it as being very similar to *Monilia fructigena* (Rorer, 1918). By the 1930s, the taxonomic status of the organism was reviewed by Ciferri who supported the provisional identification made by Smith and classified the fungus as follows: Class: Deuteromycetes, Order: Hyphales, Genus: *Monilia*, species: *roreri* (Ciferri & Parodi, 1933). The fungus was classified as an imperfect because of the apparent absence of any meiotic state.

Evans *et al.* (1978) subsequently concluded that *M. roreri* should be assigned as an anamorphic Basidiomycete based partly on a range of morphological traits, but particularly on the presence of septa with typical dolipores in the vegetative mycelium, a feature previously reported to be diagnostic of the Basidiomycetes. They accordingly created a new genus to accommodate the fungus. The generic epithet *Moniliophthora* was chosen because it was felt that this name is not unlike the previous one and could be similarly abbreviated (Evans, 1986).

Some attempts have been made to establish taxonomic relationships between *M. roreri* and other fungal species based solely on morphological traits. For instance, Evans *et al* (1978) compared cultures of *M. roreri* with a range of Basidiomycetes commonly found on cacao pods or wood in Ecuador that had been subcultured from basidiospores. According to these authors, the skeletoid hyphae of *M. roreri* show some similarities to those of some Aphylophorales, especially with species of *Pleurotus*. More recently, Ram (1989) studied fruiting bodies associated with *M. roreri* on infected pods and putatively identified a species of *Mycena* as the possible candidate representing the sexual stage of *M. roreri*. However, subsequent experimental evidence demonstrated that this hypothesis was incorrect.

There is evidence of genetic affinities between *M. roreri* and the other endemic South American cacao pathogen *Crinipellis perniciosa* (Stahel) Singer, the causal agent of witches' broom disease. *C. perniciosa* is a mushroom forming Basidiomycete, that belongs to the order Agaricales and to the family Tricholomataceae (Pereira, 2000). Both species share several features in common (Evans *et al.*, 1978; Evans, 1981; 2002). Both are indigenous to tropical South America and, attack *Theobroma* and *Herrania* species. Both fungi are also able to disrupt the hormonal balance of the host to provoke hypertrophies

and hyperplasias. Moreover, the intercellular mycelium of both fungi is typically swollen and convoluted, being distinguished from the intracellular mycelium and from that in standard culture.

Based on these similarities, Evans (1981) suggested that *M. roreri* and *C. perniciosa* have a common origin and speculated that both forms evolved into highly pathogenic species from an original saprophytic or weakly parasitic liana strain. According to this author, the analysis of conidiogenesis and hyphal ultrastructure not only elucidated the true taxonomic position of *M. roreri* within the Basidiomycetes, but also strengthened the hypothesis of a common link between this species and *C. perniciosa*. At that time, however, any attempt to further refine the classification of *M. roreri* within the Basidiomycetes was viewed as speculative (Evans *et al.*, 2002).

The apparent absence of sexual structures in *M. roreri* has seriously impeded further refinement to the classification of the species over the past 25 years. In this sense, application of molecular techniques therefore offers the prospect to overcome some of the difficulties in the classification of this taxon brought about by the lack of homologous morphological features shared with a wide range of other organisms (Hillis & Dixon, 1991). Application of a selection of DNA-based techniques has revolutionised the discipline of fungal systematics and provided tools for both the discrimination of closely related fungi and also the *in vivo* detection of fungal species at sensitivities sometimes far beyond those of traditional methods (Bridge & Arora, 1998; Mills *et al.*, 1998; Takamatsu, 1998). The renaissance in fungal taxonomy brought about by the use of molecular data sets has provided new ways of looking at relationships at all taxonomic levels and has served both to confirm supposed taxonomies and to suggest novel relationships (Hopple & Vilgalys, 1999).

Sequence data from genes encoding nuclear and mitochondrial ribosomal RNA have been an important source of taxonomic and phylogenetic information for various groups of fungi (Hibbett *et al.*, 1995; Hopple & Vilgalys, 1999; Isikhuemhen *et al.*, 2000, Moncalvo *et al.*, 2000b). The fungal nuclear rRNA genes are arranged as tandem repeats with several hundreds copies per genome, whereas an additional single copy is located on the mitochondrial genome (Bridge & Arora, 1998). In the nuclear-encoded ribosomal RNA

genes (rDNA), each copy or cluster contains the genes for the small subunit (SSU or 18S), the 5.8S and the large subunit (LSU or 25-28S) rRNA (Takamatsu, 1998). In each copy, the genes are separated by two internal transcribed spacers (ITS1 and ITS2), and two rDNA units are separated by the intergenic spacer (IGS) or nontranscribed spacer (NTS). A 5S rRNA gene may or may not be within the repeated unit, depending on the fungal taxon (Edel, 1998).

The rDNA gene cassette contains both variable and conserved regions that diverge at different rates, allowing the comparison and discrimination of organisms at several taxonomic levels (Edel, 1998). The ribosomal coding regions are the most conserved, the internal transcribed spacers (ITS) display a certain degree of variation, and the intergenic spacers (IGS) are the most variable regions (Lanfranco *et al.*, 1998). The LSU subunit contains regions with different levels of variation. Most molecular studies only utilise the first 600-900 bases from the LSU gene, which includes three divergent domains (D1, D2, D3) that are among the most variable regions within the gene (Gutell & Fox, 1988; Hillis & Dixon, 1991).

The spacer regions between the subunits are considerably more variable than the subunit sequences, and have been used widely in studies for comparing fungal species within a genus or strains within a species (Edel, 1998; Takamatsu, 1998). ITS1 and ITS2 lack a functional role, which is thought to explain the high level of sequence variation commonly found within them.

Analysis of mtDNA variation has been used to discern subspecies, vegetative incompatibility groups and different populations (Jacobs*on &* Gordon 1990; Sm*ith et al.* 1990).

The objective of the present study was to determine the taxonomic affinities of *M. roreri* within the Basidiomycetes and in particular with *C. perniciosa* using molecular tools.

5.3 MATERIALS AND METHODS

5.3.1 LOCATION OF THE EXPERIMENTAL WORK

In vitro growth of the fungal isolates and DNA extractions were carried out in the Biotechnology and Phytopathology laboratories of the Tropical Agricultural Research and Higher Educational Centre (CATIE), Turrialba, Costa Rica from November to December 1999 and in the laboratories of CABI, Egham, UK from January to May 2000. The molecular studies of the isolates were performed in the School of Plant Sciences, The University of Reading from September to December 2000.

5.3.2 EXPERIMENTAL MATERIAL

Three DNA segments (ITS regions, nLSU-rDNA and mtSSU-rDNA) were analysed using different numbers of *M. roreri* and *C. perniciosa* isolates. Isolates of *M. roreri* were mostly collected by the author and encompass the geographic range of the fungus in 1999. Details on the origin of these isolates are given in Table A2.1 (Chapter 2). Isolates of *C. perniciosa* were collected by H. Evans (CABI, UK): two from Rio Napo, Napo Province, Ecuador (Dis70 from *Liana* sp. and Dis71 from *T. cacao*) and one from Yarinacocha, Pucallpa, Ucayali Department, Peru (Dis43 from *T. bicolor*). Nucleotide sequences of one isolate of *M. roreri*, seven isolates of *C. perniciosa* and some different isolates of basidiomycetes were obtained from the NCBI database (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov) at the beginning of April 2003.

5.3.3 EXPERIMENTAL METHODOLOGY

5.3.3.1 Total Genomic DNA Extraction

Genomic DNA was extracted from dry mycelium using a DNeasy Plant Mini Kit (Qiagen, UK) according to the manufacture's instructions as described in Chapter 2. Mycelia of *M. roreri* were obtained by growing isolates in mineral liquid medium (Weising *et al.*, 1995) for eight days whereas mycelia of *C. perniciosa* isolates were grown in liquid Glucose Yeast medium (GYM) for five days (Mugnai *et al.*, 1989). DNA was quantified by

reference to known standards after electrophoresis through 1% agarose gels and also using a DyNA Quant fluorimeter (Höefer) following the manufacturer's protocol.

5.3.3.2 Analysis of Internal Transcribed Spacers (ITS regions)

The ITS region studied comprised the sections 18S (partial), ITS1, 5.8S, ITS2, 25S (partial) and was amplified using the primers ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) and ITS5 (5`-GGAAGTAAAAGTCGTAACAAGG-3`) (White *et al.*, 1990). Ninety-five isolates of *M. roreri* were analysed for this segment as explained in Chapter 4. Additionally, three isolates of *C. perniciosa* (Dis43, Dis70 and Dis71) were processed using the same methodology.

5.3.3.3 Region 25 S of Nuclear Ribosomal DNA (nLSU-rDNA)

The same procedure used for ITS regions was performed for nLSU-rDNA except for the primers and the PCR programme. The programme consisted of an initial cycle of 30 cycles of 94 °C for 1 min (denaturing), 50 °C for 45 sec (annealing) and 72 °C for 1 min (extension) and a final cycle of 72 °C for 7 min.

Primers used for PCR amplifications were 5.8SR (5`-TCGATGAAGAACGCAGCG-3`) and LR7 (5`-TACTACCACCAAGATCT-3`) and for sequencing LROR (5`-ACCCGCTGAACTTAAGC-3`); LR3R (5`-GTCTTGAAACACGGACC-3`); LR5 (5`-TCCTGAGGGAAACTTCG-3`) and LR16 (5`-TTCCACCCAAACACTCG-3`) (Hopple & Vilgalys, 1999). These primers amplified a segment comprising the following sections: 5.8S, ITS2 and 25S (partial).

Four *M. roreri* isolates were analysed for this segment: Co5, Co8, Co16 and E43. A sample of *C. perniciosa* was processed but was not included in the study because some internal fragments repeatedly failed to amplify by PCR.

5.3.3.4 Small Mitochondrial rDNA (mtSSU-rDNA)

A fungal section of the mtSSU-rDNA gene was targeted for PCR amplification using the same procedure performed for the analysis of ITS regions except for the primers and the PCR programme. The programme consisted of an initial cycle of 94 °C for 30 sec (denaturing); 35 cycles of 94 °C for 30 sec (denaturing), 52 °C for 30 sec (annealing) and 72 °C for 3 min (extension) and a final cycle of 72 °C for 7 min. The primers used were MS1 (5`-CAGCAGTCAAGAATATTAGTCAATG-3`) and MS2 (5`-GCGGATTATCGAATTAAATAAC-3`) (White *et al.*, 1990).

Three isolates of *C. perniciosa* (Dis43, Dis70 and Dis71) and 26 isolates of *M. roreri* were processed. The isolates of *M. roreri* belong to the five genetic groups of *M. roreri* as defined in Chapter 4: Co-East (Co5, Co16), Co-Central (Co7, Co9, Co17), Gileri (E42, E43), Bolívar (Co2, E2, E32, E37, P1, V2) and Co-West (Co8, Co13, E12, E16, E20, Pa7, Pa8, C3, C17, C19, N1, H1). They represented the most important areas of distribution of the pathogen in tropical America comprising eight isolates from Colombia, eight from Ecuador, three from Costa Rica, two from Panama and one from Venezuela, Peru, Nicaragua and Honduras.

5.3.3.5 Processing of the sequences

The sequences were processed using the Lasergene System Software (DNAStar Inc.). Sequences were assembled and checked for inaccurate base calling using SeqMan II. Consensus sequences of each kind were aligned manually using MegAlign. Regions of ambiguous alignment and incomplete data at the beginning and end of sequences were excluded for further analysis. Mutations were visually determined and the isolates compared accordingly.

5.3.3.6 Search of sequence similarity

In order to determine phylogenetic affinities with other fungal species and to compare the *M. roreri* and *C. perniciosa* sequences obtained in the study with those available in the NCBI database, a search of sequence similarity for each kind of sequence was conducted

on the Blast option using a representative isolate of *M. roreri* (Co5). Comparisons between this sequence and those in the database were made by using the E value, which is supplied by the Blast output. E value is an expectation value indicating the number of different aligments with scores equivalent to or better than S (raw score) that are expected to occur in a database search by chance. The lower the E value, the more significant the score.

When possible, the sequences obtained in the database were aligned and compared with those obtained in the present study.

5.4 RESULTS

5.4.1 INTERNAL TRANSCRIBED SPACERS (ITS REGIONS)

Sequences of approximately 760 bp in size were obtained for *M. roreri* and *C. perniciosa*, although this length was reduced to 741 bp after editing consensus sequences to remove anomalies.

When the Blast search of sequence similarity was carried out, the submitted sequence of *M. roreri* matched most closely with the following organisms in decreasing order of similarity (Table 5.1).

a. One sequence of *M. roreri* from Ecuador (AY194150).
b. Seven sequences of *C. perniciosa* from different hosts.
c. Different fungal species belonging to the class Basidiomycetes, order Agaricales and mainly the families Pleurotaceae (*Pleurotus lampas*); Tricholomataceae (*Marasmius* sp., *Gymnopus menehune*, *Omphalina epichysium*); Cortinariaceae (*Gymnopilus* spp.) and Psathyrellaceae (*Psathyrella candolleana* and *Coprinus* spp.).

The sequence of *M. roreri* (AY194150) and two sequences of *C. perniciosa* (from *T. subincanum* and *T. grandiflorum*) showed an E value of O when compared with the submitted sequence.

Table 5.1 Result of the Blast search to determine species similarities of *M. roreri* using the ITS regions (ten best matches).

I.D.	Order	Family	Species	Host	E value
AY194150	?	?	*M. roreri*		0
AY216469	Agaricales	Tricholomataceae	*C. perniciosa*	*T. subincanum*	0
AY216468	Agaricales	Tricholomataceae	*C. perniciosa*	*T. grandiflorum*	0
AF335590	Agaricales	Tricholomataceae	*C. perniciosa*	*T. cacao*	e-175
AY216470	Agaricales	Tricholomataceae	*C. perniciosa*	*Solanum paniculatum*	e-173
AY176316	Agaricales	Tricholomataceae	*C. perniciosa*	*Solanum paniculatum*	e-172
AY216472	Agaricales	Tricholomataceae	*C. perniciosa*	*Capsicum frutescens*	e-168
AY216471	Agaricales	Tricholomataceae	*C. perniciosa*	*Herrania sp.*	e-158
AY265837	Agaricales	Pleurotaceae	*Pleurotus lampas*	?	5e-95
AY216476	Agaricales	Tricholomataceae	*Marasmius sp.*	?	3e-95

The sequences of *M. roreri* and *C. perniciosa* obtained from the NCBI database and those generated in the present study were successfully aligned. However, it was not possible to carry out a complete alignment for the other fungal species. Mutations between *M. roreri* and *C. perniciosa* were determined and described as follows.

5.4.1.1 Sequence variation within *M. roreri*

Ten point mutations were detected within the set of 95 isolates of *M. roreri* (Table A5.1), five mutations being rare (only observed in one or two isolates), whereas the remaining clearly separated the whole set in two subspecific groups named Western and Eastern Groups (see Chapter 4 for details).

In Table A5.1, only one representative of each subspecific group is shown since the remaining isolates showed an identical nucleotide pattern. The exceptional isolates

possessing rare mutations are also included in the table. These were Co5/Co6 in the Eastern Group and Co13, E16 and E42/E43 in the Western Group.

The *M. roreri* isolate AY194150 from Ecuador showed an identical sequence than that observed in the Western Group.

5.4.1.2 Variation within *C. perniciosa*

Seventeen mutation events were scored within the set of ten *C. perniciosa* isolates (Table A5.1). Most mutations (70.6%) were observed in one isolate taken from a *Liana* sp. (Dis70), with point mutations being the most frequent form of anomaly. Two other point mutations were recorded in the isolate from *Solanum paniculatum* and another one in isolates from two *Theobroma* species: *T. grandiflorum* and *T. subincanum*. Two mutations events, a transversion G to T and an insertion TTTT related the following isolates: Dis43 (*T. bicolor*); Dis 71 (*T. cacao*); AF335590 (*T. cacao*); AY216471 *(Herrania* sp.); AY176316 (*S. paniculatum*) and AY216472 (*Capsicum frutescens*).

5.4.1.3 Comparison between *M. roreri* and *C. perniciosa* sequences

M. roreri showed a high level of sequence homology with *C. perniciosa* for ITS regions (Table A5.1). Conversely, sequence divergence with the other Agaricales was such that it was not possible to obtain a complete alignment across the entire ITS region.

In spite of their overall similarity, the nucleotide sequence variation was 16% between *M. roreri* and *C. perniciosa.* Most isolates within each species exhibited the same haplotype, however, variation between isolates was observed for five and three mutations within *M. roreri* and *C. perniciosa*, respectively. Variation observed in *M. roreri* was mentioned above since it permitted to designate the two subspecific groups within this species. Interestingly, the isolates of *C. perniciosa* registered an identical nucleotide pattern for these five mutations that mirrored that observed in the Eastern Group of *M. roreri* (Table 5.2). This establishes a clear genetic link between *C. perniciosa* and the Eastern subspecific group of *M. roreri*.

Table 5.2 Mutations observed between the two subspecific groups of *M. roreri* in relation to those observed in ten *Crinipellis perniciosa* isolates.

Position in base pairs	Mutation	Observed in	Not observed in
133	Deletion of G	Western Group [1/] (except Co13)	Eastern Group [2/] and all *C. perniciosa* isolates
432	Deletion of A	Western Group	
482	Insertion of T		
563	Transition C to T		
638	Insertion of G		

[1/] **Western Group:** Co8, Co13, Co15, Co16, E1, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, E18, E20, E24, E25, E26, E27, E28, E29, E30, E32, E42, E43, C1, C2, C3, C5, C6, C7, C9, C11, C12, C13, C14, C15, C16, C17, C18, C19, C20, C21, C22, C23, C24, C25, C26, C27, C28, C29, H1, N1, N3, N4, N5, Pa1, Pa5, Pa6, Pa7, Pa8.

[2/] **Eastern Group:** Co1, Co2, Co4, Co5, Co6, Co7, Co9, Co10, Co11, Co12, Co14, Co17, E2, E17, E21, E22, E23, E36, E37, E38, P1, P2, P3, P5, V2, V3, V4, V5.

5.4.2 REGION 25 S OF NUCLEAR RIBOSOMAL DNA (nLSU-rDNA)

Sequences of approximately 1420 bp in size were obtained, although this was reduced to 1380 bp after editing consensus sequences to remove anomalies.

When a *M. roreri* sequence was submitted to the NCBI database, the species that matched most closely with this fungus belonged to various families of the Agaricales, with greatest similarity being shown by the representative of the Tricholomataceae family (Table 5.3). An E value = 0 was found for all these species. No complete sequences of *M. roreri* and *C. perniciosa* were found in the NCBI database for the fragment analysed. Consequently, sequences of the isolates AF335590 and AY194150 from *C. perniciosa* and *M. roreri*, respectively, were used for comparison with the sequences obtained in the present study.

In spite of the considerable length of the fragment studied, sequences of five *M. roreri*, one *C. perniciosa* and five isolates from the Agaricales were successfully aligned and compared.

Table 5.3 Result of the Blast search to determine species similarities of *M. roreri* using the nLSU rDNA (ten best matches).

I.D.	Order	Family	Species	E value
AY038329	Agaricales	Strophariaceae	*Phaeomarasmius curcuma*	0
AY038310	Agaricales	Cortinariaceae	*Hebeloma olympianum*	0
ORU66451	Agaricales	Tricholomataceae	*Omphalina rivulicola*	0
AF261467	Agaricales	Tricholomataceae	*Xeromphalina kauffmanii*	0
HCU66435	Agaricales	Hygrophoraceae	*Hygrocybe citrinopallida*	0
AY038309	Agaricales	Cortinariaceae	*Galerina semilanceata*	0
AF261342	Agaricales	Tricholomataceae	*Marasmius sp.*	0
OSU66453	Agaricales	Tricholomataceae	*Omphalina sphagnicola*	0
OVU66455	Agaricales	Tricholomataceae	*Omphalina velutipes*	0
TFU76460	Agaricales	Tricholomataceae	*Tricholoma focale*	0

5.4.2.1 Sequence variation within *M. roreri*

Only two mutations were detected within the five isolates of *M. roreri* studied (Table A5.2). A transition of A to G was observed in Co16 and Co8 but not in Co5 and E43. Moreover, an insertion of T was detected in E43 at 1049 bp. Since the sequence of the *M. roreri* isolate AY194150 from Ecuador was only 617 bp in length, it was not possible to determine its state for any of these mutations.

5.4.2.2 Comparison between *M. roreri* and other fungal species

M. roreri showed the highest level of similarity with *C. perniciosa* (AF335590) in comparison with other representatives of the Agaricales studied (Table A5.2). However, the smaller size of the *C. perniciosa* sequence used meant that it was only possible for limited sequence comparison with *M. roreri* covering just 658 bp. Despite this, 14 mutations were detected between the species. Eleven were point mutations and most (64%) involved A and G nucleotides. Two inversions of CT and TC, and one insertion (TT) were also observed.

It was also possible to successfully align and compare sequences of *M. roreri* with five Agaricales isolates, with a large number of mutations of various kinds being observed between groups (Table A5.2).

5.4.3 SMALL MITOCHONDRIAL rDNA (mtSSU rDNA)

Sequences of approximately 630 bp in size were obtained for *M. roreri* and *C. perniciosa*, although this was reduced to 587 bp after editing consensus sequences to remove anomalies.

5.4.3.1 Sequence variation within *M. roreri*

Sequences of 26 isolates of *M. roreri* representing the genetic diversity of this fungus were identical. Consequently, only one sequence representing all these isolates is shown in Table A5.3.

5.4.3.2 Comparison between *M. roreri* and other fungal species

Sequences of *M. roreri* and *C. perniciosa* matched very closely for mtSSU-rDNA and so could be readily aligned. Four mutational differences were recorded between these two species (Table A5.3). Two were apparently insertions (ATAATTT and TTTTT) in *C. perniciosa* (or deletions from *M. roreri*). Moreover, an additional T in the later insertion was observed in the sequence of isolate Dis43 (*Theobroma bicolor*, Peru). The remaining mutation was a transition of G to A found only in *M. roreri*.

When the mtSSU rDNA sequence of *M. roreri* was submitted to the NCBI database, it matched most closely with fungal species belonging to the class Basidiomycetes, order Agaricales and the families Tricholomataceae and to a much lesser extent to the Agaricaceae (Table 5.4). E values from 9e-97 to 1e-83 were obtained for the first ten matches. A species belonging to the order Aphyllophorales and family Cyphellaceae also matched with the sequence of *M. roreri*. It was not possible to obtain a complete alignment between the mentioned fungi and sequences of *M. roreri* and *C. perniciosa*.

Table 5.4 Ranked sequence similarities of mtSSU-rDNA from *M. roreri* to other species contained in the NCBI database (ten best species matches).

I.D.	Order	Family	Species	E value
AF481731	Agaricales	Tricholomataceae	*Lentinula edodes*	9e-97
AF357109	Agaricales	Tricholomataceae	*Asterophora lycoperdoides*	5e-92
AF357142	Agaricales	Tricholomataceae	*Clitocybe nebularis*	3e-90
AF357108	Agaricales	Tricholomataceae	*Calocybe constricta*	5e-89
AF357110	Agaricales	Tricholomataceae	*Asterophora parasitica*	2e-88
AF426964	Aphyllophorales	Cyphellaceae	*Limnoperdon incartatum*	2e-88
AF357141	Agaricales	Tricholomataceae	*Lepista nuda*	3e-87
AF357117	Agaricales	Tricholomataceae	*Tephrocybe gibberosa*	2e-85
AF357082	Agaricales	Tricholomataceae	*Tricholoma subaureum*	1e-83
AF357081	Agaricales	Tricholomataceae	*Tricholoma portentosum*	1e-83

5.5 DISCUSSION

Results obtained in this study indicate that *M. roreri* consistently shares strong DNA sequence homology only with other members of the Basidiomycetes and apparently shows a high affinity to the order Agaricales. Significantly, the three portions of the genome analysed are widely though to exhibit different rates of molecular evolution (Edel, 1998; Moncalvo *et al*., 2000b). In this sense, it has been pointed out that the best understanding of organismal phylogenies can be gained by analyses of different genes (Felsenstein, 1978; Doyle, 1992).

These results thereby support the hypothesis of Evans *et al*. (1978) who concluded that *M. roreri* is a basidiomycete based mainly on morphological characteristics. The absence of other distinctive characters in *M. roreri* significantly delayed refined classification of the fungus for a considerable number of years. This occurred because the taxonomic status of the Agaricales has traditionally relied on morphological characters, the basidiospore with all its characters traditionally proving to be one of the most important organs used for classification and assignment to the group (Singer, 1986). In fact, the amount of distinctive morphological details of sexual sporulation has been the base of fungal classification (Carlile & Watkinson, 1994).

The limited survey performed here on the basis of rDNA sequence is most consistent with *M. roreri* belonging to the family Tricholomataceae, the largest family of order Agaricales, and for which most matches were generated when the three target fragments were compared with the NCBI database. Nevertheless, some fungi belonging to other families also showed a significant nucleotide similarity with *M. roreri*, indicating that further research is required to resolve the phylogenetic affinity of the species. This may be systematic of the largely unresolved phylogeny of many modern genera and families of Agaricales. Indeed, Moncalvo *et al.* (2000a) argued that many taxa of the order are probably artificial because of the heavy reliance placed on morphological characters in their classification, since such features are known to be subject to parallel evolution and phenotypic plasticity. As these authors mentioned, progress toward a truly phylogenetic system of classification for the agaricoid fungi has been slower than for other organisms and is still hampered by many factors, most notably the still inadequate knowledge of undiscovered taxa, especially in the tropics.

A considerable level of sequence similarity was observed between *M. roreri* and the Tricholomataceae fungus *C. perniciosa* for the three rDNA fragments analysed in this study. Moreover, whilst numerous mutations were observed between these species, the respective sequences were, in all cases, easily aligned. This is particularly significant for ITS regions, which are typically so variable between taxa that they often cannot be aligned accurately between genera (Hong *et al.*, 2002). This pattern was also observed in the present study, where it was not possible to obtain successful alignment between sequences of the *M. roreri/C. perniciosa* isolates and those of any other member of the Agaricales.

The nucleotide similarity between *M. roreri* and *C. perniciosa* thereby establishes a significant link between these two species and supports the thesis of Evans (1981, 2002) that both fungi have a common recent origin. The author based this supposition on the range of characteristics shared by both pathogens, including similarities in morphology, symptoms and host specificity and range.

The ITS sequences revealed the highest level of infraspecific variation and divergence between *M. roreri* and *C. perniciosa*. This finding is consistent with the works of many other authors who consider ITS to be one of the most variable sections of the rDNA gene (Edel, 1998; Takamatsu, 1998). In spite of the greater length of the nLSU-rDNA fragment analysed, it revealed fewer polymorphisms between these fungi, with only 14 mutations detected between

M. roreri and *C. pernicious.* Again, this observation agrees with the wide consensus that this segment is highly conserved, with much of the LSU proving invariant even across widely divergent taxa (Gutell & Fox, 1988; Hillis & Dixon, 1991). It is of interest, therefore, that two mutations were identified in LSU among the four isolates of *M. roreri* analysed. The first mutation related the Colombian isolates Co16 from El Carmen de Chucurí (Santander Department) with Co8 from western Antioquia Department. Although these isolates originated from different regions and were assigned to different genetic groups (Co-West and Co-East) on the basis of AFLP/ISSR profiles, intriguingly, they were both identified as members of the Western subgroup of monilia based on ITS profiles (see Chapter 4 for details). The second mutation was found in isolate E43, which together with E42 constitute the Gileri genetic group, for which a considerable number of rare mutations were also noted in the ITS regions.

The rate of evolution of mitochondrial DNA evolves in fungi 16 times faster than the nuclear rDNA (Bruns & Szaro, 1992) and so, a significant level of variation was expected between mitochondrial fragments analysed. However, mtSSU-rDNA showed only a very low variation between and within *M. roreri* and *C. perniciosa*; the section was invariable for the 26-*M. roreri* isolates studied and only one mutation observed within the three *C. perniciosa* isolates evaluated. In a comparable study involving 20 species representative of all known lineages in the genus *Amanita* (Agaricales), Moncalvo *et al.* (2000b) demonstrated that mtSSU-rDNA sequence data provide lower phylogenetic resolution than nLSU-rDNA sequence data.

Sequences of *C. perniciosa* and *M. roreri* were very similar for mtSSU-rDNA. Indeed, only four mutations were identified as polymorphic between these species. It was not possible to get a complete alignment between *M. roreri* and other members of the family Tricholomataceae due to the excessively large number of mutations found between them. This again supports the close affinity of *M. roreri* to *C. perniciosa.*

When samples of *C. perniciosa* isolated from different host genera (*Liana*, *Solanum*, *Capsicum*, *Herrania* and *Theobroma*) were compared for ITS regions, isolate Dis70 from *Liana* sp. showed the highest level of sequence divergence. This agrees with observation made from several other authors who noted a high level of differentiation between isolates of *C. perniciosa* collected from *Liana* spp. and those taken from other host species. For instance, Griffith & Hedger (1994) reported that Liana biotype (L-biotype) does not cause the typical symptoms of witches' broom disease on its host and exhibit a highly restricted geographical

distribution and a multiallelic outcrossing mechanism. Evans (1978) also observed that a strain from *Liana* was able to invade *Theobroma* tissues but failed to induce severe symptoms, in particular the characteristic formation of brooms.

It has been suggested that *C. perniciosa* and *M. roreri* have a common origin, both forms possibly having evolved as highly pathogenic races from a weakly parasitic liana strain (Evans, 1981) or from a forest saprophyte (Evans, 1981; Evans *et al*., 2002). Evans (1981) also suggested that *C. perniciosa* evolved in the Amazon basin and *M. roreri* in the foothills of the western Andes. In this sense, it is highly significant the fact that the Eastern subgroup of *M. roreri* and all *C. perniciosa* isolates showed a similar nucleotide profile for the five ITS mutations separating *M. roreri* isolates in two subgroups: Eastern and Western (Chapter 4). This observation supports the thesis developed in Chapter 4 suggesting a possible origin of *M. roreri* in eastern Colombia rather than in western Colombia as was proposed earlier by others (Thorold, 1975; Evans, 1981). Under the apparently reasonable premise that *C. perniciosa* and *M. roreri* share a common recent origin (Evans, 1981) and that the centre of origin of *C. perniciosa* is in the upper Amazon basin (Baker & Holliday, 1957), it can be argued that variation between both pathogens follows a geographic gradient from the closer area to the Amazon basin (Eastern Colombia) to the more distant area (Western Colombia). In Colombia, this consideration is particularly important considering that the expansion from east to west required dispersal across huge natural barriers, the most notorious being the three chains into which the Andean Cordillera splits in this country.

In conclusion, molecular evidence obtained in this study seem to confirm that *M. roreri* is a Basidiomycete and strongly suggest that it belongs to the order Agaricales and possibly to the family Tricholomataceae. Sequence analysis also demonstrated that *M. roreri* (particularly the isolates from eastern Colombia) is closely related to *C. perniciosa* and is consistent with both species sharing a recent common ancestor.

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5.7 APPENDIX

Table A5.1 Aligned DNA sequences of ITS regions of isolates of *M. roreri* and *C. perniciosa*.

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                                10        20        30        40        50        60
Mr(Eastern Group)[1/]           TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTTTTGAGCTGGCTCTTC
  Mr(Co5/Co6)[2/]               TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTTTTGAGCTGGCTCTTC
Mr(Western Group)[3/]           TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTTTTGAGCTGGCTCTTC
  Mr(Co13)                      TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTTTTGAGCTGGCTCTTC
  Mr(E16)                       TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTTTTGAGCTGGCTCTTC
  Mr(E42/E43)                   TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTTTTGAGCTGGCTCTTC
Mr (AY194150)[4/]               TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTTTTGAGCTGGCTCTTC
Cp(Dis43)[5/]                   TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTT--GAGCTGGCTCTTG
Cp(Dis71)                       TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTT--GAGCTGGCTCTTG
Cp(Dis70))                      TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTT--GAGCTGGCTCTTA
Cp (AF335590)[6/]               TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTT--GAGCTGGCTCTTG
Cp (AY176316)                   TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTT--GAGCTGGCTCTTG
Cp (AY216468)                   TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTT--GAGCTGGCTCTTG
Cp (AY216469)                   TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTT--GAGCTGGCTCTTG
Cp (AY216470)                   TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGACGTT--GAGCTGGCTCTTG
Cp (AY216471)                   TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTT--GAGCTGGCTCTTG
Cp (AY216472)                   TGAACCTGCGGAAGGATCATTATTGAAAACATTGTAAAGGAGGTT--GAGCTGGCTCTTG

                                70        80        90       100       110       120
Mr(Eastern Group)               TTAGGGGCATGTGCTCGCCGACTTTCAATCTTCATCCACCTGTGAACCTTTTGTAGTGGA
  Mr(Co5/Co6)                   TTAGGGGCATGTGCTCGCCGACTTTCAATCTTCATCCACCTGTGAACCTTTTGTAGTGGA
Mr(Western Group)               TTAGGGGCATGTGCTCGCCGACTTTCAATCTTCATCCACCTGTGAACCTTTTGTAGTGGA
  Mr(Co13)                      TTAGGAGCATGTGCTCGCCGACTTTCAATCTTCATCCACCTGTGAACCTTTTGTAGTGGA
  Mr(E16)                       TTAGGGGCATGTGCTCGCCGACTTTCAATCTTCATCCACCTGTGAACCTTTTGTAGTGGA
  Mr(E42/E43)                   TTAGGGGCATGTGCTCGCCGACTTTCAATCTTCATCCACCTGTGAACCTTTTGTAGTGGA
Mr (AY194150)                   TTAGGGGCATGTGCTCGCCGACTTTCAATCTTCATCCACCTGTGAACCTTTTGTAGTGGA
Cp(Dis43)                       T-AGGG-CATGTGCTCGCCGACTTTCAATATTCATCCACCTGTGAACCTTTTGTAGCAGA
Cp(Dis71)                       T-AGGG-CATGTGCTCGCCGACTTTCAATATTCATCCACCTGTGAACCTTTTGTAGCAGA
Cp(Dis70))                      T-AGGG-CATGTGCTCGCTGACTTTCAATATTCATCCACCTGTGAACCTTTTGTAGCAGA
Cp (AF335590)                   T-AGGG-CATGTGCTCGCCGACTTTCAATATTCATCCACCTGTGAACCTTTTGTAGCAGA
Cp (AY176316)                   T-AGGG-CATGTGCTCGCCGACTTTCAATATTCATCCACCTGTGAACCTTTTGTAGCAGA
Cp (AY216468)                   T-AGGG-CATGTGCTCGCCGACTTTCAATATTCATCCACCTGTGAACCTTTTGTAGCAGA
Cp (AY216469)                   T-AGGG-CATGTGCTCGCCGACTTTCAATATTCATCCACCTGTGAACCTTTTGTAGCAGA
Cp (AY216470)                   T-AGGG-CATGTGCTCGCCGACTTTCAATATTCATCCACCTGTGAACCTTTTGTAGCAGA
Cp (AY216471)                   T-AGGG-CATGTGCTCGCCGACTTTCAATATTCATCCACCTGTGAACCTTTTGTAGCAGA
Cp (AY216472)                   T-AGGG-CATGTGCTCGCCGACTTTCAATATTCATCCACCTGTGAACCTTTTGTAGCAGA
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[1/] **Mr = *M. roreri*.** Typical sequence showed by isolates of the **Eastern Group** except Co5 and Co6 **(**Co1, Co2, Co4, Co7, Co9, Co10, Co11, Co12, Co14, Co17, E2, E17, E21, E22, E23, E36, E37, E38, P1, P2, P3, P5, V2, V3, V4, V5).

[2/] Isolates of ***M. roreri*** Co5 and Co6 from the Eastern Group and Co13, E16, E42 and E43 from the Western Group are placed separately since they showed minor differences in the nucleotide pattern in relation to the remaining isolates of their groups.

[3/] **Mr = *M. roreri*.** Typical sequence showed by isolates of the **Western Group** except Co13, E16, E42, E43 (Co8, Co15, Co16, E1, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E18, E20, E24, E25, E26, E27, E28, E29, E30, E32, C1, C2, C3, C5, C6, C7, C9, C11, C12, C13, C14, C15, C16, C17, C18, C19, C20, C21, C22, C23, C24, C25, C26, C27, C28, C29, H1, N1, N3, N4, N5, Pa1, Pa5, Pa6, Pa7, Pa8).

[4/] ***M. roreri*** sequence **AY194150** obtained in the NCBI database and submitted by Griffith & Nicholson (Dec 2002).

[5/] **Cp** = ***C. perniciosa*** sequences. **Dis43** from *T. bicolor* (Peru); **Dis70** from *Liana* sp. (Ecuador) and **Dis71** from *T. cacao* (Ecuador).

[6/] *C. perniciosa* sequences from the NCBI data base: **AF335590** from *T. cacao* submitted by Griffith, Hedger & Nicholson (Jan. 2001); and **AY176316** from *Solanum paniculatum*; **AY216468** from *T. grandiflorum*; **AY216469** from *T. subincanum*; **AY216470** from *Solanum paniculatum*; **AY216471** from *Herrania sp.*; **AY216472** from *Capsicum frutescens* submitted by Figueira, Leal, Gómes & Tavares (Nov. 2002 AY176316 and Jan. 2003 the remaining isolates) .

Continuation Table A5.1

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                          130       140       150       160       170       180
Mr(Eastern Group)  TTCTGGAACGGGG-AGGCGCTTGCGTTT-TCTCAGTACGGAGTCTCACTATGTTTT----
  Mr(Co5/Co6)      TTCTGGAACGGGGGAGGCGCTTGCGTTT-TCTCAGTACGGAGTCTCACTATGTTTT----
Mr(Western Group)  TTCTGGAACGGG--AGGCGCTTGCGTTT-TCTCAGTACGGAGTCTCACTATGTTTT----
  Mr(Co13)         TTCTGGAACGGGG-AGGCGCTTGCGTTT-TCTCAGTACGGAGTCTCACTATGTTTT----
  Mr(E16)          TTCTGGAACGGG--AGGCGCTTGCGTTT-TCTCAGTACGGAGTCTCACTATGTTTT----
  Mr(E42/E43)      TTCTGGAACGGG--AGGCGCTTGCGTTT-TCTCAGTACGGAGTCTCACTATGTTTT----
Mr (AY194150)      TTCTGGAACGGG--AGGCGCTTGCGTTT-TCTCAGTACGGAGTCTCACTATGTTTT----

Cp(Dis43)          TTCTGGAATAGGG-AGGCGCTTGCGTTTCTCTTAGTACGGAGTCT-ACTATGTTTGTTTT
Cp(Dis71)          TTCTGGAATAGGG-AGGCGCTTGCGTTTCTCTTAGTACGGAGTCT-ACTATGTTTGTTTT
Cp(Dis70))         TTCTGGAATAGGG-AGGCGCTTGCGTCTCTCTTAGTACGGAGTCT-ACTATGTTTTTTTT
Cp (AF335590)      TTCTGGAATAGGG-AGGCGCTTGCGTTTCTCTTAGTACGGAGTCT-ACTATGTTTGTTTT
Cp (AY176316)      TTCTGGAATAGGG-AGGCGCTTGCGTTTCTCTTAGTACGGAGTCT-ACTATGTTTGTTTT
Cp (AY216468)      TTCTGGAATAGGG-AGGCGCTTGCGTTTCTCTTAGTACGGAGTCT-ACTATGTTTTTT--
Cp (AY216469)      TTCTGGAATAGGG-AGGCGCTTGCGTTTCTCTTAGTACGGAGTCT-ACTATGTTTTTT--
Cp (AY216470)      TTCTGGAATAGGG-AGGCGCTTGCGTTTCTCTTAGTACGGAGTCT-ACTATGTTTTTTT-
Cp (AY216471)      TTCTGGAATAGGG-AGGCGCTTGCGTTTCTCTTAGTACGGAGTCT-ACTATGTTTGTTTT
Cp (AY216472)      TTCTGGAATAGGG-AGGCGCTTGCGTTTCTCTTAGTACGGAGTCT-ACTATGTTTGTTTT

                          190       200       210       220       230       240
Mr(Eastern Group)  ------AACACACACTTAAT-GTATGTTTAGAATGTGTATTTTATTGGGACTTAATTGAC
  Mr(Co5/Co6)      ------AACACACACTTAAT-GTATGTTTAGAATGTGTATTTTATTGGGACTTAATTGAC
Mr(Western Group)  ------AACACACACTTAAT-GTATGTTTAGAATGTGTATTTTATTGGGACTTAATTGAC
  Mr(Co13)         ------AACACACACTTAAT-GTATGTTTAGAATGTGTATTTTATTGGGACTTAATTGAC
  Mr(E16)          ------AACACACACTTAAT-GTATGTTTAGAATGTGTATTTTATTGGGACTTAATTGAC
  Mr(E42/E43)      ------AACACACACTTAAT-GTATGTTTAGAATGTGTATTTTATTGGGACTTAATTGAC
Mr (AY194150)      ------AACACACACTTAAT-GTATGTTTAGAATGTGTATTTTATTGGGACTTAATTGAC

Cp(Dis43)          TT----AACACAC--TTAAT-GTATGTTTAGAATG--TATTTTATTGGGACTTAATTGAC
Cp(Dis71)          TT----AACACAC--TTAAT-GTATGTTTAGAATG--TATTTTATTGGGACTTAATTGAC
Cp(Dis70))         TTTTTTAACACAA--TT----GTATGTTTAGAATG--TATTTTATTGGGACTTAATTGAC
Cp (AF335590)      TT----AACACAC--TTAAT-GTATGTTTAGAATG--TATTTTATTGGGACTTAATTGAC
Cp (AY176316)      TT----AACACAC--TTAAT-GTATGTTTAGAATG--TATTTTATTGGGACTTAATTGAC
Cp (AY216468)      ------AACACAC--TTAAT-GTATGTTTAGAATG-GTATTTTATTGGGACTTAATTGAC
Cp (AY216469)      ------AACACAC--TTAAT-GTATGTTTAGAATG-GTATTTTATTGGGACTTAATTGAC
Cp (AY216470)      ------AACACAC--TTAAT-GTATGTTTAGAATG--TATTTTATTGGGACTTAATTGAC
Cp (AY216471)      TT----AACACAC--TTAATGGTATGTTTAGAATG--TATTTTATTGGGACTTAATTGAC
Cp (AY216472)      TT----AACACAC--TTAATGGTATGTTTAGAATG--TATTTTATTGGGACTTAATTGAC

                          250       260       270       280       290       300
Mr(Eastern Group)  CCTTTAAAAACTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
  Mr(Co5/Co6)      CCTTTAAAAACTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
Mr(Western Group)  CCTTTAAAAACTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
  Mr(Co13)         CCTTTAAAAACTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
  Mr(E16)          CCTTTAAAAACTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
  Mr(E42/E43)      CCTTTAAAAACTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
Mr (AY194150)      CCTTTAAAAACTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC

Cp(Dis43)          CCTTTAAAA-CTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
Cp(Dis71)          CCTTTAAAA-CTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
Cp(Dis70))         CCTTTAAAA-CTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
Cp (AF335590)      CCTTTAAAA-CTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
Cp (AY176316)      CCTTTAAAA-CTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
Cp (AY216468)      CCTTTAAAA-CTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
Cp (AY216469)      CCTTTAAAA-CTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
Cp (AY216470)      CCTTTAAAA-CTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
Cp (AY216471)      CCTTTAAAA-CTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
Cp (AY216472)      CCTTTAAAA-CTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC
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Continuation Table A5.1

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                          310        320        330        340        350        360
Mr(Eastern Group)  GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
  Mr(Co5/Co6)      GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
Mr(Western Group)  GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
  Mr(Co13)         GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
  Mr(E16)          GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
  Mr(E42/E43)      GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
Mr (AY194150)      GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA

Cp(Dis43)          GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
Cp(Dis71)          GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
Cp(Dis70))         GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
Cp (AF335590)      GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
Cp (AY176316)      GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
Cp (AY216468)      GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
Cp (AY216469)      GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
Cp (AY216470)      GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
Cp (AY216471)      GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA
Cp (AY216472)      GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA

                          370        380        390        400        410        420
Mr(Eastern Group)  CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTCTC
  Mr(Co5/Co6)      CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTCTC
Mr(Western Group)  CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTCTC
  Mr(Co13)         CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTCTC
  Mr(E16)          CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTCTC
  Mr(E42/E43)      CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTCTC
Mr (AY194150)      CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTCTC

Cp(Dis43)          CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTATC
Cp(Dis71)          CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTATC
Cp(Dis70))         CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTATC
Cp (AF335590)      CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTATC
Cp (AY176316)      CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTATC
Cp (AY216468)      CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTATC
Cp (AY216469)      CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTATC
Cp (AY216470)      CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTATC
Cp (AY216471)      CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTATC
Cp (AY216472)      CGCACCTTGCGCCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATTAAATTATC

                          430        440        450        460        470        480
Mr(Eastern Group)  AACCTCAAAAAAGCTTTTT-CGGCTTTGTTTGAGGATTGGATG-TGAGGGTTGCTGGCTT
  Mr(Co5/Co6)      AACCTCAAAAAAGCTTTTT-CGGCTTTGTTTGAGGATTGGATGGTGAGGGTTGCTGGCTT
Mr(Western Group)  AACCTCAAAAA-GCTTTTT-CGGCTTTGTTTGAGGATTGGATG-TGAGGGTTGCTGGCTT
  Mr(Co13)         AACCTCAAAAA-GCTTTTT-CGGCTTTGTTTGAGGATTGGATG-TGAGGGTTGCTGGCTT
  Mr(E16)          AACCTCAAAAA-GCTTTTT-CGGCTTTGTTTGAGGATTGGATG-TGAGGGTTGCTGGCTT
  Mr(E42/E43)      AACCTCAAAAA-GCTTTTTTTGGCTTTGTTTGAGGATTGGATG-TGAGGGTTGCTGGCTT
Mr (AY194150)      AACCTCAAAAA-GCTTTTT-CGGCTTTGTTTGAGGATTGGATG-TGAGGGTTGCTGGCTT

Cp(Dis43)          AACCTTGAATGA-CTT------G--TCATTTCAGGATTGGATG-TGAGGGCTGCCGGCTT
Cp(Dis71)          AACCTTGAATGA-CTT------G--TCATTTCAGGATTGGATG-TGAGGGCTGCCGGCTT
Cp(Dis70))         AACCTTGAATGA-CTT------G--TCATTTTAGGATTGGATG-TGAGGGCTGCCGGCTT
Cp (AF335590)      AACCTTGAATGA-CTT------G--TCATTTCAGGATTGGATG-TGAGGGCTGCCGGCTT
Cp (AY176316)      AACCTTGAATGA-CTT------G--TCATTTCAGGATTGGATG-TGAGGGCTGCCGGCTT
Cp (AY216468)      AACCTTGAATGA-CTT------G--TCATTTCAGGATTGGATG-TGAGGGCTGCCGGCTT
Cp (AY216469)      AACCTTGAATGA-CTT------G--TCATTTCAGGATTGGATG-TGAGGGCTGCCGGCTT
Cp (AY216470)      AACCTTGAATGA-CTT------G--TCATTTCAGGATTGGATG-TGAGGGCTGCCGGCTT
Cp (AY216471)      AACCTTGAATGA-CTT------G--TCATTTCAGGATTGGATG-TGAGGGCTGCCGGCTT
Cp (AY216472)      AACCTTGAATGA-CTT------G--TCATTTCAGGATTGGATG-TGAGGGCTGCCGGCTT
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Continuation Table A5.1

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                          490        500        510        520        530        540
Mr(Eastern Group)  TT-GAATAAAGAGTCGGCTCCCTTGAAATGCATTAGTGGAAACC---GGT-TGGTAGACC
  Mr(Co5/Co6)      TT-GAATAAAGAGTCGGCTCCCTTGAAATGCATTAGTGGAAACC---GGT-TGGTAGACC
Mr(Western Group)  TTTGAATAAAGAGTCGGCTCCCTTGAAATGCATTAGTGGAAACC---GGT-TGGTAGACC
  Mr(Co13)         TTTGAATAAAGAGTCGGCTCCCTTGAAATGCATTAGTGGAAACC---GGT-TGGTAGACC
  Mr(E16)          TT-GAATAAAGAGTCGGCTCCCTTGAAATGCATTAGTGGAAACC---GGT-TGGTAGACC
  Mr(E42/E43)      TTTGAATAAAGAGTCGGCTCCCTTGAAATGCATTAGTGGAAACC---GGT-TGGTAGACC
Mr (AY194150)      TTTGAATAAAGAGTCGGCTCCCTTGAAATGCATTAGTGGAAACC---GGT-TGGTAGACC

Cp(Dis43)          T--GAACAA-GAGTCGGCTCTCTTGAAATGCATTAGTGGAAACC---GCTATAGTGGACC
Cp(Dis71)          T--GAACAA-GAGTCGGCTCTCTTGAAATGCATTAGTGGAAACC---GCTATAGTGGACC
Cp(Dis70))         T--GAACAA-GAGTCGGCTCTCTTGAAATGCATTAGTGGAAACCACCGCTATAGTGGACC
Cp (AF335590)      T--GAACAA-GAGTCGGCTCTCTTGAAATGCATTAGTGGAAACC---GCTATAGTGGACC
Cp (AY176316)      T--GAACAA-GAGTCGGCTCTCTTGAAATGCATTAGTGGAAACC---GCTATAGTGGACC
Cp (AY216468)      T--GAACAA-GAGTCGGCTCTCTTGAAATGCATTAGTGGAAACC---GCTATAGTGGACC
Cp (AY216469)      T--GAACAA-GAGTCGGCTCTCTTGAAATGCATTAGTGGAAACC---GCTATAGTGGACC
Cp (AY216470)      T--GAACAA-GAGTCGGCTCTCTTGAAATGCATTAGTGGAAACC---GCTATAGTGGACC
Cp (AY216471)      T--GAACAA-GAGTCGGCTCTCTTGAAATGCATTAGTGGAAACC---GCTATAGTGGACC
Cp (AY216472)      T--GAACAA-GAGTCGGCTCTCTTGAAATGCATTAGTGGAAACC---GCTATAGTGGACC

                          550        560        570        580        590        600
Mr(Eastern Group)  ACATTGGTGTGATAATTATCTACGCCATTAG-----TCTACGTTTTTTCGAATGTTGGC-
  Mr(Co5/Co6)      ACATTGGTGTGATAATTATCTACGCCATTAG-----TCTACGTTTTTTCGAATGTTGGC-
Mr(Western Group)  ACATTGGTGTGATAATTATCTATGCCATTAG-----TCTACGTTTTTTCGAATGTTGGC-
  Mr(Co13)         ACATTGGTGTGATAATTATCTATGCCATTAG-----TCTACGTTTTTTCGAATGTTGGC-
  Mr(E16)          ACATTGGTGTGATAATTATCTATGCCATTAG-----TCTACGTTTTTTCGAATGTTGGC-
  Mr(E42/E43)      ACATTGGTGTGATAATTATCTATGCCATTAG-----TCTACGTTTTTTCGAATGTTGGC-
Mr (AY194150)      ACATTGGTGTGATAATTATCTATGCCATTAG-----TCTACGTTTTTTCGAATGTTGGC-

Cp(Dis43)          ACATTGGTGTGATAATTATCTACGCCATTGGGTTTATCTACGCTTT--CGAATGTTGGTG
Cp(Dis71)          ACATTGGTGTGATAATTATCTACGCCATTGGGTTTATCTACGCTTT--CGAATGTTGGTG
Cp(Dis70))         ATATTGGTGTGATAATTATCTACGCCATTGGGTTTATCTACGCTTT--CGAATGTTGGCG
Cp (AF335590)      ACATTGGTGTGATAATTATCTACGCCATTGGGTTTATCTACGCTTT--CGAATGTTGGTG
Cp (AY176316)      ACATTGGTGTGATAATTATCTACGCCATTGGGTTTATCTACGCTTT--CGAATGTTGGTG
Cp (AY216468)      ACATTGGTGTGATAATTATCTACGCCATTGGGTTTATCTACGCTTT--CGAATGTTGGTG
Cp (AY216469)      ACATTGGTGTGATAATTATCTACGCCATTGGGTTTATCTACGCTTT--CGAATGTTGGTG
Cp (AY216470)      ACATTGGTGTGATAATTATCTACGCCATTGGGTTTATCTACGCTTT--CGAATGTTGGTG
Cp (AY216471)      ACATTGGTGTGATAATTATCTACGCCATTGGGTTTATCTACGCTTT--CGAATGTTGGTG
Cp (AY216472)      ACATTGGTGTGATAATTATCTACGCCATTGGGTTTATCTACGCTTT--CGAATGTTGGTG

                          610        620        630        640        650        660
Mr(Eastern Group)  ---CT------AG--CT-AACACTTCGGTTTGGGGGG-CTTGCAAGTTTGTAATGAACCT
  Mr(Co5/Co6)      ---CT------AG--CT-AACACTTCGGTTTGGGGGG-CTTGCAAGTTTGTAATGAACCT
Mr(Western Group)  ---CT------AG--CT-AACACTTCGGTTTGGGGGGGCTTGCAAGTTTGTAATGAACCT
  Mr(Co13)         ---CT------AG--CT-AACACTTCGGTTTGGGGGGGCTTGCAAGTTTGTAATGAACCT
  Mr(E16)          ---CT------AG--CT-AACACTTCGGTTTGGGGGGGCTTGCAAGTTTGTAATGAACCT
  Mr(E42/E43)      ---CT------AG--CT-AACACTTCGGTTTGGGGGGGCTTGCAAGTTTGTAATGAACCT
Mr (AY194150)      ---CT------AG--CT-AACACTTCGGTTTGGGGGGGCTTGCAAGTTTGTAATGAACCT

Cp(Dis43)          GCTCTTTAATTAGGTCTCAACACTTCGGTTTGGAGGG--TTGCAAGTT-GTAATGA-CCT
Cp(Dis71)          GCTCTTTAATTAGGTCTCAACACTTCGGTTTGGAGGG--TTGCAAGTT-GTAATGA-CCT
Cp(Dis70))         GCTCTTTAATTAGGTCTCAACACTTCGGTTTGGAGGG--TTGCAAGTT-GTAATGA-CCT
Cp (AF335590)      GCTCTTTAATTAGGTCTCAACACTTCGGTTTGGAGGG--TTGCAAGTT-GTAATGA-CCT
Cp (AY176316)      GCTCTTTAATTAGGTCTCAACACTTCGGTTTGGAGGG--TTGCAAGTT-GTAATGA-CCT
Cp (AY216468)      GCTCTTTAATTAGGTCTCAACACTTCGGTTTGGAGGG--TTGCAAGTT-GTAATGA-CCT
Cp (AY216469)      GCTCTTTAATTAGGTCTCAACACTTCGGTTTGGAGGG--TTGCAAGTT-GTAATGA-CCT
Cp (AY216470)      GCTCTTTAATTAGGTCTCAACACTTCGGTTTGGAGGG--TTGCAAGTT-GTAATGA-CCT
Cp (AY216471)      GCTCTTTAATTAGGTCTCAACACTTCGGTTTGGAGGG--TTGCAAGTT-GTAATGA-CCT
Cp (AY216472)      GCTCTTTAATTAGGTCTCAACACTTCGGTTTGGAGGG--TTGCAAGTT-GTAATGA-CCT
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Continuation Table A5.1

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                          670       680       690       700       710       720
Mr(Eastern Group)  GCTCTCTCTGTTATTGAACGGTATCTGCTTCAAACTGTCCTAAGTTTATTGGACAA--CA
  Mr(Co5/Co6)      GCTCTCTCTGTTATTGAACGGTATCTGCTTCAAACTGTCCTAAGTTTATTGGACAA--CA
Mr(Western Group)  GCTCTCTCTGTTATTGAACGGTATCTGCTTCAAACTGTCCTAAGTTTATTGGACAA--CA
  Mr(Co13)         GCTCTCTCTGTTATTGAACGGTATCTGCTTCAAACTGTCCTAAGTTTATTGGACAA--CA
  Mr(E16)          GCTCTCTCTGTTATTGAACGGTATCTGCTTCAAACTGTCCTAAGTTTATTGGACAA--CA
  Mr(E42/E43)      GCTCTCTCTGTTATTGAACGGTATCTGCTTCAAACTGTCCTAAGTTTATTGGACAA--CA
Mr (AY194150)      GCTCTCTCTGTTATTGAACGGTATCTGCTTCAAACTGTCCTAAGTTTATTGGACAA--CA

Cp(Dis43)          GCTCTCTCTG-----G---GGTATCTGCTTCGAACCGTCCAAAGTT-ACTGGACAATGAA
Cp(Dis71)          GCTCTCTCTG-----G---GGTATCTGCTTCGAACCGTCCAAAGTT-ACTGGACAATGAA
Cp(Dis70))         GCTCTCTCTG-----G---GGTATCTGCTTCGAACCGTCCCAAGTT-ACTGGACAATGAA
Cp (AF335590)      GCTCTCTCTG-----G---GGTATCTGCTTCGAACCGTCCAAAGTT-ACTGGACAATGAA
Cp (AY176316)      GCTCTCTCTG-----G---GGTATCTGCTTCGAACCGTCCAAAGTT-ACTGGACAATGAA
Cp (AY216468)      GCTCTCTCTG-----G---GGTATCTGCTTCGAACCGTCCAAAGTT-ACTGGACAATGAA
Cp (AY216469)      GCTCTCTCTG-----G---GGTATCTGCTTCGAACCGTCCAAAGTT-ACTGGACAATGAA
Cp (AY216470)      GCTCTCTCTG-----G---GGTATCTGCTTCGAACCGTCCAAAGTT-ACTGGACAATGAA
Cp (AY216471)      GCTCTCTCTG-----G---GGTATCTGCTTCGAACCGTCCAAAGTT-ACTGGACAATGAA
Cp (AY216472)      GCTCTCTCTG-----G---GGTATCTGCTTCGAACCGTCCAAAGTT-ACTGGACAATGAA

                          730       740
Mr(Eastern Group)  ATTAGATCATTTTGACCTCAA
  Mr(Co5/Co6)      ATTAGATCATTTTGACCTCAA
Mr(Western Group)  ATTAGATCATTTTGACCTCAA
  Mr(Co13)         ATTAGATCATTTTGACCTCAA
  Mr(E16)          ATTAGATCATTTTGACCTCAA
  Mr(E42/E43)      ATTAGATCATTTTGACCTCAA
Mr (AY194150)      ATTAGATCATTTTGACCTCAA

Cp(Dis43)          CTTAGATCATTTTGACCTCAA
Cp(Dis71)          CTTAGATCATTTTGACCTCAA
Cp(Dis70))         TTTAGATCATTTTGACCTCAA
Cp (AF335590)      CTTAGATCATTTTGACCTCAA
Cp (AY176316)      CTTAGATCATTTTGACCTCAA
Cp (AY216468)      CTTAGATCATTTTGACCTCAA
Cp (AY216469)      CTTAGATCATTTTGACCTCAA
Cp (AY216470)      CTTAGATCATTTTGACCTCAA
Cp (AY216471)      CTTAGATCATTTTGACCTCAA
Cp (AY216472)      CTTAGATCATTTTGACCTCAA
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Table A5.2 Aligned nLSU-rDNA sequences of isolates of *M. roreri* and *C. perniciosa*.

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                        10        20        30        40        50        60
Mr(Co5) 1/      GGATTCCCCTAGTAACTGCGAGTGAAGAGGGAAAAGCTCAAATTTGAAATCTGGCAGTCT
Mr(Co16)        GGATTCCCCTAGTAACTGCGAGTGAAGAGGGAAAAGCTCAAATTTGAAATCTGGCAGTCT
Mr(Co8)         GGATTCCCCTAGTAACTGCGAGTGAAGAGGGAAAAGCTCAAATTTGAAATCTGGCAGTCT
Mr(E43)         GGATTCCCCTAGTAACTGCGAGTGAAGAGGGAAAAGCTCAAATTTGAAATCTGGCAGTCT
Mr(AY194150) 2/ GGATTCCCCTAGTAACTGCGAGTGAAGAGGGAAAAGCTCAAATTTGAAATCTGGCAGTCT
CP(AF335590) 3/ GGATTCCCCTAGTAACTGCGAGTGAAGAGGGAAAAGCTCAAATTTAAAATCTGGCAGTCT

Bm(AY038329) 4/ GGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCAAATTTAAAATCTGGCAGTTT
Bm(AY038310)    GGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCAAATTTAAAATCTGGCGGTCT
Bm(HCU66435)    GGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCAAATTTAAAATCTGGCGGTCC
Bm(ORU66451)    GGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCAAATTTAAAATCTGGCGGTCC
Bm(AF261467)    GGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCAAATTTAAAATCTGGCGGTCT

                        70        80        90       100       110       120
Mr(Co5)         CTG--GCTGTCCGAGTTGTAATTTAGAGAAGTGTT-ACCCGTGCTGGACCGTGTGTAAGT
Mr(Co16)        CTG--GCTGTCCGAGTTGTAATTTAGAGAAGTGTT-ACCCGTGCTGGACCGTGTGTAAGT
Mr(Co8)         CTG--GCTGTCCGAGTTGTAATTTAGAGAAGTGTT-ACCCGTGCTGGACCGTGTGTAAGT
Mr(E43)         CTG--GCTGTCCGAGTTGTAATTTAGAGAAGTGTT-ACCCGTGCTGGACCGTGTGTAAGT
Mr(AY194150)    CTG--GCTGTCCGAGTTGTAATTTAGAGAAGTGTT-ACCCGTGCTGGACCGTGTGTAAGT
CP(AF335590)    CTG--GCTGTCCGAGTTGTAATTTAGAGAAGTGTT-ACCCGTGCTGGACCGTGTATAAGT

Bm(AY038329)    C----ACTGTCCGAGTTGTAATCTAGAGAAGTGTT-ATCCGCGCTGGACCGTGTACAAGT
Bm(AY038310)    CTG--GCCGTCCGAGTTGTAATCTAGAGAAGTGTT-ATCCGCGCTGGACCGTGTACAAGT
Bm(HCU66435)    TTGCGGCTGTCCGAGTTGTAATCTAGAGAAGTGTTT-TCCGTGCTGGACCGTGTACAAGT
Bm(ORU66451)    TTGTGGCCGTCCGAGTTGTAATCTAGAGAAGTGTT-ATCCGCGCTGGACCGTGTACAAGT
Bm(AF261467)    TTG--GCCGTCCGAGTTGTAATTTAGAGAAGTGTTTATCCGCGCTGGACCGTGTACAAGT

                       130       140       150       160       170       180
Mr(Co5)         CTCCTGGAATGGAGCGTCATAGAGGGTGAGAATCCCGTCTTTGACACGGACTCCCAGTGC
Mr(Co16)        CTCCTGGAATGGAGCGTCATAGAGGGTGAGAATCCCGTCTTTGACACGGACTCCCAGTGC
Mr(Co8)         CTCCTGGAATGGAGCGTCATAGAGGGTGAGAATCCCGTCTTTGACACGGACTCCCAGTGC
Mr(E43)         CTCCTGGAATGGAGCGTCATAGAGGGTGAGAATCCCGTCTTTGACACGGACTCCCAGTGC
Mr(AY194150)    CTCCTGGAATGGAGCGTCATAGAGGGTGAGAATCCCGTCTTTGACACGGACTCCCAGTGC
CP(AF335590)    CTCCTGGAATGGAGCGTCATAGAGGGTGAGAATCCCGTCTTTGACGCGGACTCCCAGTGC

Bm(AY038329)    CTCCTGGAATGGAGCGTCATAGAGGGTGAGAATCCCGTCTTTGACACGGACTGCCAGGGC
Bm(AY038310)    CTCCTGGAATGGAGCGTCATAGAGGGTGAGAATCCCGTCTTTGACACGGACTGCCAGGGC
Bm(HCU66435)    CTCTTGGAATAGAGCGTCATAGAGGGTGAGAATCCCGTCTTTGACACGGACTACCAGTGC
Bm(ORU66451)    CTCCTGGAATGGAGCGTCATAGAGGGTGAGAATCCCGTCTTTGACACGGACTACCAGGGC
Bm(AF261467)    CTCCTGGAACGGAGCGTCATAGAGGGTGAGAATCCCGTCTTTGACACGGACCACCAGTGC
```

[1/] **Mr = *M. roreri*** isolate.

[2/] ***M. roreri*** sequence **AY194150** from Ecuador. Submitted to the NCBI database by Griffith & Nicholson in December, 2002.

[3/] **Cp = *C. perniciosa*** isolate. Sequence **AF335590** from *T. cacao* submitted to the NCBI database by Griffith, Hedger & Nicholson in January, 2001.

[4/] **Bm = Basidiomycete**. Sequences from the NCBI database which matched most closely with ***M. roreri***: **AY038329** from *Phaeomarasmius curcuma*; **AY038310** from *Hebeloma olympianum*; **HCU66435** from *Hygrocybe citrinopallida*; **ORU66451** from *Omphalina rivulicola* and **AF261467** from *Xeromphalina kauffmanii.*

Continuation Table A5.2

```
                    190       200       210       220       230       240
Mr(Co5)         ATTGTGGTGCGCTCTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGTAA
Mr(Co16)        ATTGTGGTGCGCTCTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGTAA
Mr(Co8)         ATTGTGGTGCGCTCTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGTAA
Mr(E43)         ATTGTGGTGCGCTCTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGTAA
Mr(AY194150)    ATTGTGGTGCGCTCTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGTAA
CP(AF335590)    ATTGTGGTACGCTCTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGTAA

Bm(AY038329)    TTTGTGATGCGCTCTCAAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAATGGGTGGTAA
Bm(AY038310)    TTTGTGATGCGCTCTCAAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAATGGGTGGTAA
Bm(HCU66435)    TTTGTGATGCGCTCTCAAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAATGGGTGGTAA
Bm(ORU66451)    TTTGTGATGCGCTCTCAAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAATGGGTGGTAA
Bm(AF261467)    TTTGTGATGCGCTCTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAATGGGTGGTAA

                    250       260       270       280       290       300
Mr(Co5)         ATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAA
Mr(Co16)        ATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAA
Mr(Co8)         ATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAA
Mr(E43)         ATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAA
Mr(AY194150)    ATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAA
CP(AF335590)    ATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAA

Bm(AY038329)    ATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAA
Bm(AY038310)    ATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAA
Bm(HCU66435)    ATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAA
Bm(ORU66451)    ATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAA
Bm(AF261467)    ATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAA

                    310       320       330       340       350       360
Mr(Co5)         GATGAAAAGAACTTTGGAAAGAGAGTTAAACAGTACGTGAAATTGCTGAAAGGGAAACGC
Mr(Co16)        GATGAAAAGAACTTTGGAAAGAGAGTTAAACAGTACGTGAAATTGCTGAAAGGGAAACGC
Mr(Co8)         GATGAAAAGAACTTTGGAAAGAGAGTTAAACAGTACGTGAAATTGCTGAAAGGGAAACGC
Mr(E43)         GATGAAAAGAACTTTGGAAAGAGAGTTAAACAGTACGTGAAATTGCTGAAAGGGAAACGC
Mr(AY194150)    GATGAAAAGAACTTTGGAAAGAGAGTTAAACAGTACGTGAAATTGCTGAAAGGGAAACGC
CP(AF335590)    GATGAAAAGAACTTTGGAAAGAGAGTTAAACAGTACGTGAAATTGCTGAAAGGGAAACGC

Bm(AY038329)    GATGAAAAGAACTTTGGAAAGAGAGTTAAACAGTACGTGAAATTGCTGAAAGGGAAACGC
Bm(AY038310)    GATGAAAAGAACTTTGGAAAGAGAGTTAAACAGTACGTGAAATTGCTGAAAGGGAAACGC
Bm(HCU66435)    GATGAAAAGAACTTTGGAAAGAGAGTTAAACAGTACGTGAAATTGTTGAAAGGGAAACGC
Bm(ORU66451)    GATGAAAAGAACTTTGGAAAGAGAGTTAAACAGTACGTGAAATTGTTGAAAGGGAAACGC
Bm(AF261467)    GATGAAAAGAACTTTGGAAAGAGAGTTAAACAGTACGTGAAATTGTTGAAAGGGAAACGC

                    370       380       390       400       410       420
Mr(Co5)         TTGAAGTCAGTCGCGTTGGCCAGGGATCAACCTTGCTTTTTAG--TGAGGCGTACTTCCT
Mr(Co16)        TTGAAGTCAGTCGCGTTGGCCAGGGATCAACCTTGCTTTTTAG--TGAGGCGTACTTCCT
Mr(Co8)         TTGAAGTCAGTCGCGTTGGCCAGGGATCAACCTTGCTTTTTAG--TGAGGCGTACTTCCT
Mr(E43)         TTGAAGTCAGTCGCGTTGGCCAGGGATCAACCTTGCTTTTTAG--TGAGGCGTACTTCCT
Mr(AY194150)    TTGAAGTCAGTCGCGTTGGCCAGGGATCAACCTTGCTTTTTAG--TGAGGCGTACTTCCT
CP(AF335590)    TTGAAGTCAGTCGCGTTATCCAGGGATCAACCTTGCTTT--AG--TGAGGCGTACTTCCT

Bm(AY038329)    TTGAAGTCAGTCGCGTTGGCTGGGAATCAACCTTGCTTTT--GC-TG-GGCGTATTTTCT
Bm(AY038310)    TTGAAGTCAGTCGCGTTGGCCGGGGATCAACCTTGCTTTT--GC-TG-GGTGTACTTTCC
Bm(HCU66435)    TTGAAGTCAGTCGCGTTGGCCGAGGATCAACCTTGCAATTT-GC-TG-GGCGTACTTCTC
Bm(ORU66451)    TTGAAGTCAGTCGCGTTGGTCAGAAATCAACCTTGCTTTTTTG--TGGGGTGAACTTTCT
Bm(AF261467)    TTGAAGTCAGTCGCGTTGTCCGGGGATCAACCTTGCATTCGTGCTTGGCGT--ACTTCCC
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Continuation Table A5.2

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                    430        440        450        460        470        480
Mr(Co5)         GGTTGACGGGTCAGCATCAGTTTTGACCGCTGGAGAAAGGCTTGGGGAATGTGGCATCTC
Mr(Co16)        GGTTGACGGGTCAGCATCAGTTTTGACCGCTGGAGAAAGGCTTGGGGAATGTGGCATCTC
Mr(Co8)         GGTTGACGGGTCAGCATCAGTTTTGACCGCTGGAGAAAGGCTTGGGGAATGTGGCATCTC
Mr(E43)         GGTTGACGGGTCAGCATCAGTTTTGACCGCTGGAGAAAGGCTTGGGGAATGTGGCATCTC
Mr(AY194150)    GGTTGACGGGTCAGCATCAGTTTTGACCGCTGGAGAAAGGCTTGGGGAATGTGGCATCTC
CP(AF335590)    GGATGACGGGTCAGCATCAGTTTTGACCGTCGGAAAAAGGCTTAGGGAATGTGGCATCTC

Bm(AY038329)    GGTCGACGGGTCAACATCAGTTTTGACCGTTGGATAAAGTCTTTGGGAATGTGGCATCTT
Bm(AY038310)    GGTTGACGGGTCAGCATCAATTTTGACCGTTGGATAAAGTCCAGGGGAATGTGGCATCTT
Bm(HCU66435)    GGTTGATGGGTCAGCATCAATTTTGACCGTTGGATAAAGGTTGGGGGAATGTGGCATCTT
Bm(ORU66451)    GGTTGACGGGTCAGCATCAATTTTGACCGTTGGAAAAAAGTTGGGGGAATGTGGCATCTT
Bm(AF261467)    GGTCGACGGGTCAGCATCAATTTTGACCGCTGGATAAAGGTCAGGGGAATGTGGCATCCT

                    490        500        510        520        530        540
Mr(Co5)         CGGATGTGTTATAGCCTCTTGTCGTATACAGCGGTTGGGACTGAGGAACTCAGCACGCCG
Mr(Co16)        CGGATGTGTTATAGCCTCTTGTCGTATACAGCGGTTGGGACTGAGGAACTCAGCACGCCG
Mr(Co8)         CGGATGTGTTATAGCCTCTTGTCGTATACAGCGGTTGGGACTGAGGAACTCAGCACGCCG
Mr(E43)         CGGATGTGTTATAGCCTCTTGTCGTATACAGCGGTTGGGACTGAGGAACTCAGCACGCCG
Mr(AY194150)    CGGATGTGTTATAGCCTCTTGTCGTATACAGCGGTTGGGACTGAGGAACTCAGCACGCCG
CP(AF335590)    CGGATGTGTTATAGCCCTTTGTCGTATACGGCGGTTGGGACTGAGGAACTCAGCACGCCG

Bm(AY038329)    AGGATGTGTTATAGCCCTTTGTCGCATACAACGGTTGGGACTGAGGAACTCAGCGCGCCG
Bm(AY038310)    CGGATGTGTTATAGCCCTTGGTCGCATACAACGGTTGGGATTGAGGAACTCAGCACGCCG
Bm(HCU66435)    CGGATGTGTTATAGCCCTTAGTTGTATACAACGGTTGGGATTGAGGAACTCAGCACGCCG
Bm(ORU66451)    CGGATGTGTTATAGCCCCTGATTGTATACAACGGTTGGGATTGAGGAACTCAGCAT.CCG
Bm(AF261467)    CGGATGTGTTATAGCCTCTGGCTGCATACAGCGGTTGGGATTGAGGAACTCAGCACGCCG

                    550        560        570        580        590        600
Mr(Co5)         CAAGGCCGGGTTTTTA-ACCACGTT-CGTGCTTAGGATGCTGGCATAA-TGGCTTTAAGC
Mr(Co16)        CAAGGCCGGGTTTTTA-ACCACGTT-CGTGCTTAGGATGCTGGCATAA-TGGCTTTAAGC
Mr(Co8)         CAAGGCCGGGTTTTTA-ACCACGTT-CGTGCTTAGGATGCTGGCATAA-TGGCTTTAAGC
Mr(E43)         CAAGGCCGGGTTTTTA-ACCACGTT-CGTGCTTAGGATGCTGGCATAA-TGGCTTTAAGC
Mr(AY194150)    CAAGGCCGGGTTTTTA-ACCACGTT-CGTGCTTAGGATGTTGGCATAA-TGGCTTTAAGC
CP(AF335590)    CAAGGCCGGGTTTTTA-ACCACGTT-CGTGCTTAGGATGTTGGCATAA-TGGCTTTAAGC

Bm(AY038329)    CAAGGCCGGGTCTTTG-ACCACGTT-CGCGCTTAGGATGTTGGCATAA-TGGCTTTAATC
Bm(AY038310)    CAAGGCCGGGTACTTGTACCACGTT-CGTGCTTAGGATGCTGGCATAA-TGGCTTTAATC
Bm(HCU66435)    CAAGGCCGGG-GTTTAC-CCACGTT-CGTGCTTAGGATGCTGGCATAA-TGGCTTTAATC
Bm(ORU66451)    CAAGGACGGGT-TTTA-ACCACGTAACATGCTTAGGATGCTGGCAAAA-TGGCTTTAATC
Bm(AF261467)    CAAGGCCGGGTTTTTA-ACCACGTA-CGTGCTTAGGATGCTGGCATAA-TGGCTTTAATC

                    610        620        630        640        650        660
Mr(Co5)         GACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGCTTGCGAGTGTTTGGGTGGAAAA
Mr(Co16)        GACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGCTTGCGAGTGTTTGGGTGGAAAA
Mr(Co8)         GACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGCTTGCGAGTGTTTGGGTGGAAAA
Mr(E43)         GACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGCTTGCGAGTGTTTGGGTGGAAAA
Mr(AY194150)    GACCCGTCTTGAAACAC----------------------------------------
CP(AF335590)    GACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGCTTGCGAGTGTTTGGGTGGAA--

Bm(AY038329)    GACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGCCTGCGAGTGTTTGGGTGGAAAA
Bm(AY038310)    GACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGCCTGCGAGTGTTTGGGTGGAAAA
Bm(HCU66435)    GACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGCCTGCGAGTGTTTGGGTGGAAAA
Bm(ORU66451)    GACCCGTCTTGAA.CACGGACCAAGGAGTCTAACATGCCTGCGAGTGTTTGGGTGGTAAA
Bm(AF261467)    GACCCGTCTTGAAACACGGACCAAGGAGTCTAACATGCCCGCGAGTGTTTGGGTGGAAAA
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Continuation Table A5.2

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                    670       680       690       700       710       720
Mr(Co5)         CCCGAGCGCGTAATGAAAGTGAAAGTTGGGATCTCTGTCGTGGAGAGCACCGACGCCCGG
Mr(Co16)        CCCGAGCGCGTAATGAAAGTGAAAGTTGGGATCTCTGTCGTGGGGAGCACCGACGCCCGG
Mr(Co8)         CCCGAGCGCGTAATGAAAGTGAAAGTTGGGATCTCTGTCGTGGGGAGCACCGACGCCCGG
Mr(E43)         CCCGAGCGCGTAATGAAAGTGAAAGTTGGGATCTCTGTCGTGGAGAGCACCGACGCCCGG

Bm(AY038329)    CCCGAGCGCATAATGAAAGTGAAAGTTGAGATCCCTGTCGTGGGGAGCATCGACGCCCGG
Bm(AY038310)    CTCGGACGCGTAATGAAAGTGAAAGTTGAGATCCCTGTCGCGGGGAGCATCGACGCCCGG
Bm(HCU66435)    CCCGAGCGCGTAATGAAAGTGAAAGTTGAGATCCCTGTCGTGGGGAGCATCGACGCCCGG
Bm(ORU66451)    CCCGAGCGCACAATGAAAGTGAAAGTTGAGATCTCTGTCGTGGAGAGCATCGACGCCCGG
Bm(AF261467)    CCCGAGCGCGTAATGAAAGTGAAAGTTGAGATCTCTGTCGTGGAGAGCATCGACGCCCGG

                    730       740       750       760       770       780
Mr(Co5)         ACCTGATGTTTACTGACGGATCTGCGGTAGAGCACGTATGTTGGGACCCGAAAGATGGTG
Mr(Co16)        ACCTGATGTTTACTGACGGATCTGCGGTAGAGCACGTATGTTGGGACCCGAAAGATGGTG
Mr(Co8)         ACCTGATGTTTACTGACGGATCTGCGGTAGAGCACGTATGTTGGGACCCGAAAGATGGTG
Mr(E43)         ACCTGATGTTTACTGACGGATCTGCGGTAGAGCACGTATGTTGGGACCCGAAAGATGGTG

Bm(AY038329)    ACCAGACCTTTTGTGACGGATCCGCGGTAGAGCATGTGTGTTGGGACCCGAAAGATGGTG
Bm(AY038310)    ACCAGACCTTTTGTGACGGATCCGCGGTAGAGCATGTATGTTGGGACCCGAAAGATGGTG
Bm(HCU66435)    ACCAGACCTTTTGTGACGGATCCGCGGTTGAGCATGTATGTTGGGACCCGAAAGATGGTG
Bm(ORU66451)    ACCAGACCTTTTGTGACGGTCCCGCGGTAGAGCATGTATGTTGGGACCCGAAAGATGGTG
Bm(AF261467)    ACCAGACCTTCTGTGACGGATCCGCGGTAGAGCGTGTATGTTGGGACCCGAAAGATGGTG

                    790       800       810       820       830       840
Mr(Co5)         AACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCTCGTAGCGATTCTG
Mr(Co16)        AACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCTCGTAGCGATTCTG
Mr(Co8)         AACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCTCGTAGCGATTCTG
Mr(E43)         AACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCTCGTAGCGATTCTG

Bm(AY038329)    AACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCTCGTAGCGATTCTG
Bm(AY038310)    AACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCTCGTAGCGATTCTG
Bm(HCU66435)    AACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCTCGTAGCGATTCTG
Bm(ORU66451)    AACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCTCGTAGCGATTCTG
Bm(AF261467)    AACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCTCGTAGCGATTCTG

                    850       860       870       880       890       900
Mr(Co5)         ACGTGCAAATCGATCGTCGAATTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGT
Mr(Co16)        ACGTGCAAATCGATCGTCGAATTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGT
Mr(Co8)         ACGTGCAAATCGATCGTCGAATTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGT
Mr(E43)         ACGTGCAAATCGATCGTCGAATTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGT

Bm(AY038329)    ACGTGCAAATCGATCGTCAAATTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGT
Bm(AY038310)    ACGTGCAAATCGATCGTCAAATTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGT
Bm(HCU66435)    ACGTGCAAATCGATCGTCGAATTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGT
Bm(ORU66451)    ACGTGCAAATCGATCGTCGAATTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGT
Bm(AF261467)    ACGTGCAAATCGATCGTCGAATTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGT

                    910       920       930       940       950       960
Mr(Co5)         AGCTGGTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAAACTCATTGTCAGATTTATGTGG
Mr(Co16)        AGCTGGTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAAACTCATTGTCAGATTTATGTGG
Mr(Co8)         AGCTGGTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAAACTCATTGTCAGATTTATGTGG
Mr(E43)         AGCTGGTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAAACTCATTGTCAGATTTATGTGG

Bm(AY038329)    AGCTGGTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAAACTCATT-TCAGATTTATGTGG
Bm(AY038310)    AGCTGGTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAAACTCATT-TCAGATTTATGTGG
Bm(HCU66435)    AGCTGGTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAAACTCAT-GTCAGATTTATGTGG
Bm(ORU66451)    AGCTGGTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAAACTCAT-GTCAGATTTATGTGG
Bm(AF261467)    AGCTGGTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAAACTCATTATCAGATTTATGTGG
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Continuation Table A5.2

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                    970       980       990       1000      1010      1020
Mr(Co5)         TAAAGCGAATGATTAGAGGCCTTGGGGTTGTAACAACCTTAACCTATTCTCAAACTTTAA
Mr(Co16)        TAAAGCGAATGATTAGAGGCCTTGGGGTTGTAACAACCTTAACCTATTCTCAAACTTTAA
Mr(Co8)         TAAAGCGAATGATTAGAGGCCTTGGGGTTGTAACAACCTTAACCTATTCTCAAACTTTAA
Mr(E43)         TAAAGCGAATGATTAGAGGCCTTGGGGTTGTAACAACCTTAACCTATTCTCAAACTTTAA

Bm(AY038329)    TAAAGCGAATGATTAGAGGCCTTGGGGTTGAAACAACCTTAACCTATTCTCAAACTTTAA
Bm(AY038310)    TAAAGCGAATGATTAGAGGCCTTGGGGTTGAAACAACCTTAACCTATTCTCAAACTTTAA
Bm(HCU66435)    TAAAGCGAATGATTAGAGGCCTTGGGGTTGAAACAACCTTAACCTATTCTCAAACTTTAA
Bm(ORU66451)    TAAAGCGAATGATTAGAGGCCTTGGGGTTGAAACAACCTTAACCTATTCTCAAACTTTAA
Bm(AF261467)    TAAAGCGAATGATTAGAGGCCTTGGGGTTGAAACAACCTTAACCTATTCTCAAACTTTAA

                    1030      1040      1050      1060      1070      1080
Mr(Co5)         ATATGTAAGAACGAGCCGTCTCTTAATT-GGACCGCTCGGCGATTGAAGAGTTTCTAGTG
Mr(Co16)        ATATGTAAGAACGAGCCGTCTCTTAATT-GGACCGCTCGGCGATTGAAGAGTTTCTAGTG
Mr(Co8)         ATATGTAAGAACGAGCCGTCTCTTAATT-GGACCGCTCGGCGATTGAAGAGTTTCTAGTG
Mr(E43)         ATATGTAAGAACGAGCCGTCTCTTAATTTGGACCGCTCGGCGATTGAAGAGTTTCTAGTG

Bm(AY038329)    ATATGTAAGAACGAGCCGTCTCTTGATT-GGACCGCTCGGCGATTGA-GAGTTTCTAGTG
Bm(AY038310)    ATATGTAAGAACGAGCCGTCTCTTGATT-GGACCGCTCGGCGATTGA-GAGTTTCTAGTG
Bm(HCU66435)    ATATGTAAGATCGAGCCGTCTCTTGATT-GGACCGCTCG.CGATTGA-GAGTTTCTAGTG
Bm(ORU66451)    ATATGTAAGAACGAGCCGTCTCTTGATT-GGACCGCTCGGCGATTGA-GAGTTTCTAGTG
Bm(AF261467)    ATATGTAAGAACGAGCCGTCTCTTGATT-GGACCGCTCGGCGATTGA-GAGTTTCTAGTG

                    1090      1100      1110      1120      1130      1140
Mr(Co5)         GGCCATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAACCGAACGCGAGGTTAAGGTGC
Mr(Co16)        GGCCATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAACCGAACGCGAGGTTAAGGTGC
Mr(Co8)         GGCCATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAACCGAACGCGAGGTTAAGGTGC
Mr(E43)         GGCCATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAACCGAACGCGAGGTTAAGGTGC

Bm(AY038329)    GGCCATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAACCGAACGCGAGGTTAAGGTGC
Bm(AY038310)    GGCCATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAACCGAACGCGAGGTTAAGGTGC
Bm(HCU66435)    G-CCATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAACCGAACGTGAGGTTAAGGTGC
Bm(ORU66451)    GGCCATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAACCGAACGCGAGGTTAAGGTGC
Bm(AF261467)    GGCCATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAACCGAACGCGAGGTTAAGGTGC

                    1150      1160      1170      1180      1190      1200
Mr(Co5)         CGGAATTCACGCTCATCAGACACTACAAAAGGTGTTAGTTCATCTAGACAGCAGGACGGT
Mr(Co16)        CGGAATTCACGCTCATCAGACACTACAAAAGGTGTTAGTTCATCTAGACAGCAGGACGGT
Mr(Co8)         CGGAATTCACGCTCATCAGACACTACAAAAGGTGTTAGTTCATCTAGACAGCAGGACGGT
Mr(E43)         CGGAATTCACGCTCATCAGACACTACAAAAGGTGTTAGTTCATCTAGACAGCAGGACGGT

Bm(AY038329)    CGGAATTCACGCTCATCAGACACCACAAAAGGTGTTAGTTCATCTAGACAGCAGGACGGT
Bm(AY038310)    CGGAATTCACGCTCATCAGACACCACAAAAGGTGTTAGTTCATCTAGACAGCAGGACGGT
Bm(HCU66435)    CGGAATTCACGCTCATCAGACACCACAAAAGGTGTTAGTTCATCTAGACAGCAGGACGGT
Bm(ORU66451)    CGGAATTTACGCTCATCAGACACCACAAAAGGTGTTAGTTCATCTAGACAGCAGGACGGT
Bm(AF261467)    CGGAATTCACGCTCATCAGACACCACAAAAGGTGTTAGTTCATCTAGACAGCAGGACGGT

                    1210      1220      1230      1240      1250      1260
Mr(Co5)         GGCCATGGAAGTCGGAACCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAATGAACTAG
Mr(Co16)        GGCCATGGAAGTCGGAACCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAATGAACTAG
Mr(Co8)         GGCCATGGAAGTCGGAACCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAATGAACTAG
Mr(E43)         GGCCATGGAAGTCGGAACCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAATGAACTAG

Bm(AY038329)    GGCCATGGAAGTCGGAATCCGCTAAGGAATGTGTAACAACTCACCTGCCGAATGAACTAG
Bm(AY038310)    GGCCATGGAAGTCGGAATCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAATGAACTAG
Bm(HCU66435)    GGCCATGGAAGTCGGAACCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAATGAACTAG
Bm(ORU66451)    GGCCATGGAAGTCGGAATCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAATGAACTAG
Bm(AF261467)    GGCCATGGAAGTCGGAATCCGCTA.GGAGTGTGTAACAACTCACCTGCCGAATGAACTAG
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Continuation Table A5.2

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                    1270      1280       1290      1300       1310      1320
Mr(Co5)         CCCTGAAAATGGATGGCGCTTAAGCGTGATACCCATACCTCGCCGTCAGCGTTGAAGTGA
Mr(Co16)        CCCTGAAAATGGATGGCGCTTAAGCGTGATACCCATACCTCGCCGTCAGCGTTGAAGTGA
Mr(Co8)         CCCTGAAAATGGATGGCGCTTAAGCGTGATACCCATACCTCGCCGTCAGCGTTGAAGTGA
Mr(E43)         CCCTGAAAATGGATGGCGCTTAAGCGTGATACCCATACCTCGCCGTCAGCGTTGAAGTGA

Bm(AY038329)    CCCTGAAAATGGATGGCGCTTAAGCGTGATACCCATACCTCGCCGTCAGCGTTGAAGTGA
Bm(AY038310)    CCCTGAAAATGGATGGCGCTTAAGCGTGATACCCATACCTCGCCGTCAGCGTTGAAGTGA
Bm(HCU66435)    CCCTGAAAATGGATGGCGCTCAAGCGTGATACCCATACCTCACCGTCAGTGTTAAAGTGA
Bm(ORU66451)    CCCTGAAAATGGATGGCGCTTAAGCGTGATACCCATACCTCGCCGTCAGCGTTGAAGTGA
Bm(AF261467)    CCCTGAAAATGGATGGCGCTTAAGCGTGATACCCATACCTCGCCGTCAGCGTTGAAGTGA

                    1330      1340       1350      1360       1370      1380
Mr(Co5)         CGCGCT-GACGAGTAGGCAGGCGTGGAGGTCGGTGAAGAAGCCTTGGCAGCGATGCTGGG
Mr(Co16)        CGCGCT-GACGAGTAGGCAGGCGTGGAGGTCGGTGAAGAAGCCTTGGCAGCGATGCTGGG
Mr(Co8)         CGCGCT-GACGAGTAGGCAGGCGTGGAGGTCGGTGAAGAAGCCTTGGCAGCGATGCTGGG
Mr(E43)         CGCGCT-GACGAGTAGGCAGGCGTGGAGGTCGGTGAAGAAGCCTTGGCAGCGATGCTGGG

Bm(AY038329)    CGCGCT-GACGAATAGGCAGGCGTGGAGGTCAGTGAAAAAACCTTGGCAGTGATGCTGGG
Bm(AY038310)    CCCGCTTGACGAGTAGGCAGGCGTGGAGGTCAGTGAAAAA--------------------
Bm(HCU66435)    TGCGCT-GACGAGTAGGCAGGCGTGGAGGTCAGTGAAGAAGCCTCGGCAGTGATGCTGGG
Bm(ORU66451)    CGCGCT-GACGAGTAGGCAGGCGT.GAGGTCAGTGAAGAAGCCTTGGCAGTGATGCTGGG
Bm(AF261467)    C------------------------------------------------------------
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Table A5.3 Aligned mtSSU-rDNA sequences of 26 isolates of *M. roreri* and three of *C. perniciosa*.

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                    10        20        30        40        50        60
M. roreri 1/        GCTAACTGAAATCACATTTTGATCTTCGGGAGAAGTGATAAATGTCCATAGTAAGGGAAA
Cp (Dis43) 2/       GCTAACTGAAATCACATTTTGATCTTCGGGAGAAGTGATAAATGTCCATAGTAAGGGAAA
Cp (Dis70)          GCTAACTGAAATCACATTTTGATCTTCGGGAGAAGTGATAAATGTCCATAGTAAGGGAAA
Cp (Dis71)          GCTAACTGAAATCACATTTTGATCTTCGGGAGAAGTGATAAATGTCCATAGTAAGGGAAA

                    70        80        90        100       110       120
M. roreri           AAAATGATGCTACCTTACTCTTAGTGTTGTCCAAATCTGGTGCCAGAAGACTCGGTAAGG
Cp (Dis43)          AAAATGATGCTACCTTACTCTTAGTGTTGTCCAAATCTGGTGCCAGAAGACTCGGTAAGG
Cp (Dis70)          AAAATGATGCTACCTTACTCTTAGTGTTGTCCAAATCTGGTGCCAGAAGACTCGGTAAGG
Cp (Dis71)          AAAATGATGCTACCTTACTCTTAGTGTTGTCCAAATCTGGTGCCAGAAGACTCGGTAAGG

                    130       140       150       160       170       180
M. roreri           CCAGGAACGCGAACGTTAGTCACATTGACCAGGCGTAAAGGGTATGTAGGCAGCTTGAAA
Cp (Dis43)          CCAGGAACGCGAACGTTAGTCACATTGACCAGGCGTAAAGGGTATGTAGGCAGCTTGAAA
Cp (Dis70)          CCAGGAACGCGAACGTTAGTCACATTGACCAGGCGTAAAGGGTATGTAGGCAGCTTGAAA
Cp (Dis71)          CCAGGAACGCGAACGTTAGTCACATTGACCAGGCGTAAAGGGTATGTAGGCAGCTTGAAA

                    190       200       210       220       230       240
M. roreri           GTTTTATTTTTATCATAGCTATTTCAATTTAAAATAAATAATTT ---- ---- TAAATATT
Cp (Dis43)          GTTTTATTTTTATCATAGCTATTTCAATTTAAAATAAATAATTTAATAATTTTAAATATT
Cp (Dis70)          GTTTTATTTTTATCATAGCTATTTCAATTTAAAATAAATAATTTAATAATTTTAAATATT
Cp (Dis71)          GTTTTATTTTTATCATAGCTATTTCAATTTAAAATAAATAATTTAATAATTTTAAATATT

                    250       260       270       280       290       300
M. roreri           TATTTTTTT------GATTTTAAAAATAAAATGAATTAAAGATAGAATCAAATTGAGGAT
Cp (Dis43)          TATTTTTTTTTTTTTGATTTTAAAAATAAAATGAATTAAAGATAGAATCAAATTGAGGAT
Cp (Dis70)          TATTTTTTTTTTTT-GATTTTAAAAATAAAATGAATTAAAGATAGAATCAAATTGAGGAT
Cp (Dis71)          TATTTTTTTTTTTT-GATTTTAAAAATAAAATGAATTAAAGATAGAATCAAATTGAGGAT

                    310       320       330       340       350       360
M. roreri           AAGCCGAATAATATTTAGAGTAGGGATTAAATATGCAAATACTAAATGGAATACTAAAGG
Cp (Dis43)          AAGCCGAATAATATTTAGAGTAGGGATTAAATATGCAAATACTAAATGGAATACTAAAGG
Cp (Dis70)          AAGCCGAATAATATTTAGAGTAGGGATTAAATATGCAAATACTAAATGGAATACTAAAGG
Cp (Dis71)          AAGCCGAATAATATTTAGAGTAGGGATTAAATATGCAAATACTAAATGGAATACTAAAGG

                    370       380       390       400       410       420
M. roreri           TGAAAGCTTTTTATCTAATATTGATCGACGCTGAGATACTAAGGTGGGAAAAGGAAATAG
Cp (Dis43)          TGAAAGCTTTTTATCTAATATTGATCGACGCTGAGATACTAAGGTGGGAAAAGGAAATAG
Cp (Dis70)          TGAAAGCTTTTTATCTAATATTGATCGACGCTGAGATACTAAGGTGGGAAAAGGAAATAG
Cp (Dis71)          TGAAAGCTTTTTATCTAATATTGATCGACGCTGAGATACTAAGGTGGGAAAAGGAAATAG

                    430       440       450       460       470       480
M. roreri           GATTAGAGACCCTGGTACCTCCCACAGTCAACGATGAATGGTGGATTATTAGTTTTATTT
Cp (Dis43)          GATTAGAGACCCTGGTACCTCCCACAGTCAACGATGAATGGTGGATTGTTAGTTTTATTT
Cp (Dis70)          GATTAGAGACCCTGGTACCTCCCACAGTCAACGATGAATGGTGGATTGTTAGTTTTATTT
Cp (Dis71)          GATTAGAGACCCTGGTACCTCCCACAGTCAACGATGAATGGTGGATTGTTAGTTTTATTT

                    490       500       510       520       530       540
M. roreri           AAAACTAGTACCGATGTTAACGCGATAACCATTCCGCCTTGAGAGTACGGTTGCAAAACT
Cp (Dis43)          AAAACTAGTACCGATGTTAACGCGATAACCATTCCGCCTTGAGAGTACGGTTGCAAAACT
Cp (Dis70)          AAAACTAGTACCGATGTTAACGCGATAACCATTCCGCCTTGAGAGTACGGTTGCAAAACT
Cp (Dis71)          AAAACTAGTACCGATGTTAACGCGATAACCATTCCGCCTTGAGAGTACGGTTGCAAAACT

                    550       560       570       580
M. roreri           GGAACCAAAAAAATTAGTCGGTCTCGGAGCAAACGAAGTGAAGCATG
Cp (Dis43)          GGAACCAAAAAAATTAGTCGGTCTCGGAGCAAACGAAGTGAAGCATG
Cp (Dis70)          GGAACCAAAAAAATTAGTCGGTCTCGGAGCAAACGAAGTGAAGCATG
Cp (Dis71)          GGAACCAAAAAAATTAGTCGGTCTCGGAGCAAACGAAGTGAAGCATG
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1/ ***M. roreri***: sequence observed in the whole set of isolates analysed: Co5, Co16, Co7, Co9, Co17, E42, E43, Co2, E2, E32, E37, P1, V2, Co8, Co13, E12, E16, E20, Pa7, Pa8, C3, C17, C19, N1 and H1.

2/ **Cp** = ***C. perniciosa*** sequences. **Dis43** from *T. bicolor* (Peru); **Dis70** from *Liana* sp. (Ecuador) and **Dis71** from *T. cacao* (Ecuador).

CHAPTER 6

Evaluation of cacao (*Theobroma cacao* L.) clones against seven Colombian isolates of *Moniliophthora roreri* (Cif.) Evans *et al.*, representing the global genetic diversity of the fungus.

6.1 ABSTRACT

The virulence of seven Colombian isolates of *Moniliophthora roreri* was determined by artificially inoculating pods of five cacao genotypes (ICS-1, ICS-95, TSH-565, SCC-61, and CAP-34) in La Suiza Experimental Farm (500 m.a.s.l., 25°C, 2,000 mm), Santander Department, Colombia. The isolates used for this work represent the global genetic diversity of the pathogen.

Isolates were evaluated by spraying two- to three-month-old pods of each genotype with a spore suspension. The following variables were determined nine weeks after inoculation: a) Disease incidence (DI), b) External Severity (ES) and c) Internal Severity (IS). IS was found to be of greatest value in classifying the reaction of the host genotype against *M. roreri*. Highly significant positive correlations were found between IS and both the ES ($r = 0.92$, 111 degrees of freedom) and DI ($r = 0.64$, 111 degrees of freedom).

Genetic variation detected between isolates was not matched by similar diversity in their virulence. Indeed, there was apparently only limited variation in virulence between isolates, with all isolates showing high levels of virulence against most cacao genotypes and only two isolates evoking slightly reduced IS and ES symptoms. It follows that, under appropriate conditions, all isolates are capable of inflicting similarly high levels of damage to cacao pods. Isolates Co5 (Rionegro, Santander Department), and in particular Co1 (Zulia, North of Santander Department) registered the lowest average values for the three measures of infection used, but nevertheless caused significant pod losses.

The seven *M. roreri* isolates induced the disease in all clones although there was considerable variation in the IS and ES. The clones could be categorised into two

statistically distinctive groups (Duncan Test, $P<0.05$) on the basis of their differential resistance reactions. Clone ICS-95 exhibited a significant resistant reaction against all the isolates ($IS \leq 1.25$) whereas most of the remaining clones were either susceptible ($IS \geq 3.76$) or moderately susceptible ($3.75 \geq IS \geq 2.51$). An intermediate condition was found in clone SCC-61, although it was only possible to inoculate this genotype using four isolates.

The identification of one cultivated clone (ICS-95) displaying some level of resistance against all 7 isolates, together with a moderately resistant cultivated clone (SCC-61) with good field resistance is highly relevant since it opens the possibility to select genotypes with a durable resistance.

Keywords: artificial inoculation, cacao, Colombia, disease resistance, frosty pod disease, moniliasis, *Moniliophthora roreri*, *Theobroma cacao*.

6.2 INTRODUCTION

Moniliasis or frosty pod rot of cacao is caused by the fungus *M. roreri* (Cif.) Evans *et al.* and is one of the most destructive diseases of cacao in Latin America. The dramatic social and economical consequences caused by the fungus in different countries (Rorer, 1926; Enríquez *et al.*, 1982; Krauss & Soberanis, 2001; Anon., 2001) clearly demonstrate the scale of damage that could result from the eventual dispersal of *M. roreri* into the major cacao producing countries.

For close to a century, various cultural, chemical and biological strategies have been tested for the control of moniliasis and, whilst some of these measures have been very effective on an experimental scale (Barros, 1980; Porras *et al*., 1990; Argüello, 2000a; Krauss & Soberanis, 2001), none have been widely adopted by the smallholders that typically grow cacao. The frequency and cost requirements of these practices have played a major part in discouraging their use, particularly during the long periods of low cacao prices. A similar situation has been described for other cacao diseases (Pereira, 1996).

An integrated and 'environmentally-friendly' approach, based on the use of improved genotypes, is a feasible strategy to obtain long-term control of cacao diseases for smallholder farmers. Resistant genotypes could provide a more durable, permanent and less costly control, which could be used with other complementary control measures. There has already been some progress towards identifying cacao material with field resistance to *M. roreri*, with resistant genotypes being reported from several countries (Delgado *et al*., 1960; Suárez-Capello, 1996; Argüello, 1997; Phillips-Mora, 1996; Arévalo *et al.*, 1996). There is also at least one breeding programme currently focussing on the development of resistant material (Phillips-Mora, 1996).

The use of genotypes with a suitable combination of desirable properties, in particular resistance and high yields, can significantly reduce disease incidence and increase the yield, as has been observed on the Atlantic coast of Costa Rica under an high natural inoculum pressure of *M. roreri.* Clones with a moderate level of resistance against the fungus, such as CC-137 and EET-183, registered a disease incidence close to 45% and a seven-year average yield of 998 and 725 kg ha^{-1} yr^{-1}, respectively, whilst the widely used international clones Pound-7, UF-613 and Catongo had a disease incidence higher than 84% and yields lower than 178 kg ha^{-1} yr^{-1} (A. Mora and W. Phillips, CATIE, Costa Rica, unpublished results).

Extensive knowledge of the genetic variability of pathogens is an important aid for the selection of clones that have the potential for durable and stable resistance in the field. This is particularly important for perennial crops such as cacao since individual plants may remain in production for more than 25 years under constant disease pressure. Zadocks (1997) analysed the world status of disease resistance in cacao and concluded that variability of the different cacao pathogens is often considerable but has yet to be satisfactorily explored. This author considered pathotype variability to be of utmost importance to cacao growers since, at any time, it may undermine their livelihood by imposing a 'boom-and-bust' economic cycle.

There has been extensive research on the genetic basis of the interaction between varieties of important crop plants and strains of their major fungal pathogens (Carlile & Watkinson, 1994). However, there are relatively few studies on the reaction of cacao genotypes

against different isolates of any pathogen of this crop given its economic importance. Phillips & Galindo (1991) reported a significant interaction between cacao clones and different isolates of *Phytophthora palmivora*. Surujdeo-Maharaj *et al.* (2001) similarly investigated the resistance of leaves from 18 cacao genotypes to 10 isolates of *P. palmivora* and found a 5-to 6-fold difference in aggressiveness among isolates. In contrast to the earlier work, however, the host genotype x isolate interaction was not significant.

There have also been several notable studies on other pathogens of cacao including *Crinipellis perniciosa* (Wheeler & Mepsted, 1988; Laker, 1990; Wheeler, 1996) and *Oncobasidium theobromae*, causal pathogens of witches´ broom disease and vascular streak dieback (VSD) (Bong *et al.*, 1996), respectively. Wheeler & Mepsted (1988) reported a considerable variation amongst isolates of *C. perniciosa* from six tropical American countries in their ability to induce disease symptoms. The authors suggested that two pathotypes of the fungus exist on cultivated cacao each with a particular geographical distribution and different capability to induce symptoms on SCA-6, a traditionally highly resistant clone to *C. perniciosa*. Laker (1990) found significant differences between isolates of *C. perniciosa* collected in Trinidad for the morphological reactions caused on cacao seedlings, however, those differences were identified on a open-pollinated progeny but not confirmed on clonal plants. He concluded that only one pathotype of *C. perniciosa* is present in Trinidad for which clone SCA-6 was highly resistant (Laker, 1990). Wheeler (1996) investigated the interactions between different cacao genotypes and five isolates of this pathogen. He reported a fairly constant ranking of isolates of the fungus in relation to the limited range of cacao types tested. Finally, Bong *et al.* (1996) investigated the incidence of disease in eleven cacao genotypes when challenged with eleven isolates of *Oncobasidium theobromae* and found significant interaction between the cacao clones and isolates of the pathogen. To date, however, there has been no study of this kind for *M. roreri*.

Information on the genetic affinities of 96 *M. roreri* isolates in this thesis (Chapter 4) has allowed the selection of seven isolates that are representative of the genetic variability of the species. In this chapter, these seven isolates were used to challenge five diverse cultivated clones of cacao.

6.3 MATERIALS AND METHODS

6.3.1 EXPERIMENT LOCATION

The experiment was carried out at *La Suiza (Salamaga)* Experimental Farm in different steps from June to December 2001. La Suiza is located in the Municipality of Rionegro, 33 km from Bucaramanga, the capital of the Department of Santander, Colombia. La Suiza is situated at 500 m.a.s.l., 7° 22´ north and 73° 10´ west. Annual average temperature is 25°C, the annual precipitation is 2,000 mm, and the mean relative humidity 77%. This 32.6 hectare experimental farm is owned by the Colombian Corporation for Agricultural and Livestock Research (CORPOICA) and houses an important cacao germplasm collection that contains approximately 250 local and 100 introduced clones.

6.3.2 DESCRIPTION OF THE EXPERIMENTAL MATERIAL

Seven isolates of *M. roreri* representing the genetic diversity of the pathogen in tropical America, the only continent where this pathogen presently occurs, were selected for this study (Table 6.1). These isolates represent most of the major genetic groupings identified by cluster analysis generated using AFLP and ISSR data, and described in Chapter 4. The one group not represented comprised two Ecuadorian genotypes from *Theobroma gileri*. These samples were omitted from the study because of quarantine restrictions.

Table 6.1 Origin and genetic group of the Colombian isolates of *M. roreri* inoculated onto five cacao clones in Santander, Colombia.

Isolate	Municipality	Department	Genetic group
Co1	Zulia	North of Santander	Bolívar
Co5	Rio Negro	Santander	Co-East
Co8	San Jerónimo	Antioquia	Co-West
Co10	Palestina	Caldas	Co-Central
Co13	Carepa, Urabá	Antioquia	Co-West
Co14	San Vicente de Chucurí	Santander	Co-East
Co16	El Carmen de Chucurí	Santander	Co-East

The cacao clones ICS-95, SCC-61, TSH-565, CAP-34 and ICS-1 were selected for evaluation against the *M. roreri* isolates because of their current economic importance in Colombia, informal reports of differential field reactions against *M. roreri* and the availability of sufficient trees at La Suiza for meaningful field tests of resistance. Some features of the selected clones based on field observations made by Argüello (2000b, c) are shown in Table 6.2.

Table 6.2 Characteristics of five cacao clones evaluated against different isolates of *M. roreri* in Santander, Colombia. [1/]

Trait	TSH-565	ICS-1	CAP-34	ICS-95	SCC-61
Geographic origin					
	Trinidad	Imperial College, Trinidad	Pichilingue, Ecuador	Imperial College, Trinidad	Chucurí, Santander, Colombia
Reaction against cacao pathogens in Santander, Colombia [2/]					
M. roreri	S	MS	MR	R	R
Crinipellis perniciosa	T	MS	S	T	R
***Phytophthora* sp.**	S	S	S	S	R
Ceratocystis fimbriata	T	MS	R	S	R
***Rosellinia* spp.**	S	S	S	S	
Other traits					
Self-compatibility [3/]	-	+	-	+	+
Pod colour	Red	Red	Red	Red	Red
Pod shape	Angoleta	Amelonado	Cundeamor	Amelonado	Oblong
Seeds pod^{-1}	39	40	40	41	41
Seed shape	Cylindrical	Cylindrical	Cylindrical	Cylindrical	Oval
Seed colour	Purple	Purple	Purple	Purple	Purple
Pods/tree/year	55	46	47	60	137
Kg/tree/year	2.3	2.4	3.1	3.3	10.5
Pod index [4/]	24	19	15	18	13
Seed weight (g)	1.1	1.3	1.8	1.4	2.1
Recommended altitude (m.a.s.l.)	> 800	> 800	> 400	100-1.200	Not available

[1/] Modified from Argüello (2000b, 2000c).

[2/] Disease reaction: R = resistant, T = tolerant, MR = moderately resistant, MS = moderately susceptible, S = susceptible
[3/] '-' = self-incompatible; '+' = self-compatible
[4/] Pod index = number of pods to prepare 1 kg of dry cacao.

6.3.3 ARTIFICIAL INOCULATION

Artificial pollination was carried out in the whole set of clones during the period June/July 2001 in order to increase the availability of pods for the inoculations. Artificial inoculations were performed using the methodology described by Phillips-Mora (1996) with the following minor modifications:

Isolates were grown in plastic Petri dishes (5.2 cm diameter) containing 20% v/v, V8 juice (Campbell Soup Company, Camden, NJ, USA), 0.1% w/v asparagine, 2% w/v maltose and 1.8% w/v agar culture medium for 11 d. Cultures were maintained in an incubator at constant 24°C and with 12/12 alternate periods of light/darkness. Light was provided by fluorescent lamps.

Immediately before inoculation, a spore suspension was prepared for each isolate by adding in three steps, 20 ml of distilled water to each Petri dish and gently removing the spores with a paintbrush. Filtration of the suspensions was not necessary because of the low frequency presence of hyphal fragments. Two dishes were used for each isolate and replicate. The suspension was placed in a beaker to which 200 ml of distilled water and two drops of Tween 80 were added. The mixture was stirred continuously for 10 minutes. The spore concentration of each isolate was determined using a compound light microscope (200 x) and a haemocytometer and then adjusted to 1.2×10^5 spores ml^{-1} by adding distilled water.

Two- to three-month-old pods were sprayed in the field with approximately 0.5 ml of the freshly prepared suspension using a simple manual sprayer. Fruits were then covered with a transparent plastic bag containing a paper towel and 50 ml water, which acted as a humid chamber. The water and the paper towels were removed after two days and then autoclaved. Artificial inoculations were divided into four replicates, all of which were performed in September 2002. Two replicates were carried out on September 17th and the

remaining two on September 18th. Between eight and ten pods per clone and isolate were inoculated in each replicate depending on pod availability. There were insufficient pods of clone SSC-61 to allow evaluation against all seven isolates. For this reason, only two replicates were performed for this clone using isolates Co1, Co5, Co8 and Co10.

The following variables were measured nine weeks after inoculation:

a. **Disease incidence (DI):** Percentage of diseased fruits in relation to the total evaluated. This variable is useful to determine both the effectiveness of the artificial inoculations and the reaction of the materials.

b. **External severity (ES):** Based on the external appearance of each pod using the following six-point-scale that considers the development of symptoms and pathogen signs: 0 = healthy pod, 1 = hydrosis, 2 = swellings and/or premature ripening, 3 = necrosis, 4 = mycelium covering no more than one fourth of the necrosis and 5 = mycelium covering more than one quarter of the necrosis. This variable measures the level of external damage caused by the fungus and the ability of the fungus to produce propagules.

c. **Internal severity (IS):** Based on the internal necrosis percentage observed in each fruit when sectioned longitudinally and measured with the following scale: 0 = 0 %, 1 = 1-20 %, 2 = 21-40 %, 3 = 41-60 %, 4 = 61-80 % and 5 = >81% (Sánchez *et al.*, 1987). This variable shows the capacity of damage that the fungus can cause to the commercial product of the cacao tree, the beans.

The clones were classified using the average internal severity as follows: resistant (IS = 0 to 1.25), moderately resistant (1.26 to 2.50), moderately susceptible (2.51 to 3.75) and susceptible (3.76 to 5.0).

6.3.4 DATA ANALYSIS:

A split-plot arrangement was used with sub-sampling (clones as factor A (units), isolates as factor B (subunits) and pods as sub-samples) on a randomised complete-block design with four blocks and ten pods/block/treatment. The mathematical model employed was:

$$\mathbf{Y_{ijkl} = \mu + P_i + C_j + PC_{ij} + I_k + IC_{jk} + PCI_{ijk} + e_{ijkl}}$$

$\mathbf{Y_{ijkl}}$	=	Variable to be analysed for the i^{th} level of P, the j^{th} level of C and the k^{th} level of I
$\boldsymbol{\mu}$	=	Overall mean
$\mathbf{P_i}$	=	Effect of the i^{th} plot
$\mathbf{C_j}$	=	Effect of the j^{th} clone
$\mathbf{PC_{ij}}$	=	Error A or plot error
$\mathbf{I_k}$	=	Effect of the k^{th} isolate
$\mathbf{IC_{jk}}$	=	Interaction clone with isolate
$\mathbf{PCI_{ijk}}$	=	Error B or sub-plot error
$\mathbf{e_{ijkl}}$	=	sampling error

When necessary, the model was reduced to the appropriate number of sources of variation, e.g. in the analysis of the disease incidence and in the analysis of the clone SSC-61. The model for disease incidence did not consider the error B or sub-plot error (Plot x Clone x Isolate), because the calculation of this parameter is based on the percentage of diseased fruits within each replicate. The model for the analysis of clone SSC-61 did not include the sources of variation clone, error A and the interaction clone with isolate because the analysis was performed only for this single genotype.

The following transformations were applied to normalise the raw data:

a). **Arcsin (percentage/100)^{½}**: Angular transformation was used for the moniliasis incidence.

b) **(value+0.5)^{½}** : For both the External and the Internal Severity.

Data analysis was undertaken using the SAS software Version 8.00 (SAS Institute, North Carolina, USA). An analysis was performed for each variable applying the GLM (General Linear Model) procedure. When a treatment effect was found to be significant, a Duncan test was performed to determine statistical differences among the means of the treatments.

6.4 RESULTS

Significant differences for the three evaluated variables (DI, ES and IS) were found between both the seven isolates of *M. roreri* ($P<0.05$) and also between the four cacao clones ($P<0.01$) used although there was no significant interaction between clone and isolate (Table A1). For clone SSC-61, no significant differences were found among the four isolates evaluated for any of the three variables scored (Table A2).

High levels of moniliasis were obtained in all replications. Furthermore, each of the seven isolates was able to reproduce the disease in all clones and to inflict considerable damage. A significant and positive correlation was obtained among the three variables, in particular between the IS and ES ($r = 0.92$, 111 degrees of freedom), and to a lesser degree between ES and DI ($r = 0.64$, 111 degrees of freedom) and between IS and DI ($r = 0.64$, 111 degrees of freedom).

A clear distinction was obtained between the less aggressive isolate Co-1 and the remaining group of isolates, and between cacao clone ICS-95 and the remaining group of clones for the three analysed variables. The reaction of the cacao clones used was divided into two categories based on the IS of the disease (Duncan, $P<0.05$). ICS-95 displayed a resistant reaction against all isolates ($IS < 1.1$), and so was clearly distinct from the remaining clones that registered a susceptible ($IS > 4$) or moderately susceptible reaction ($4 > IS > 3$). Clone SCC-61 showed reaction of intermediate intensity. A more detailed breakdown of the data relating to each of the variables is given below.

6.4.1 INTERNAL SEVERITY

Significant differences were found for both clones (P<0.01) and isolates (P<0.05). The interaction clone with isolate was not significant for IS (Table A1). The mean values of this variable ranged from 2.3 in isolate Co1 to 3.6 in isolate Co10 and from 1.0 in clone ICS-95 to 3.9 in clone TSH-565 (Table 6.3).

Table 6.3 Pod internal severity reaction of four cacao clones challenged with seven isolates of *M. roreri,* Santander, Colombia, 2001.

	TSH-565		ICS-1		CAP-34		ICS-95		
Isolate	**IS**	**Re.[1/]**	**IS**	**Re.**	**IS**	**Re.**	**IS**	**Re.**	**Average**
Co10	4.3 a [2/]	S	4.3 a	S	4.6 a	S	1.0 a	R	**3.6 a**
Co13	4.3 a	S	4.6 a	S	3.3 ab	MS	1.0 a	R	**3.5 a**
Co16	4.0 a	S	4.2 a	S	4.2 a	S	1.1 a	R	**3.3 a**
Co14	4.4 a	S	4.3 a	S	3.0 ab	MS	1.1 a	R	**3.3 a**
Co8	3.6 a	MS	3.8 a	S	4.1 ab	S	0.8 a	R	**3.1 ab**
Co5	3.4 a	MS	4.0 a	S	2.4 b	MR	0.9 a	R	**2.6 bc**
Co1	3.2 a	MS	2.3 b	MR	2.6 ab	MS	1.1 a	R	**2.3 c**
Avg. [3/]	**3.9 a**		**3.8 a**		**3.5 a**		**1.0 b**		**3.1**

[1/] Disease reaction (Re.): R = resistant (IS < 1.26), MR = moderately resistant (IS = 1.26-2.50), MS = moderately susceptible (2.51-3.75), S = susceptible (> 3.76).

[2/] Values in each column followed by the same letter do not differ statistically (Duncan Test, P<0.05).

[3/] Values in this row followed by the same letter do not differ statistically (Duncan Test, P<0.05).

There was no significant difference between mean IS values of pods from isolates Co10, Co13, Co16, Co14 and Co8 (Duncan test, P<0.05) (Table 6.3). Pods infected with isolate Co1 exhibited lower IS values than this group of isolates (P<0.05) but not than isolate Co5.

Pods of clone ICS-95 showed a mean IS of 1.0, whereas the remaining clones produced average IS values of between 3.5 and 3.9 (Table 6.3). When the performance of isolates was compared separately for each clone, no differences between isolates were found for TSH-565 or ICS-95, although there was variation in clones CAP-34 and ICS-1 (Table 6.3).

Thus, isolate Co5 induced lower IS values than isolates Co10 and Co16 in clone CAP-34, and isolate Co1 produced lower IS scores than all other isolates in clone ICS-1 ($P<0.05$).

Interestingly, clone ICS-95 showed a visible resistant reaction against all isolates ($IS<1.26$). The remaining isolates caused a susceptible or moderately susceptible reaction in clones TSH-565, ICS-1 and CAP-34. Isolates Co1 and Co5 caused a moderately resistant reaction in ICS-1 and CAP-34, respectively.

6.4.2 EXTERNAL SEVERITY

Significant differences were found for both clones and isolates ($P<0.01$). The interaction clone with isolate was not significant for ES (Table A1). This variable varied between 1.8 and 3.3 in isolates Co1 and Co10, respectively, and between 0.6 and 3.6 in clones ICS-95 and TSH-565, respectively (Table 6.4).

Table 6.4 Pod external severity reaction of four cacao clones inoculated with seven isolates of *M. roreri*, Santander, Colombia, 2001.

Clones / Isolates	TSH-565	ICS-1	CAP-34	ICS-95	Average
Co10	4.2 a [1/]	4.3 a	4.1 a	0.6 a	**3.3 a**
Co16	3.8 ab	4.1 a	4.2 a	0.5 a	**3.1 a**
Co13	4.0 ab	4.2 a	2.4 ab	0.5 a	**2.9 ab**
Co8	3.5 ab	3.2 b	3.7 ab	0.6 a	**2.8 ab**
Co14	3.9 ab	3.0 b	2.7 ab	0.6 a	**2.6 ab**
Co5	3.3 ab	3. 8 ab	2.2 b	0.7 a	**2.4 b**
Co1	2.7 b	1.8 c	2.6 ab	0.5 a	**1.8 c**
Avg. [2/]	**3.6 a**	**3.4 a**	**3.2 a**	**0.6 b**	2.7

[1/] Values in each column followed by the same letter do not differ statistically (Duncan, $P<0.05$).

[2/] Values in this row followed by the same letter do not differ statistically (Duncan, $P<0.05$).

The mean value for external severity (ES) across all isolates used in the experiment was 2.7 (Table 6.4). Isolate Co1 induced a significantly lower mean ES value than all other

isolates, and Co5 produced significantly lower mean ES values than Co10 and Co16 (Duncan, $P<0.05$). There was also a clear distinction between the reaction of different cacao clones with respect to ES scores. The mean ES value of clone ICS-95 was significantly lower (Duncan, $P<0.05$) than the remaining three clones (Table 6.4).

When the performance of isolates was considered separately within each clone, the ES values ranged from 0.5 (for isolates Co16, Co13 and Co1 in clone ICS-95) to 4.3 (isolate Co10 in clone ICS-1) (Table 6.4). All isolates induced similar ES values in clone ICS-95 (Duncan, $P<0.05$) although significant variation was observed between isolates (Duncan, $P<0.05$) for all of the remaining clones (Table 6.4). In general, isolate Co10 induced high ES symptoms whereas Co1 induced relatively low ES scores, with these two isolates generating significantly different ES values for clones TSH- 565 and ICS-1 but not quite for CAP-34 (Table 6.4). Within clone CAP-34, isolate Co5 also induced significantly lower ES scores than either isolates Co10 or Co16. Three groups of isolates were obtained within ICS-1: isolate Co1 produced significantly lower ES scores than all other isolates; Co8, Co14 and Co5 were similar ($P<0.05$) and only the last (Co5) did not show statistical differences ($P<0.05$) with the third group which had the highest ES values and comprised isolates Co10, Co16 and Co13 (Table 6.4).

Evaluated pods mostly showed contrasting extreme grades of ES, with pods either showing severe grades of ES or remaining healthy. Necrosis with or without mycelium (grades 3, 4, and 5) occurred in more than 50% of pods taken from all the isolates except in isolate Co1, where 38.3 % of fruits were with this condition. The clones had more than 64% of necrotic pods except in clone ICS-95 in which only 9.7% of pods were severely affected. A total of 12.6% of the fruits showed the initial symptom of hydrosis, but very few (0.4%) had swellings and/or premature ripening. The percentage of healthy pods recorded at the end of the experiment was 31.7%. This was largely attributed to the high percentage of healthy pods obtained from clone ICS-95 (69.5%), with the remaining group of clones all possessing less than 25% asymptomatic fruits. The highest percentage of healthy pods was obtained by isolates Co5 and Co1 with 42.2 and 45.1% respectively

6.4.3 DISEASE INCIDENCE

Significant differences were found for clones and isolates (P<0.01). The interaction of clone with isolate was not significant for DI (Table A1). This variable ranged from 55.9 in isolate Co1 to 80.6 in isolate Co16 and from 29.5 in clone ICS-95 to 84.4 in clone TSH-565 (Table 6.5).

Table 6.5 Incidence of moniliasis caused by seven isolates of *M. roreri* on four cacao clones, Santander, Colombia, 2001.

Clones / Isolates	TSH-565	ICS-1	CAP-34	ICS-95	Average
Co16	90.2 ab[1/]	95.0 a	95.8 a	41.4 a	**80.6 a**
Co10	92.5 ab	82.3 a	95.0 a	25.0 a	**73.7 ab**
Co14	100 a	92.7 a	59.0 a	32.4 a	**71.0 ab**
Co8	79.8 ab	80.1 a	84.2 a	29.2 a	**68.3 ab**
Co13	87.5 ab	91.7 a	72.3 a	18.8 a	**66.2 ab**
Co5	68.7 b	86.0 a	59.2 a	31.5 a	**61.3 ab**
Co1	73.1 b	60.1 a	61.7 a	28.6 a	**55.9 b**
Average [2/]	**84.4 a**	**84.0 a**	**75.3 a**	**29.5 b**	68.2

[1/] Values in each column followed by the same letter do not differ statistically (Duncan Test, P<0.05).
[2/] Values in this row followed by the same letter do not differ statistically (Duncan Test, P<0.05).

The average incidence for the experiment was 68.2% (Table 6.5). Isolate Co16 generated the maximum value for DI (80.6%), whilst isolate Co1 produced the lowest (55.9%). These two isolates were the only pair that differed significantly (Duncan Test, P<0.05) across all cacao genotypes. Variation was less pronounced in DI within individual cacao clones and generally DI values of isolates within particular clones did not vary, except within clone TSH-565, where Co14 generated a significantly higher estimate for DI than either Co5 or Co1 (P<0.05).

There was considerable variation between the mean values of DI of different cacao genotypes, with TSH-565 (84.4%), ICS-1 (84.0%), and CAP-34 yielding significantly higher estimates for DI (P<0.05) than ICS-95 (29.5%) (Table 6.5).

6.4.4 REACTION OF CLONE SSC-61

The mean values of DI, ES and IS for clone SSC-61 were 76.9%, 1.4 and 2.0 respectively (Table 6.6), with no variation between any of the isolates evaluated, resulting in a moderately susceptible to resistant reaction.

Table 6.6 Internal severity, external severity, and disease incidence of the clone SCC-61 when inoculated with four isolates of *M. roreri*, Santander, Colombia, 2001.

Isolates	Incidence	External severity	Internal severity	Reaction
Co10	86.9 a [1/]	1.8 a	3.0 a	MS [2/]
Co8	96.2 a	1.8 a	2.8 a	MS
Co1	64.6 a	1.2 a	1.4 a	MR
Co5	60.0 a	0.8 a	0.9 a	R
Average	**76.9**	**1.4**	**2.0**	

[1/] Values in each column followed by the same letter do not differ statistically (Duncan Test, P<0.05).

[2/] Disease reaction: R = resistant (IS < 1.25), MR = moderately resistant (IS = 1.26-2.50), MS = moderately susceptible (2.51-3.75), S = susceptible (> 3.76).

6.5 DISCUSSION

A working knowledge of the genetic variation amongst isolates is essential in order to establish effective strategies for the genetic control of plant pathogens. Until recently, such information was scarce for *M. roreri*, but molecular evidence presented in Chapter 4 allowed the identification of five distinct genetic groups of the pathogen.

The next phase in a comprehensive study in this field is to determine the effect of variability on the reaction of different genetic races of the host, which is the main goal of the present chapter. Seven isolates of *M. roreri* were chosen to represent the major genetic groups previously identified. Only Colombian isolates were considered here due to quarantine reasons, but this only slightly affected the objective of the experiment because

all of the significant genetic groups except one (*Gileri* group comprising two genotypes from Ecuador) are present in Colombia. Indeed, the highest genetic variation of the pathogen is found in this country.

When the isolates were artificially inoculated onto different field-grown clones using an established methodology (Sánchez *et al.* 1987, Sánchez & González, 1989, Phillips-Mora 1996), significant differences between the isolates and the clones were detected for the three variables studied.

Differences in virulence were identified among the evaluated isolates, although, all caused significant pod damage. Six of the seven evaluated isolates were highly virulent, causing severe internal and external grades of disease on pods in the majority of clones. Considering that these isolates represent the most important genetic groups of the pathogen, this result could help to explain the severe effects that *M. roreri* has produced in all countries that have been exposed to the disease and why movement into new areas has invariably had devastating consequences on cacao yield (Rorer, 1926; Enríquez *et al.*, 1982; Krauss & Soberanis, 2001; Anon, 2001). This behaviour could be attributed to the high level of specialisation that *M. roreri* had reached throughout a long period of coevolution with its hosts, which comprise species of the two closely related genera *Theobroma* and *Herrania* (Whitlock & Baum, 1999).

Although isolates Co1, and to some extent Co5, caused lower levels of internal and external damage than the other isolates, nevertheless their effect on cacao yield could be very severe under the perspective that they were still able to infect more than 59% of the fruits in all the clones, except from ICS-95. The infection caused by *M. roreri* usually results in the total pod loss.

It is perhaps significant to note that whilst isolate Co5 was obtained locally from the same farm where the experiment was carried out, it did not exhibit a higher virulence on cacao than did isolates obtained from distant sites. Thus, there was no evidence here of strong adaptation of this strain to local conditions. Isolate Co5 belongs to the same genetic group as isolates Co16 and Co14 (Eastern Colombia group), however, and their virulence

differed from this isolate with respect to the internal severity and, to some extent, external severity.

Co1 was the isolate that consistently induced lower levels of disease across the cacao clones investigated. This isolate was collected in the 'North of Santander' Department and belongs to the 'Bolivar group', which has also been found in Ecuador, and is apparently the only group present in Venezuela and Peru. It is not possible to eliminate the possibility that the inoculation and/or environmental conditions under which the experiment was carried out could have impeded the performance of this isolate, but under the premise that Co-1 effectively represents its group and that no other external factors affected its performance during the experiment, some implications could be deducted from its reduced virulence. First, it means that local breeding efforts, particularly in Venezuela and Peru should consider the possibility of renewed invasion by a second, more virulent *M. roreri* genotype group. If members of the group all share similar levels of virulence, then it is a cause for concern that the isolate representing the group from Peru seemingly has relatively poor virulence compared with other isolate groupings and yet has still inflicted tremendous yield losses in Peru (Krauss & Soberanis, 2001). Second, these findings raise the question as to whether virulence has been partly lost during the course of the spread southwards or whether the original invasive strain or strains simply happened to be less virulent. In this respect, it is relevant to notice that *M. roreri* has been relatively easy to control using biological and cultural measurements in Peru but not under similar climatic conditions in Central America where a different genetic group (Co-West) is present (U. Krauss, CATIE, Costa Rica, personal communication). It follows that this work should be followed by a more extensive survey of the variability of virulence within genetic groupings of *M. roreri*.

In spite of the relatively severe effects caused by all isolates on the cacao clones, one cacao genotype was identified with a significant level of resistance against all the isolates (ICS-95). This result opens the possibility of directly selecting genotypes with durable resistance against the pathogen for cultivation and makes feasible the use of such materials to breeding programmes. Multilocational experiments to determine the stability of the reaction against *M. roreri* of this and other outstanding clones are strongly recommended.

Clone ICS-95 has been reported as having considerable levels of resistance against *M. roreri* in Colombia (Argüello, 1997), Costa Rica (Phillips-Mora, 1996) and Peru (Evans *et al*., 1998), but it shows a susceptible reaction in Ecuador (C. Suárez, INIAP, Ecuador, personal communication). A molecular study using 13 microsatellites was carried out to determine if the differential reaction observed in Ecuador is genuine or due to clone misidentification (J.C. Motamayor, USDA-ARS, USA, personal communication). After studying some samples from CATIE (Turrialba, Costa Rica), Pichilingue Tropical Experimental Station (Los Ríos, Ecuador) and *La Suiza* Experimental Farm (Santander, Colombia), it was found that samples of ICS-95 from Costa Rica, Colombia and one Pichilingue were identical. However, another genotype with this name from Pichilingue possessed a different microsatellite profile. This opens the possibility that in Ecuador may exist at least two genotypes named ICS-95 with a different reaction to *M. roreri*.

Clone ICS-95 has been widely used in Latin America for a long time and was the most favoured clone among Trinidad planters (Hardy, 1957). It is a high-yielding cacao clone (Wood, 1959) and is tolerant to witches´ broom disease, caused by *C. perniciosa* (Argüello 2000c). It is considered as a promising material in Peru (Evans *et al*., 1998) and is recommended for new plantings in all the cacao producing areas of Colombia (Rondón, 2000). It was used as a standard variety against which local varieties were assessed for yield in different Latin American countries in the 1950s and early 1960s (Young, 1994).

Another interesting genotype was clone SSC-61 which was selected in Santander, Colombia because of its field resistance to different disease, self-compatibility and its remarkable yield potential (Table 6.2). In this study, SSC-61 registered a moderate reaction against *M. roreri* that ranged from resistant to moderately susceptible depending on the virulence of the isolate that was used. Although the reduced number of replications and isolates tested for SSC-61 could have limited the study of this clone, its apparently considerable level of resistance to *M. roreri* together with the other attributes mentioned above make it possible to consider it as promising commercial material.

Conversely, clones ICS-1 and TSH-565 are widely grown in many countries but exhibited a susceptible reaction to the disease. This result agrees with observations of these genotypes in Costa Rica and Colombia (Sánchez & Cubillos, 1984; Argüello, 2000c), so

that the possible argument that the virulence of these clones have been disadvantaged by local environmental conditions in this study, such as, sub-optimal altitude, appears to be unlikely. Interestingly, clone CAP-34 was considered as moderately resistant by Argüello (2000c) but showed a susceptible reaction against all isolates here.

High levels of DI were obtained in all the replications, thus confirming both the capability of the isolates to reproduce the disease and the effectiveness of the inoculation method. The mean DI separated both the susceptible from the resistant clones and the more virulent from the less virulent isolates, but it was less able than IS and ES to separate the reaction of the isolates within individual cacao clones. Under conditions of natural infection (Galindo & Enríquez 1985) and artificial inoculations (Phillips & Galindo, 1987) disease incidence has also been useful to identify the extreme classes of host genotypes in terms of their reaction to *M. roreri*.

It was possible to discriminate between cacao clones and isolates on the basis of their differential external severity (ES) reaction against *M. roreri*. However, the usefulness of ES as a routine screen in an evaluation programme is limited by the fact that ES not detect hidden infections that are quite common in cacao (Ampuero, 1967; Evans, 1981). External severity could be useful to keep track of the development of the symptoms of moniliasis during a resistance trial, and provides and indication of the potential scale of infection. Thus, in the present study, the high percentage of pods exhibiting a severe grade of external severity (sporulating pod or close to sporulation) when inoculated with the different isolates of *M. roreri* shows the high epidemiological potential that these isolates could reach under appropriate conditions. Here it is important to take into account that a single infected pod may produce over 7,000 million spores and that each of these has a high infective capability (Campuzano, 1976), and that, under field conditions, the spores remain viable on mummified hanging pods for more than nine months (Evans, 1981).

It can be argued that internal severity (IS) is the most important variable to measure the effect of *M. roreri* on cacao, because it best defines the capacity of damage that the fungus can cause to the commercial product of the cacao tree, the seeds (Phillips & Galindo, 1987; Suárez-Capello, 1996). The highly significant and positive correlation between the ES and

IS found here agrees with a previous study of the disease (Phillips & Galindo, 1987) and implies an implicit consideration of ES whenever IS is measured.

In this study, the absence of an interaction between the variability of the pathogen and the variability of the host is perhaps more consistent with a polygenic mode of resistance against *M. roreri*. The absence of this kind of interaction has been also reported for other cacao pathogens such as *P. palmivora* (Surujdeo-Maharaj *et al.*, 2001), and *C. perniciosa* (Wheeler 1996), but apparently is not the rule in this plant because other authors have detected interactions cacao-pathogen working with *P. palmivora* (Phillips & Galindo, 1991) and with *Oncobasidium theobromae* (Bong *et al.*, 1996). It is particularly interesting to compare *C. perniciosa*, a basidiomycete, with *M. roreri* due to the strong genetic relationship existing between them (Chapter 5) and in which two pathotypes have been discovered with contrasting capabilities to induce symptoms on clone SCA-6 and with a particular geographical distribution in South America (Wheeler & Mepsted, 1988; Laker, 1990). This perhaps suggests a possible vertical mechanism of resistance can act against *C. perniciosa* in cacao.

The influence of the environmental conditions on the expression of resistance, the detection of different grades of resistance and the fact that no immune material has thus far been identified, suggests that cacao has horizontal resistance against *M. roreri*. Zadocks (1997) recommended concentrating breeding efforts, on the exploitation of this kind of resistance as it supposedly, contributes to the durability of resistance.

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6.7 APPENDIX

Table A6.1 Analysis of variance: disease incidence, external severity and internal severity of four cacao clones challenged with seven isolates of *M. roreri*.

Variation Source	Variables								
	Disease incidence			External severity			Internal severity		
	DF[1/]	MS[2/]	Signif.[3/]	DF	MS	Signif.	DF	MS	Signif.
Plot	3	0.149	n.s.	3	0.760	n.s.	3	0.882	n.s.
Clone	3	3.548	**	3	32.916	**	3	30.905	**
Plot x clone	9	0.093	n.s.	9	0.413	n.s.	9	0.529	n.s.
Isolate	6	0.278	**	6	1.467	**	6	1.310	*
Clone x isolate	18	0.124	n.s.	18	0.552	n.s.	18	0.539	n.s.
Plot x clone x isolate	--[4/]	--	--	71	0.413	*	71	0.483	n.s.
Error	71	0.085	--	565	0.311	--	565	0.367	--

[1/] DF = degrees of freedom

[2/] MS = means square

[3/] Signif. = Significance: n.s.= non significant, *=significant (P<0.05), **=highly significant (P<0.01).

[4/] '--' = not applicable

Table A6.2 Analysis of variance: disease incidence, external severity and internal severity of clone SSC-61 challenged with four isolates of *M. roreri*.

Variation Source	Variables								
	Disease incidence			External severity			Internal severity		
	DF[1/]	MS[2/]	Signif.[3/]	DF	MS	Signif.	DF	MS	Signif.
Plot	1	0.041	n.s.	1	0.359	n.s.	1	0.138	n.s.
Isolate	3	0.159	n.s.	3	0.514	n.s.	3	1.217	n.s.
Plot x Isolate	--	--	--	3	0.255	n.s.	3	0.363	n.s.
Error	3	0.086	--	42	0.260	--	42	0.406	--

[1/] DF = degrees of freedom

[2/] MS = means square

[3/] Signif. = Significance: n.s.= non significant, *=significant (P<0.05), **=highly significant (P<0.01).

[4/] '--' = not applicable

CHAPTER 7

In vitro effect of three temperatures on morpho-physiological traits of the cacao (*Theobroma cacao* L.) fungal pathogen *Moniliophthora roreri* (Cif.) Evans *et al.*

7.1 ABSTRACT

In vitro cultures of 15 isolates of *M. roreri* representing the genetic variability of the pathogen were grown under three temperature regimes (18 ºC, 24 ºC and 30 ºC) to study their effect on eight morpho-physiological traits: radial growth, first appearance of sporulation, ring intensity, and the following spores based characteristics: production, density, germination, shape and size. A total of 11 variables were analysed: radial growth at 10 d, days to sporulation, ring intensity, spore production, spore density, percentage of germinated spores after 24 h, percentage of globose and ellipsoid spores, globose spore diameter, ellipsoid spore width and ellipsoid spore length.

The isolates were able to grow and sporulate at all three temperatures tested, although most parameters were severely reduced at 18 ºC. Conversely, spore density and those variables related to the size of the spore (e.g. globose spore diameter, ellipsoid spore width and ellipsoid spore length) increased at 18 ºC compared to the other two temperatures. Optimal performance in terms of radial growth and spore production was observed at 24 ºC. Whilst radial growth was moderately but significantly depressed at 30 ºC, all other parameters were essentially unchanged from 24 ^{o}C.

There was significant overall variation among temperature treatments, and also between isolates cultured at the same temperature. When the genetic affinity of isolates was considered, it was possible to detect differences in growth and reproductive capacities between major genetic groups of isolates. The power of discrimination was most evident at 30 ºC in the variables radial growth, days to sporulation, ring intensity, spore production and spore size. The significance of these results is discussed.

Keywords: cacao, frosty pod disease, genetic diversity, moniliasis, radial growth, spore morphology, *Moniliophthora roreri*, *Theobroma cacao*

7.2 INTRODUCTION

Moniliophthora roreri (Cif.) Evans *et al*., the causal agent of moniliasis or frosty pod disease, is a highly specialised fungus that invades only actively-growing pods of cacao and other related species and causes total destruction of the fruits. The fungus shows a wide environmental adaptation ranging from sea level to over 1000 m and from very dry environments (where cacao trees are irrigated) to very humid zones (Evans, 1981).

Temperature is an extremely important variable that influences the rate fungal growth (Moore-Landecker, 1996). Increasing temperature above a minimal threshold has the general effect of increasing enzyme and chemical activity, although at highly elevated temperatures enzymes eventually become heat inactivated. The majority of filamentous fungi, such as *M. roreri,* are mesophilic, growing at temperatures within the range of 10-35 ºC, with optimum temperatures between 15 ºC and 30 ºC (Smith, 2002).

Temperature is also one of the most important physical factors affecting fungal reproduction, as it may influence a number of metabolic and physiological processes related to this phenomenon (Moore-Landecker, 1996). Most fungi grow and sporulate well at temperatures between 15 ºC and 25 ºC, the sporulation range usually being smaller than the growth range (Hawker, 1950). The minimum and maximum temperatures for reproduction are generally within 5-10 ºC of the optimum range. However, different fungal species and strains may have contrasting temperature requirements (Moore-Landecker, 1996).

Dispersal of the spores of *M. roreri* is favoured by dry conditions (Porras & González, 1982; Schmitz, 1985), whereas their germination and penetration is highly dependent on the availability of free water on the pod (López, 1954; Merchán, 1981; Campuzano, 1981b). Both phenomena are affected by temperature, with spore liberation and their permanence in the air being favoured by temperatures higher than 26 ºC (Schmitz, 1985)

whilst germination is favoured by lower temperatures in the range of 22-24 ºC (López, 1954; Campuzano, 1981b; Evans, 1981; Merchán, 1981; Ram *et al*., 1987). Cabanilla (1966) found that spores of *M. roreri* survive freeze-drying and subsequently become pathogenic after 15, 75, 135 or 315 days.

Several studies have investigated how temperature can affect *in vitro* mycelium growth, sporulation and germination of *M. roreri* (López, 1954; Chacín, 1975, Pérez & Posada, 1978; Campuzano, 1981b; Merchán, 1981; Evans, 1981; Phillips & Galindo, 1985; Herrera, 1988; Ram *et al*., 1987) although such studies have typically used only a small number of local isolates. The optimum temperature range for growth and sporulation of *M. roreri* isolates was established as 25-27 °C in Venezuela (Chacín, 1975), 25-26 °C in Ecuador (Evans, 1981) and 24-28 °C in Costa Rica (Herrera, 1988). Growth occurred up to 33 °C (Evans, 1981). A reduction in the spore production occurs at temperatures above 26 °C (Pérez & Posada, 1978; Phillips & Galindo, 1985; Herrera, 1988), with no sporulation being reported above 32 °C (Herrera, 1988). In Venezuela, *M. roreri* was able to grow at *in vitro* temperatures as low as 12 °C and as high as 42 °C, but very significant growth reductions were observed at these temperatures, with lower being more limiting than higher ones (Chacín, 1975).

None of these studies considered the variation in response to temperature between genetically diverse isolates of the pathogen. Results on the genetic variation of the pathogen reported in Chapter 4 opened the novel possibility to investigate how a group of isolates representing the global genetic variability of the fungus interact with different *in vitro* temperatures. The objective of this chapter is to determine the effect of three temperatures on morpho-physiological traits in isolates of *M. roreri* that represent the global genetic diversity of the fungus.

7.3 MATERIALS AND METHODS

7.3.1 LOCATION OF THE EXPERIMENT

The experiment was carried out in the Laboratories of Biotechnology and Phytopathology of the Tropical Agricultural Research and Higher Education Centre (CATIE) located in Turrialba, Costa Rica during the period October-December 2001.

7.3.2 EXPERIMENTAL MATERIAL

Fifteen isolates of *M. roreri* representing the most important genetic groups of the fungus as identified in Chapter 4 were included in the study (Figure 7.1). The isolates studied and their respective groups were: Co5, Co14 and Co16 (Eastern Colombia group = 'Co-East'), Co10, Co11, Co12 (Central Colombia group = 'Co-Central'), P5, V3 and E22 (Bolívar group = 'Bolívar') and Co8, Co13, C19, N3, E3, E-20 (Western Colombia group = 'Co-West') (Figure 7.1). Only the 'Gileri' group, comprising two isolates from *Theobroma gileri* from Ecuador was not included in the study.

7.3.3 METHODOLOGY

7.3.3.1 Preparation of the plates

A set of Petri dishes (8.5 cm diam.) containing 20 ml of a modified V8 culture medium (20% v/v V8 juice, Campbell Soup Company, Camden, NJ, USA, 0.1% w/v asparagine, 2% w/v maltose and 1.8% w/v agar), was prepared five days before the establishment of the experiment. The plates were kept within polyethylene bags under laboratory temperature conditions (16-23 ºC) to detect and discard any possible contamination.

Genetic group, isolate name, geographic origin, elevation, annual precipitation and annual average temperature of the places of collection:

Co-East (Eastern Colombia group)

Co5 Río Negro, Santander, Colombia (500 m.a.s.l., 2000 mm yr^{-1}, 25.0ºC)
Co14 San Vicente de Chucurí, Santander, Colombia (750 m.a.s.l., 1867 mm yr^{-1} 21.4ºC)
Co16 El Carmen de Chucurí, Santander, Colombia (460 m.a.s.l., 2245 mm yr^{-1}, 23.2ºC)

Co-Central (Central Colombia group)

Co10 Palestina, Caldas, Colombia (1520 m.a.s.l., 2294 mm yr^{-1}, 18.6ºC)
Co11 Palestina, Caldas, Colombia (1350 m.a.s.l., 2294 mm yr^{-1}, 20.4ºC)
Co12 Palestina, Caldas, Colombia (1200 m.a.s.l., 2294 mm yr^{-1}, 21.9ºC)

Bolivar group

P5 Castillo Papayal, Huánuco, Perú (666 m.a.s.l., 3000 mm yr^{-1}, 25.6ºC)
V3 Playa Grande, Zulia, Venezuela (105 m.a.s.l., 1859 mm yr^{-1}, 27.3ºC)
E22 Bolívar, Manabí, Ecuador (200 m.a.s.l., 961 mm yr^{-1}, 25.8ºC)

Co-West (Western Colombia group

Co8 San Jerónimo, Antioquia, Colombia (703 m.a.s.l., 1343 mm yr^{-1}, 24.9ºC)
Co13 Urabá, Antioquia, Colombia (132 m.a.s.l., 5511 mm yr^{-1}, 27.1ºC)
E3 El Empalme, Guayas, Ecuador (120 m.a.s.l., 2087 mm yr^{-1}, 24.4ºC)
E20 San Pablo, Los Ríos, Ecuador (120 m.a.s.l., 2087 mm yr^{-1}, 24.4ºC)
C19 Punta Uvita, Costa Rica (0 m.a.s.l., 3970 mm yr^{-1}, 25.8ºC)
N3 Río San Juan, Nicaragua (40 m.a.s.l., 1857 mm yr^{-1}, 26.5ºC)

Figure 7.1 Geophysical information on the sites of collection for fifteen isolates of *M. roreri* (Cif.) Evans *et al*.

7.3.3.2 Isolate transfer

In a laminar flow chamber (Edge Card Hood Model EG-4320), a six-millimetre in diameter disc of agar-mycelium was extracted with a punch #3 from the advancing zone of a *M. roreri* culture grown in similar conditions as those used in the experiment. The disc was immediately placed in the centre of each Petri dish which were then closed with Parafilm tape (American National).

7.3.3.3 Experimental conditions

Three incubators (Percival Boone 50036 model I-35LL) were adjusted to three different temperatures (18 ºC, 24 ºC and 30 ºC) five days before the experiment took place. Four Petri dishes per isolate, prepared as previously described, were randomly placed in each incubator. Cultures were incubated at 12/12 alternate periods of light/darkness, with light provided by fluorescent lamps. The light intensity was 646 lux measured with a Simpson Illumination Level Meter, model 408-2.

7.3.3.4 Characters of evaluation

7.3.3.4.1 Radial growth

The diameter of the colony was measured in two perpendicular directions and then an average was calculated. Four measurements were carried out during the 20-day-growth period at intervals of five days. These data were used to construct a growth curve for each temperature. As it will be explained in the results section, a single measurement of radial growth was chosen for comparison between the isolates based on this curve.

7.3.3.4.2 Days to sporulation

This trait was determined by a visual check for the presence of sporulation on the plates after 5, 7, 10, 15 and 20 d.

7.3.3.4.3 Ring intensity

Ring intensity was determined at 20 d using a six-grade visual scale in which 0 was absence of rings, and 5 was the presence of very intense rings.

7.3.3.4.4 Spore production

The following steps were carried out to determine the spore production of each replication (Petri dish):

a. **Preparation of the spore suspension**: Spores were removed from the 20-day-old cultures by adding in three steps, 50 ml of a 0.01% Tween-80 solution in distilled water and gently brushing the surface of the colony with a paintbrush. Each suspension was then placed in an Erlenmeyer flask and stirred continuously for 10 min to separate the spores.

b. **Sample**: One ml of spore suspension was aliquoted into a labelled Eppendorf tube and this was immediately stored at 8°C until measurement of the spore concentration and recording of the size and shape of the spores. Another 1 ml aliquote was used immediately to test germination.

c. **Spore counting**: A 1:10 dilution was prepared to simplify spore counting. Spore concentration was measured using a haemocytometer and a Bausch and Lomb light microscope (100x magnification). The same microscope was used to determine spore shape and size. Spore concentration measurements took one week to be finished.

7.3.3.4.5 Spore density

This trait was calculated by dividing the number of spores per plate by the area of colony showing sporulation as measured for diameter of sporulation at 20 d.

7.3.3.4.6 Spore germination

One ml of the fresh spore suspension, as previously prepared, was placed into a Petri dish containing 5 ml of water-agar (3%) (Umaña & González, 1981). The suspension was uniformly distributed across the plate by rotating hand movements. The dishes were then stored in the dark at 24 °C. The percentage of germinated spores was determined after 24 h by examining the bottom of the plates with the light microscope (100x magnification) and counting the spores that showed a recognisable germ tube (Hawker, 1950; Etten *et al*., 1983). For each measurement, 200 spores were scored per plate in random fields of view.

7.3.3.4.7 Spore shape

This characteristic was determined using a haemocytometer and a light microscope (400x magnification). Using the spore suspension previously described, twenty random spores per plate were examined and their shape classified. The spores were classified in three types: globose, ellipsoid and abnormal.

7.3.3.4.8 Spore size

Spore width and length were measured for the same group of spores used to assess shape with a 12.5x ocular micrometer Leitz Wetzlar (Germany) previously calibrated using a plaque micrometer. The following variables were analysed based on this information: diameter of the globose spores and ellipsoid spore width and length. Spore size and shape determinations took two weeks to be finished.

7.3.3.5 Variables of evaluation

Based on the characters described before, the following 11 variables were analysed: radial growth at 10 d and days to sporulation, and after 20 days growth, ring intensity, spore production (spore plate^{-1}), spore density (spore cm^{-2}), percentage of germinated spores after 24 h, percentage of globose and ellipsoid spores, globose spore diameter, ellipsoid spore width and ellipsoid spore length. The variables were selected based on results

obtained by other authors, in particular by Chacín (1975) and Herrera (1988) and in previous experiences of the author.

7.3.4 DATA ANALYSIS

A nested (hierarchical) arrangement (isolates within temperatures) was used on a completely random design with four replications. The mathematical model used was:

$$Y_{ijk} = \mu + T_i + I_{j \mid i} + e_{k \mid j,i}$$

Y_{ijkl} = Response variable of the i^{th} temperature, the j^{th} isolate and the k^{th} observation

μ = Overall mean

T_i = Effect of the i^{th} temperature

$I_{j \mid i}$ = Effect of the j^{th} isolate within each temperature

$e_{k \mid j,i}$ = Random error of the i^{th} temperature, the j^{th} isolate and the k^{th} observation

In order to normalise the data, the following transformations were applied to the raw data:

a) ***Arcsin (percentage/100)^{½}:*** Angular transformation was used for the variables expressed in terms of percentage such as: percentage of germinated spores, percentage of globose spores and percentage of ellipsoid spores.

b) **(value+0.5)^{½}**: For ring intensity, a non parametric variable.

Data analysis was performed using the SAS software Version 8.00 (SAS Institute, North Caroline, USA). A variance analysis was performed for each variable using the GLM (General Linear Model) procedure. Then, a Tukey test was carried out to determine significant differences among the means of the treatments. The same procedures were used to analyse the isolates in accordance with their correspond genetic group. In this case, the mathematical model only included as source of variation the overall mean, the effect of the genetic groups and the random error.

7.4 RESULTS

7.4.1 GROWTH CURVES OF *M. RORERI AT DIFFERENT TEMPERATURES*

Based on the radial growth of all isolates measured every five days, an average growth curve of *M. roreri* was obtained for each temperature. Growth of *M. roreri* was highest at 24 ºC followed by 30 ºC, with a considerable reduction of growth at 18 ºC (Figure 7.2).

Although measurement of radial growth at 10 or 15 d provided equally good separation of colonies at any temperature, the earlier recording date (10 d) was selected to compare the isolates because it allowed a faster evaluation of experiments. The measurement at five days did not adequately separate the effect of the temperatures whilst that at 20 d, the growth of some colonies was limited by the size of the Petri dishes. The deflection experienced by the curve for 24 ºC between 15 and 20 d illustrates this adverse effect (Figure 7.2).

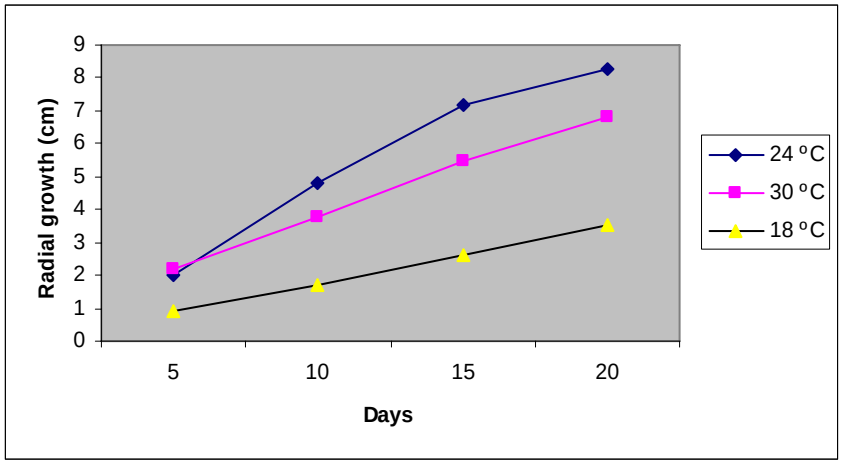


Figure 7.2 ***In vitro*** **effect of three temperatures (18 ºC, 24 ºC and 30 ºC) on the average radial growth of** ***M. roreri*** **grown on modified V8 medium for 20 d.**

7.4.2 GENERAL EFFECT OF TEMPERATURE ON THE VARIABLES

Significant differences (P<0.05) were detected between temperatures for all the morpho-physiological variables except the percentage of globose or ellipsoid spores and the percentage of germinated spores (Table A7.1).

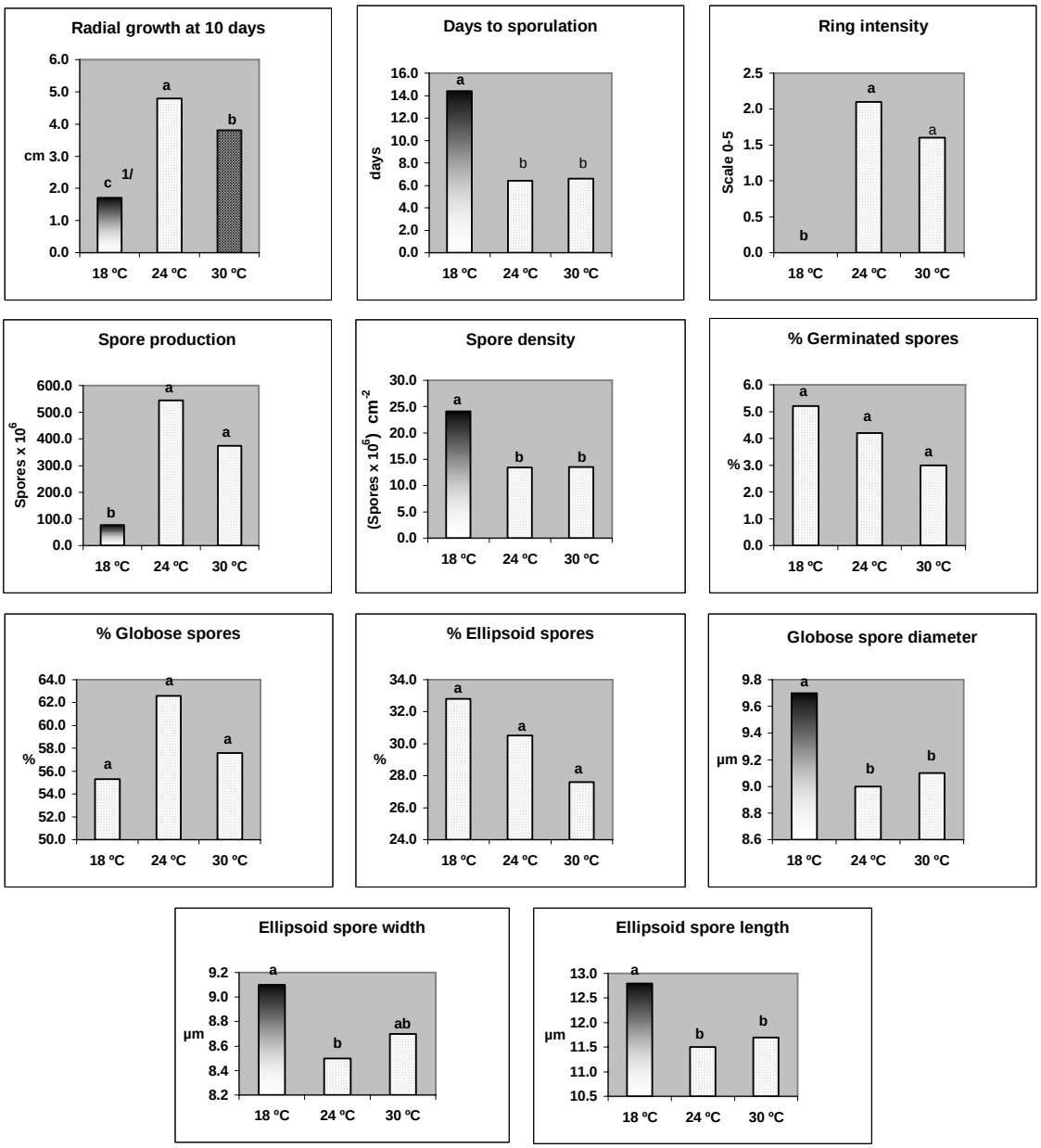
All variables exhibiting significant differences between temperatures showed a clear distinction between results at 18 ºC and those at both 24 ºC and 30 ºC, with the exception of ellipsoid spore width for which differences (P<0.05) were only found between 18 ºC and 24 ºC (Figure 7.3).

The following variables were significantly raised (Tukey test, P<0.05) at 24 ºC and 30 ºC but inhibited at 18 ºC: radial growth, days to sporulation (it shows an inverse relationship in the graph), ring intensity and spore production (Figure 7.3). No differences (P<0.05) were found between results at 24 ºC and 30 ºC for all variables except radial growth, where a higher value was observed at 24 ºC. Conversely, spore density and those variables related to the size of the spore (e.g. globose spore diameter, ellipsoid spore width and ellipsoid spore length) were higher at 18 ºC than of the other two temperatures (Figure 7.3). Ellipsoid spore width did not vary (P<0.05) between 18 ºC and 30 ºC.

7.4.3 EFFECT OF TEMPERATURE ON THE ISOLATES

The following work only makes comparisons between isolates within the same temperature because the experimental design used does not allow statistical comparison of traits of isolates across different temperatures.

Isolates within each temperature registered highly significant differences (P<0.01) for all parameters except for ellipsoid spore length (Table A7.1).



[1/] Columns with the same letter within each variable do not have significant differences (Tukey Test, P<0.05)

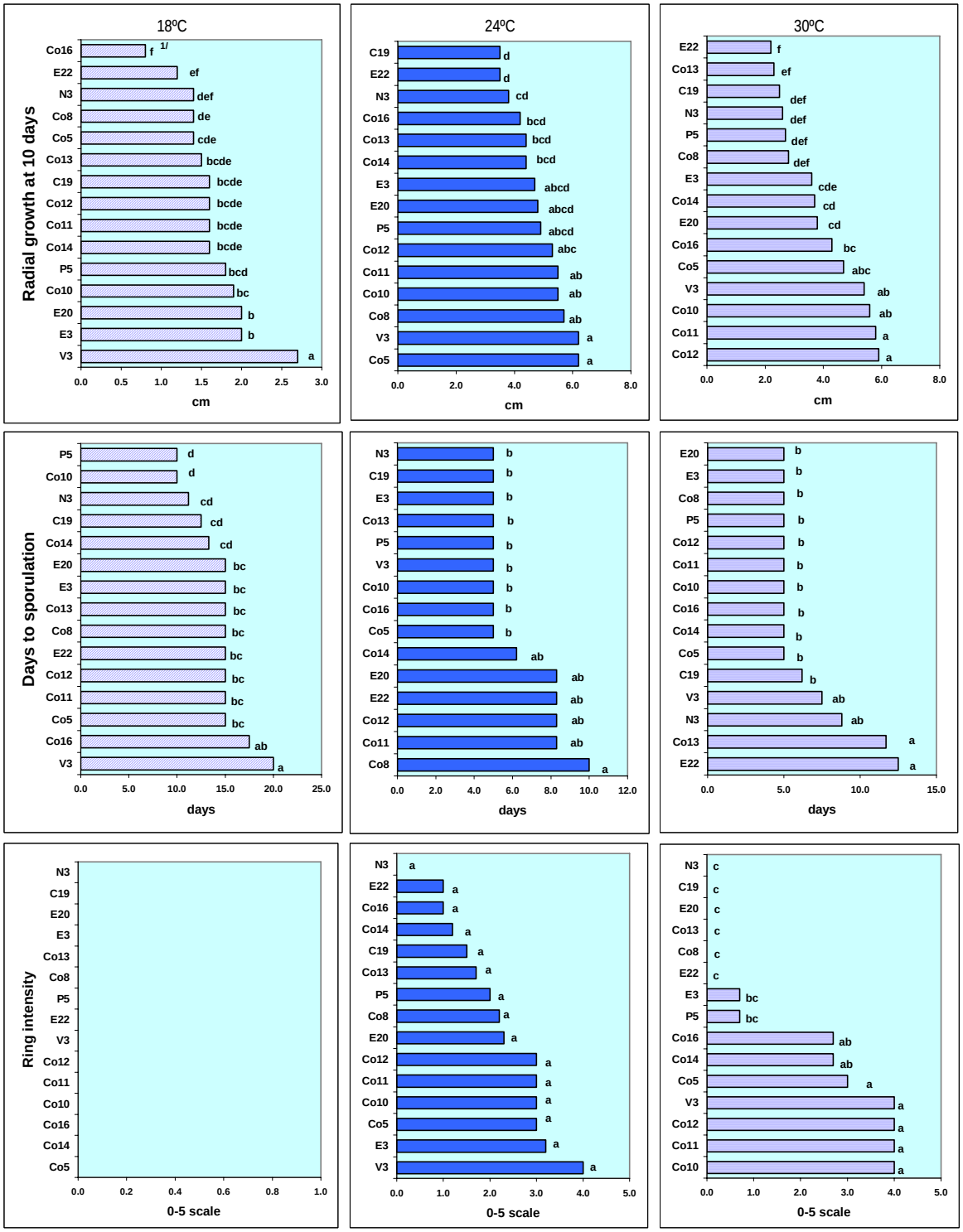
Figure 7.3 *In vitro* effect of three temperatures on eleven morpho-physiological variables of isolates of *M. roreri*.

Isolates showed a considerable variation ($P<0.05$) for radial growth at 10 d (Figure 7.4). This variable varied between isolates with ranges from 0.8 to 2.7 cm, 3.5 to 6.2 cm and 2.2 to 5.9 cm at 18 ºC, 24 ºC and 30 ºC, respectively. Isolates V3 and E-22 consistently registered among the highest or lowest values, respectively, at all temperatures. Isolates Co10, Co11 and Co12 showed good growth at all the three temperatures. The relative performance of the remaining isolates varied between temperatures.

In all isolates, radial growth measurements at 18 ºC was only 20% to 50% of those obtained at 24 ºC (Figure 7.4). Most isolates also showed a slightly reduced growth at 30 ºC in comparison to 24 ºC. On the other hand, isolates Co16, Co10, Co11 and Co12 registered a slightly superior value at 30 ºC to 24 ºC, although a statistical comparison cannot be made in this instance.

Days to sporulation ranged from 10.0 to 20.0, 5.0 to 10.0 and 5.0 to 12.5 for 18 ºC, 24 ºC and 30 ºC, respectively (Figure 7.4). For all isolates, sporulation appeared sooner at 24 ºC and 30 ºC and only later at 18 ºC. At 18 ºC, sporulation was observed first in isolates P5 and Co10 (10 d) and last in isolates V3 and Co16 (20 and 17.5 d, respectively). Differences between these groups were significant ($P<0.05$). At the other temperatures, the only isolates to differ significantly ($P<0.05$) from a considerable number of isolates were Co8 (10 d) at 24 ºC and E22 and Co13 (12.5 and 11.7 d, respectively) at 30 ºC.

Rings were not observed in any isolate at 18 ºC nor in the following isolates at 30 ºC: N3, C19, E20, Co13, Co8 and E22 (Figure 7.4). No significant differences were obtained for ring intensity between isolates at 24 ºC. At 30 ºC, isolates E3 and P5 produced a ring intensity of 0.7 whereas isolates Co5, V3, Co12, Co11 and Co10 registered significantly higher values ($P<0.05$) ≥ 3.



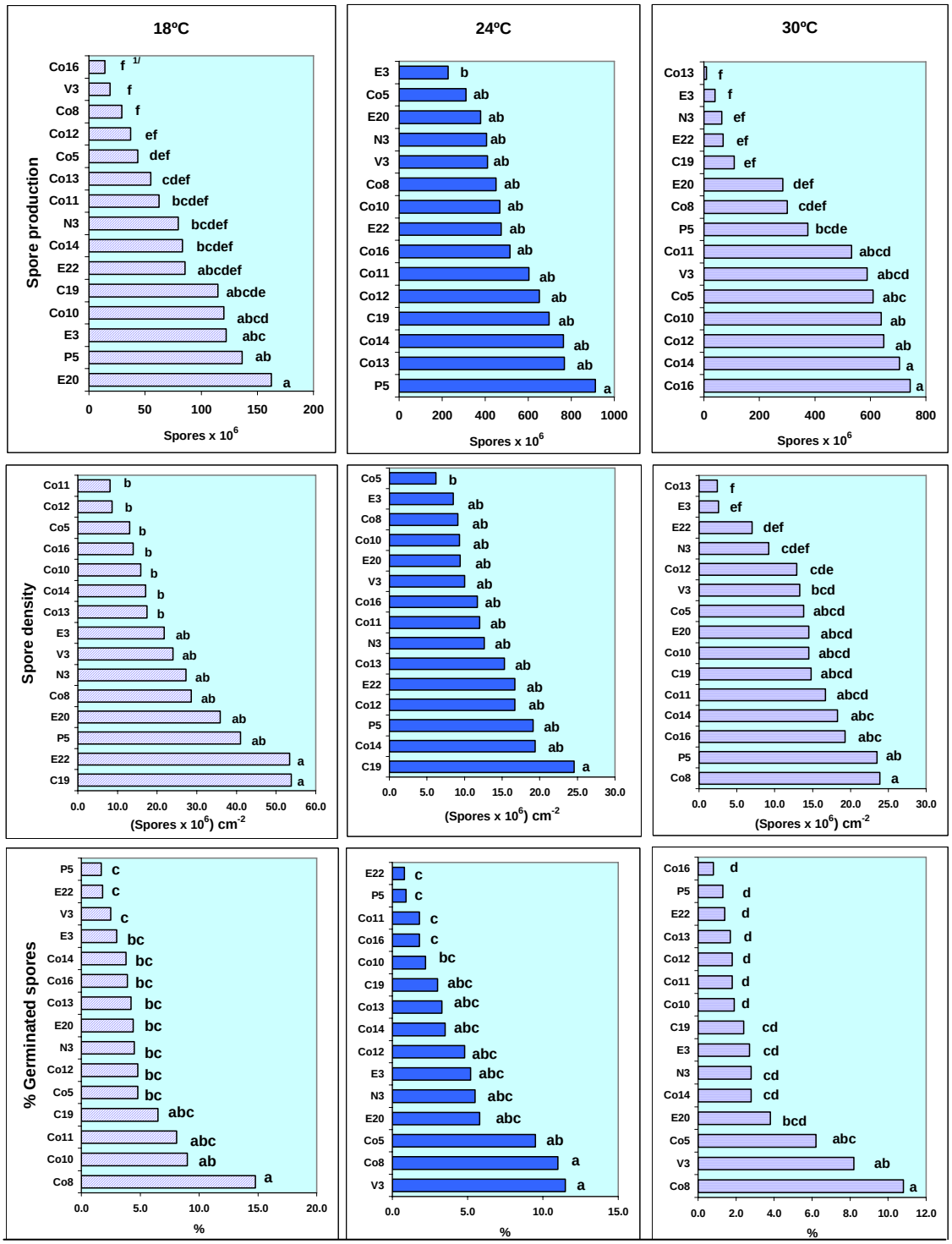
1/ **Isolates within each variable and temperature with the same letter do not have significant differences (Tukey, P<0.05).**

Figure 7.4 Radial growth at 10 d, days to sporulation and ring intensity of 15 isolates of *M. roreri* grown at three different growth temperatures.

Spore production varied considerably between temperatures ($P<0.05$) (Figure 7.5). This variable ranged from 14.3 to 162.5, 226.9 to 911.9 and 9.4 to 743.8 million spores plate^{-1} at 18 ºC, 24 ºC and 30 ºC, respectively. At 18 ºC, the lowest values were obtained by isolates Co16, V3 and Co8 (14.3, 18.9 and 29.2 million spores plate^{-1}, respectively) and the highest by isolates E20, P5, E3, Co10 and C19 (162.5, 136.4, 122.2, 120.3 and 115.0 million spores respectively). These groups were significantly different from each other ($P<0.05$). At 24 ºC, only isolates E3 (226.9 x 10^6 spores) and P5 (911.9 x 10^6 spores) differed significantly ($P<0.05$). At 30 ºC, the following isolates registered the highest spore production: Co16, Co14, Co12, Co10, Co5, V3 and Co11 ($743.8 \geq x \geq 532.1$ million spores). These isolates were significantly different ($P<0.05$) to the spore production by isolates Co13, E3, N3, E22 and C19 ($9.4 \leq x \leq 109.8$ million spores).

Spore density ranged from 8.1 to 53.8, 6.2 to 24.6 and 2.4 to 23.9 million spores cm^{-2} at 18 ºC, 24 ºC and 30 ºC, respectively (Figure 7.5). At 18 ºC, the densities observed in isolates C19 and E22 (53.8 and 53.4 million spores cm^{-2}) were significantly higher ($P<0.05$) than those in isolates Co11, Co12, Co5, Co16, Co10, Co14 and Co13 ($8.1 \leq x \leq 17.4$ million spores cm^{-2}). At 24 ºC, isolate Co5 (6.2 x 10^6 spores cm^{-2}) had a much lower density ($P<0.05$) than C19 (24.6 x10^6 spores cm^{-2}) but other isolates did not differ. The highest values at 30 ºC were obtained by isolates Co8 and P5 (23.9 and 23.5 million spores cm^{-2}, respectively) and these were significantly higher than those of isolates Co13, E3, E22, N3 and Co12 ($2.4 \leq x \leq 12.9$ million spores cm^{-2}) (Figure 7.5).

The percentage of germinated spores after 24 h incubated at 24 ºC failed to exceed 14.8% in any treatment (Figure 7.5). This variable ranged from 1.7% to 14.8%, 0.8% to 11.5% and 0.8% to 10.8% for spores produced at 18 ºC, 24 ºC and 30 ºC, respectively. Isolate Co8 consistently registered high percentages of germinated spores at the three temperatures evaluated, whereas isolates P5 and E22 registered low values at the same temperatures (Figure 7.5). Isolate V3 had a good performance at 24 ºC and 30 ºC but not at 18 ºC. The lowest percentages of germinated spores were obtained for isolates P5, E22 and V3 (1.7%, 1.8% and 2.5%, respectively) at 18 ºC, for isolates E22, P5, Co11 and Co16 (0.8%, 0.9%, 1.8% and 1.8%, respectively) at 24 ºC and for isolates Co16, P5, E22, Co-13, Co12, Co11 and Co10 ($0.8 \leq x \leq 1.9$ million spores cm^{-2}) at 30 ºC (Figure 7.5).



[1/] Isolates within each variable and temperature with the same letter do not have significant differences (Tukey, P<0.05).

Figure 7.5 Spore production, spore density and percentage of germinated spores after 24h of 15 isolates of *M. roreri* grown at three different temperatures.

The percentage of globose, ellipsoid and abnormal spores are shown together in Figure 7.6. The globose and ellipsoid spores were the most frequently observed; however, spores with other diverse spore shapes (most of them abnormal) were also seen but in a lower proportion (Figure 7.6).

The percentage of globose spores ranged from 21.2% to 85.0%, from 18.3% to 95.0% and from 5% to 93.3 % at 18 ºC, 24 ºC and 30 ºC, respectively (Table A7.2). The percentage of ellipsoid spores ranged from 11.7% to 57.5%, from 5.0% to 55.0% and from 3.3% to 45.0 % at the same temperatures.

Isolate E20 showed the lowest percentages of globose spores at 18 ºC, 24 ºC and 30 ºC with 21.2%, 18.3% and 5%, respectively (Table A7.2). This isolate differed statistically ($P<0.05$) from most other isolates but in particular from isolate P5 which consistently registered the highest percentages (85% at 18 ºC, 95% at 24 ºC and 93.3% at 30 ºC).

A considerable proportion of abnormal spores were obtained for isolate E20 at 18 ºC, 24 ºC and 30 ºC (Figure 7.6). Isolates E22, C19 and N3 at 30 ºC and V3 at 18 ºC also registered a considerable percentage of abnormal spores. For isolate V3, fewer abnormal spores were observed as the temperature increased, but an inverse behaviour was observed for isolate E20.

Globose spore diameter ranged from 8.5µm to 10.4µm, 8.5µm to 9.8µm and 7.5µm to 10.5µm at 18 ºC, 24 ºC and 30 ºC, respectively (Figure 7.7). Significant differences ($P<0.05$) were found at 18 ºC between isolates Co5, E22, E3 and P5 that registered the highest diameters ($10.4 \geq x \geq 10.3$ µm) and isolates V3 and N3 with the lowest (8.5µm and 9.1µm, respectively). Isolate E20 showed the lowest value recorded at 24 ºC and 30 ºC (8.5µm and 7.5µm, respectively) which differed significantly ($P<0.05$) from isolates that showed the highest diameters and in particular with Co5 and Co8 at 24 ºC (9.8µm and 9.6 µm, respectively) and E22 at 30 ºC (10.5µm) (Figure 7.7).

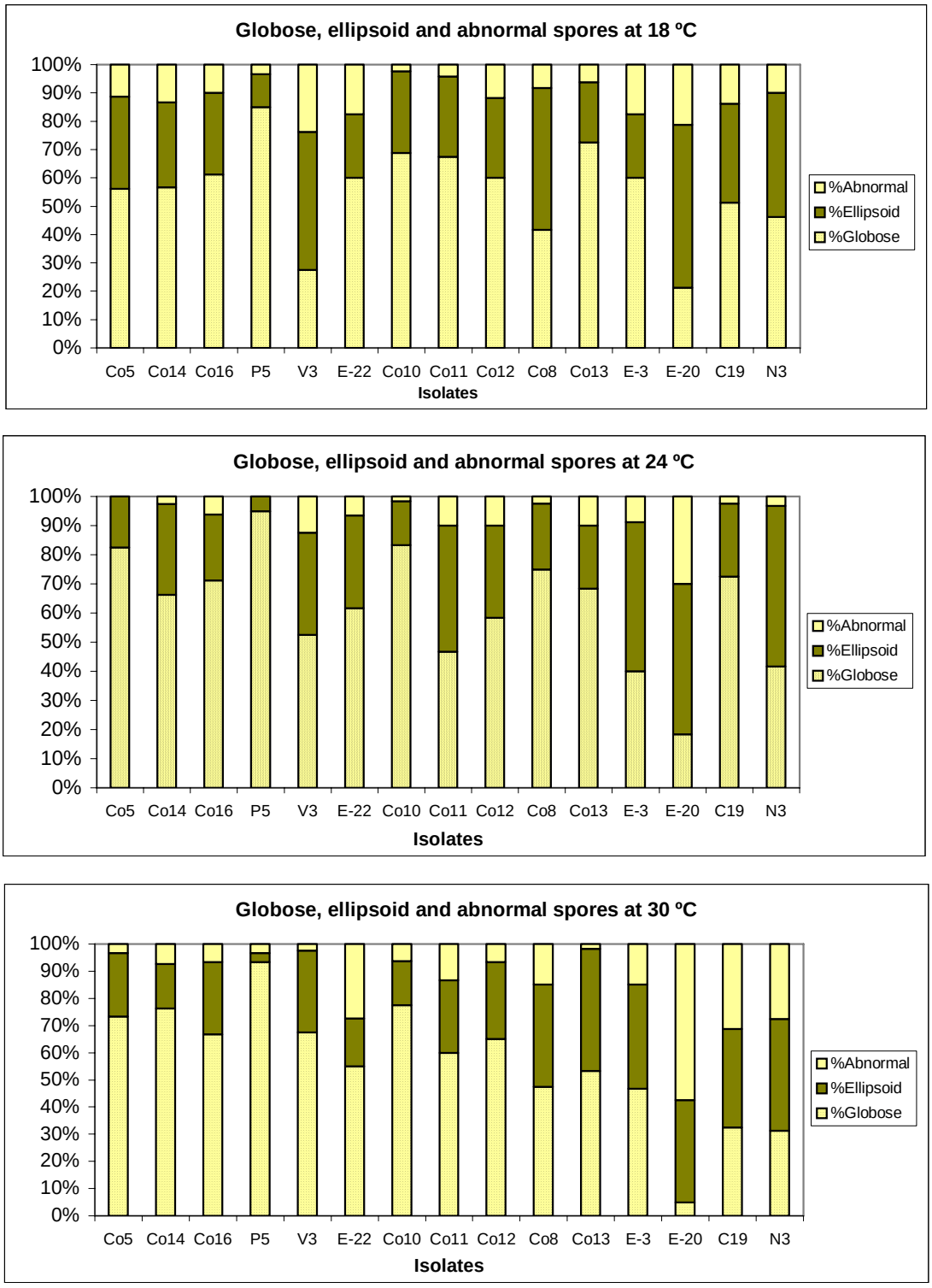
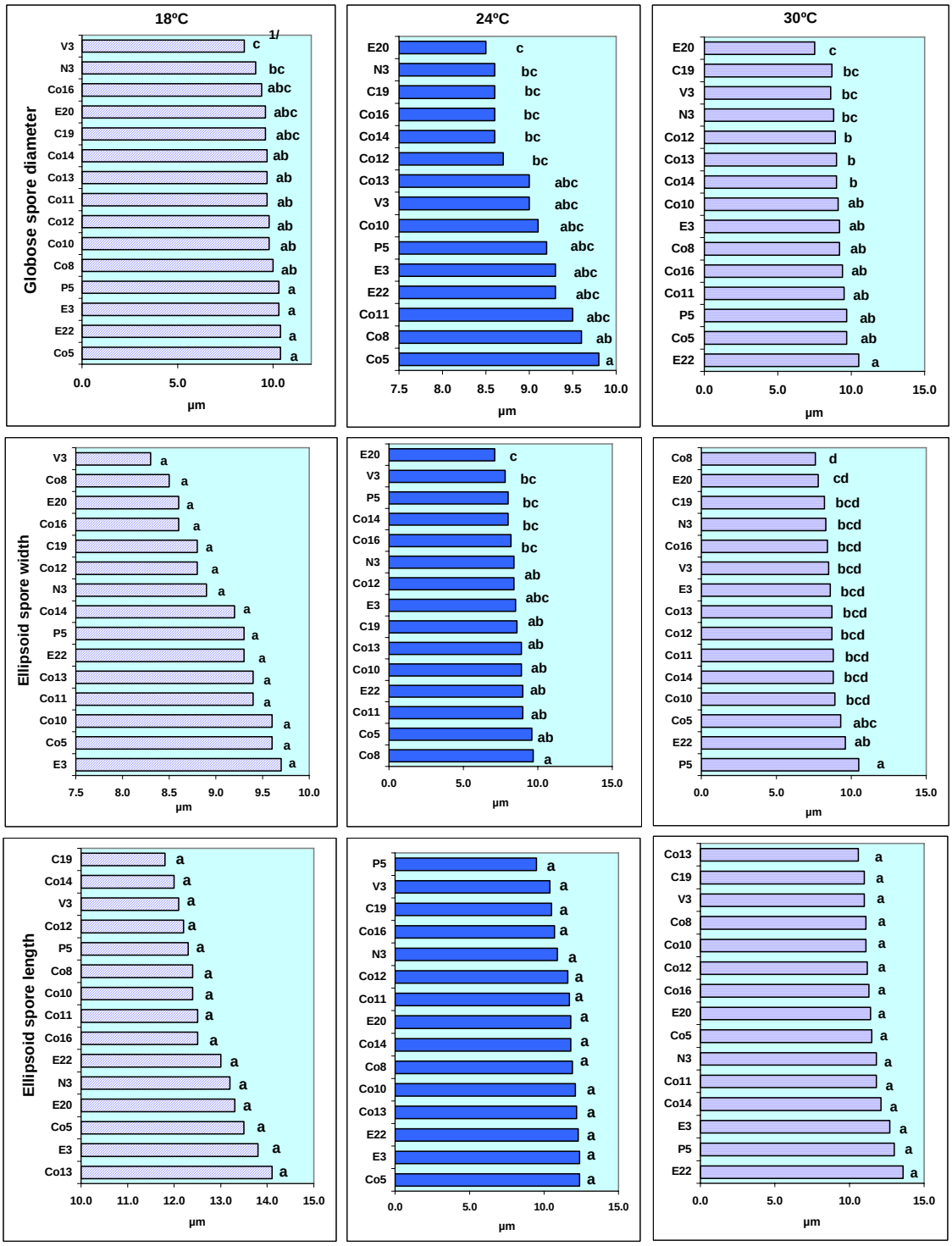


Figure 7.6 Percentage of globose, ellipsoid and abnormal spores of 15 isolates of *M. roreri* grown at three different temperatures.



[1/] **Isolates within each variable and temperature with the same letter do not have significant differences (Tukey, P<0.05).**

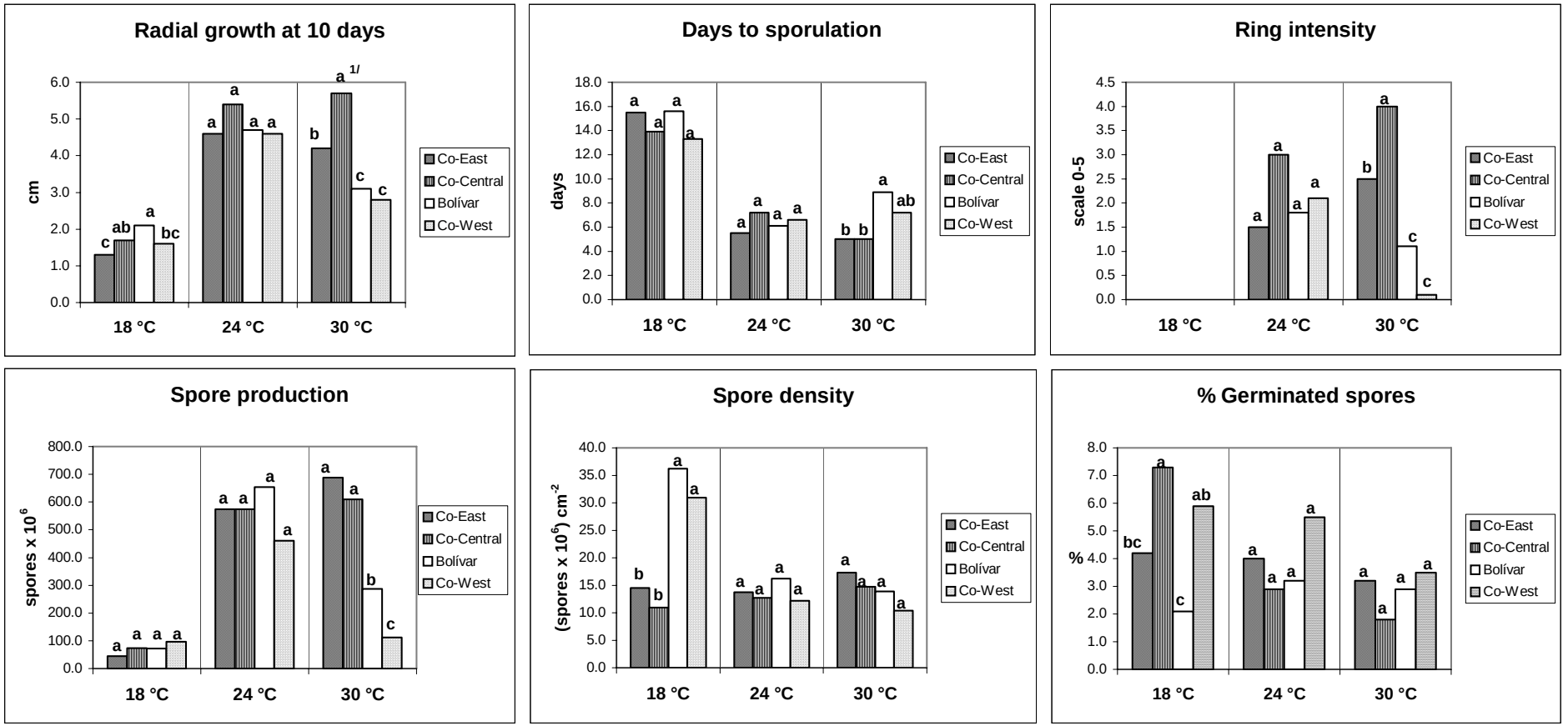
Figure 7.7 Globose spore diameter, ellipsoid spore width and ellipsoid spore length of 15 isolates of *M. roreri* grown at three different temperatures.

Ellipsoid spore width ranged from 8.3µm to 9.7µm, from 7.1µm to 9.7µm and from 7.6µm to 10.5µm at 18 ºC, 24 ºC and 30 ºC, respectively (Figure 7.7). There was no significant variation ($P<0.05$) in this variable at 18 ºC. At 24 ºC, the highest values were obtained for isolate Co8 (9.7µm) which was significantly higher ($P<0.05$) than those of isolates E20, V3, P5, Co14 and Co16 ($7.1 \leq x \leq 8.2$µm). At 30 ºC, isolates with the highest ellipsoid spores width P5, E22 and Co5 ($10.5 \geq x \geq 9.3$µm) were significantly broader ($P<0.05$) than the isolate showing the lowest value, Co8 (7.6µm). No significant differences ($P<0.05$) between isolates were obtained for ellipsoid spores length at any temperature (Figure 7.7).

7.4.4 ANALYSIS BY GENETIC GROUP

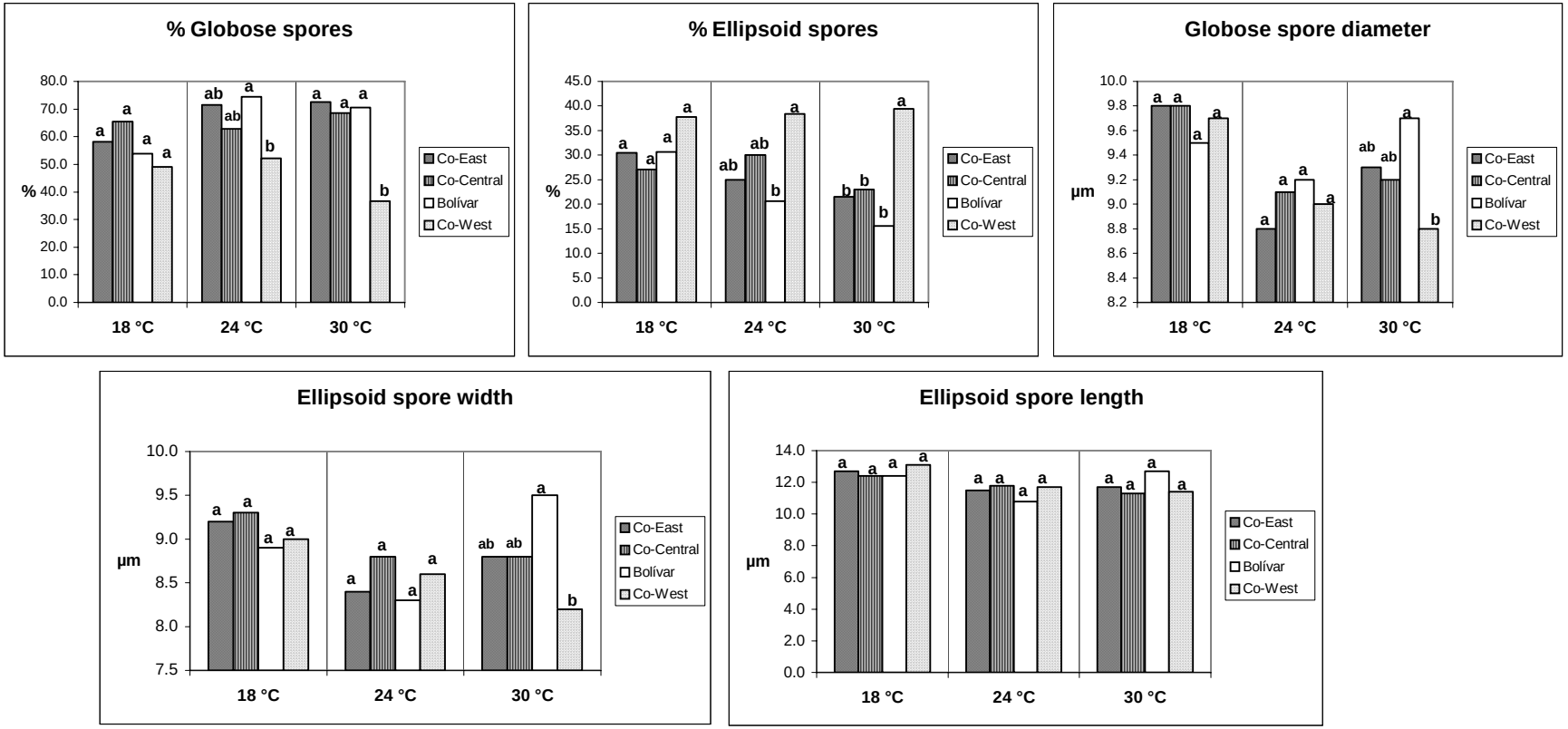
When the isolates were analysed in accordance with their correspondent genetic group, highly significant ($P<0.01$) or significant ($P<0.05$) differences were found for variables radial growth, spore production, spore density and the percentage of germinated spores at 18 ºC (Table A7.3) and significant differences ($P<0.05$) were identified for the percentage of globose and ellipsoid spores at 24 ºC (Table A7.4). At 30 ºC, highly significant differences ($P<0.01$) were found for variables radial growth, days to sporulation, ring intensity, spore production, percent globose and ellipsoid spores, globose spore diameter and ellipsoid spore width (Table A7.5).

For all variables except ellipsoid spore length, significant differences ($P<0.05$) were obtained between the major genetic groupings of isolates for at least one of the three temperature tested (Figures 7.8 and 7.9). Three variables at 18 ºC (radial growth, spore density and germinated spores), two variables at 24 ºC (globose spores and ellipsoid spores) and eight variables at 30 ºC (radial growth, days to sporulation, ring intensity, spore production, globose spores, ellipsoid spores, globose spore diameter and ellipsoid spore width) registered significant differences ($P<0.05$) among the four genetic groups (Figures 7.8 and 7.9).



[1/] Genetic groups within each variable and temperature with the same letter do no have significant differences (Tukey, P<0.05)

Figure 7.8 Radial growth at 10 d, days to sporulation, ring intensity, spore production, spore density and percentage of germinated spores of four genetic groups of *M. roreri*.



[1/] **Genetic groups within each variable and temperature with the same letter do no have significant differences (Tukey, P<0.05).**

Figure 7.9 Percentage of globose and ellipsoid spores, globose spore diameter, ellipsoid spore width and ellipsoid spore length of four genetic groups of *M. roreri*.

The only three variables that made a distinction between Co-East and Co-Central groups were radial growth at 18 ºC and 30 ºC, ring intensity at 30 ºC and % germinated spores at 18 ºC (Figure 7.8 and 7.9). In all cases values were higher in Co-Central. These two groups differed ($P<0.05$) from Bolívar group for radial growth, days to sporulation, ring intensity, spore production at 30 ºC and spore density at 18 ºC. Moreover, Bolívar differed ($P<0.05$) from Co-East in radial growth and from Co-Central in germinated spores at 18 ºC.

Co-Central and Co-East groupings separated significantly ($P<0.05$) from the Co-West group for most variables at 30 ºC, viz.: globose spore diameter, ellipsoid spore width, ring intensity, spore production, % globose spore, % ellipsoid spore and radial growth and only for spore density at 18 ºC. Moreover, at 18 ºC, the Co-West group differed ($P<0.05$) in radial growth from the Co-East group. A significant distinction ($P<0.05$) was observed between Bolívar and Co-West groups in globose spore diameter, ellipsoid spore width, % globose and % ellipsoid spore at 30 ºC, in % ellipsoid spore at 24 ºC and in radial growth and % germinated spores at 18 ºC. The Bolívar group showed significantly higher radial growth (2.1 cm) than Co-East (1.3 cm) after 10 d at 18 ºC ($P<0.05$) (Figure 7.8). At 30 ºC, the Co-Central group showed the highest radial growth (5.7 cm) and the Co-West group the lowest (2.8 cm). The Co-East group was intermediate and significantly different ($P<0.05$) for the other groups

No differences ($P<0.05$) were observed between groups in the days to sporulation, ring intensity and spore production at 18 ºC and 24 ºC (Figure 7.8). At 30 ºC, Co-Central and Co-East groups showed a significantly shorter time to sporulation (5 d) in comparison to the Bolívar isolates (8.9 d). At the same temperature, ring intensity was at maximal intensity in the Co-Central group (4.0 value) and minimal in Co-West and Bolívar groups (0.1 and 1.1 values, respectively). These extreme groups were significantly different ($P<0.05$) for both variables.

Spore production and spore density followed inverse patterns of variation at 30 ºC and 18 ºC (Figure 7.8). At 30 ºC, spore production was highest in Co-East and Co-Central groups (687.6 and 609.6 million spores, respectively) and lowest in Co-West and Bolívar isolates (111.9 and 286.5 million spores, respectively). At 18 ºC, however, spore density was

significantly higher in Bolívar and Co-West isolates (36.2 and 30.9 million spores, respectively) than in Co-Central and Co-East isolates (10.9 and 14.5 million spores, respectively). Differences were significant ($P<0.05$) in both cases.

Percentage of spore germination at 18 ºC was highest in Co-West (7.3%) and lowest in Co-East (4.2%) (Figure 7.8). These values differed significantly ($P<0.05$).

Differences between groups in the percentage of globose and ellipsoid spores were significant ($P<0.05$) only at 24 ºC and 30 ºC (Figure 7.9). At both temperature, the percentage of globose spores was significantly reduced ($P<0.05$) in the Co-West group (52.1 and 36.7%, respectively). Not surprisingly, a more or less inverse of this pattern was observed for % ellipsoid spores.

Variation in globose spore diameter and ellipsoid spore width were only significant between groups at 30 ºC. A similar ranking between groups was observed for both variables: Bolívar had significantly higher values (9.7 and 9.5 µm for globose diameter and ellipsoid width, respectively) to Co-West (8.8 and 8.2 µm) (Figure 7.9). As mentioned above, no significant differences were found between groups for ellipsoid spore length.

7.5 DISCUSSION

Temperature had a significant effect on the isolates of *M. roreri* evaluated in this study, which represented the global genetic diversity of this fungus. The isolates showed optimal average performance in terms of growth and sporulation at 24 ºC. This closely matches mean daily temperatures in the tropics and so it is possibly a consequence of adaptation to the environmental conditions where the hosts of *T. cacao* (*Theobroma* and *Herrania* species) normally grow. In this sense, the minimum annual average temperature for the cacao tree has been established at 21 ºC with optimums for the different physiological processes between 22-28 ºC (Hardy, 1960). At the primary centre of diversity of *T. cacao*, in the headwaters of the River Amazon, temperatures vary between 20 ºC and 30 ºC, but for trees in the lower storey of the rain forest the diurnal variation is reduced to about 6 ºC (Wood & Lass, 1985).

Although the growth and sporulating capability of *M. roreri* were severely reduced at 18 ºC, all isolates were nevertheless able to complete their life cycle at the three temperature tested. This result helps to explain the ability of the fungus to thrive under a wide range of environmental conditions, from sea level to over 1000 m and from very dry (irrigated) to very humid zones (Evans, 1981). This result also suggests that *M. roreri* could be capable of surviving in areas beyond its current geographic range and is sufficiently robust to accommodate for climatic change. Significantly, temperature in the tropics is projected to rise between 1 ºC and 3 ºC by 2100 and it is considered as a factor that will impact strongly on disease management and research policy (Chakraborty, 2002).

The highest level of morpho-physiological differentiation among the genetic groups was found between Co-East and Co-Central groups on one hand and Bolívar group and, in particular, the Co-West group on the other. The former two groups are genetically related and currently still restricted to part of Colombia whereas the latter two groups are dispersed in the following countries: Bolívar group in Colombia, Venezuela, Ecuador and Peru, and Co-West group in Colombia, Ecuador, Panama, Costa Rica, Nicaragua and Honduras (Chapter 4).

It is relevant to mention that most differentiation among genetic groups was found when the fungus was cultured at temperatures other than its optimum (24 ºC), in particular at 30 ºC, which is consistent with the possible adaptation of isolates to conditions at the extreme of its natural range of temperatures.

In general, Co-Central group showed the most vigorous behaviour in comparison with the other groups at 30 °C suggesting that these isolates are perhaps less sensitive to raised temperatures and possibly even that the optimum temperature for these isolates could be higher than that for other isolates. At this temperature, growth and sporulation were considerably favoured in this group in terms of the following variables: radial growth, days to sporulation, ring intensity and spore production. Similar results were obtained for Co-East but to a lesser extent for radial growth and ring intensity. Isolates from these two groups were collected at considerable altitudes ($1520 \geq x \geq 460$ m.a.s.l.) where the

pathogen is possibly adapted to grow and sporulated faster when temperatures are more favourable in particular for the dispersal of the spores.

Co-Central and Co-East groups only differed in three variables in the entire experiment: radial growth and % germinated spores at 18°C and ring intensity at 30°C. On the other hand, Co-West and Bolívar groups showed a considerable level of differentiation in relation to Co-Central and Co-East groups and both of them performed poorly at 30°C for the variables mentioned. Overall, however, there were far fewer differences between the Co-West and Bolívar groups than similarities. On average, Co-West was the less vigorous group showing both the lowest radial growth and spore production at 30°C. Moreover, this group showed the lowest percentage of globose spores at 24°C and 30°C and their spores were smaller at 30°C. In contrast, Bolívar group registered similar percentages of globose spores as Co-East and Co-Central and the biggest spores at the same temperatures.

Although value of the described traits to distinguish between genetic groups has debatable practical utility, the ability to distinguish between the major genetic groups of *M. roreri* on the basis of morpho-physiological characters has significance in that it appears to demonstrate detectable differences in growth and reproductive capacities. This unexpected variability may allow the fungus the flexibility to occupy subtly different endoenvironments and perhaps to adapt to hosts exhibiting different pod growth characteristics.

The present results suggest that much of the morpho-physiological differentiation of *M. roreri* could have originated from adaptation processes. The fact that greatest morpho-physiological diversity seemingly occurred between Bolívar and Co-West groups in relation with Co-East and Co-Central may be of significance. These data are consistent with *M. roreri* originating in Colombia but showing morpho-physiological adaptation as it dispersed eastwards and westwards from the hypothetical centre of origin in north eastern Colombia into areas where the climatic conditions were different, in particular for humidity and temperature.

It is possible that the most significant changes coincide with altitudinal changes, in particular when *M. roreri* dispersed from the original environment in sites possibly at more

than 500 m.a.s.l. to lower altitudes, for instance, westwards near to the north-western coast of Colombia where annual precipitation and average temperature in certain areas can be as high as 5.000 mm yr^{-1} and 26 °C, respectively, or eastwards in the north-eastern Colombia region where precipitation is much lower and temperatures higher. The two described places are, respectively, possible candidates sites where the genetic groups Co-West and Bolívar may have originated.

The optimal performance of *M. roreri,* in terms of different parameters related to growth and spore production obtained at 24 ºC, agrees with the findings of Chacín (1975), Evans (1981) and Herrera (1988) who established the optimum growth and sporulation of this fungus within the range 24-28 ºC. As mentioned above, although *M. roreri* showed a similar behaviour for the same parameters at 30 ºC, its radial growth was reduced in most isolates. This result suggests that *M. roreri* has, on average, a higher range of optimal temperatures for sporulation than for mycelial growth. However, this is not a general rule for all isolates. For instance, the sporulation capability of the Costa Rican isolates was significantly reduced in this experiment at 30 ºC which agrees with the findings of Phillips and Galindo (1985), and also Herrera (1988) who reported a reduction in spore production of Costa Rican isolates above 26 ºC.

Growth and sporulation of *M. roreri* were seriously depressed at 18 ºC. The level of reduction produced at this temperature was as high as 7-fold for total spore difference production, 3-fold for radial growth and nearly 2-fold for days to sporulation. These reductions indicate that 18 ºC is suboptimal for vegetative and reproductive growth of this fungus. Interestedly, variables related to the size of the spores (globose spore diameter, ellipsoid spore width and ellipsoid spore length) were significantly favoured at 18 ºC over 24 ºC and 30 ºC. This observation may be indicative of a so far uncharacterised mechanism for survival against cold. It is possible that this or similar mechanisms could also be activated under other conditions of stress. Moreover, the enlargement of spore walls in *M. roreri* after 40-50 days may be connected with the formation of resistant structures (Campuzano, 1981a). The larger spores of the Bolívar group at 30 ºC could have similar significance.

Spore density was also increased at 18 ºC. This means that, although the growth and sporulation of *M. roreri* was severely reduced at this temperature, the production of spores was more concentrated. A possible explanation of this result may arise from the reduced growth rate at this temperature could lead to a higher density of hyphae.

In summary, the results obtained show that the isolates of *M. roreri* have a considerable level of variation in their response to different temperatures. This variation has a genetic base that may have originated in the natural adaptation of certain isolates to specific environmental conditions and, is possibly related with the historical dispersal of *M. roreri* from its hypothetical centre of origin into the ecological diverse environments that its currently occupies.

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7.7 APPENDIX

Table A7.1 Analysis of variance for eleven morpho-physiological variables measured on 15 isolates of *M. roreri*.

Source of variation	Radial growth			Days to sporulation			Ring intensity			Spore production		
	DF[1/]	Mean Square	Signif. [2/]	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.
Temperature	2	132.570	**	2	1042.011	**	2	8.788	**	2	2664,392	**
Isolate (temperature)	42	2.854	**	42	19.777	**	42	0.558	**	42	126,728	**
Error Plate (isolate(temp.))	104	0.163		104	262.500		104	0.138		104	1885,329	

Source of variation	Spore density			% germinated spores			% globose spores			% ellipsoid spores		
	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.
Temperature	2	2,288	**	2	0.025	n.s.	2	0.085	n.s.	2	0.029	n.s.
Isolate (temperature)	42	309	**	42	0.015	**	42	0.159	**	42	0.083	**
Error Plate (isolate(temp.))	104	7,797		102	1,020.002		104	0.024		104	0.018	

Source of variation	Globose spore diameter			Ellipsoid spore width			Ellipsoid spore length		
	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.
Temperature	2	7.532	**	2	3.467	*	2	21.969	**
Isolate (temperature)	42	0.866	**	42	1.119	**	42	2.041	n.s.
Error Plate (isolate(temp.))	103	0.170		100	0.317		100	2.020	

[1/] **DF = degrees of freedom**

[2/] **Sign. = Statistical significance: ** = highly significant (P<0.01), * = significant (P<0.05), n.s. = non significant.**

Table A7.2 Results of the Tukey test for the percentage of globose and ellipsoid spores evaluated for 15 isolates of *M. roreri* grown under three temperatures.

	% globose spores			% ellipsoid spores		
Isolate	**18 °C**	**24 °C**	**30 °C**	**18 °C**	**24 °C**	**30 °C**
Co5	56.2 abcd	82.5 ab	73.3 abcd	32.5 ab	17.5 de	23.3 ab
Co14	56.7 abcd	66.2 bc	76.2 abc	30.0 ab	31.2 abcd	16.5 ab
Co16	61.2 abc	71.2 abc	66.7 ab	28.8 ab	22.5 cd	26.7 a
P5	85.0 a	95.0 a	93.3 a	11.7 c	5.0 e	3.3 b
V3	27.5 cd	52.5 bc	67.5 ab	48.8 ab	35.0 abcd	30.0 a
E-22	60.0 abc	61.7 bc	55.0 abc	22.5 ab	31.7 abcd	17.5 ab
Co10	68.8 ab	83.3 ab	77.5 ab	28.8 ab	15.0 de	16.2 ab
Co11	67.5 ab	46.7 cd	60.0 ab	28.2 ab	43.3 abc	26.7 a
Co12	60.0 abc	58.3 bc	65.0 ab	28.2 ab	31.7 abcd	28.3 a
Co8	41.7 bcd	75.0 abc	47.5 abc	50.0 a	22.5 cd	37.5 a
Co13	72.5 ab	68.3 bc	53.3 ab	21.2 ab	21.7 cde	45.0 a
E-3	60.0 abc	40.0 cd	46.7 abc	22.5 ab	51.2 ab	38.3 a
E-20	21.2 d	18.3 d	5.0 e	57.5 a	51.7 ab	37.5 a
C19	51.2 abcd	72.5 abc	32.5 bc	35.0 ab	25.0 bcd	36.2 a
N3	46.2 bcd	41.7 cd	31.2 de	43.8 ab	55.0 a	41.2 a

[1/] Values in each column followed by the same letter do not have significant differences (Tukey Test, P<0.05).

Table A7.3 Analysis of variance by genetic group for eleven morpho-physiological variables measured on 15 isolates of *M. roreri* at 18 ºC.

Source of variation	Radial growth			Days to sporulation			Ring intensity			Spore production		
	DF[1/]	Mean Square	Signif[2/]	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.
Group	3	1.113	**	3	14.428	n.s	3	0		3	7117.938	*
Error	51	0.150		51	8.028		51	0		51	2415.397	

Source of variation	Spore density			% germinated spores			% globose spores			% ellipsoid spores		
	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.
Group	3	1832.094	**	3	0.038	**	3	0.082	n.s.	3	0.043	n.s.
Error	51	11090.820		51	0.005		51	0.044		51	0.037	

Source of variation	Globose spore diameter			Ellipsoid spore width			Ellipsoid spore length		
	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.
Group	3	0.213	n.s.	3	0.553	n.s.	3	2.166	n.s.
Error	51	0.377		50	0.554		50	2.686	

[1/] **DF = degrees of freedom**

[2/] **Sign. = Statistical significance: ** = highly significant (P<0.01), * = significant (P<0.05), n.s. = non significant.**

Table A7.4 Analysis of variance by genetic group for eleven morpho-physiological variables measured on 15 isolates of *M. roreri* at 24 ºC.

Source of variation	Radial growth			Days to sporulation			Ring intensity			Spore production		
	DF[1/]	Mean Square	Signif[2/]	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.
Group	3	1.542	n.s.	3	5.177	n.s.	3	0.710	n.s.	3	86101.865	n.s.
Error	43	0.816		43	5.106		43	0.339		43	69035.365	

Source of variation	Spore density			% germinated spores			% globose spores			% ellipsoid spores		
	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.
Group	3	35.424	n.s.	3	0.019	n.s.	3	0.199	*	3	0.121	*
Error	43	46.576		41	0.007		43	0.054		43	0.32	

Source of variation	Globose spore diameter			Ellipsoid spore width			Ellipsoid spore length		
	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.
Group	3	0.265	n.s.	3	0.334	n.s.	3	1.986	n.s.
Error	43	0.262		42	0.595		42	1.601	

[1/] **DF = degrees of freedom**

[2/] **Sign. = Statistical significance: ** = highly significant (P<0.01), * = significant (P<0.05), n.s. = non significant.**

Table A7.5 Analysis of variance by genetic group for eleven morpho-physiological variables measured on 15 isolates of *M. roreri* at 30 ºC.

Source of variation	Radial growth			Days to sporulation			Ring intensity			Spore production		
	DF[1/]	Mean Square	Signif[2/]	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.
Group	3	20.012	**	3	35.106	**	3	4.471	**	3	947939.018	**
Error	43	0.608		43	6.977		43	0.178		43	23086.398	

Source of variation	Spore density			% germinated spores			% globose spores			% ellipsoid spores		
	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.
Group	3	108.012	n.s.	3	0.004	n.s.	3	0.497	**	3	0.244	**
Error	43	41.030		43	0.004		43	0.053		43	0.019	

Source of variation	Globose spore diameter			Ellipsoid spore width			Ellipsoid spore length		
	DF	Mean Square	Signif.	DF	Mean Square	Signif.	DF	Mean Square	Signif.
Group	3	1.780	**	3	2.953	**	3	3.082	n.s.
Error	42	0.395		41	0.366		41	1.573	

[1/] DF = degrees of freedom

[2/] Sign. = Statistical significance: ** = highly significant (P<0.01), * = significant (P<0.05), n.s. = non significant.

CHAPTER 8

Morpho-physiological variation of the cacao (*Theobroma cacao* L.) pathogen *Moniliophthora roreri* (Cif.) Evans *et al*. in tropical America

8.1 ABSTRACT

The morpho-physiological variation of *M. roreri*, causal agent of the moniliasis or frosty pod diseases in cacao, was studied using 88 isolates collected from the eight countries and major areas currently affected by the pathogen. The fungus was grown in Petri dishes containing a modified V8 culture medium during 20 d at 24 °C and 12/12 h alternate periods of light/darkness. Eleven variables were analysed: radial growth, days to sporulation, ring intensity, spore production, spore density, spore germination, percentage of globose and ellipsoid spores, globose spore diameter, ellipsoid spore width and ellipsoid spore length.

Significant disparity was found among the isolates for all variables. A Mantel test revealed a poor fit exists between the morpho-physiological variation and the molecular variation described previously (r = 0.20) and between the morpho-physiological variation and the geographic distances between the collection sites of the isolates (r =0.13).

The analysis of the isolates according to the genetic groups previously defined using the AFLP and ISSR analyses detected however, significant differences for most variables studied, suggesting a genetic base in the expression of the characters. On average, isolates from Central Colombia (Co-Central group) grew faster and showed the highest ring intensity whereas isolates from Bolívar (group that comprises some isolates from Colombia and Ecuador and all isolates from Venezuela and Peru) were the most vigorous in terms of production, density and size of spores. Isolates from the Santander Department of Colombia (Co-East group) showed a high production and density of spores and their mycelial growth was intermediate. Two wild isolates from north-western Ecuador (Gileri group) and a group of genotypes comprising the Central American, the Central Colombian and two Western Colombian isolates (Co-West group) registered the lowest radial growth

and production of spores and produced the smallest spores. The possibility that these differences originated from the adaptation of the isolates to specific environmental conditions is explored.

A cluster analysis carried out on all 88 isolates on the basis of five selected traits (radial growth, days to sporulation, ring intensity, spore production and globose spore diameter), separate most isolates with a western Colombian origin (Co-West and Gileri) from those with a eastern Colombian origin (Co-Central, Co-East, Bolívar). There was a tendency for isolates from the same genetic group to cluster together, although several divergent isolates were also observed.

When isolates were analysed in accordance with their country of origin, significant differences were also found for most variables. The Peruvian, Colombian and Venezuelan isolates were on average the most vigorous in terms of growth, sporulation and size of spores. At the opposite extreme, the less vigorous isolates were from Central Ecuador and Central America.

Keywords: cacao, frosty pod disease, genetic diversity, moniliasis, *Moniliophthora roreri*, radial growth, spore morphology, *Theobroma cacao*.

8.2 INTRODUCTION

Moniliophthora roreri (Cif.) Evans *et al*., the causal agent of moniliasis or frosty pod diseases is currently one of the most important factors limiting cacao production in several Latin American countries (Colombia, Ecuador, Venezuela, Peru, Panama, Costa Rica, Nicaragua and Honduras) and soon threatens to spread to other important cacao producing countries in the region.

Knowledge of the biology of *M. roreri* is limited particularly with relation to the phenotypic and genetic diversity of the pathogen and to the geographical distribution of this diversity.

Certain morpho-physiological characteristics of the fungus have been evaluated in previous works although these studies have invariably examined only one or a few isolates. The morpho-physiological features of the fungus were first described by Ciferri & Parodi (1933) from diseased cacao pods collected in Ecuador. The authors observed that spores were highly diverse in shape, but more frequently spheroid, 7.5 µm to 10 µm in size, to subhelicoidal, (8-10.5 µm) x (9-14 µm) in size. Evans *et al*. (1978) also used samples from Ecuador to describe the fungus in more detail. They reported that growth on malt extract agar was slow, with colonies attaining a diameter of 8-15 mm only after 2 weeks of growth. Mat was described as diverse both in colour and appearance. Spores were easily separable, thick-walled, pale yellow, brown coloured en masse, typically globose to subglobose (6.5-) 8-15 (-25) µm in diameter sometimes ellipsoid 8-20 µm x 5-14 µm, with walls up to 2 µm thick. Cylindrical thin-walled spores were also observed and probably derived from immature chains.

Castaño (1952) and Campuzano (1976) both described the growth and morphology of isolates collected from the Department of Caldas, Colombia. Castaño (1952) observed that colonies showed concentric growth areas of different colours with a compact sporulating area in the centre of the colony when grown on PDA at 28ºC. Spores were heteromorphic with the predominating shapes being spheroid, ellipsoid and elliptic ovoid, and the spore size was (5-10 µm) x (5-15 µm). On the other hand, Campuzano (1976) observed that spore density formed on diseased pods increased most rapidly during the first nine days of the colony. The peak of spore production was reached after 15-16 d with 4.4×10^7 spores cm^{-2} or 7.7×10^9 spores per pod.

In Venezuela, Chacín (1975) grew samples of *M. roreri* on PDA and reported that colonies covered the plates in 7-8 d. The colony was initially white-cream-coloured but became light yellow when older. Spores were generally ovoid or ellipsoid and exceptionally globose. Spores were (2.7-5.4 µm) x (6.8-9.4 µm) in size with an average of 4.6 µm x 7.6 µm.

Several authors have examined the germination behaviour of spores of *M. roreri* and have found that the percentage of germinated spores can be very variable, ranging from 0% when the spores are kept under a 100% moisture environment but without free water

(Bastidas, 1953) to 80% when the spores were placed in Petri dishes with Yeast Extract Agar medium (Oxoid Yeast extract 1%, Difco agar 1.5%) (Ram *et al.,* 1987). Considerable variation has been also noted between samples (Bastidas, 1953). Several environmental factors have been suggested to contribute to this variability, including humidity (Bastidas, 1953; Chacín, 1975), spore age (López, 1954; Ram *et al.* 1987), source of spores (López, 1954), pH (López, 1954; Chacín, 1975; Ram *et al*., 1987), temperature (López, 1954; Chacín, 1975; Merchán, 1981; Ram *et al.*, 1987) and substratum (Bastidas, 1953, López, 1954; Umaña & González, 1981). Ram (1989) observed differences in the germination of spores of isolates from different countries. Evans (1981) mentioned that there appears to be no relationship between germination in the laboratory and pod penetration and infection in the field, since spores which failed to germinate in the laboratory nevertheless proved to be infective following inoculation of pods in the field. He concluded that there may be a dormancy mechanism in older thick-walled spores.

Some authors have reported differences among isolates of *M. roreri* in respect to their morpho-physiological phenotypes (Jiménez *et al*., 1986; Ram, 1989; Herrera *et al.*, 1989). Chacín (1975) suggested the existence of physiological races of this fungus in Venezuela, although she failed to present experimental evidence to support this claim.

Jiménez *et al.* (1986) reported significant differences among isolates of *M. roreri* from Costa Rica when evaluating their *in vitro* growth in the presence and absence of the antagonistic bacterium *Pseudomonas aeruginosa*, but also failed to give details about their findings. However, the most extensive studies on the morpho-physiological variation of *M. roreri* were carried out by Herrera *et al*. (1989) and Ram (1989). Herrera *et al.* (1989) evaluated *in vitro* growth and morphology of 16 isolates from various cacao-producing zones of Costa Rica when cultured on a modified V8 culture medium (20% V8, 2% maltose 0.1% asparagine and 2% agar) and grown at 25ºC, in 90% relative humidity and 12/12 alternate periods of light/darkness. Significant differences were found after a 14 d-growth-period for all four variables evaluated (colony diameter, diameter of the sporulated area, spores plate^{-1} and spores area^{-1}).

Ram (1989) studied *in vitro* growth, spore production and germination of nine isolates from Colombia, Ecuador, Costa Rica and Venezuela grown in three different media

(potato-carrot-dextrose-agar, V-8 juice-maltose-asparagine-agar and malt extract-maltose-agar) cultured at 24 ºC in 12/12 alternate periods of light/darkness for 15 d. He found that isolates from Napo (Ecuador) and Manizales (Colombia) grew more rapidly on all three media used whereas the isolate from Bajo Cauca (Colombia) was the slowest growing. All isolates produced concentric zones of sporulation. Sporulation appeared 2 d after the initiation of colonies of isolates from Costa Rica and 2-4 d later in other isolates. Sporulation was estimated quantitatively on media potato-carrot-dextrose-agar. Isolates from Costa Rica sporulated most abundantly (21-35 x 10^6 conidia cm^{-2}) and the Venezuelan isolate produced the least number of conidia (13 x 10^6 conidia cm^{-2}) and lost its sporulating capacity very quickly (after 3 or 4 transfers). Differences were also noted in the morphology of conidia. The spores from Costa Rica and Urabá (Colombia) were generally oval whereas other isolates produced mostly spherical spores. The conidia of most isolates germinated well on yeast extract agar, yielding between 65% and 86% germination but isolates from Ecuador had relatively low percentages of germination, 35-36% (Ram, 1989).

Ram (1989) also studied the mycelial interactions of the nine isolates by growing them in Petri dishes in all possible combinations. Isolates from Ecuador were distinct but had some commonality with the isolate from Manizales (Colombia). Costa Rican isolates produced some evidence of commonality only with isolates from Urabá (Colombia) which was used to infer a common origin for both groups of isolates. The Venezuelan isolate showed some similarity with isolates from Ecuador and Manizales but not with isolates from Costa Rica.

The objectives of the present research were to determine the level of morpho-physiological variation of *M. roreri* in tropical America by analysing 88 isolates collected across its entire geographic range, to detect relationships among the isolates based on these characters and to relate the geographic and genetic origin of the isolates to the morpho-physiological variation. Results on the genetic variation of the pathogen reported in Chapter 4 opened the possibility for a study of the relationship between the phenotypic and the genetic variation of *M. roreri*.

8.3 MATERIALS AND METHODS

8.3.1 LOCATION OF THE EXPERIMENT

The morpho-physiological study of the isolates was carried out in the Laboratories of Biotechnology and Phytopathology of the Tropical Agricultural Research and Higher Education Centre (CATIE) located in Turrialba, Costa Rica during the period June-August 2001. As indicated below, a mycelial interaction test comprising eight isolates was also achieved at CATIE in March 2002.

8.3.2 EXPERIMENTAL MATERIAL

A set of 88 isolates of *M. roreri* mostly collected by the author in 1999 was used in the study. Isolates from all countries and most of the major areas currently affected by the disease were represented in the study set (Figure 8.1). Sixteen isolates were from Colombia, 35 from Ecuador, three from Venezuela, four from Peru, two from Panama, 23 from Costa Rica, four from Nicaragua and one from Honduras.

Seventy nine isolates (90%) were obtained from *Theobroma cacao,* with the remaining being taken from the following species of *Theobroma* and *Herrania* held in the International Germplasm Collection of CATIE: *T. grandiflorum* (C14), *T. bicolor* (C20), *T. speciosum* (C22), *T. mammosum* (C25), *H. nitida* (C18) and *H. albiflora* (C23). Three isolates taken from *T. cacao* growing in the same locality were also studied, one collected in 1987 (C1) and two in 1999 (C21 and C24). Furthermore, two isolates were collected from the Napo Province in Ecuador, one from a clone of *T. bicolor* (E36) and the other from *T. cacao* (E37), and two additional isolates growing on *T. gileri* (E42 and E43) were also collected in Ecuador and included in the study.

The isolates belong to the following five genetic groups of *M. roreri* as determined in Chapter 4: 56 to the Western Colombia (Co-West), 18 to the Bolívar (Bolívar), two to the Gileri (Gileri), six to the Eastern Colombia (Co-East) and six to the Central Colombia group (Co-Central).

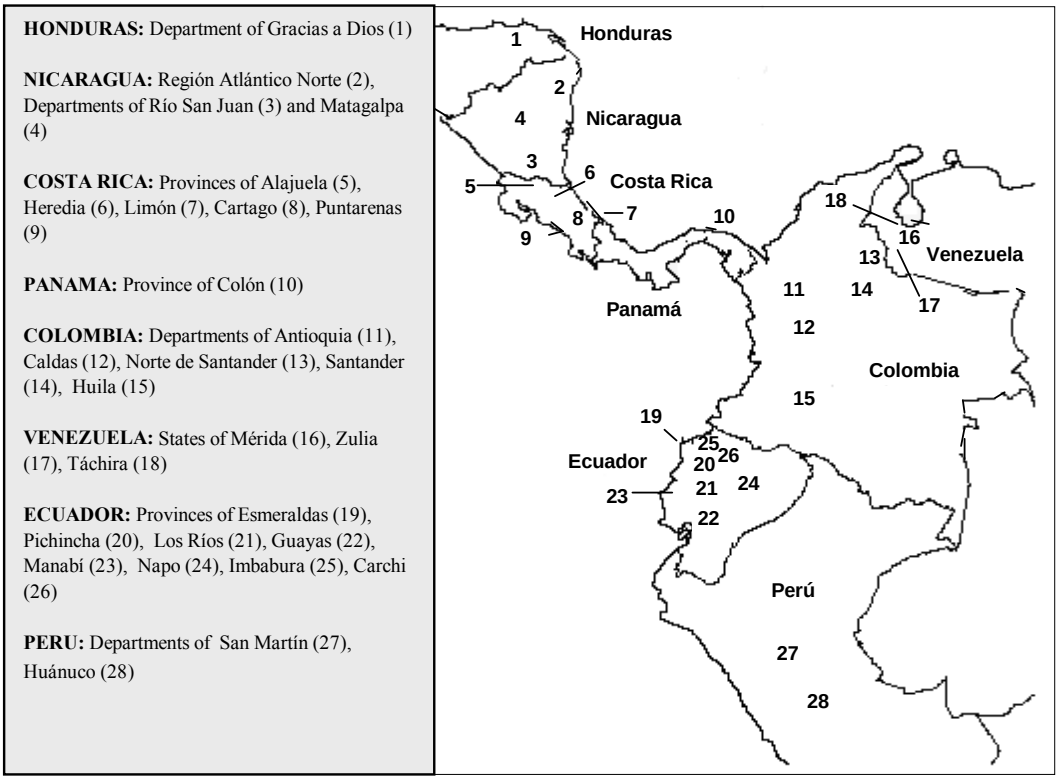


Figure 8.1 Countries and major areas of origin (indicated by numbers on the map) of 88 isolates of *M. roreri*.

The genetic groups, the origin of the isolates and basic climatic information (averaged annual temperature and annual precipitation) of the sites of collection are shown in Table 8.1.

8.3.3 METHODOLOGY

All aseptic work was performed in a laminar flow chamber (Edge Card Hood Model EG-4320). First, a six-millimetre-in diameter disc of agar-mycelium was extracted with a punch #3 from the advancing zone of a *M. roreri* culture grown previously in similar conditions to those used in the experiment. The disc was immediately placed in the centre of a Petri dish (8.5 cm diam.) containing 20 ml of modified V8 medium (20% v/v V8 juice, Campbell Soup Company, Camden, NJ, USA, 0.1% w/v asparagine, 2% w/v maltose and 1.8% w/v agar) and sealed with Parafilm tape (American National).

Table 8.1 Genetic group and geographic origin of 88 isolates of *M. roreri* and climatic information of the places of origin.

Isolate	Genetic group	Country	Major political division	Place of collection	Altitude	Annual [1/] Precipitation	Avg. annual Temperature
					(m.a.s.l.)	(mm)	(°C)
C1	Co-West	Costa Rica	Province Cartago	CATIE, Cantón Turrialba (year 1987)	602	2645	22.5
C2	Co-West	Costa Rica	Province Limón	La Lola Farm, Cantón Matina	40	3560	24.5
C5	Co-West	Costa Rica	Province Puntarenas	Km 7, La Purruja, Cantón Golfito	50	4211	28.3
C6	Co-West	Costa Rica	Province Limón	Keköldi Indian Reservation, Cantón Talamanca	180	2415	25.8
C7	Co-West	Costa Rica	Province Puntarenas	Buenos Aires, Cantón Osa	350	3666	23.5
C9	Co-West	Costa Rica	Province Alajuela	Upala, Cantón Upala	70	2500	24.0
C11	Co-West	Costa Rica	Province Limón	Earth, Cantón Pococí	250	4450	24.5
C13	Co-West	Costa Rica	Province Alajuela	Monterrey, La Fortuna, Cantón San Carlos	250	3608	24.0
C14	Co-West	Costa Rica	Province Cartago	CATIE, Cantón Turrialba, *T. grandiflorum*	602	2645	22.5
C16	Co-West	Costa Rica	Province Heredia	Puerto Viejo, Cantón Sarapiquí	37	4374	25.1
C17	Co-West	Costa Rica	Province Limón	Penshurt, Valle de la Estrella, Cantón Limón	18	2770	26.0
C18	Co-West	Costa Rica	Province Cartago	CATIE, Cantón Turrialba. *H. nitida*	602	2645	22.5
C19	Co-West	Costa Rica	Province Puntarenas	Punta Uvita, Distrito Bahía Ballena, Cantón de Osa	0	3970	25.8
C20	Co-West	Costa Rica	Province Cartago	CATIE, Cantón Turrialba. *T. bicolor*	602	2645	22.5
C21	Co-West	Costa Rica	Province Cartago	CATIE, Cantón Turrialba. *T. cacao*, APA-4	602	2645	22.5
C22	Co-West	Costa Rica	Province Cartago	CATIE, Cantón Turrialba. *T. speciosum*	602	2645	22.5
C23	Co-West	Costa Rica	Province Cartago	CATIE, Cantón Turrialba. *H. albiflora*	602	2645	22.5
C24	Co-West	Costa Rica	Province Cartago	CATIE, Cantón Turrialba. *T. cacao*, SPA-11	602	2645	22.5
C25	Co-West	Costa Rica	Province Cartago	CATIE, Cantón Turrialba. *T. mammosum*	602	2645	22.5
C26	Co-West	Costa Rica	Province Cartago	Guayabo, Cantón Turrialba. *T. cacao*	602	2645	22.5
C27	Co-West	Costa Rica	Province Limón	Playa Manzanillo, Cantón Talamanca	0	2415	25.8
C28	Co-West	Costa Rica	Province Limón	Betania, Cantón Siquirres	70	4103	25.2
C29	Co-West	Costa Rica	Province Limón	Cahuita, Cantón Talamanca	0	2770	26.0

[1/] Annual precipitation and average annual temperature from FAO (2000) and complemented with local sources when necessary.

Continuation Table 8.1

Isolate	Genetic group	Country	Major political division	Place of collection	Altitude (m.a.s.l)	Annual Precipitation (mm)	Avg. annual Temperature (°C)
Co1	Bolívar	Colombia	Dep. Norte de Santander	Vereda Pedregales, Municipality of Zulia	90	1800	27.2
Co2	Bolívar	Colombia	Dep. Norte de Santander	Vereda Punta del Palo, Municipality of Tibú	98	2341	28.1
Co4	Co-East	Colombia	Dep. Santander	La Suiza CORPOICA´s Farm, Municipality of Río Negro	500	2000	25.0
Co5	Co-East	Colombia	Dep. Santander	La Suiza CORPOICA´s Farm, Municipality of Río Negro	500	2000	25.0
Co6	Co-East	Colombia	Dep. Santander	Bijagual, Vereda Puente Piedra, Municip. of Bucaramanga	800	1651	23.5
Co7	Co-Central	Colombia	Dep. Antíoquia	La Nacional Farm, Municipality of Támesis	1190	2272	21.8
Co8	Co-West	Colombia	Dep. Antioquia	Agricultural Institute, Leticia, Municipality of San Jerónimo	703	1343	24.9
Co9	Co-Central	Colombia	Dep. Caldas	Lucker Farm, Corregimiento Arauca, Municip. of Palestina	1120	2294	21.9
Co10	Co-Central	Colombia	Dep. Caldas	Vereda La Inquisición, Municipality of Palestina	1520	2294	18.6
Co11	Co-Central	Colombia	Dep. Caldas	Vereda La Inquisición, Municipality of Palestina	1350	2294	20.4
Co12	Co-Central	Colombia	Dep. Caldas	Vereda La Inquisición, Municipality of Palestina	1200	2294	21.9
Co13	Co-West	Colombia	Dep. Antioquia	Municipality of Carepa, Urabá	132	5511	27.1
Co14	Co-East	Colombia	Dep. Santander	Vereda Santa Inés, Municipality of San Vicente de Chucurí	750	1867	21.4
Co15	Co-East	Colombia	Dep. Santander	Vereda Barranco Amarillo, Municip. El Carmen de Chucuri	820	2245	23.2
Co16	Co-East	Colombia	Dep. Santander	Vereda Rancho Grande, Municipality El Carmen de Chucuri	460	2245	23.2
Co17	Co-Central	Colombia	Dep. Huila	Vereda El Guadual, Municipality of Rivera	800	1443	24.0
E1	Co-West	Ecuador	Province Guayas	Municipality of Naranjal	30	1210	24.9
E2	Bolívar	Ecuador	Province Guayas	Recinto Rio Congo, Cantón Balzar	60	1709	25.3
E3	Co-West	Ecuador	Province Guayas	Recinto San Juan, Cantón El Empalme	120	2087	24.4
E4	Co-West	Ecuador	Province Los Rios	Recinto Cuatro Vientos, Cantón Mocache	120	2087	24.4
E5	Co-West	Ecuador	Province Los Rios	Recinto La Carmela, Cantón Mocache	120	2087	24.4
E6	Co-West	Ecuador	Province Los Rios	Recinto Agua Prieta, Cantón Mocache	120	2087	24.4
E7	Co-West	Ecuador	Province Los Rios	Recinto San Pedro, Cantón Mocache	120	2087	24.4
E8	Co-West	Ecuador	Province Los Rios	Recinto San Ignasio, Cantón Mocache	120	2087	24.4
E9	Co-West	Ecuador	Province Los Rios	Recinto Come Gallo, Cantón Quevedo	120	2087	24.4
E10	Co-West	Ecuador	Province Los Rios	Recinto Toquillal, Cantón Quevedo	120	2087	24.4

Continuation Table 8.1

Isolate	Genetic group	Country	Major political division	Place of collection	Altitude (m.a.s.l)	Annual Precipitation (mm)	Avg. annual Temperature (°C)
E11	Co-West	Ecuador	Province Los Rios	Pichilingue, Lote Caseta, Cantón Quevedo	120	2087	24.4
E12	Co-West	Ecuador	Province Los Rios	Km30 on the Quevedo-Babahoyo road	60	2109	25.9
E13	Co-West	Ecuador	Province Los Rios	Recinto Ventanilla Sur, Cantón Ventanas	60	2109	25.9
E14	Co-West	Ecuador	Province Los Rios	Recinto Juana de Oro	150	2312	24.3
E15	Co-West	Ecuador	Province Los Rios	Recinto Las Pampas, Cantón Vinces	41	1401	25.2
E16	Co-West	Ecuador	Province Los Rios	University of Guayaquil, Cantón Vinces	41	1401	25.2
E17	Bolívar	Ecuador	Province Los Rios	Isla Bejucal, Cantón Baba	41	1401	25.2
E18	Co-West	Ecuador	Province Los Rios	Pichilingue, Lote Adentro, Cantón Quevedo	120	2087	24.4
E20	Co-West	Ecuador	Province Los Rios	Nestlé Farm, Recinto San Pablo	120	2087	24.4
E21	Bolívar	Ecuador	Province Manabi	Parroquia Chone, Cantón Chone	69	1157	25.5
E22	Bolívar	Ecuador	Province Manabi	Parroquia Quiroga, Cantón Bolívar	200	961	25.8
E23	Bolívar	Ecuador	Province Manabi	Parroquia Calceta, Cantón Bolívar	180	961	25.8
E24	Co-West	Ecuador	Province Esmeraldas	Recinto El Control, Cantón San Mateo	50	1946	25.6
E25	Co-West	Ecuador	Province Esmeraldas	Parroquia Chinca, Cantón Quinindé	95	2404	25.1
E26	Co-West	Ecuador	Province Esmeraldas	Recinto Viche, Cantón Quinindé	30	1946	25.6
E27	Co-West	Ecuador	Province Esmeraldas	Recinto El Consuelo, Cantón Quinindé	60	3129	23.7
E28	Co-West	Ecuador	Province Pichincha	Recinto Urbano, Cantón Santo Domingo	660	3227	22.0
E29	Co-West	Ecuador	Province Pichincha	Recinto Urbano, Cantón Santo Domingo	660	3227	22.0
E30	Co-West	Ecuador	Province Los Rios	La Variante, Cantón Buena Fe	120	2087	24.4
E32	Bolívar	Ecuador	Province Guayas	Tenguel	15	777	25.1
E33	Bolívar	Ecuador	Province Guayas	Tenguel	15	777	25.1
E36	Bolívar	Ecuador	Province Napo	Nueva Primavera. *T. bicolor*	215	2719	24.1
E37	Bolívar	Ecuador	Province Napo	Anangu, Rio Napo. *T. cacao*	215	2719	24.1
E42	Gileri	Ecuador	Province Carchi	El Rocio, Guadual. *T. gileri*	571	3611	22.8
E43	Gileri	Ecuador	Province Imbabura	Lita-Alto Tambo. *T. gileri*	571	3611	22.8
H1	Co-West	Honduras	Dep. Gracias a Dios	Planyare, Wampusirpe, Mosquitia	10	2856	26.9

Continuation Table 8.1

Isolate	Genetic group	Country	Major political division	Place of collection	Altitude (m.a.s.l)	Annual Precipitation (mm)	Avg. annual Temperature (°C)
N1	Co-West	Nicaragua	Región Atlántico Norte	Kosuly, Municipality of Waslala	118	2449	25.3
N3	Co-West	Nicaragua	Dep. Río San Juan	Municipality of San Carlos	40	1857	26.5
N4	Co-West	Nicaragua	Región Atlántico Norte	Municipality of Puerto Cabezas	28	777	25.1
N5	Co-West	Nicaragua	Department Matagalpa	Paiwas, Municipality Rio Blanco	118	2449	25.3
P1	Bolívar	Peru	Department Huánuco	Province Leoncio Prado, Tingo Maria	669	3203	24.8
P2	Bolívar	Peru	Department San Martín	Province Mariscal Cáceres, Tocache	669	3203	24.8
P3	Bolívar	Peru	Department Huánuco	Province Leoncio Prado, Marona	666	3000	24.0
P5	Bolívar	Peru	Department Huánuco	Province Leoncio Prado, Castillo Papayal	666	3000	24.0
Pa6	Co-West	Panama	Province Colón	Coclecito	30	3103	27.2
Pa7	Co-West	Panama	Province Colón	Corregimiento Buena Vista	38	3700	26.5
V2	Bolívar	Venezuela	State Mérida	La Rocolita, Municip. of Caracido Parra y Olmedo	130	1808	27.3
V3	Bolívar	Venezuela	State Zulia	Playa Grande, Municipality of Heras	130	1808	27.3
V5	Bolívar	Venezuela	State Táchira	Municipality of Colón	105	1859	25.6

Five Petri dishes per isolate were prepared in the same way. One Petri dish was cultured in darkness by wrapping it with aluminium foil to provide preliminary data on the effect of darkness on the growth and sporulation of the isolates. Cultures were then incubated for 20 d in a Percival Boone 50036 model I-35LL incubator at 24 ºC and 12/12 alternate periods of light/darkness, with light provided by fluorescent lamps. Light intensity was 646 lux, as measured using a Simpson Illumination Level Meter, model 408-2.

The following characters were evaluated from the resulting colonies:

a. Radial growth: The diameter of the colony was measured in two perpendicular directions and an mean was calculated. Four measurements were carried out for each plate during the 20-day-growth period at five days intervals. These data were used to assemble growth curves for a subset of isolates from each country. As it will be explained in the results section, a single measurement time for radial growth was eventually chosen to compare all isolates.

b. Days to sporulation: This trait was determined by a visual check of the presence of sporulation on the plates after 5, 7, 10, 15 and 20 d.

c. Ring intensity: Ring intensity was determined after 20 d using a six-grade visual scale in which 0 was denoted as absence of rings and 5 represented the presence of very intense rings.

d. Spore production: The following steps were carried out to determine the spore production of each Petri dish:

- **Preparation of the spore suspension**: Spores were removed from the 20-day-old cultures by adding in three steps, 50 ml of a 0.01% Tween-80 solution in distilled water and gently brushing the surface of the colony with a paintbrush. Each suspension was then placed in an Erlenmeyer flask and stirred continuously for 10 min to separate the spores.

- **Sample**: One ml of spore suspension was aliquoted into an Eppendorf tube. The tubes were stored at 8 °C until the sample was used to measure spore concentration and to determine the size and shape of the spores. Another 1 ml aliquot was immediately used to test germination.

- **Spore counting**: A 1:10 dilution was prepared to simplify spore counting. Spore concentration was measured using a haemocytometer and a Bausch and Lomb light microscope (100x magnification). The same microscope was used to determine spore shape and size. Spore concentration measurements took two weeks to be finished.

e. Spore density: This trait was calculated by dividing the number of spores per plate by the area of colony showing sporulation as measured for diameter of sporulation at 20 d.

f. Spore germination: One ml of the fresh spore suspension, as previously prepared, was placed into a Petri dish containing 5 ml of water-agar (3%) in accordance with the methodology described by Umaña & González (1981). The suspension was uniformly distributed across the plate by rotating hand movements. The dishes were then stored in the dark at 24 °C. The percentage of germinated spores was determined after 24 h by examining the bottom of the plates under a compound light microscope (100x magnification) and counting the frequency of spores that showed a recognisable germ tube (Hawker, 1950; Etten *et al*., 1983). For each measurement, 200 spores were scored per plate in random fields of view.

g. Spore shape: This characteristic was observed using a haemocytometer and a compound light microscope (400x magnification). Using the spore suspension previously described, twenty random spores per plate were examined and their shape classified. The spores were classified in three types: globose, ellipsoid and abnormal.

h. Spore size: Spore width and length were measured for the same group of spores used to assess shape with a 12.5x ocular micrometer Leitz Wetzlar (Germany) previously calibrated using a plaque micrometer. The following variables were analysed based on this

information: diameter of the globose spores and width and length of the ellipsoid spores. Spore size and shape determinations took two weeks to be finished.

i. General appearance of the colony: The appearance of the colonies was determined at 20 d by a simple observation of the colonies. Annotations were made on the most distinctive characteristics such as growth pattern, colour and presence of sectoring.

8.3.4 VARIABLES EVALUATED

Based on the characters described above, the following 11 variables were analysed: radial growth at 10 d and days to sporulation, and after 20 d growth, ring intensity, spore production (spore $plate^{-1}$), spore density (spore cm^{-2}), percentage of germinated spores after 24 h, percentage of globose and ellipsoid spores, globose spore diameter, ellipsoid spore width and ellipsoid spore length. These variables were selected based on results obtained by other authors, in particular by Chacín (1975) and Herrera *et al*. (1989) and also from previous experience of the author.

8.3.5 MYCELIAL INTERACTION TEST

A preliminary study of the mycelial interaction between a representative group of isolates was carried out at CATIE in March 2002. Eight isolates representing the five major genetic groups of the fungus were studied: Co5 (Co-East group), Co10 (Co-Central), V3 and P3 (Bolívar), E43 (Gileri) and Co13, E8 and H1 (Co-West).

Two Petri dishes (8.5 cm diam.) containing 20 ml of modified V8 were prepared per isolate. For each isolate, a six-millimetre-in diameter disc of agar-mycelium was placed in the centre of each plate, then a similar disc of the following isolates were place in each cardinal position and separated 1.3 cm from the edge: E8, Co13, Co10 and Co5 in the first plate and H1, V3, P1 and E43 in the second. Cultures were incubated for 20 d in similar conditions as those described in the morpho-physiological study. After this period, the mycelial interactions between isolates were determined and documented by apical and basal photographs of the plates. The mycelial interaction was recorded as compatible (+) where mycelia intermingled or incompatible (-) where the opposing mycelia ceased to

grow before they met so that there was a gap between the colonies (Ram, 1989). The information obtained in this test was not subjected to further data analysis.

8.3.6 CLIMATIC INFORMATION

Climatic information of some localities within or near to the hypothetical centre of differentiation of the genetic groups was obtained from FAO (2000). This information was used to relate basic climatic parameters with the possible arising of the morpho-physiological traits.

8.3.7 DATA ANALYSIS

A randomised complete-block design with four blocks (Petri dishes) was used for the morpho-physiological study. The mathematical model was:

$$\mathbf{Y_{ij} = \mu + B_i + I_j + e_{ij}}$$

$\mathbf{Y_{ij}}$ = Variable to be analysed from the i^{th} level of B and the j^{th} level of I.
$\boldsymbol{\mu}$ = Overall mean
$\mathbf{B_i}$ = Effect of the i^{th} block
$\mathbf{I_j}$ = Effect of the j^{th} isolate
$\mathbf{e_{ij}}$ = Experimental error

In order to normalised the data, the following transformations were applied to the raw data:

a) ***Arcsin (percentage/100)^{½}:*** Angular transformation was used for the variables expressed in terms of percentage such as: percentage of germinated spores, percentage of globose spores and percentage of ellipsoid spores.

b) **(value+0.5)^{½}**: For ring intensity, a variable based on a non-parametric evaluation scale.

Data analysis was carried out using the SAS software Version 8.00 (SAS Institute, North Caroline, USA). A variance analysis was performed for each variable using the GLM (General Linear Model) procedure. Then, a Tukey test was carried out to evaluate for significant differences among the means of the treatments. No analyses were made for the percentage of abnormal spores, the general appearance of the colonies and the mycelial interaction test.

Variance analysis and Tukey tests were also used to analyse the isolates in accordance to the country of origin and the genetic group. In both cases, the mathematical model only included the following sources of variation: the overall mean, the effect of the country or the genetic group and the random error. In the analysis by country, the single isolate from Honduras was analysed together with the isolates from Nicaragua.

Paired correlation analyses were performed between three geo-climatic parameters (altitude, annual precipitation and average annual temperature) of the places of collection of the isolates (Table 8.1) and the morpho-physiological variables. The percentage of ellipsoid spores was not considered due to the high dependence of this variable on the percentage of globose spores.

A Ward's Minimum Variance Cluster Analysis using squared euclidian distances was performed to group the set of 88 isolates in accordance to the following variables: radial growth, days to sporulation, ring intensity, spore production and globose spore diameter. The selection of the variables was based on both the coefficient of correlation between the morpho-physiological variables and the criteria of the author. The variables were standardised before the analysis.

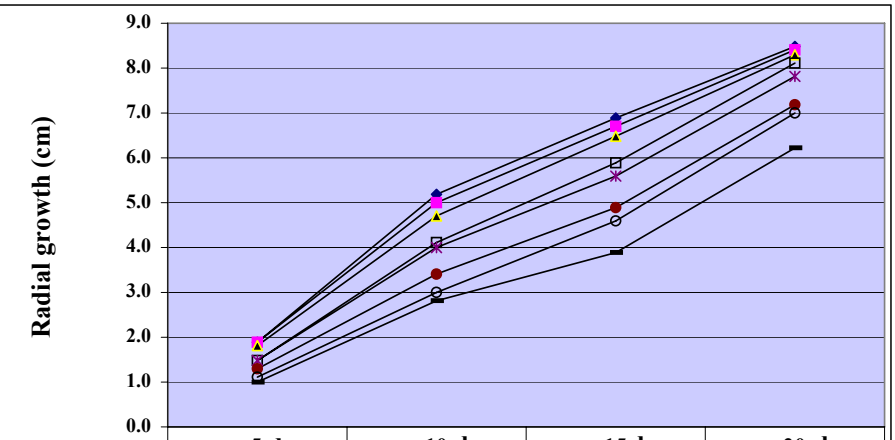
The Mantel test (Mantel, 1967) was used to test for correlation between the dissimilarity matrix obtained using morpho-physiological characters and either the dissimilarity matrix based on the geographic distances between isolates or on the molecular characters. Genetix software (Laboratoire Génome et Populations, Université de Montpellier II, France) and 1000 permutations were used. The morpho-physiological dissimilarity matrix was obtained using squared euclidian distances, Ward's method, and the same variables used in the cluster analysis. The geographic matrix was calculated using the geographic

distances between the places of collection of the isolates. The molecular matrix was obtained using the AFLP and ISSR jointly band profiles of the isolates (Chapter 4) compiled with Jaccard coefficient and subjected to the Unweighted Pair-Group Method, Arithmetic Mean (UPGMA).

8.4 RESULTS

8.4.1 GROWTH CURVES BY COUNTRY

A growth curve of *M. roreri* was obtained for each country based on the radial growth of the isolates measured every five days. Curves showed a similar slope but significant differences ($P<0.05$) in magnitude were observed among countries within each replicate time point (Figure 8.2).



	5 days	10 days	15 days	20 days
Venezuela	1.9 a 1/	5.2 a	6.9 a	8.5 a
Colombia	1.9 a	5.0 a	6.7 ab	8.4 a
Peru	1.8 ab	4.7 ab	6.5 ab	8.3 a
Panama	1.5 bc	4.1 bc	5.9 bc	8.1 a
Ecuador	1.5 bc	4.0 bc	5.6 cd	7.8 ab
Costa Rica	1.3 cd	3.4 cd	4.9 de	7.2 b
Nicaragua	1.1 de	3.0 d	4.6 ef	7.0 b
Honduras	1.0 e	2.8 d	3.9 f	6.2 c

[1/] **Values in each column followed by the same letter do not have significant differences (Duncan Test, $P<0.05$)**

Figure 8.2 Radial growth by country of *M. roreri* grown on modified V8 medium at 24 °C and 12/12 alternate periods of light/darkness.

Although a single radial growth measurement after 10 or 15 d could provide a good estimation of this variable, measures recorded after 10 d was selected to compare the isolates because it allowed a more rapid assimilation of data. Measurements made after five days did not adequately distinguish between isolates whereas those made after 20 d faced the problem that growth of some colonies was limited by the size of the Petri dishes (Figure 8.2).

In general, isolates from South America had a higher radial growth than isolates from Central America. Furthermore, a significant reduction ($P<0.05$) in the radial growth of the Central American isolates occurred from Panama northwards (Figure 8.2).

8.4.2 GENERAL VARIATION OF THE ISOLATES

Highly significant differences ($P<0.01$) were found among the isolates for all variables studied except for ellipsoid spore length for which differences were significant only at the 5% level ($P<0.05$) (Table A8.1). Results of the Tukey test for each variable are described as follows.

Radial growth: This variable ranged from 1.3 cm to 6.2 cm (Table 8.2). The highest values were obtained for isolates Co11, Co10, Co9, E17, Co8, E37, Co17, V2, V3 and E22 ($6.2 \text{ cm} \geq x \geq 5.2 \text{ cm}$), the first three being from Central Colombia. This group of isolates differed significantly ($P<0.05$) from the isolates with the lowest radial growths from Ecuador and Central America: E3, C24, N3, N5, N1, E28, H1, E23, C27, E5, C17 and E11 ($3.1 \text{ cm} \geq x \geq 1.3 \text{ cm}$). Isolate E11 showed a very slow growth (1.3 cm) that differed from all other isolates ($P<0.05$).

Days to sporulation: This variable ranged from 5.0 d to 13.8 d (Table 8.2). The highest values were obtained for the Ecuadorian isolates E17, E24, E11, E10 and E23 ($13.8 \text{ d} \geq x \geq 9.8 \text{ d}$). The lowest values were found in isolates E27, C28, Co17, E13, E16, Co11, E29, P1, P2, P3 and P5 ($5.7 \text{ d} \geq x \geq 5.0 \text{ d}$), which includes all Peruvian isolates. These two groups differed significantly ($P<0.05$).

Table 8.2 Radial growth, days to sporulation and ring intensity of 88 isolates of *M. roreri* grown in Petri dishes with modified V8 medium at 24 °C and 12/12 alternate periods of light/darkness.

Radial growth at 10 d (cm) [1/]		Days to sporulation		Ring intensity at 20 d	
Co11	6.2 a	**E17**	13.8 a	**Co17**	5.0 a
Co10	6.0 ab	**E24**	13.8 a	**V2**	5.0 a
Co9	5.8 abc	**E11**	12.5 ab	**Co2**	4.8 ab
E17	5.8 abcd	**E10**	11.2 abc	**V3**	4.8 ab
Co8	5.7 abcde	**E23**	9.8 bcd	**E37**	4.5 abc
E37	5.7 abcde	**C5**	9.2 bcde	**C21**	4.5 abcd
Co17	5.5 abcdef	**N1**	9.2 bcde	**Co12**	4.2 abcde
V2	5.4 abcdefg	**E21**	8.5 cdef	**Co4**	4.0 abcdef
V3	5.4 abcdefgh	**C11**	8.5 cdef	**Co7**	4.0 abcdef
E22	5.2 abcdefgh	**C14**	8.5 cdef	**Co9**	4.0 abcdef
Co2	5.1 bcdefghi	**C17**	8.0 cdef	**E29**	4.0 abcdef
Co4	5.1 bcdefghi	**C27**	8.0 cdef	**C25**	4.0 abcdef
E12	5.0 cdefghi	**E5**	7.8 cdef	**E26**	3.8 abcdefg
E36	5.0 cdefghij	**E18**	7.8 cdef	**P3**	3.8 abcdefg
P2	5.0 cdefghijk	**E20**	7.8 cdef	**C9**	3.8 abcdefg
Co6	4.9 cdefghijk	**E25**	7.8 cdef	**Co5**	3.7 abcdefh
E16	4.9 cdefghijkl	**E28**	7.8 cdef	**E2**	3.5 abcdefgh
E32	4.9 cdefghijkl	**C21**	7.8 cdef	**C16**	3.5 abcdefgh
P1	4.9 cdefghijk	**C24**	7.8 cdef	**Co10**	3.2 abcdefgh
C1	4.9 defghijkl	**Co1**	7.0 def	**C11**	3.2 abcdefgh
Co14	4.8 defghijklm	**Co4**	7.0 def	**Co11**	3.0 abcdefghi
Co7	4.8 efghijklmn	**Co5**	7.0 def	**E6**	3.0 abcdefghi

[1/] Values for each variable followed by the same letter do not have significant differences (Tukey Test, P<0.05).

Continuation Table 8.2

Radial growth at 10 d (cm)			Days to sporulation			Ring intensity at 20 d	
Co12	4.8	efghijklmn	**Co6**	7.0	def	**E7**	3.0 abcdefghi
V5	4.8	efghijklmn	**Co8**	7.0	def	**E12**	3.0 abcdefghi
Co16	4.7	fghijklmno	**Co10**	7.0	def	**E16**	3.0 abcdefghi
E1	4.7	fghijklmno	**Co13**	7.0	def	**E20**	3.0 abcdefghi
E10	4.7	fghijklmno	**Co14**	7.0	def	**E28**	3.0 abcdefghi
E20	4.7	fghijklmno	**Co15**	7.0	def	**E33**	3.0 abcdefghi
E27	4.7	fghijklmno	**Co16**	7.0	def	**P1**	3.0 abcdefghij
P5	4.7	fghijklmno	**E3**	7.0	def	**P5**	3.0 abcdefghij
PA6	4.7	fghijklmnop	**E4**	7.0	def	**N3**	3.0 abcdefghi
E26	4.5	ghijklmnopq	**E6**	7.0	def	**E4**	2.8 abcdefghij
Co15	4.4	hijklmnopqr	**E7**	7.0	def	**E8**	2.8 abcdefghi
Co1	4.4	hijklmnopqrs	**E8**	7.0	def	**E14**	2.8 abcdefghi
E25	4.3	ijklmnopqrst	**E9**	7.0	def	**E30**	2.8 abcdefghi
E6	4.3	ijklmnopqrstu	**E12**	7.0	def	**C14**	2.8 abcdefghi
E13	4.3	ijklmnopqrstu	**E14**	7.0	def	**E10**	2.5 abcdefghij
Co13	4.2	ijklmnopqrstuv	**E15**	7.0	def	**E15**	2.5 abcdefghij
E7	4.2	ijklmnopqrstuv	**E26**	7.0	def	**E25**	2.5 abcdefghij
E43	4.2	ijklmnopqrstuv	**E30**	7.0	def	**C7**	2.5 abcdefghij
E15	4.0	jklmnopqrstuvw	**E33**	7.0	def	**PA6**	2.5 abcdefghij
P3	4.0	klmnopqrstuvw	**E42**	7.0	def	**E1**	2.5 abcdefghijk
C5	4.0	lmnopqrstuvwx	**E43**	7.0	def	**Co1**	2.2 abcdefghijk
E42	3.9	mnopqrstuvwxy	**V2**	7.0	def	**Co8**	2.2 abcdefghijk
E18	3.9	nopqrstuvwxy	**V3**	7.0	def	**Co16**	2.2 abcdefghijk
E30	3.9	nopqrstuvwxy	**V5**	7.0	def	**E3**	2.2 abcdefghijk

Continuation Table 8.2

Radial growth at 10 d (cm)			Days to sporulation			Ring intensity at 20 d		
E21	3.8	opqrstuvwxyz	**C1**	7.0	def	**E5**	2.2	abcdefghijk
C2	3.8	pqrstuvwxyzA	**C2**	7.0	def	**E23**	2.2	abcdefghijk
C13	3.8	qrstuvwxyzA	**C6**	7.0	def	**E24**	2.2	abcdefghijk
E2	3.7	qrstuvwxyzAB	**C7**	7.0	def	**C1**	2.2	abcdefghijk
E14	3.7	qrstuvwxyzAB	**C16**	7.0	def	**C20**	2.2	abcdefghijk
E29	3.7	qrstuvwxyzAB	**C18**	7.0	def	**C23**	2.2	abcdefghijk
C22	3.7	qrstuvwxyzAB	**C19**	7.0	def	**N4**	2.2	abcdefghijk
C23	3.7	qrstuvwxyzABC	**C20**	7.0	def	**E9**	2.0	abcdefghijk
E8	3.6	qrstuvwxyzABCD	**C22**	7.0	def	**C22**	2.0	abcdefghijk
C9	3.6	rstuvwxyzABCD	**C23**	7.0	def	**C24**	2.0	abcdefghijk
C18	3.5	rstuvwxyzABCD	**C25**	7.0	def	**C29**	2.0	abcdefghijk
C29	3.5	rstuvwxyzABCD	**C26**	7.0	def	**PA7**	2.0	abcdefghijk
C16	3.5	stuvwxyzABCD	**H1**	7.0	def	**E17**	1.8	abcdefghijk
C20	3.5	stuvwxyzABCD	**N3**	7.0	def	**C13**	1.8	abcdefghijk
Co5	3.5	tuvwxyzABCD	**N4**	7.0	def	**Co13**	1.5	abcdefghijk
E4	3.5	tuvwxyzABCD	**N5**	7.0	def	**N1**	1.5	abcdefghijk
C25	3.4	tuvwxyzABCD	**PA6**	7.0	def	**N5**	1.5	abcdefghijk
C28	3.4	tuvwxyzABCD	**PA7**	7.0	def	**E18**	1.5	bcdefghijk
E24	3.4	uvwxyzABCDE	**Co2**	6.8	def	**E21**	1.5	bcdefghijk
C6	3.3	vwxyzABCDE	**E1**	6.8	def	**C6**	1.5	bcdefghijk
C11	3.3	vwxyzABCDE	**Co7**	6.5	def	**Co15**	1.3	cdefghijk
C21	3.3	vwxyzABCDE	**Co9**	6.5	def	**C28**	1.3	cdefghijk
N4	3.3	vwxyzABCDE	**Co12**	6.5	def	**C19**	1.2	cdefghijk
PA7	3.3	vwxyzABCDE	**E2**	6.5	def	**C2**	1.2	defghijk

Continuation Table 8.2

Radial growth at 10 d (cm)			Days to sporulation			Ring intensity at 20 d		
E9	3.2	vwxyzABCDEF	**E22**	6.5	def	**V5**	1.2	efghijk
C7	3.2	wxyzABCDEF	**C13**	6.5	def	**E36**	1.0	efghijk
C14	3.2	vwxyzABCDEF	**C9**	6.5	def	**Co6**	1.0	efghijk
C19	3.2	wxyzABCDEF	**C29**	6.5	def	**C27**	1.0	efghijk
C26	3.2	wxyzABCDEF	**E32**	6.0	ef	**H1**	1.0	efghijk
E33	3.1	wxyzABCDEF	**E36**	6.0	ef	**E13**	1.0	fghijk
E3	3.1	xyzABCDEF	**E37**	6.0	ef	**E27**	1.0	fghijk
C24	3.0	yzABCDEF	**E27**	5.7	f	**E22**	1.0	ghijk
N3	3.0	yzABCDEF	**C28**	5.7	f	**E32**	0.8	hijk
N5	3.0	zABCDEF	**Co17**	5.5	f	**E42**	0.8	hijk
N1	2.9	zABCDEF	**E13**	5.5	f	**P2**	0.8	hijk
E28	2.8	ABCDEF	**E16**	5.5	f	**E43**	0.7	hijk
H1	2.8	BCDEF	**Co11**	5.0	f	**Co14**	0.5	ijk
E23	2.7	CDEF	**E29**	5.0	f	**C17**	0.2	jk
C27	2.6	DEF	**P1**	5.0	f	**E11**	0.0	k
E5	2.4	EF	**P2**	5.0	f	**C5**	0.0	k
C17	2.3	F	**P3**	5.0	f	**C18**	0.0	k
E11	1.3	G	**P5**	5.0	f	**C26**	0.0	k

Ring intensity: The variable varied between 0 and 5.0 (Table 8.2). Isolates Co17, V2, Co2, V3, E37, C21, Co12, Co4, Co7, Co9, E29 and C25 displayed the highest ring intensities ($5.0 \geq x \geq 4.0$), whereas isolates E32, E42, P2, E43, Co14, C17, E11, C5, C18 and C26 ($0.8 \geq x \geq 0.0$) yielded the lowest values. The two groups differed significantly ($P<0.05$).

Spore production: Mean number of spores ranged from 3.6 to 564.4 million spores plate^{-1} (Table 8.3). The highest values were obtained for a group of isolates with a diverse origin (P5, E36, P2, E22, Co16, Co15, E27, E37, C23, V5, E32, P3, E33, C24, Co9 and E42) ($564.4 \geq x \geq 280.4$ million spores plate^{-1}) whereas the lowest values ($P<0.05$) were restricted to some isolates from Costa Rica and Ecuador (E18, E25, C5, C2, C17, E9, E10, E17 and E21) ($41.9 \geq x \geq 3.6$ million spores plate^{-1}).

Spore density: This variable ranged from 0.3 to 11.8 million spores cm^{-2} (Table 8.3). The highest values were obtained for a diverse array of isolates with widely separated geographic origins (E33, E11, P5, E36, E37, C26, Co15, P2, Co16, E22, C23 and P3) ($11.8 \geq x \geq 8.8$ million spores cm^{-2}). The lowest values ($P<0.05$) were found among a group of isolates that largely originated from Ecuador (E18, C9, E8, E14, E26, Co2, E7, E17, E6, E16, E25, E9, E10 and E21) ($1.8 \geq x \geq 0.3$ million spores cm^{-2}).

Germinated spores: The percentage of germinated spores varied from 0% to 25.5% (Table 8.3). Significant differences ($P<0.05$) were found among the group of isolates with the highest values, mostly Ecuadorian isolates (E24, E14, C1, E26 and E20) ($25.5\% \geq x \geq 15.0\%$) and a more diverse group of isolates (P2, E36, E30, E27, E23, E21, E1, Co16 and Co15) all of which failed to germinate.

Percentage of globose spores: This variable ranged from 21.7 % to 95.6% (Table 8.4). The highest percentages were obtained for Ecuadorian and Peruvian isolates such as E27, E1, E36, P3, E12, P1, P5, E32 and E37 ($95.6\% \geq x \geq 86.7\%$). This group of isolates contained significantly more globose spores ($P<0.05$) than a group comprised of mostly Ecuadorian and Costa Rican isolates (E42, E4, C2, Co2, C27, E18, E25, E20 and C6) ($36.7\% \geq x \geq 21.7\%$).

Table 8.3 Spore production, spore density and percentage of germinated spores of 88 isolates of *M. roreri* grown in Petri dishes with modified V8 medium at 24 °C and 12/12 alternate periods of light/darkness.

Spores plate^{-1} (x 10^6) [1/]			Spores cm^{-2}(x 10^6)			% germinated spores		
P5	564.4	a	**E33**	11.8	a	**E24**	25.5	a
E36	541.2	ab	**E11**	11.1	ab	**E14**	17.5	ab
P2	516.2	ab	**P5**	10.8	abc	**C1**	16.5	abc
E22	465.0	abc	**E36**	10.1	abcd	**E26**	15.5	abcd
Co16	460.2	abcd	**E37**	9.9	abcdef	**E20**	15.0	abcd
Co15	452.5	abcde	**C26**	9.9	abcdef	**Co9**	14.2	abcde
E27	438.3	abcdef	**Co15**	9.6	abcdefg	**E25**	13.5	abcde
E37	437.5	abcdef	**P2**	9.5	abcdefgh	**Co13**	13.4	abcde
C23	378.1	abcdefg	**Co16**	9.4	abcdefghi	**Co8**	13.0	abcde
V5	360.6	abcdefgh	**E22**	9.2	abcdefghij	**E4**	13.0	abcde
E32	353.1	abcdefghi	**C23**	9.2	abcdefghijk	**E16**	13.0	abcde
P3	353.1	bcdefghij	**P3**	8.8	abcdefghijkl	**E10**	12.5	abcde
E33	332.8	bcdefghijk	**E5**	8.6	abcdefghijklm	**C9**	12.5	abcde
C24	288.1	cdefghijkl	**E27**	8.5	abcdefghijklm	**E6**	12.1	abcde
Co9	287.4	cdefghijkl	**Co5**	8.2	abcdefghijklmno	**P3**	11.0	abcdef
E42	280.4	cdefghijklm	**E23**	8.2	abcdefghijklmno	**C2**	10.7	abcdef
V3	267.0	cdefghijklmn	**C7**	8.2	abcdefghijklmno	**N3**	10.4	abcdef
Co5	264.8	cdefghijklmn	**C24**	7.7	abcdefghijklmnop	**PA7**	10.4	abcdef
V2	260.4	cdefghijklmnop	**E42**	7.2	abcdefghijklmnopq	**Co5**	9.3	abcdef
C26	252.5	cdefghijklmnopq	**V5**	7.1	abcdefghijklmnopq	**C20**	8.8	abcdef
E2	251.2	cdefghijklmnopq	**E2**	6.9	abcdefghijklmnopqr	**E5**	8.7	abcdef
E20	238.8	defghijklmnopq	**E32**	6.8	abcdefghijklmnopqr	**Co2**	8.0	abcdef
C7	236.4	efghijklmnopqr	**C28**	6.8	abcdefghijklmnopqr	**E8**	8.0	abcdef

[1/] **Values for each variable followed by the same letter do not have significant differences (Tukey Test, P<0.05).**

Continuation Table 8.3

Spores plate^{-1} (x 10^6)			Spores cm^{-2}(x 10^6)			% germinated spores		
P1	216.8	fghijklmnopqrs	**E3**	6.7	abcdefghijklmnopqr	**E28**	8.0	abcdef
Co10	215.9	ghijklmnopqrs	**E4**	6.5	abcdefghijklmnopqr	**C6**	7.9	abcdef
E4	213.8	ghijklmnopqrs	**C25**	6.2	abcdefghijklmnopqr	**Co12**	7.8	abcdef
E3	200.4	ghijklmnopqrs	**N3**	6.2	abcdefghijklmnopqr	**Co10**	7.4	abcdef
E12	197.5	ghijklmnopqrs	**E28**	6.1	abcdefghijklmnopqr	**H1**	7.4	abcdef
E1	191.2	ghijklmnopqrs	**E24**	5.6	abcdefghijklmnopqr	**C21**	7.3	abcdef
E5	187.5	ghijklmnopqrs	**E20**	5.4	abcdefghijklmnopqr	**E2**	7.0	abcdef
Co13	183.8	ghijklmnopqrs	**C27**	5.4	abcdefghijklmnopqr	**E18**	7.0	abcdef
Co11	183.4	ghijklmnopqrs	**Co9**	5.1	bcdefghijklmnopqr	**N4**	7.0	abcdef
Co8	179.8	ghijklmnopqrs	**E1**	5.0	bcdefghijklmnopqr	**C18**	6.8	abcdef
E28	179.8	ghijklmnopqrs	**V3**	4.7	bcdefghijklmnopqr	**E29**	6.0	abcdef
C25	179.8	ghijklmnopqrs	**V2**	4.6	bcdefghijklmnopqr	**C14**	6.0	abcdef
Co4	175.2	ghijklmnopqrs	**E43**	4.5	bcdefghijklmnopqr	**C25**	6.0	abcdef
E30	174.6	ghijklmnopqrs	**E30**	4.4	bcdefghijklmnopqr	**C26**	5.8	abcdef
C13	167.5	ghijklmnopqrs	**C6**	4.2	cdefghijklmnopqr	**C28**	5.8	abcdef
PA6	167.5	ghijklmnopqrs	**C13**	4.2	cdefghijklmnopqr	**N1**	5.4	abcdef
Co14	164.2	ghijklmnopqrs	**PA7**	4.1	defghijklmnopqr	**V3**	5.0	abcdef
E43	162.8	ghijklmnopqrs	**Co10**	3.8	defghijklmnopqr	**C29**	5.0	abcdef
C28	156.0	ghijklmnopqrs	**E12**	3.8	defghijklmnopqr	**Co14**	4.9	abcdef
Co7	149.2	hijklmnopqrs	**P1**	3.8	defghijklmnopqr	**C11**	4.8	abcdef
C22	148.3	hijklmnopqrs	**H1**	3.8	defghijklmnopqr	**C13**	4.7	abcdef
C1	140.2	hijklmnopqrs	**C1**	3.6	defghijklmnopqr	**E7**	4.5	abcdef
E24	137.0	jklmnopqrs	**Co13**	3.5	defghijklmnopqr	**C23**	4.5	abcdef
Co6	135.1	jklmnopqrs	**C18**	3.5	defghijklmnopqr	**Co17**	4.4	abcdef
E13	128.7	jklmnopqrs	**C19**	3.5	defghijklmnopqr	**Co1**	4.3	abcdef

Continuation Table 8.3

Spores plate^{-1} (x 10^6)			Spores cm^{-2}(x 10^6)			% germinated spores		
PA7	126.0	jklmnopqrs	**C22**	3.5	defghijklmnopqr	**C16**	4.3	abcdef
C18	125.6	jklmnopqrs	**N5**	3.5	defghijklmnopqr	**E3**	4.0	abcdef
C21	120.6	jklmnopqrs	**C21**	3.3	efghijklmnopqr	**N5**	3.9	bcdef
Co17	114.8	jklmnopqrs	**Co8**	3.2	fghijklmnopqr	**C22**	3.4	bcdef
E29	111.8	klmnopqrs	**Co11**	3.2	fghijklmnopqr	**PA6**	3.4	bcdef
N4	110.1	klmnopqrs	**E13**	3.2	fghijklmnopqr	**C27**	3.2	bcdef
C27	109.2	lmnopqrs	**Co4**	3.1	ghijklmnopqr	**E11**	3.0	bcdef
N5	107.6	lmnopqrs	**E29**	3.1	ghijklmnopqr	**E13**	3.0	bcdef
C14	107.4	lmnopqrs	**PA6**	3.1	ghijklmnopqr	**E15**	3.0	bcdef
C20	106.9	lmnopqrs	**Co6**	2.9	hijklmnopqr	**E33**	3.0	bcdef
Co12	106.7	lmnopqrs	**Co14**	2.9	hijklmnopqr	**Co11**	2.6	bcdef
C6	105.5	lmnopqrs	**C17**	2.9	hijklmnopqr	**E17**	2.5	bcdef
C19	100.0	lmnopqrs	**C11**	2.8	ijklmnopqr	**V2**	2.3	bcdef
H1	98.0	lmnopqrs	**C14**	2.8	ijklmnopqr	**Co6**	2.2	bcdef
E23	96.0	lmnopqrs	**C20**	2.8	ijklmnopqr	**E42**	2.0	bcdef
Co1	91.5	lmnopqrs	**Co7**	2.7	jklmnopqr	**E43**	2.0	bcdef
E15	82.8	lmnopqrs	**N4**	2.6	jklmnopqr	**Co7**	1.8	bcdef
C11	81.5	lmnopqrs	**C2**	2.5	klmnopqr	**P1**	1.6	bcdef
N3	74.7	lmnopqrs	**E15**	2.2	lmnopqr	**C24**	1.6	bcdef
C16	71.9	lmnopqrs	**C16**	2.2	mnopqr	**E32**	1.5	bcdef
E11	63.8	mnopqrs	**C29**	2.1	mnopqr	**V5**	1.5	bcdef
C29	61.2	mnopqrs	**N1**	2.1	mnopqr	**C19**	1.4	bcdef
C9	60.4	mnopqrs	**Co17**	2.0	mnopqr	**C17**	1.2	bcdef
E8	60.0	mnopqrs	**C5**	2.0	mnopqr	**Co4**	1.0	bcdef
E26	56.5	nopqrs	**Co1**	1.9	mnopqr	**E9**	1.0	cdef

Continuation Table 8.3

Spores plate^{-1} (x 10^6)			Spores cm^{-2}(x 10^6)			% germinated spores		
E16	55.4	nopqrs	**Co12**	1.9	mnopqr	**E12**	1.0	cdef
E7	53.5	nopqrs	**E18**	1.8	nopqr	**C5**	0.8	def
E6	53.1	nopqrs	**C9**	1.8	nopqr	**C7**	0.8	def
E14	53.0	nopqrs	**E8**	1.6	nopqr	**E22**	0.5	ef
N1	51.6	nopqrs	**E14**	1.5	opqr	**E37**	0.5	ef
Co2	47.8	nopqrs	**E26**	1.5	opqr	**P5**	0.5	ef
E18	41.9	opqrs	**Co2**	1.4	pqr	**Co15**	0.0	f
E25	40.1	pqrs	**E7**	1.4	pqr	**Co16**	0.0	f
C5	39.5	pqrs	**E17**	1.4	pqr	**E1**	0.0	f
C2	37.6	pqrs	**E6**	1.3	pqr	**E21**	0.0	f
C17	34.2	qrs	**E16**	1.3	pqr	**E23**	0.0	f
E9	15.0	rs	**E25**	1.0	qr	**E27**	0.0	f
E10	14.0	rs	**E9**	0.8	qr	**E30**	0.0	f
E17	12.1	s	**E10**	0.4	r	**E36**	0.0	f
E21	3.6	s	**E21**	0.3	r	**P2**	0.0	f

Table 8.4 Percentage of globose spores, percentage of ellipsoid spores, globose spore diameter and ellipsoid spore width and length of 88 isolates of *M. roreri* grown in Petri dishes with modified V8 medium at 24 °C and 12/12 alternate periods of light/darkness.

% globose spores[1/]		% ellipsoid spores		Globose spore diameter (µm)		Ellipsoid spore width (µm)		Ellipsoid spore length (µm)	
E27	95.6 a	**Co5**	55.6 a	**E24**	10.4 a	**P1**	10.6 a	**Co1**	14.4 a
E1	93.3 a	**C27**	48.9 ab	**Co1**	10.3 ab	**P2**	10.3 ab	**P1**	14.3 a
E36	93.3 a	**E4**	48.3 ab	**C5**	10.3 abc	**Co1**	10.2 abc	**P3**	14.0 a
P3	95.0 ab	**E42**	48.3 ab	**E37**	10.2 abcd	**E37**	10.2 abc	**Co4**	13.6 a
E12	93.3 abc	**C2**	46.7 ab	**P1**	10.2 abcd	**E32**	10.0 abcd	**E33**	13.5 a
P1	91.7 abcd	**C6**	45.0 ab	**E26**	10.2 abcde	**Co5**	9.8 abcde	**P2**	13.4 a
P5	91.7 abcde	**E29**	43.3 ab	**Co5**	10.1 abcdef	**Co8**	9.7 abcdef	**Co17**	13.3 a
E32	88.3 abcdef	**E20**	41.7 ab	**E30**	10.0 abcdefg	**V3**	9.7 abcdef	**Co7**	13.1 a
E37	86.7 abcdefg	**C23**	41.7 ab	**E27**	9.9 abcdefg	**E15**	9.6 abcdef	**E7**	13.1 a
E24	85.0 abcdefgh	**Co7**	40.0 abc	**P3**	9.9 abcdefg	**E22**	9.6 abcdef	**C17**	13.0 a
P2	85.0 abcdefgh	**E5**	40.0 abc	**P5**	9.8 abcdefgh	**Co6**	9.4 abcdef	**E15**	12.8 a
Co1	83.3 abcdefgh	**E10**	40.0 abc	**Co7**	9.8 abcdefghi	**Co7**	9.4 abcdef	**E30**	12.7 a
E22	83.3 abcdefgh	**E25**	40.0 abc	**E32**	9.8 abcdefghi	**E9**	9.4 abcdef	**E24**	12.5 a
C14	83.3 abcdefgh	**E30**	40.0 abc	**Co17**	9.8 abcdefghij	**E26**	9.4 abcdef	**E27**	12.5 a
N5	81.7 abcdefgh	**V5**	40.0 abc	**Co6**	9.7 abcdefghij	**C17**	9.4 abcdef	**N3**	12.5 a
V2	80.0 abcdefgh	**E3**	38.3 abcd	**E11**	9.7 abcdefghij	**PA7**	9.4 abcdef	**Co5**	12.4 a
C16	78.3 abcdefghi	**Co11**	38.3 abcde	**P2**	9.7 abcdefghij	**E24**	9.3 abcdef	**Co6**	12.4 a
H1	77.8 abcdefghi	**C9**	38.3 abcde	**Co8**	9.6 abcdefghij	**E36**	9.2 abcdef	**Co12**	12.4 a
N1	77.7 abcdefghi	**Co2**	36.7 abcdef	**E9**	9.6 abcdefghij	**Co4**	9.1 abcdef	**E5**	12.4 a
E11	76.7 abcdefghij	**E15**	36.7 abcdef	**E12**	9.5 abcdefghijk	**Co9**	9.1 abcdef	**E22**	12.4 a
E23	75.0 abcdefghi	**E21**	36.7 abcdef	**E22**	9.5 abcdefghijkl	**Co12**	9.1 abcdef	**E37**	12.4 a

[1/] Values for each variable followed by the same letter do not have significant differences (Tukey Test, P<0.05).

Continuation Table 8.4

% globose spores		% ellipsoid spores		Globose spore diameter (µm)		Ellipsoid spore width (µm)		Ellipsoid spore length (µm)	
Co8	75.0 abcdefghijk	**E28**	36.7 abcdef	**E23**	9.5 abcdefghijkl	**E11**	9.1 abcdef	**E1**	12.2 a
PA6	71.7 abcdefghijkl	**C24**	36.7 abcdef	**Co2**	9.4 abcdefghijkl	**E27**	9.0 abcdef	**E8**	12.2 a
V3	70.0 abcdefghijklm	**PA7**	36.7 abcdefg	**Co9**	9.4 abcdefghijkl	**P5**	9.0 abcdef	**E12**	12.2 a
C1	70.0 abcdefghijklm	**Co15**	35.6 abcdefg	**E1**	9.4 abcdefghijkl	**Co17**	8.9 abcdef	**E26**	12.2 a
N4	70.0 abcdefghijklm	**E43**	35.6 abcdefg	**E36**	9.4 abcdefghijkl	**E33**	8.9 abcdef	**E11**	12.1 a
C13	68.9 abcdefghijklm	**C19**	35.6 abcdefg	**Co14**	9.3 abcdefghijklm	**C14**	8.9 abcdef	**E23**	12.1 a
N3	68.9 abcdefghijklm	**Co10**	35.0 abcdefg	**C17**	9.3 abcdefghijklm	**E8**	8.8 abcdef	**C2**	12.1 a
Co6	68.3 abcdefghijklm	**E8**	35.0 abcdefg	**E14**	9.2 abcdefghijklm	**E14**	8.8 abcdef	**N5**	12.1 a
E13	68.3 abcdefghijklm	**E16**	35.0 abcdefg	**E17**	9.2 abcdefghijklm	**E16**	8.8 abcdef	**E9**	12.0 a
C21	68.3 abcdefghijklm	**Co14**	33.3 abcdefg	**E33**	9.2 abcdefghijklm	**E21**	8.8 abcdef	**E29**	12.0 a
E26	66.7 abcdefghijklmn	**E6**	33.3 abcdefg	**V2**	9.2 abcdefghijklm	**E23**	8.8 abcdef	**C5**	12.0 a
C25	66.7 abcdefghijklmn	**E7**	33.3 abcdefg	**V3**	9.2 abcdefghijklm	**E30**	8.8 abcdef	**C14**	12.0 a
E17	65.2 abcdefghijklmn	**E18**	33.3 abcdefg	**C1**	9.2 abcdefghijklm	**C20**	8.8 abcdef	**H1**	12.0 a
C7	65.0 abcdefghijklmn	**C22**	33.3 abcdefg	**C25**	9.2 abcdefghijklm	**C21**	8.8 abcdef	**Co2**	11.9 a
C17	64.4 abcdefghijklmno	**C28**	33.3 abcdefg	**N5**	9.2 abcdefghijklm	**N5**	8.8 abcdef	**Co8**	11.9 a
C22	64.4 abcdefghijklmno	**Co6**	31.7 abcdefg	**E15**	9.1 abcdefghijklm	**PA6**	8.8 abcdef	**C1**	11.8 a
C18	63.3 abcdefghijklmno	**E2**	31.7 abcdefg	**Co10**	9.0 abcdefghijklm	**Co10**	8.7 abcdef	**C6**	11.8 a
Co4	62.2 bcdefghijklmno	**E14**	31.7 abcdefg	**Co11**	9.0 abcdefghijklm	**P3**	8.7 abcdef	**C9**	11.8 a
E33	61.7 bcdefghijklmno	**E26**	31.7 abcdefg	**E21**	9.0 abcdefghijklm	**C5**	8.7 abcdef	**PA7**	11.8 a
Co16	61.7 cdefghijklmno	**C7**	31.7 abcdefg	**E43**	9.0 abcdefghijklm	**N3**	8.7 abcdef	**Co9**	11.7 a
C20	61.7 cdefghijklmno	**C18**	31.7 abcdefg	**C26**	9.0 abcdefghijklm	**Co2**	8.6 abcdef	**Co16**	11.7 a
Co7	60.0 defghijklmno	**C20**	31.7 abcdefg	**E7**	8.9 abcdefghijklm	**Co11**	8.6 abcdef	**E4**	11.7 a
Co12	60.0 defghijklmno	**C29**	31.7 abcdefg	**E28**	8.9 abcdefghijklm	**E5**	8.6 abcdef	**E32**	11.7 a
Co14	60.0 defghijklmno	**Co4**	31.1 abcdefg	**V5**	8.9 abcdefghijklm	**E29**	8.6 abcdef	**V5**	11.7 a
E16	60.0 defghijklmno	**Co9**	30.0 abcdefg	**E42**	8.8 abcdefghijklm	**C25**	8.6 abcdef	**C21**	11.7 a

Continuation Table 8.4

% globose spores			% ellipsoid spores		Globose spore diameter (µm)			Ellipsoid spore width (µm)		Ellipsoid spore length (µm)	
C5	60.0	defghijklmno	**Co12**	30.0 abcdefg	**C14**	8.8	abcdefghijklm	**Co13**	8.5 abcdef	**Co11**	11.6 a
C24	60.0	defghijklmno	**Co13**	30.0 abcdefg	**N1**	8.8	abcdefghijklm	**Co15**	8.5 abcdef	**E6**	11.6 a
Co10	58.3	defghijklmno	**Co17**	30.0 abcdefg	**N3**	8.8	abcdefghijklm	**E4**	8.5 abcdef	**E16**	11.6 a
Co13	58.3	defghijklmno	**C1**	30.0 abcdefg	**PA6**	8.8	abcdefghijklm	**E7**	8.5 abcdef	**E42**	11.6 a
C19	57.8	defghijklmno	**C5**	30.0 abcdefg	**Co4**	8.7	abcdefghijklm	**E28**	8.5 abcdef	**C20**	11.6 a
E15	56.7	efghijklmno	**C21**	30.0 abcdefg	**Co13**	8.7	abcdefghijklm	**V2**	8.5 abcdef	**E2**	11.5 a
C11	56.7	efghijklmno	**C26**	30.0 abcdefg	**E5**	8.7	abcdefghijklm	**C1**	8.5 abcdef	**E3**	11.5 a
C29	56.7	efghijklmno	**Co16**	26.7 abcdefg	**E8**	8.7	abcdefghijklm	**C13**	8.5 abcdef	**E17**	11.5 a
Co9	55.0	fghijklmno	**E33**	26.7 abcdefg	**C16**	8.7	abcdefghijklm	**C29**	8.5 abcdef	**E28**	11.5 a
E6	55.0	fghijklmno	**N3**	26.7 abcdefg	**E16**	8.6	abcdefghijklm	**Co16**	8.4 abcdef	**V3**	11.5 a
E28	55.0	fghijklmno	**PA6**	26.7 abcdefg	**N4**	8.6	abcdefghijklm	**E3**	8.4 abcdef	**C22**	11.5 a
Co11	53.3	fghijklmno	**E17**	26.5 abcdefg	**E4**	8.6	bcdefghijklm	**E42**	8.4 abcdef	**C27**	11.5 a
Co17	53.3	fghijklmno	**V3**	25.0 abcdefg	**E6**	8.6	bcdefghijklm	**C19**	8.4 abcdef	**C29**	11.5 a
E2	53.3	fghijklmno	**C11**	25.0 abcdefg	**C9**	8.6	bcdefghijklm	**C23**	8.4 abcdef	**Co10**	11.4 a
E5	53.3	fghijklmno	**C13**	24.4 abcdefg	**Co16**	8.5	bcdefghijklm	**N1**	8.4 abcdef	**E14**	11.4 a
E7	53.3	fghijklmno	**C17**	24.4 abcdefg	**E29**	8.5	bcdefghijklm	**V5**	8.3 abcdef	**C16**	11.4 a
E8	53.3	fghijklmno	**N4**	23.3 abcdefg	**C18**	8.5	bcdefghijklm	**C16**	8.3 abcdef	**E18**	11.3 a
C23	53.3	fghijklmno	**Co8**	21.7 abcdefg	**C27**	8.5	bcdefghijklm	**C24**	8.3 abcdef	**E20**	11.2 a
PA7	53.3	fghijklmno	**E9**	21.7 abcdefg	**C24**	8.5	cdefghijklm	**C9**	8.2 abcdef	**C13**	11.2 a
E10	51.1	fghijklmno	**E13**	21.7 abcdefg	**C13**	8.5	defghijklm	**C26**	8.2 abcdef	**N4**	11.2 a
V5	50.0	fghijklmno	**N1**	21.7 abcdefg	**Co12**	8.4	defghijklm	**E2**	8.1 abcdef	**PA6**	11.2 a
C26	50.0	fghijklmno	**E11**	20.0 abcdefg	**Co15**	8.4	defghijklm	**E17**	8.1 abcdef	**Co13**	11.1 a
Co15	48.9	fghijklmno	**E23**	20.0 abcdefg	**E3**	8.4	defghijklm	**E43**	8.1 abcdef	**Co14**	11.1 a
E9	48.3	fghijklmno	**C16**	20.0 abcdefg	**C20**	8.4	defghijklm	**C18**	8.1 abcdef	**E21**	11.0 a
E29	48.3	fghijklmno	**C25**	20.0 abcdefg	**PA7**	8.4	defghijklm	**C22**	8.1 abcdef	**E36**	11.0 a
C9	48.3	fghijklmno	**Co1**	16.7 abcdefg	**C29**	8.4	fghijklm	**E1**	8.0 abcdef	**E43**	11.0 a

Continuation Table 8.4

% globose spores			% ellipsoid spores			Globose spore diameter (µm)			Ellipsoid spore width (µm)			Ellipsoid spore length (µm)		
E21	46.7	ghijklmno	**E22**	16.7	abcdefg	**C22**	8.3	fghijklm	**C27**	8.0	abcdef	**C19**	11.0	a
E14	45.0	hijklmno	**V2**	16.7	abcdefg	**C23**	8.3	fghijklm	**C28**	8.0	abcdef	**C23**	11.0	a
E30	45.0	hijklmno	**C14**	16.7	abcdefg	**H1**	8.3	fghijklm	**E6**	8.0	bcdef	**C24**	11.0	a
Co5	44.4	hijklmno	**H1**	15.6	abcdefg	**C21**	8.3	ghijklm	**Co14**	7.9	bcdef	**N1**	11.0	a
E43	44.4	hijklmno	**E37**	13.3	abcdefg	**C7**	8.2	ghijklm	**E13**	7.9	bcdef	**E25**	10.9	a
C28	44.4	hijklmno	**P2**	13.3	abcdefg	**C19**	8.2	ghijklm	**H1**	7.9	bcdef	**C11**	10.9	a
E3	43.3	hijklmno	**N5**	13.3	bcdefg	**C28**	8.2	ghijklm	**C7**	7.8	bcdef	**C28**	10.9	a
E42	36.7	ijklmno	**E24**	11.7	bcdefg	**E2**	8.0	hijklm	**E10**	7.7	bcdef	**C7**	10.8	a
E4	33.3	jklmno	**E12**	6.6	cdefg	**E18**	8.0	hijklm	**C6**	7.7	bcdef	**C26**	10.8	a
C2	31.7	klmno	**P3**	5.0	cdefg	**C11**	8.0	ijklm	**N4**	7.7	bcdef	**E10**	10.7	a
Co2	30.0	lmno	**E36**	6.7	defg	**E13**	8.0	jklm	**E25**	7.6	cdef	**V2**	10.7	a
C27	28.9	lmno	**E32**	5.0	defg	**E25**	8.0	jklm	**C11**	7.4	def	**C18**	10.5	a
E18	26.7	mno	**P1**	6.7	efg	**C2**	8.0	jklm	**C2**	7.3	ef	**E13**	10.4	a
E25	25.0	no	**E27**	5.0	fg	**E10**	7.8	klm	**E18**	7.2	ef	**Co15**	10.2	a
E20	21.7	o	**E1**	5.0	g	**C6**	7.7	lm	**E12**	7.0	f	**C25**	10.1	a
C6	21.7	o	**P5**	5.0	g	**E20**	7.5	m	**E20**	7.0	f	**P5**	10.0	a

Percentage of ellipsoid spores: This variable ranged from 5.0% to 55.6% (Table 8.4). Not surprisingly given that most spores were either globose or ellipsoid, the pattern of variation observed was complementary to that recorded for the frequency of globose spores. Moreover, the highest percentages were mostly obtained for a group of isolates containing mostly Ecuadorian and Costa Rican isolates (Co5, C27, E4, E42, C2, C6, E29, E20 and C23) (55.6% ≥ x ≥ 41.7%) whereas the lowest values ($P<0.05$) were seen among a group comprising Ecuadorian and Peruvian isolates (E36, E32, P1, E27, E1 and P5) (6.7% ≥ x ≥ 5.0%).

Globose spore diameter: This variable ranged from 7.5 µm to 10.4 µm (Table 8.4). The highest values were obtained for a group of isolates with diverse origins (E24, Co1, C5, E37, P1, E26 and Co5) (10.4 µm ≥ x ≥ 10.1 µm). This group produced spores with significantly greater spore diameters ($P<0.05$) than a second group from Ecuador and Costa Rica (E2, E18, C11, E13, E25, C2, E10, C6 and E20) (8.0 µm ≥ x ≥ 7.5 µm).

Ellipsoid spore width: This variable ranged from 7.0 µm to 10.6 µm (Table 8.4). The highest values were obtained for isolates P1, P2, Co1 and E37 (10.6 µm ≥ x ≥ 10.2 µm) and the lowest values for isolates C2, E18, E12 and E20 (7.3 µm ≥ x ≥ 7.0 µm). Differences between these two groups were significant ($P<0.05$).

Ellipsoid spore length: The range of this variable was from 10.0 µm (P5) to 14.4 µm (Co1) although no differences were found between any isolate or group of isolates (Table 8.4).

Colony appearance: The general appearance of the colonies was very diverse and difficult to score as can be seen in a sample of 16 isolates shown in Plate 8.1. Evans (1981) and Herrera *et al*. (1989) also reported a high level of variability for this trait for isolates from Ecuador and Costa Rica, respectively. It was not possible within the timeframe of the present study to adequately describe this variability in a quantitative or semi-quantitative manner. Nevertheless, it was possible to make the following general observations. Colombian isolates were the most variable in appearance and very difficult to categorise into groups, whereas both the Peruvian and the Nicaraguan/Honduran isolates were very uniform. Although some differences were detected for the Costa Rican and Panamanian

isolates, most of the Central American isolates were similar in their general appearance. Isolates from Venezuela showed a considerable level of variation with particular types of growth not observed for other isolates. As described by Chacín (1975) isolates from Venezuela were, in general, initially white-cream-coloured but became light yellow when older some exhibiting a 'cottony' growth. Although Ecuadorian isolates also showed a considerable variation, it was possible to classify some of them in groups based on their similarities. In fact, some isolates from Ecuador were similar to the type most common in Central America. In this country, the three isolates from the Province of Manabí (E21, E22, E23) exhibited very diverse colony morphologies.

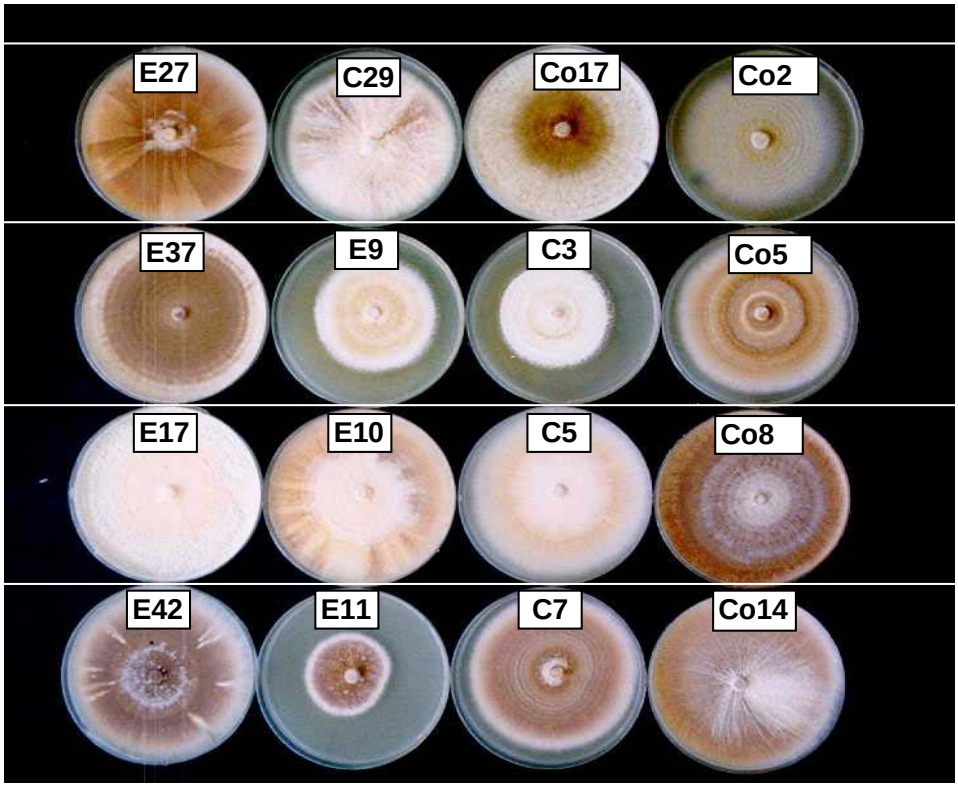


Plate 8.1 Appearance of 16 isolates of *M. roreri* grown in modified V8 medium for 20 days.

Sectoring is defined as a sector of the circular colony that is obviously different from the rest (Hawker, 1950) and was observed during the experiment or in the routinely transfer of the cultures. The phenomenon was seen in some plates of the following isolates: E12, E20, E23, E27, E29, E30, E32, E43, C7, C23, C29, P2, P3, Co9 and particularly in Co14.

8.4.3 MORPHO-PHYSIOLOGY OF ISOLATES FROM DIFFERENT SPECIES OF *THEOBROMA* SPP. AND *HERRANIA* SPP.

Significant differences ($P<0.05$) were only observed for ring intensity and spore production between isolates from *Theobroma* spp. and *Herrania* spp. collected in the CATIE's International Germplasm Collection. The absence of rings in the *H. nitida* isolate (C18) also separated this isolate from the remaining (Table 8.2). The only distinction for spore production was obtained between the isolate taken from *H. albiflora* (C23) (564.4 million spores plate^{-1}) and those collected from the following tree species ($148.3 \geq x \geq 106.9$ million spores plate^{-1}): *T. speciosum* (C22), *H. nitida* (C18), *T. grandiflorum* (C14), *T. bicolor* (C20) and two isolates of *T. cacao* (C1 and C21) (Table 8.3). The two isolates collected from *T. cacao* (E37) and *T. bicolor* (E36) in the Napo Province of Ecuador were significantly different ($P<0.05$) for ring intensity, with E37 possessing a high ring intensity of 4.5 and E36 with a low intensity of 1 (Table 8.2). The two isolates taken from *T. gileri* (E42 and E43) were not significantly different from each other for any of the parameters measured.

8.4.4 EFFECT OF DARKNESS ON THE MORPHO-PHYSIOLOGICAL TRAITS

It was not possible to subject isolates culture in darkness to statistical analysis since only one plate per isolate was cultured. However, the following observations were made from the plates kept under total darkness after 20 d. Radial growth was, on average, higher in these plates (average 8.5 cm) than in plates cultured during the same period under a regime of 12/12 alternate periods of light/darkness (average 7.7 cm). Presence of rings was observed in 68.2% of the plates with an average intensity of rings of 1.6 under total darkness in comparison with a 83.3% and 2.4 in the illuminated set of plates. Finally, the production of spores and the density of spores in darkness were 42.1 million spores plate^{-1} and 2.4 million spores cm^{-2}, respectively, whereas, 176.0 million spores plate^{-1} and 4.6 million spores cm^{-2} respectively, were obtained when light was provided. In summary, presence and intensity of rings and production and density of spores were favoured in illuminated conditions, radial growth being the only variable favoured in darkness.

8.4.5 RESULTS BY COUNTRY

When isolates were compared in accordance to their country of origin highly significant differences ($P<0.01$) were found for all variables studied except for the percentage of germinated spores (Table A8.2).

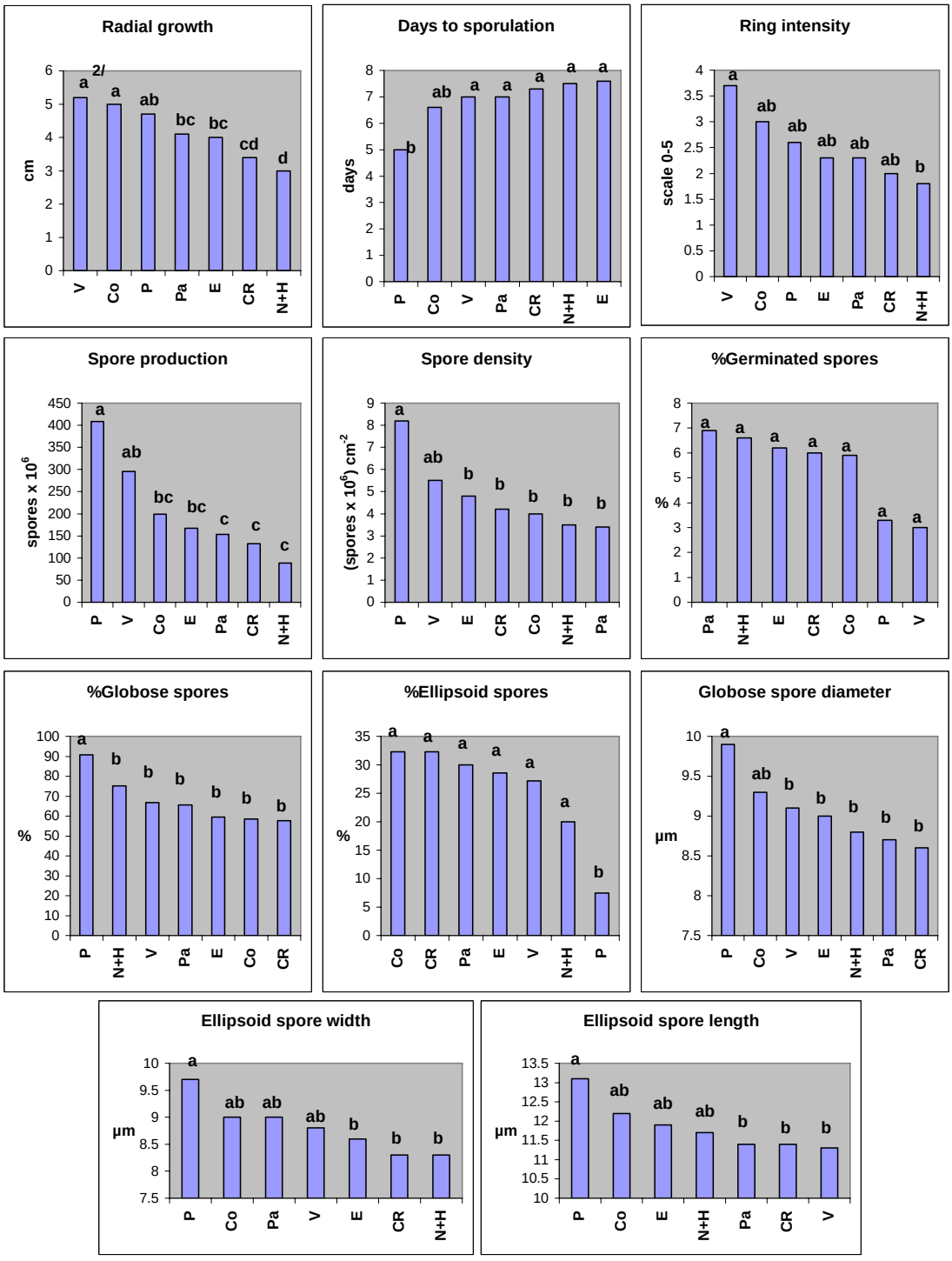
Radial growth ranged from 3.0 cm to 5.2 cm (Figure 8.3). Isolates from Venezuela and Colombia (5.2 cm $\geq x \geq$ 4.7 cm) were significantly faster growing ($P<0.05$) than the remaining isolates (except for Peruvian isolates). The lowest levels of radial growth were obtained for the Central American and Ecuadorian isolates (4.1 cm $\geq x \geq$ 3.0 cm).

Days to sporulation ranged from 5.0 d to 7.6 d (Figure 8.3). Isolates from Peru showed the most rapid progression to sporulation (5 d). This rate was faster than for isolates from all other countries ($P<0.05$) except Colombia (6.6 d). Ring intensity ranged from 1.8 to 3.7, being most intense for Venezuelan isolates and least so for Nicaraguan/Honduran isolates. These two groups were significantly different ($P<0.05$, statistical test) (Figure 8.3).

Spore production ranged from 88.6 to 408.2 million spores plate^{-1} (Figure 8.3, statistical test). Isolates from Peru (408.2 million spores plate^{-1}) produced significantly more ($P<0.05$) spores than isolates from any other country isolates (198.6 million spores plate$^{-1} \geq x \geq$ 88.6 million spores plate^{-1}) except Venezuela (296.0 million spores plate^{-1}). Spore density ranged from 3.4 to 8.2 million spores cm^{-2} (Figure 8.3). Peruvian isolates produced significantly more ($P<0.05$, statistical test) spores cm^{-2} (8.2 million spores cm^{-2}) than isolates from any other country except Venezuela (5.5 million spores cm^{-2}).

Percentage of germinated spores ranged from 3.0% to 6.9%. No significant differences were found among isolates from any country (Figure 8.3).

The percentage of globose spores and the percentage of ellipsoid spores ranged from 57.7% (Costa Rica) to 90.8% (Peru) and from 7.5% (Peru) to 32.3% (Colombia, Costa Rica). The Peruvian isolates produced significantly more ellipsoid spores ($P<0.05$, statistical test) from those from all other countries (Figure 8.3).



[1/] Countries: Co = Colombia; CR = Costa Rica; E = Ecuador, H = Honduras; N = Nicaragua; P = Peru; Pa = Panama and V = Venezuela.

[2/] Values within each variable followed for the same letter do not have significant differences (Tukey Test, P<0.05).

Figure 8.3 Results by country [1/] of eleven morpho-physiological traits evaluated *in vitro* in 88 isolates of *M. roreri*.

Globose spore diameter, ellipsoid spore width and ellipsoid spore length ranged from 8.6 µm to 9.9 µm, from 8.3 µm to 9.7 µm and from 11.4 µm to 13.1 µm, respectively, with the Peruvian isolates producing the highest values (Figure 8.3). Peruvian isolates had greater globose spore diameter ($P<0.05$) than any of the remaining countries except Colombia. In terms of ellipsoid spore width, Peruvian isolates were wider ($P<0.05$) than those from Ecuador, Costa Rica and Nicaragua/Honduras isolates. For ellipsoid spore length, Peruvian isolates produced higher values than those from Venezuela, Panama and Costa Rica

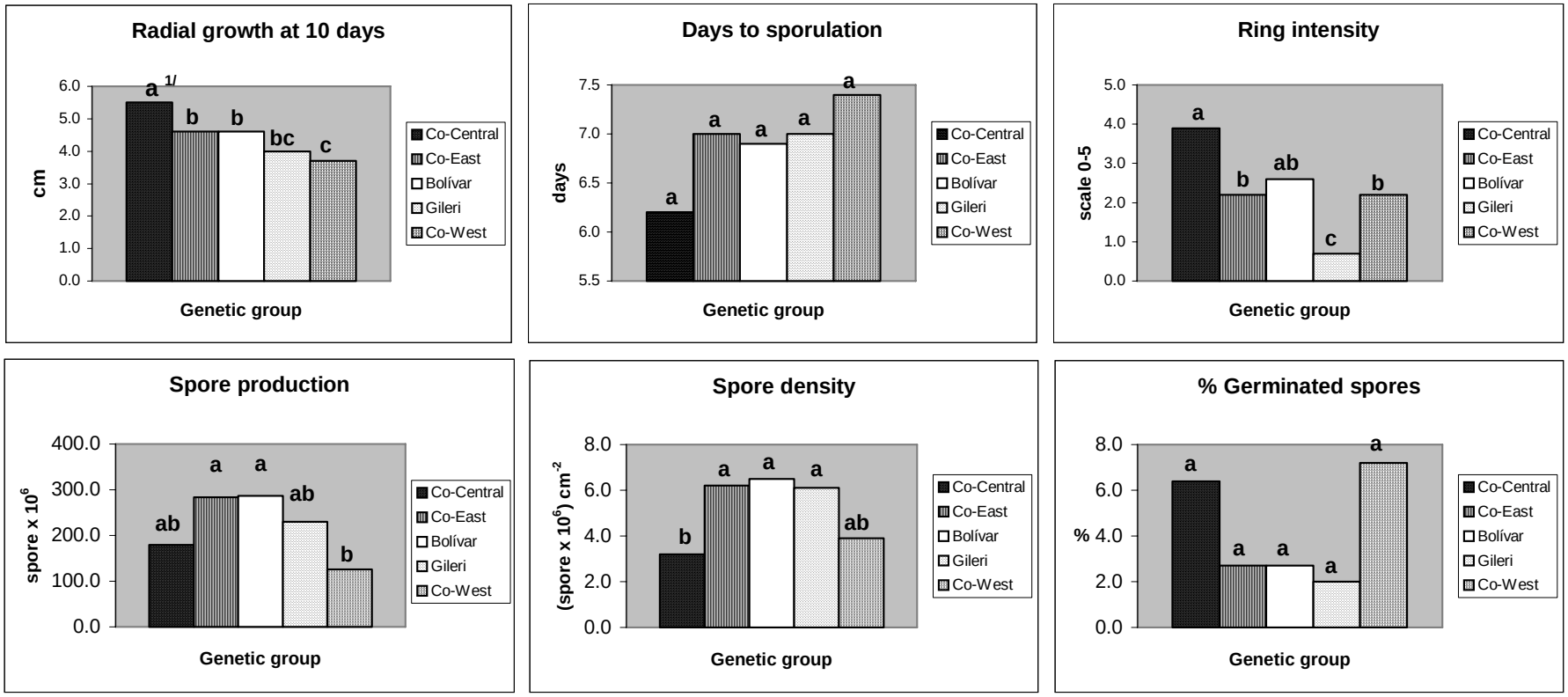
8.4.6 MORPHO-PHYSIOLOGICAL VARIATION BY GENETIC GROUP

Highly significant or significant differences ($P<0.01$) were found between genetic groups for all variables studied (Table A8.3). However, when the mean test was carried out, no significant differences were found for the following variables: days to sporulation, % germinated spores and ellipsoid spore length (Tukey, $P<0.05$).

Radial growth ranged from 3.7 cm to 5.5 cm (Figure 8.4). Co-Central (5.5 cm) was significantly faster growing ($P<0.05$) than the remaining groups. Conversely, Co-West registered the lowest value (3.7 cm) and showed significant differences ($P<0.05$) with the other groups except with Gileri.

Ring intensity ranged from 0.7 to 3.9 (Figure 8.4). Co-Central (5.5) showed the highest intensity which was significantly different ($P<0.05$) to those obtained by the other groups except by Bolívar (2.6). No differences ($P<0.05$) were found between the latter group and Co-East and Co-West, but all of them had differences with Gileri (0.7) (Figure 8.4).

Spore production ranged from 125.6 to 286.3 million spores $plate^{-1}$ (Figure 8.4). Significant differences ($P<0.05$) were only detected between Bolívar and Co-East (286.3 and 283.6 million spores $plate^{-1}$, respectively) in relation to Co-West (125.6 million spores $plate^{-1}$). Spore density ranged from 3.2 to 6.5 million spores cm^{-2} (Figure 8.4). Bolívar, Co-East and Gileri showed the highest densities which had significant differences ($P<0.05$) with Co-Central (3.2 million spores cm^{-2}).



[1/] Genetic groups within each variable with the same letter do no have significant differences (Tukey, P<0.05)

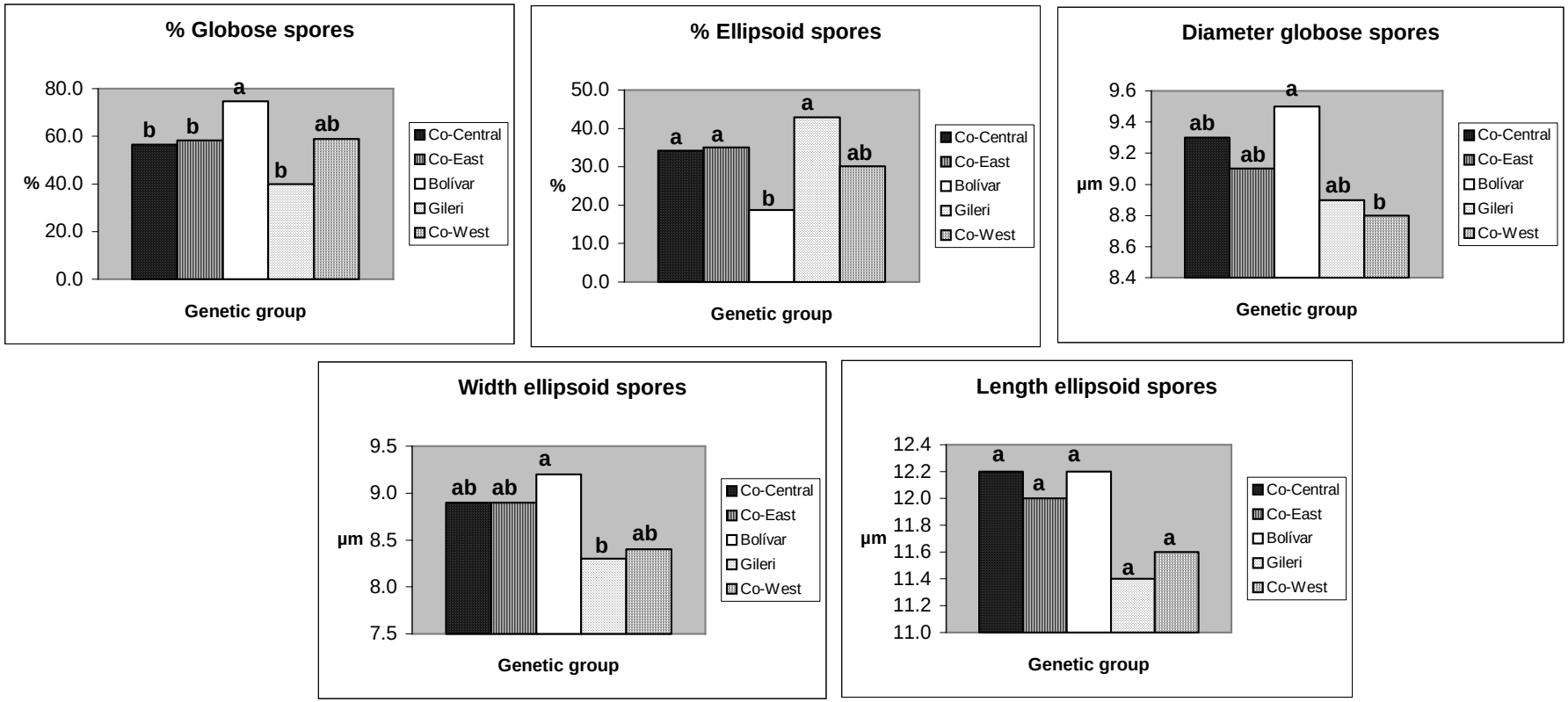
Figure 8.4 Radial growth at 10 days, days to sporulation, ring intensity, spore production, spore density and percentage of germinated spores of five genetic groups of *M. roreri*.

The percentage of globose spores ranged from 40.0% (Gileri group) to 74.7% (Bolívar group) (Figure 8.5). Bolívar had significantly more globose spores ($P<0.05$, statistical test) than the Co-Central (56.4%), Co-East (58.2%) and Gileri (40.0%) groups but not with Co-West group (58.9%).

Globose spore diameter and ellipsoid spore width ranged from 8.8 µm to 9.5 µm and from 8.3 µm to 9.2 µm, respectively, with Bolívar isolates producing the highest values and those belonging to the Co-West and Gileri groups generating the lowest (Figure 8.5). Significant differences ($P<0.05$) were only found between Bolívar and Co-West and between Bolívar and Gileri for the Globose spore diameter and the ellipsoid spore width, respectively.

8.4.7 SPECIES DESCRIPTION OF THE MORPHO-PHYSIOLOGICAL TRAITS OF *M. RORERI*.

Under the experimental conditions of this research, *M. roreri* registered the following mean values and standard errors. Radial growth at 10 d 4.06±0.10 cm; days to sporulation 7.18±0.16 d and ring intensity at 20 d 2.36±0.13. The production and density of spores were as follows: 176.12±14.00 million spores plate^{-1} and 4.65±0.31 million spores cm^{-2}. The percentage of germinated spores was 5.77±0.54. The percentage of shapes of spores were: percentage of globose spores 61.45±1.16 %, ellipsoid spores 28.76±1.43 % and abnormal spores 9.79±0.85 %. Finally, the size of spores were as follows: globose spore diameter 8.97±0.07 µm, ellipsoid spore width 8.65±0.08 µm, ellipsoid spore length 11.79±0.09 µm, abnormal spore width 8.10±1.9 µm and abnormal spore length 13.9±5.0 µm.



[1/] **Genetic groups within each variable and temperature with the same letter do no have significant differences (Tukey, P<0.05).**

Figure 8.5 Percentage of globose and ellipsoid spores, globose spore diameter, ellipsoid spore width and ellipsoid spore length of five genetic groups of *M. roreri*.

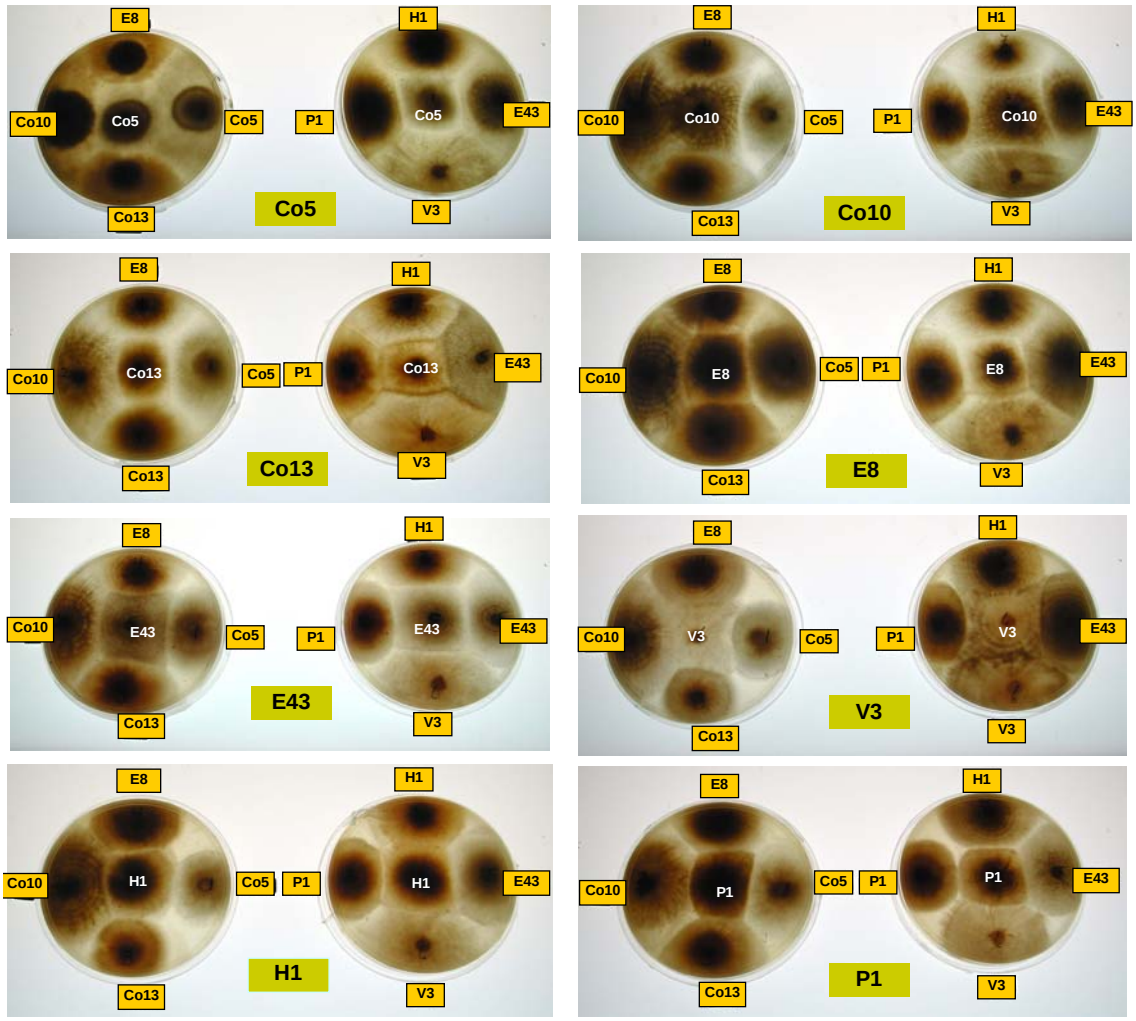
8.4.8 MYCELIAL INTERACTION TEST

Plate 8.2 shows the basal views of Petri dishes where different combinations of eight isolates of *M. roreri* were grown together to reveal their mycelial interactions. Basal views of the plates were more helpful to detect the reaction between isolates than apical views. All isolates showed an incompatible reaction (-) to each other, the only compatible reactions (+) being observed when the isolates Co5, Co10, Co13 and H1 were challenged by themselves.

Some of the compatibility reactions were sufficiently clear to be scored with a high level of confidence. In other cases, however, relationships were more difficult to assess. For instance, the incompatibility reaction of Co5 in relation to the other isolates or the self-compatibility reaction of Co10 both proved to be problematic to asses reliably score. In some cases, the gap between two incompatible isolates was very slight and in other cases a line of mycelium of different colour was observed in the encounter zone being the reaction scored as incompatible. This happened for example, when Co13 was grown in the centre of the plate surrounded by H1, P1, E43 and V3. New studies on this subject considering more replications and isolates are strongly recommended.

8.4.9 CORRELATION AMONG VARIABLES

When the morpho-physiological parameters were correlated with three geo-climatic variables (altitude, annual precipitation and average annual temperature), highly significant correlations ($P<0.01$) were obtained between altitude and the variables: average annual temperature ($r = -0.79$, $n = 88$), radial growth ($r = 0.37$) and spore production ($r = 0.29$), and a significant correlation ($P<0.05$) between altitude and days to sporulation ($r = -0.26$) (Table A8.4). A negative and significant correlation ($P<0.05$) was found between annual precipitation and the variables related with the spore size ($r = -0.21$ to -0.25).



	Co5	Co10	Co13	E8	E43	V3	H1	P1
Co5	+ [1/]	-	-	-	-	-	-	-
Co10		+	-	-	-	-	-	-
Co13			-	-	-	-	-	-
E8				-	-	-	-	-
E43					-	-	-	-
V3						+	-	-
H1							+	-
P1								-

[1/] Mycelial compatibility (+) or incompatibility (-).

Plate 8.2 Basal view of petri dishes showing mycelial interactions between eight isolates of *M. roreri* representing the global genetic variability of the fungus.

Other relevant correlations in terms of their magnitude ($r > 0.5$) and significance ($P<0.01$) were found between production and density of spores ($r = 0.81$, $n = 88$) and also between the different variables related to spore size ($r = 0.43$-0.70) (Table A8.4). Correlations between size and the percentage of globose or ellipsoid spores were positive and either highly significant ($P<0.01$) or significant ($P<0.05$). Highly significant correlation ($P<0.01$) were also obtained between radial growth and the following variables: days to sporulation ($r = -0.30$), ring intensity ($r = 0.32$), spore production ($r = 0.33$) and globose spore diameter ($r = 0.28$). Spore production correlated with all variables except with ring intensity and ellipsoid spore length showing a positive and highly significant correlation against radial growth, spore density and % globose spores and a negative and highly significant correlation against days to sporulation and % germinated spores (Table A8.4). A negative and highly significant correlation was obtained between the percentage of germinated spores and both spore production ($r = -0.34$) and spore density ($r = -0.28$).

8.4.10 CLUSTER ANALYSIS

The Ward's Minimum Variance Cluster Analysis was performed using the following five variables: radial growth, days to sporulation, ring intensity, spore production and globose spore diameter. The remaining variables were not considered because they showed a correlation coefficient of $r \geq 0.5$ against another variable already included in the analysis. The percentage of germination was excluded because it was determine that its inclusion produced a lower resolution of the groups probably due to the low values obtained for this variable in the present study.

The cluster analysis separated the isolates into two major clusters (Figure 8.6). Cluster 1 comprised 45 isolates belonging to Co-West (80%), the two isolates of Gileri (100%) and four isolates of Bolívar (22.2%). The only isolates of Co-West absent in Cluster 1 were Co8, C1, C25, Pa6 and seven Ecuadorian isolates without any particular characteristic in common that could help to explain their behaviour. The only isolates from Bolívar in this cluster were E2, E17, E21 and E23, all collected in the relatively near provinces of Guayas, Los Rios and Manabí in Ecuador. Cluster 1 divided into two subgroups: Cluster 1a that comprised entirely of four Ecuadorian isolates and Cluster 1b that showed various subgroups without any particular arrangement (Figure 8.6).

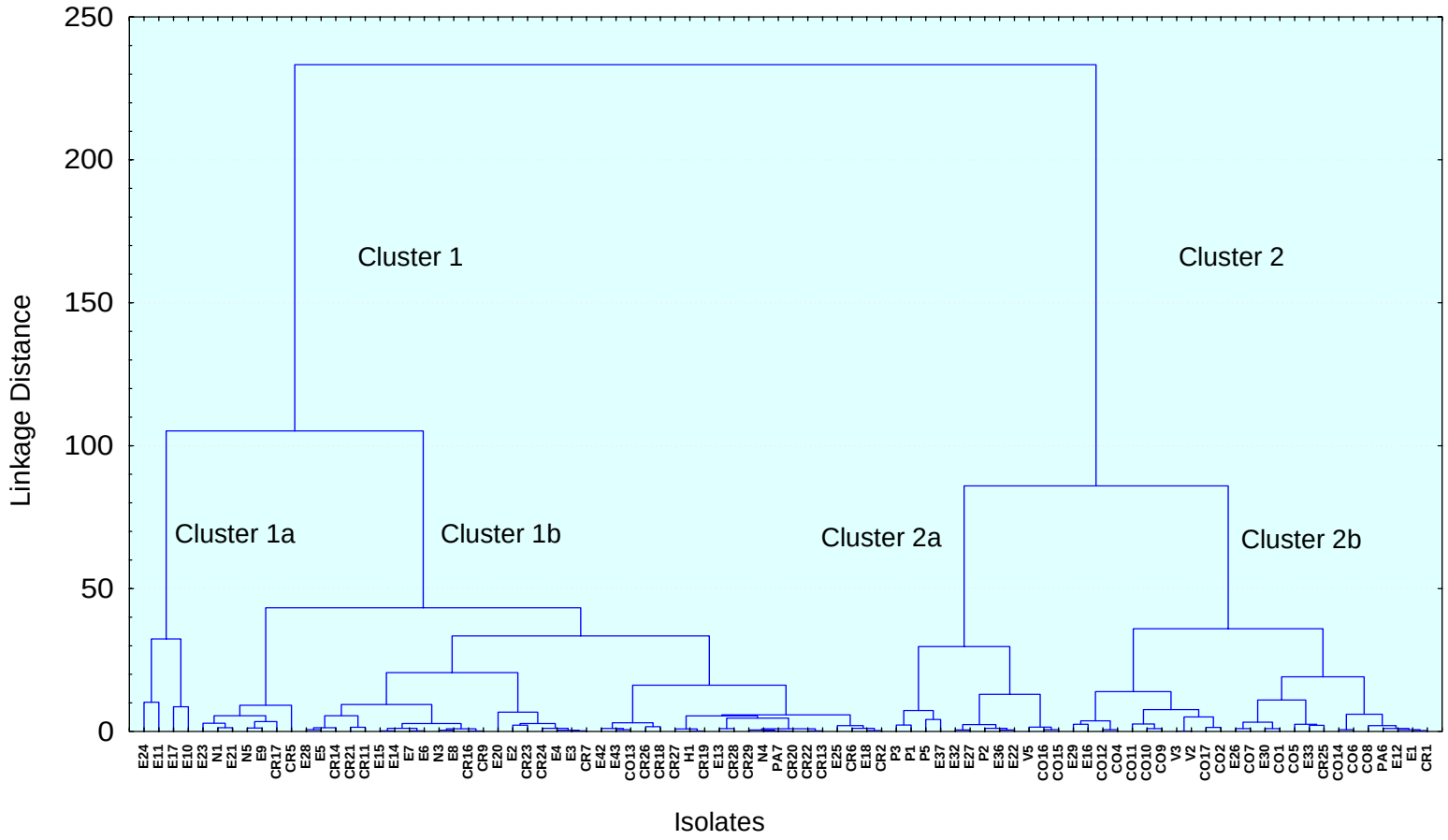


Figure 8.6 Ward's Minimum Variance Cluster Analysis using squared Euclidian distances and five morpho-physiological variables of 88 isolates of *M. roreri.*

Cluster 2 comprised all isolates of Co-East (100%) and Co-Central (100%), 14 isolates of Bolívar (77.8%) and the 11 isolates (20%) of Co-West already mentioned (Figure 8.6). Cluster 2 divided in two subgroups: Cluster 2a comprised nine Bolívar isolates including all the isolates from Peru and from the Napo Province of Ecuador and Cluster 2b included the two Bolívar isolates from Colombia and two isolates from Venezuela (66%). Cluster 2b also comprised all isolates from the Co-Central and Co-East groupings except two isolates from San Vicente de Chucurí in Colombia (Co15 and Co16) fell in Cluster 2a (Figure 8.6).

8.4.11 MANTEL TEST

When a Mantel test was performed to test for correlation between the dissimilarity matrices of morpho-physiological variation and geographic distances, a Pearson correlation coefficient of r =0.13 with a probability of 0.004 of independence between the matrices was found (Table 8.5). Similarly, a correlation coefficient of r = 0.20 with a probability of 0.006 was obtained between the morpho-physiological and the molecular matrices of dissimilarity.

Table 8.5 Results of the Mantel test for comparisons between the morpho-physiological and either the geographic or molecular dissimilarity matrices.

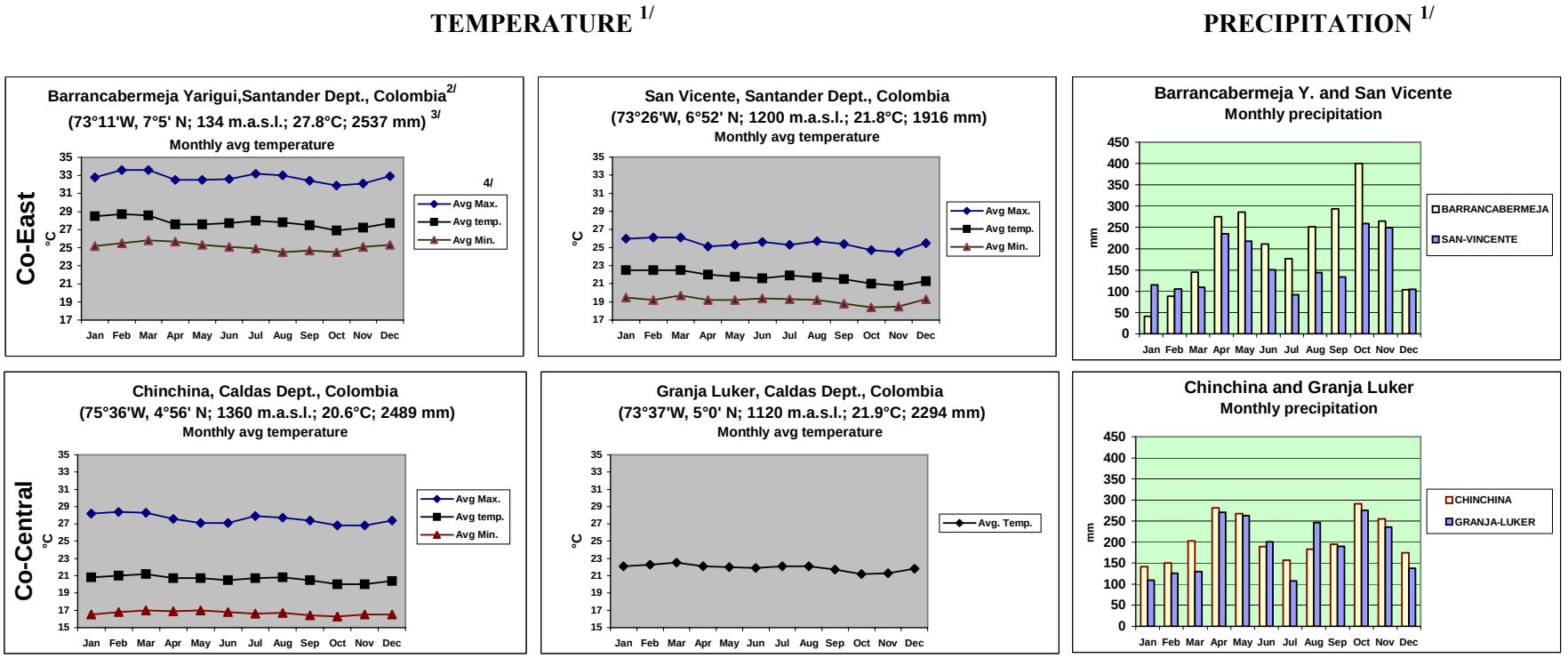
Comparison between	Coefficient of correlation (r)	Interpretation	Probability
Morpho-physiological and geographic dissimilarity matrices	0.13	Poor fit	0.004
Morpho-physiological and molecular dissimilarity matrices	0.20	Poor fit	0.006

8.4.12 CLIMATIC INFORMATION

Climatic information was collected for some localities within or close to the hypothetical centres of differentiation of the genetic groups in Colombia and Ecuador. Since the meteorological stations are not abundant in these areas, the stations that better matched with the environmental conditions where the cacao tree normally grows were selected.

In the case of Co-East, the two stations selected were situated in the Department of Santander, Colombia (Barrancabermeja-Yariguí and San Vicente) and represent the two extremes in altitude, temperature and precipitation where cacao frequently grows in this area. For the Co-Central group two stations (Chinchina and Granja Luker) were selected in the Department of Caldas, Colombia, where cacao frequently grows at more than 1100 m.a.s.l. The Chinchina meteorological station is probably outside the normal altitudinal range for cacao, but the information is included as reference of the variation of the minimum an maximum temperatures in this area. The stations representing the possible area of differentiation of Bolívar are located in the Norte de Santander Department in Colombia (Cúcuta-Camilo Daza) and close to the Catatumbo river in Venezuela (Encontrados-MOP). These locations represent the range in precipitation usually encountered in this area. In the case of the Co-West group, Villa Arteaga station represents the possible environmental conditions where this group differentiated in Western Colombia, whereas, San Jerónimo station, the particular climatic conditions where isolate Co-8 was collected. Two meteorological stations were considered for Gileri located at different heights, one in Colombia (Tumaco-El Mira) at 16 m.a.s.l. and the other in Ecuador (Lita) at 571 m.a.s.l.

For most stations mentioned, only very basic climatic information was available. Average monthly temperature and monthly precipitation together with the name of the meteorological station and its geographical position, annual average temperature and annual precipitation are shown in Figure 8.7. Average monthly minimum and maximum temperatures were included when available. The information collected was used to interpret the possible relationship between the climatic parameters and the distinctive morpho-physiological traits obtained for the genetic groups.

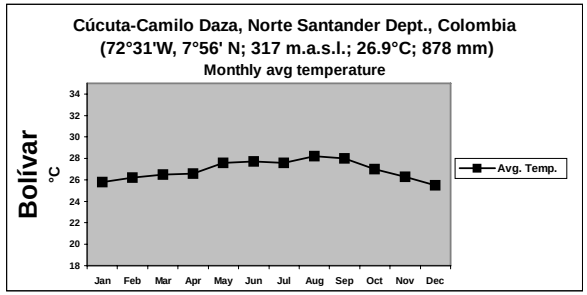


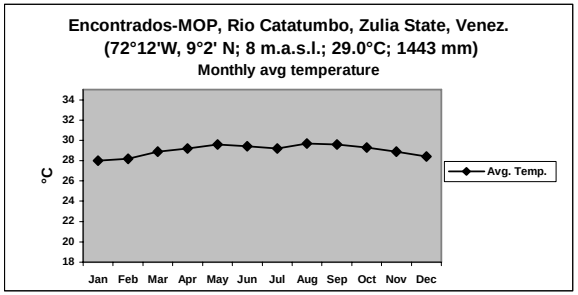
[1/] Climatic data source: FAO (2000).
[2/] Name and location of the meteorological station.
[3/] Co-ordinates, altitude, annual average temperature and annual total precipitation.
[4/] Avg Max.: average of monthly maximum temperatures; Avg Temp.: average of monthly temperatures; Avg Min.: average of monthly minimum temperatures

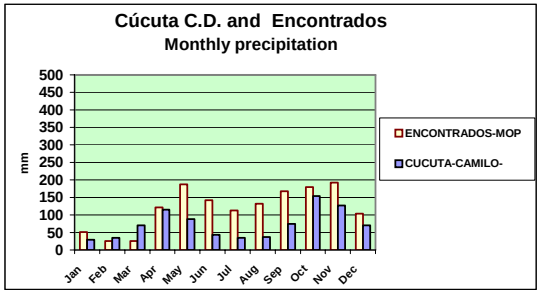
Figure 8.7 Monthly average temperature, monthly precipitation and other geo-climatic information of localities within or close to possible areas of differentiation of the genetic groups of *M. roreri*.

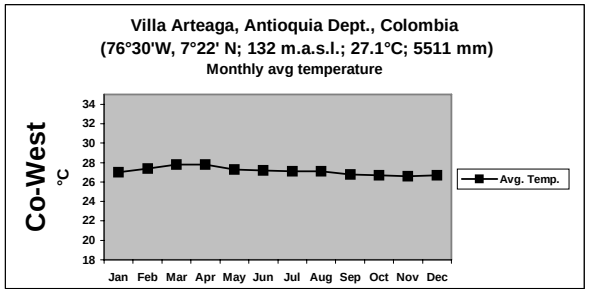
Continuation Figure 8.7

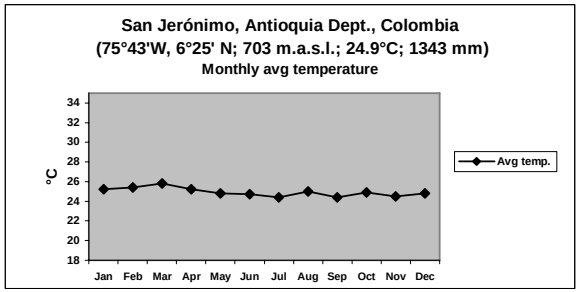
TEMPERATURE **PRECIPITATION**

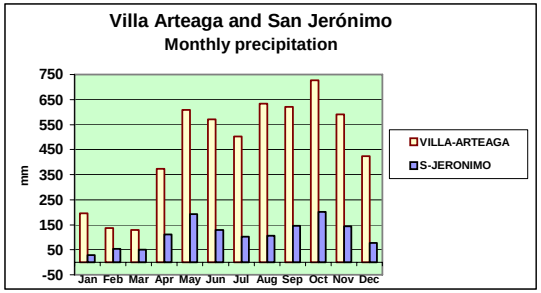


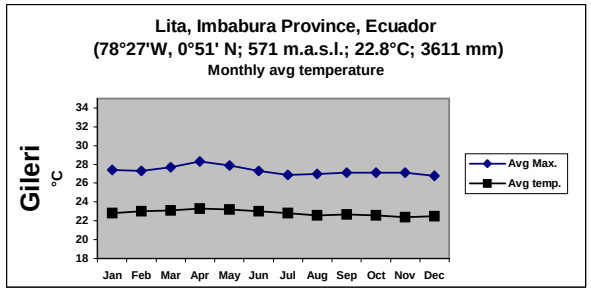


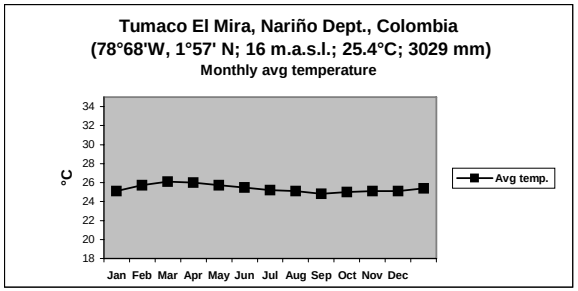


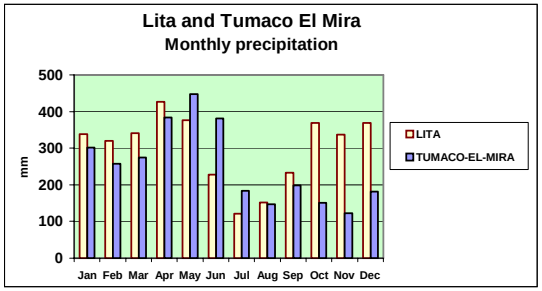












8.5 DISCUSSION

This study clearly showed that *M. roreri* has a significant level of morpho-physiological variation. This is the first study of this type that comprises isolates representing the entire dispersal range of *M. roreri*. The few previous investigations have only involved a limited number of genotypes from a restricted geographic range (Ram, 1989; Herrera *et al.*, 1989). In this sense, it has been pointed out that an adequate representation of the isolates is fundamental to determine the extent to which a fungal species varies in nature (Carlile & Watkinson, 1994).

A considerable level of variation was obtained among isolates for all evaluated variables, which comprised 11 parameters related with the growth and sporulation of *M. roreri*. Differences and congruencies were observed in relation with previous studies. For instance, levels of production of spores observed here were significantly lower than those reported previously by Herrera *et al.* (1989) and Ram (1989). Since these authors estimated the production and density of the spores using small disks of the colonies and in the present research the whole colony was used to determine these parameters, the most plausible explanation for the disparity lie in the different methodologies applied. The difference does nevertheless serve to highlight the difficulty in making direct comparisons between studies of sporulation or growth characteristics because of the strong influence of environment and scoring methodology, as well as genetics in determining the recorded phenotype (Hawker, 1950). Comparisons are easier to make between quantitative characters that relate directly to anatomical structure. For instance, although spore size registered a considerable variation among isolates, the averaged values for the size of globose and ellipsoid spores were within the ranges reported previously by Ciferri & Parodi (1933) and Evans *et al*. (1978) for isolates from Ecuador and by Castaño (1954) for isolates from Colombia.

The considerable variation in spore shapes observed in this study agrees with previous reports from Ecuador (Ciferri & Parodi, 1933; Evans *et al*., 1978), Colombia (Castaño, 1952) and Venezuela (Chacín, 1975). These works described the spores of *M. roreri* as being highly diverse in shape, with the types that can be classified as globose/subglobose and ellipsoid predominating. A similar result was found in the present study where

globose spores were predominant in most isolates but they never comprised 100% of the spores. Differences were consequently found in the percentage of shapes of spores between this research and previous studies (Chacín, 1975; Ram, 1989). Caution should again be exercised, however, in making direct inferences between such studies because there are external factors influencing this variable. Evans *et al*. (1978) for example, noted that variation in the shapes of spores can be influenced by their level of maturity and López (1954) suggested that spore age and the position that they occupy in the chain can affect their shape.

Interestingly, rings and spores were observed in many isolates not only under alternate periods of light/darkness but also under complete darkness. The biological importance of rings is that whenever a fungus is growing in a substrate that is large with relation to the size of the colony, 'fairy ring' formation improves the reproductive success of the colony, yet at the same time allowing the mycelial extension for the acquisition of new substrate and nutrients (Jennings & Lysek, 1999). Since production of rings under darkness is not very frequent in the Basidiomycetes (Jennings & Lysek, 1999), results shown here suggest that *M. roreri* has a particular ability to grow and sporulate under variable conditions of light, which may be an adaptation to the variable conditions of light found in the lower story of the rain forest. However, it should be remembered that the relationship between light and reproduction in fungi are generally extremely complex, and may differ between closely related species or even between different isolates of the same species (Moore-Landecker, 1996).

When the isolates were analysed in accordance to the country of origin, the isolates from Peru (Bolívar group) were the most vigorous in terms of growth, sporulation and size of spores. These isolates also registered the highest and the lowest percentages of globose and ellipsoid spores, respectively. Venezuelan isolates (Bolívar group) similarly showed high values for radial growth and spore production but spores were smaller than in Peru. The lowest values for growth and sporulation and size of spores were obtained for the Central American isolates, and in particular for the Nicaraguan/Honduran isolates, all of which belong to the same genetic group (Co-West).

The reduction in the radial growth observed from Panama northwards when isolates of four Central American countries were evaluated during 20 d in Petri dishes may have be originated in the progressive natural selection of isolates with a reduced metabolic rate due to the favourable environmental conditions prevailing in this region. Also, the phenomenon could be explained as a non-intentional selection of slow-growing isolates mediated by human beings when moving pods with hidden infections, because those isolates that grow slower have less opportunity to be detected prior to transport and the pods discarded. In this sense, the long period of pod colonisation prior to the manifestation of visible symptoms demonstrates how an apparently healthy, systematically infected pod can be selected and transported for use as a source of planting material or, more dangerously, for trade or demonstration purposes to friends or family where no selection operates (Evans, 1986). That said, cognisance must be taken of the relative invariability of the Central American isolates as this provides only limited opportunity for selection. In this context, the accumulation of mutations (leading to slower growth) or sexual recombination may provide a more attractive explanation for the apparent cline.

The morpho-physiological similarities observed in the present study between the Peruvian isolates and the isolates from the Province of Napo in Ecuador were manifest not only in terms of growth and sporulation but also in the percentage of the globose and ellipsoid spores and in the spore size. These isolates were also molecularly similar as determined in Chapter 4 and belong to the same genetic group (Bolívar). These findings support the hypothesis of Hernández *et al*. (1990) and Evans *et al*. (1998) that isolates from the Province of Napo spread into Peru.

The analysis of the morpho-physiological parameters according to the genetic groups of the isolates apparently supports the assertion that the variation observed in the characters studied has a genetic basis. On average, Co-Central grew faster and showed the highest ring intensity although Bolívar was the most vigorous group in terms of production, density and size of spores. This group also registered a similar ring intensity than Co-Central and produced the highest percentage of globose spores. The Co-East group showed a high production and density of spores and its mycelial growth was intermediate. Gileri and particularly Co-West groups registered the lowest radial growth, the minimum production of spores and the smallest spores.

It is tempting to speculate that the adaptation of *M. roreri* to new habitats characterised by striking differences in environmental conditions, particularly in altitude, temperature and precipitation could explain not only the divergence of the genetic groups but also the acquisition of some of their distinctive morpho-physiological traits. At this point, it is relevant to emphasise that, since *M. roreri* is a highly sophisticated, obligate parasite (Evans, 1986) naturally adapted to only invade actively-growing pods of *Theobroma* and *Herrania*, the environmental conditions have a dual effect on the fungus, by affecting it directly or by their influence on the host.

The isolates of Co-Central typically grew faster at 24 °C than other groups as observed in this research, and are apparently fostered in its growth and sporulation at 30 °C but performed poorly at 18 °C (Chapter 7). As was postulated in Chapter 7, this may be due to an adaptive modification of the group to readily grow when temperatures become more favourable for the production and liberation of the spores, phenomena usually occurring at temperatures higher than 26 ºC (Porras & González, 1982; Schmitz, 1985). This adaptation is probably essential for the survival of *M. roreri* at high altitudes where the lower temperatures can limit the activity of both the fungus and the host (see as reference Figure 8.7). In this sense, the minimum annual mean temperature for cacao have been established in 21 °C at sea level, however, within mountainous countries near to the Equator, such as Colombia, non favourable temperatures can prevail at an altitude of about 1300 metres (Hardy, 1960). In Central Colombia where Co-Central probably originated, cacao frequently grows at altitudes higher than 1100 m.a.s.l. under temperatures near to the minimum established for cacao although under adequate regimens of precipitation.

On the other hand, the distinctive characters of Bolívar group quite possibly arose when *M. roreri* dispersed into the north-eastern flanks of the Cordillera Oriental of Colombia colonising habitats characterised by low altitudes, high temperatures, moderate to low precipitation and a marked drought period of 3-4 consecutive months (<100 mm precipitation month^{-1}) (Figure 8.7). These conditions have important consequences on both the host and the pathogen. During the drought period the growth and pod production of cacao may be arrested (Wood & Lass, 1985) and, although high temperatures and low humidity during this periods are favourable for spore liberation of *M. roreri* (Porras &

González, 1982; Schmitz, 1985), they are not propitious for the germination and penetration because these processes are highly dependant on the availability of free water on the pod and favoured by temperatures in the range of 22-24 ºC (López, 1954; Merchán, 1981; Campuzano, 1981, Evans, 1981).

In the present study carried out at 24 °C, Bolívar isolates were the most vigorous group in terms of spore production and spore size. However, as was observed in Chapter 7, the growth and sporulation of this group were apparently depressed and the diameter of the globose and the width of the ellipsoid spores apparently increased at the extreme temperatures studied (18 °C and 30 °C) when compared with performance at 24 °C. These results is indicative that Bolívar isolates may be adapted to sporulate intensely in moderate temperatures and to reduce their growth and sporulation when temperatures become extreme. This behaviour could permit the fungus to survive in this environment by reducing the metabolic activity during the dry and hot periods, and by increasing the activity when conditions are more suitable for the germination and penetration of the spores.

M. roreri survives as spores in infected pods between the wet and dry seasons (Ampuero, 1967). Since fungal spores are readily killed by sun, drought and excessive cold (Wellman, 1972), the larger spore size observed in Bolívar isolates and their capability to increase the spore size at 30 °C as observed in Chapter 7, possibly permit these spores to prolong survival under the adverse conditions commonly encountered in the area where this group differentiated, in particular during the drought periods. In this respect, it has been pointed out for fungi in general, that resistance of the fungal propagules to extreme temperatures depends on their water content (Jennings & Lysek, 1999) and that small thin-walled spores may be liable to die through desiccation under dry conditions, whereas, larger thick-walled spores may be nearly immune to desiccation (Carlile & Watkinson, 1994).

An increase of spore size was also observed in Chapter 7 when isolates grew at 18 °C in comparison to the apparently optimal temperature of 24 °C. Since an enlargement of the spore wall of *M. roreri* has been observed in spores 40-50 day-old and is apparently related to the formation of resistant structures (Campuzano, 1981), it is possible that the

enlargement capability of the spores in *M. roreri* is a survival mechanism activated under diverse stress conditions. This dormancy mechanism however, could delay the germination of the spores as suggested by Evans (1981) for older thick-walled spores.

In the case of the Bolívar isolates, the relatively extreme environmental conditions found in the area occupied by the group probably exerted selective pressure for genotypes with permanently larger spores. However, this characteristic does not consistently confer higher fitness to the isolates of this group, because aerodynamic constraints restrict dispersal capacity (Meerts, 1999) and may delay spore germination. Thus, this adaptation, as with others, probably imposes both a cost and benefit to an organism depending on the environmental conditions in which the fungus finds itself (Cook & Callow, 1999).

Co-West and Gileri isolates most probably differentiated in Western Colombia and north-western Ecuador, respectively. The predominating conditions in these regions are low altitudes, high temperatures, very high precipitation and the marked absence of a dry season (Figure 8.7). In this environment, where conditions are very favourable for both the plant and the fungus (Wood & Lass, 1985), selection possibly favours isolates with the capability to growth and sporulate less intensely perhaps as a mechanism of metabolic economy. These two groups also possessed the smallest spores, which likely improves their releasing and dispersal since smaller spores become drier sooner and are more easily transported by the wind.

Interestingly, isolate Co8 which belongs to Co-West but was collected in San Jerónimo, Antíoquia, Colombia under atypical environmental conditions for the group such as a relatively high altitude (703 m.a.s.l.), low annual precipitation (1343 mm) and three or four consecutive drought months (Figure 8.7), showed a very fast colony growth and large spore sizes which contrasts with the general characteristics observed among Co-West isolates. These traits are apparently more suited to periodically unfavourable conditions as explained above for Co-Central (colony growth) and Bolívar (spore size).

The positive and highly significant correlation found between altitude and both radial growth and spore production apparently supports the hypothesis that, in the process of adaptation to new environments, *M. roreri* has undergone morpho-physiological

modifications to adapt to the changes in climate associated with increasing altitude. Apparently, rapid growth and sporulation tends to increase at high altitudes and to decrease at low altitudes. Since there is a strong relationship between altitude and temperature, it is probable that temperature and its influence on other climatic parameters has been the determinant factor in the occurrence of this phenomenon. However, the absence of correlation between the morpho-physiological variables and the mean annual temperature indicates that this measurement does not give a good estimation of the effect of temperature. Perhaps, other related parameters such as the minimum and maximum temperatures could better explain this effect. Regrettably, it was not possible to achieve these comparisons because only basic climatic information was available for most of the meteorological stations of interest in Colombia.

The negative correlation between the annual precipitation and the size of the spores supports the fact mentioned above that *M. roreri* has also suffered adaptations in the spore sizes to survive in environments with different precipitation regimes. Thus, bigger spore sizes are apparently better fitted for the survival of the fungus in very dry conditions and small spores in wet environments.

The cluster analysis provided evidence of a tendency for isolates belonging to the same genetic groups to co-cluster, although there were notable exceptions. Two main reasons could explain the presence of isolates from one group in different clusters: the significant range of variation observed for all variables as is expected for continuous variables, and adaptive changes suffered by some isolates when exposed to differential environmental conditions for a significant period of time. The importance of phenotypic plasticity also warrants future attention.

The five variables used in the Ward's cluster analysis (radial growth, days to sporulation, ring intensity, spore production and globose spore diameter) separated most isolates with a western Colombian origin (Co-West and Gileri groups) from those with an eastern Colombian origin (Co-Central, Co-East and Bolívar groups). Similar distinctions were noted when the isolates were assessed for genetic variability using AFLP/ISSR and ITS data sets (Chapter 4). Nevertheless, morpho-physiological diversity in *M. roreri* has a relatively low level of correlation (Mantel test, r = 0.4) with the molecular diversity. This

phenomenon could be consequence of the fact that small molecular changes are not easily detected using the traditional molecular tools and yet could produce disproportionately large phenotypic changes. Moncalvo *et al*. (2000) argued that there are several examples in which rates of molecular and morphological evolution are uncoupled in fungi, most probably because morphological evolution of certain traits is under stronger natural selection than is the genome as a whole, as revealed by general measures of molecular evolution.

It is concluded after analysing samples from the entire dispersal range of *M. roreri,* that this fungus exhibits a considerable level of morpho-physiological variation. Significant differences were observed for most variables when isolates were considered collectively or analysed in accordance to their genetic group or country of origin. There was some indication of possible morpho-physiological adaptations in terms of growth, sporulation intensity and spore size among the genetic groups. Such adaptations may have arisen when the fungus spread from its hypothetical centre of origin in north-eastern Colombia and moved into new areas with different climatic conditions, most notably contrasting profiles of altitude, temperature and humidity.

Finally, it is important to take in account that, although suitable models and indispensable in this kind of research, experiments on Petri dishes are artificial systems and may not necessarily resemble the performance of fungal mycelia in their natural environments (Jennings & Lysek 1999). It follows that, new studies under natural conditions should be performed at a later date to confirm the findings described here .

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8.7 APPENDIX

Table A8.1 Analyses of variance for 11 morpho-physiological variables evaluated on 88 isolates of *M. roreri*.

		Radial Growth			Days to sporulation			Ring intensity			Spore production	
Source	**DF[1/]**	**Mean Square**	**Signif.[2/]**	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**
Block	3	0.28	*	3	1.223	n.s.	3	1.296	**	3	13,673	*
Isolate	87	3.481	**	87	9.424	**	87	0.596	**	87	66,378	**
Error	245	0.087		245	1.289		245	0.092		237	4,854	

		Spore density			% germinated spores			% globose spores			% ellipsoid spores	
Source	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**
Block	3	13.3	*	3	1.099	n.s.	3	0.048	n.s.	3	482.113	**
Isolate	87	32.425	**	87	55.721	**	87	0.171	**	87	494.9	**
Error	237	4,853.62		96	0.006		234	0.021		234	0.022	

		Globose spore diameter			Ellipsoid spore width			Ellipsoid spore length	
Source	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**
Block	3	1.08	*	3	0.567	n.s.	3	5.697	*
Isolate	87	1.728	**	87	1.794	**	87	3.148	*
Error	234	0.31		220	0.645		220	1.809	

[1/] DF = degrees of freedom

[2/] Sign. = Statistical significance: ** = highly significant (P<0.01), * = significant (P<0.05), n.s. = non significant.

Table A8.2 Analyses of variance by country for 11 morpho-physiological variables evaluated on 88 isolates of *M. roreri*.

		Radial Growth			Days to sporulation			Ring intensity			Spore production	
Source	**DF[1/]**	**Mean Square**	**Signif.[2/]**	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**
Country	6	21.77	**	6	20.32	**	6	0.86	**	6	228747.42	**
Error	329	0.59		329	3.09		329	0.22		321	17453.87	

		Spore density			% germinated spores			% globose spores			% ellipsoid spores	
Source	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**
Country	6	48.8	**	6	0.03	n.s.	6	0.51	**	6	0.36	**
Error	321	3605.94		180	0.17		318	0.05		318	0.03	

		Globose spore diameter			Ellipsoid spore width			Ellipsoid spore length	
Source	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**
Country	6	5.98	**	6	5.61	**	6	7.36	**
Error	318	0.60		304	0.88		304	1.95	

[1/] DF = degrees of freedom

[2/] Sign. = Statistical significance: ** = highly significant (P<0.01), * = significant (P<0.05), n.s. = non significant.

Table A8.3 Analyses of variance by genetic group for 11 morpho-physiological variables evaluated on 88 isolates of *M. roreri*.

		Radial Growth			Days to sporulation			Ring intensity			Spore production	
Source	**DF[1/]**	**Mean Square**	**Signif.[2/]**	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**
Group	4	28.73	**	4	10.46	*	4	2.00	**	4	410164.22	**
Error	331	0.64		331	3.32		331	0.21		323	16515.54	

		Spore density			% germinated spores			% globose spores			% ellipsoid spores	
Source	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**
Group	4	117.30	**	4	0.14	**	4	0.67	**	4	0.49	**
Error	323	10.62		182	0.01		320	0.05		320	0.04	

		Globose spore diameter			Ellipsoid spore width			Ellipsoid spore length	
Source	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**	**DF**	**Mean Square**	**Signif.**
Group	4	7.36	**	4	4.61	**	4	7.95	*
Error	320	0.62		306	0.88		306	0.88	

[1/] DF = degrees of freedom

[2/] Sign. = Statistical significance: ** = highly significant (P<0.01), * = significant (P<0.05), n.s. = non significant.

Table A8.4 Matrix of correlation between geo-climatic parameters and 10 morpho-physiological variables of 88 isolates of *M. roreri*.

Variable	Altitude	Annual precip.	Avg Annual temp.	Radial growth	Days to sporulation	Ring intensity	Spore production	Spore density	% germinated spores	% globose spores	Globose spore diameter	Ellipsoid spore width	Ellipsoid spore length
Altitude	**1**	0.07 n.s	**-0.79**[1/]**	**0.37****	-0.26*	0.19 n.s	**0.29****	0.12 n.s	-0.06 n.s	0.04 n.s	0.12 n.s	0.18 n.s	0.03 n.s
Annual precip.		**1**	-0.05 n.s	-0.21 n.s	-0.13 n.s	-0.15 n.s	0.01 n.s	0.01 n.s	0.02 n.s	-0.08 n.s	-0.23*	-0.22*	-0.25*
Avg annual temp			**1**	-0.13 n.s	0.16 n.s	-0.08 n.s	-0.19 n.s	-0.13 n.s	-0.01 n.s	0.08 n.s	0.10 n.s	0.09 n.s	0.15 n.s
Radial growth				**1**	**-0.30****	**0.32****	**0.33****	-0.14 n.s.	-0.06 n.s.	0.17 n.s.	**0.28 ****	0.20 n.s.	0.09 n.s.
Days to sporulation					**1**	-0.21 n.s.	**-0.39****	-0.11 n.s.	0.20 n.s.	-0.05 n.s.	0.01 n.s.	-0.16 n.s.	-0.11 n.s.
Ring intensity						**1**	-0.03 n.s.	-0.16 n.s.	**0.24***	0.01 n.s.	0.01 n.s.	0.12 n.s.	0.20 n.s.
Spore production							**1**	**0.81****	**-0.34****	**0.36****	**0.24***	**0.27***	-0.02 n.s.
Spore density								**1**	**-0.28****	**0.25***	0.18 n.s.	0.16 n.s.	-0.01 n.s.
% germinated spores									**1**	**-0.26***	-0.15 n.s.	-0.15 n.s.	-0.04 n.s.
% globose spores										**1**	**0.55****	**0.43****	**0.25***
Globose spore diameter											**1**	**0.70****	**0.50****
Ellipsoid spore width												**1**	**0.51****
Ellipsoid spore length													**1**

[1/] **Significance: n.s.= non significant, *=significant (P<0.05), **=highly significant (P<0.01).**

CHAPTER 9

GENERAL DISCUSSION

Moniliasis is a largely unresolved problem in tropical America where the disease is one of the most important factors limiting cacao cultivation in many areas. Historical evidence presented in Chapter 3 clearly demonstrates the devastating effects caused by *M. roreri* throughout its range over almost 200 years. Moreover, the recent appearance and the aggressiveness showed by this pathogen in the two latitudinal extremes of its range (Honduras and Peru) indicates that this pathogen is in an intense invasive phase and leads to the reasonable expectation of further spread in the near future. Considered collectively, the aggressiveness of this airborne fungus, its outstanding capacity to survive different environmental conditions, the apparent susceptibility of most comercial cacao genotypes, the rapid rate of expansion of its range and its propensity for man-mediated dispersal all mean that moniliasis presents a substantial threat for cacao cultivation not only in surrounding regions but also on a world-wide scale.

In spite of the economic importance of *M. roreri*, several aspects of its biology including its origin, genetic diversity, biogeography and systematic position have remained unresolved for many years. These aspects were addressed in the present research using a multidisciplinary approach comprising of molecular, morpho-physiological and phytopathological studies. The application of these strategies to a very broad selection of isolates representing the entire geographic range of the fungus permitted the study to reach generic conclusions on the global status of the species as suggested by Carlile & Watkinson (1994). This breadth has led to some surprising findings that are outlined below.

9.1 THE ORIGIN OF *M. RORERI*

Prior to this study, there was a general belief that the centres of origin and diversity of the fungus is located in Ecuador (Rorer, 1918; Briton-Jones, 1934; Erneholm, 1948; Barros, 1977; Delgado & Suárez, 1993). However, molecular data presented here (AFLP and

ISSR) provides strong evidence that the geographic region containing the greatest genetic diversity (centre of diversity) resides in north-eastern Colombia, probably within or close to the Middle Magdalena area (see Chapter 4). Importantly, there are good grounds for reasoning that this area also constitutes the centre of origin of the species. For instance, there was a clear decline in diversity with distance from this region (Chapter 4). Support is also provided by the presence of two ITS haplotypes in the Middle Magdalena area that apparently divide *M. roreri* isolates in two subspecific groupings ("Western" and "Eastern") (Chapter 5). Intriguingly, the closer genetic relationship revealed by ITS between the "Eastern group" and *C. perniciosa* (Chapter 5) suggests that isolates containing this eastern haplotype may be closer to the original progenitor of both *M. roreri* and *C. perniciosa*. Again, this suggests a centre of origin that lies within Colombia. Likewise, the presence of seemingly ancient populations of wild species of *Theobroma* and *Herrania* in the Middle Magdalena area (Chapter 3), and the existence of historical references accounting for a apparent early appearance of the disease in north-eastern Colombia (Chapter 3) also point towards this region containing the centre of origin for the fungus.

The scarcity of cacao clones exhibiting resistance to *M. roreri,* coupled with the propensity of R- gene resistance to show race-specificity means that it is important for breeders to have access to appropriate and diverse sources of isolates for resistance screening. Evidence presented here shows that the Middle Magdalena area of Colombia contains the widest diversity of isolates of the disease and so should be targeted as a region of high priority for future collection expeditions for the disease and also for resistant wild cacao trees to identify new sources of resistance.

Information on the origin of *M. roreri* could be also useful to target the search for antagonist agents for biocontrol. In fact, the selection of mycoparasites for biocontrol of *M. roreri* in its centre of origin is a strategy currently in place in western Ecuador (Evans, 2002a).

9.2 EARLY DISPERSAL OF *M. RORERI*

The spread of *M. roreri* from its centre of origin possibly occurred over a long period of time, hypothetically starting with an ancient natural spread of the pathogen between *Theobroma* and *Herrania* wild populations possibly aided by the intervention of human activity for invasion of new areas. This spread occurred despite the presence of substantial physical barriers in Colombia but also in the remaining northern Andean countries.

After this phase, *M. roreri* probably remained in the wild in different regions of Colombia, Ecuador and perhaps in Venezuela until more recent historical times when, as mentioned by Evans (2002b), the fungus rose to prominence when countries in northern South America began to cultivate indigenous forest tree species on a large scale. This activity permitted the previously unknown fungal pathogen to emerge from the obscurity of their forest habitats to wreak havoc in these new monocultures.

Other outbreaks have been registered more recently in Central America and Peru. These epidemics are almost certainly the product of the anthropologically mediated invasion of the pathogen from infected cacao areas into cultivated stands of the crop in countries previously free of the disease.

9.3 ADAPTATION OF *M. RORERI*

Molecular analyses revealed a considerable level of genetic variation in *M. roreri*. However, genetic uniformity was also observed over extensive areas such as Central America, Central Ecuador and Peru, where apparently only one or very few introductions have occurred and where the pathogen has probably been predominantly clonally dispersed. In this sense, Kohn (1994) and Anderson & Kohn (1995) pointed out that the repeated recovery of the same multilocus genotype for a series of loci known to be polymorphic, especially over long distances, is a clear evidence of clonality. This was certainly the case in the present study, where identical AFLP and ISSR profiles were noted from *M. roreri* isolates collected from several locations covering considerable areas of Central America.

A considerable level of morpho-physiological variation was also detected for this fungus, even within the comparatively uniform genetic populations of Central America and Central Ecuador. The low correlation observed between the morpho-physiological and the genotypes revealed by molecular analyses when isolates were collectively analysed, agrees with several observations in other fungi, in which rates of molecular and morphological evolution have been uncoupled (Moncalvo *et al*., 2000). It is possibly that small genetic changes produce morpho-physiological variants of the fungus that are not easily detected through changes to the nuclear and cytoplasmic genomes.

No morpho-physiological variation between isolates could be interpreted as providing possible adaptations of *M. roreri* to a specific host and so it may be that all strains of *M. roreri* are equally well suited to effectively attack *Theobroma* and *Herrania*.

9.4 THE SOURCE OF VARIABILITY IN *M. RORERI*

The identification of significant levels of molecular and morpho-physiological variation is particularly interesting for a fungus that until recently had been identified as being the asexual state of an unknown Basidiomycete (Evans, 1986). Mutations could explain part of the observed variation in *M. roreri* since this mechanism is the ultimate source of genetic variability in fungi, occurring in 1% of the cells in each generation (Carlile & Watkinson, 1994). Certainly, point mutations between isolates of *M. roreri* were observed in this study when DNA fragments were sequenced and compared. Minor mutations also provide a plausible explanation for the frequent rare bands observed when isolates were analysed using ISSR and AFLP.

In Central America where apparently only a single founder event occurred and where the very few polymorphisms observed were unique bands, mutations are the cause of at least some of the limited genetic variation observed. Favourable conditions in this area for both the fungus and the host have likely favoured the predominance of clonality, although the phenomenon was also observed in other contrasting environments such as Central Ecuador and Peru. In fact, asexual reproduction predominates in the majority of plant pathogenic fungi (Milgroom, 1996) probably due to the fact that among this kind of fungi, the prevalence of efficient asexual reproduction affords the opportunity for invasive, epidemic,

clonal colonisation and spread (Kohn, 1994). Clonality is probably most extreme in monoculture, although it could be expected to be important in wild plants and in transfers of adaptive pathogenic genotypes between wild and cultivated plants (Kohn, 1994).

A meiotic recombination mechanism recently identified by Evans *et al*. (2002) could explain some of the variation observed between isolates of *M. roreri*. Evidence was collected by these authors on the occurrence of meiosis during the sporogenesis and the germination of the spores of *M. roreri* which results in the production of a four-celled, uninucleate "metabasidium", later developing sterigmatal-like side branches which may function as infective hyphae.

At one level, the genetic uniformity observed in some areas recently colonised by the pathogen such as Central America and Peru could be taken to suggest the complete absence of sexual recombination under certain conditions or that its effects are inconspicuous. On the other hand, the variability observed within both newly invaded regions requires careful consideration. For dominantly inherited marker systems such as AFLP and ISSR, both sexual and parasexual segregation could lead to the loss of some heterozygous loci (by fixation of the null allele). Thus, either mechanism could easily explain the apparent loss of bands from founder profiles. Mutations can lead to the appearance of novel bands by several mechanisms including the formation of a new restriction site (for AFLP), primer binding site (AFLP and ISSR) or the formation of an indel causing a change in locus size. Equally, mutations can also lead to loss of amplicons where restriction sites or primer binding sites are changed such that amplification is no longer possible. The appearance of novel bands is more difficult to explain by sexual or parasexual mechanisms and would require fresh invasion or some form of recombination with a second, undiscovered strain. The latter would lead to a sudden, simultaneous appearance of several novel bands coupled with loss of heterozygous bands in some recombinant clones. Segregation would then lead to further heterozygous band losses as the recombinant isolate spreads, with the novel bands exhibiting a high tendency for elimination (since all will be heterozygous). A different pattern would be expected when mutation be the chief cause of changes to the band profile. Here, novel bands are more likely to appear randomly and gradually accumulate as invasion progresses. These alternative possibilities can be most readily compared by reference to the Central American

isolates, where there are good historical records of the spread of the disease. Interestingly, a few isolates did contain a disproportionate number of novel bands, with isolate Pa8 possessing 8 and C19 with 6, respectively. Significantly, these isolates were collected in Panama and Costa Rica, respectively, rather than at the leading edge of the invasion front. This observation is suggestive that band loss and gain has occurred mostly by either repeated invasion or by sexual or parasexual recombination rather than by changes attributable to mutation.

There is more direct evidence of the possibility of sex provided by close examination of isolates E20 and E32 from the central and peripheral groupings of Ecuador. Both of these strains contained several AFLP and ISSR bands that were otherwise typically diagnostic of the other genetic grouping perhaps suggesting natural recombination may have occurred between the two groups. However, far more research is necessary in this field to demonstrate the presence and role of sexual or parasexual recombination in *M. roreri* evolution.

9.5 PATTERNS OF GENETIC VARIATION IN *M. RORERI*

Collectively, genetic distance analyses using AFLP and ISSR data sets suggested that *M. roreri* contains five major genetic infraspecific groupings (Chapter 4). These groups could also be distinguished on the basis of detectable differences in growth and reproductive capacity. It is possible that these groups have adaptive significance and that such variability provides the fungus with the flexibility to occupy subtly different endoenvironments and perhaps to adapt to hosts exhibiting different pod growth characteristics.

The genetic groups varied in their geographical distribution: the Bolívar and Co-West groups being widely spread, whereas the remaining aggregates being endemic to either Colombia (Co-Central and Co-East groups) or Ecuador (Gileri group). All genetic groups except Gileri were represented in Colombia, further supporting this country as the centre of diversity (and origin) for *M. roreri*. Moreover, it is plausible that further sampling would reveal that even the Gileri group is present in Colombia since isolates of this group were found close from the Colombian-Ecuadorian border. Three genetic groups were identified

in Ecuador (Co-West, Gileri and Bolívar). Reference to historical records suggests that each of these probably correspond to independent invasions. Conversely, only one group was found in Venezuela (Bolívar), Peru (Bolívar) and Central American (Co-West).

Within Colombia, the Co-East group contained the greatest genetic diversity. As is mentioned above, this is consistent with the centre of diversity and possibly the centre of origin of the disease being located within or close to the area holding these isolates. The Co-Central assemblage also showed a significant degree of molecular variation, and was appears to be genetically and morpho-physiologically affiliated to the Co-East group. It might be argued, therefore, that these isolates merely represent a subset of Co-East that have possibly become adapted to the environmental conditions found in Central Colombia. These two groups occupy areas that are adjacent although located at different altitudes: one in the Middle Magdalena area (Co-East) and the other on the Central Andean Cordillera (Co-Central).

The Gileri and Co-West groups occupy adjacent areas with similar environmental conditions and the morpho-physiology of these groups was found to be very similar. They also showed an analogous ITS pattern and some similarities in the AFLP/ISSR bands. It seems likely then that Gileri and Co-West originated from a relatively recent common ancestor. Whilst the sequence data generated in this study is insufficiently robust to assign one as the more ancestral group, it appears most probable that the common ancestor invaded from north-east Colombia into western Colombia and afterwards spread southwards into north-western Ecuador. However, it is important to consider that representatives of the Gileri group were collected in the wild whereas those of Co-West were obtained from cultivation and that the Gileri group shared more alleles in common with eastern Colombia than does Co-West. This factor may indicate that the Gileri group is the most ancestral. It remains possible though, that more extensive collections from wild host populations in the Co-West region may unveil an ancestral stock from which the cultivated isolates are derived.

Although Bolívar and Co-West showed clear molecular and morphological differences, they also registered some affinities including a strong divergence from the Co-East and Co-Central groupings. This divergence probably arose when isolates from the hypothetical

place of origin in Eastern Colombia dispersed eastwards and westwards into areas separated by huge barriers, the Eastern Andean Cordillera in the case of Bolívar and the Central and Western Andean Cordilleras in the case of Co-West. It is possible that both dispersal events were historically connected, which could explain the similarities found between Bolívar and Co-West.

9.6 EVOLUTION OF THE INFRASPECIFIC GROUPINGS

It is tempting to speculate that the adaptation of *M. roreri* to new habitats characterised by striking differences in environmental conditions, particularly in altitude, temperature and precipitation following a period of isolation, could explain not only the evolution of the genetic groups but also the acquisition of some of their distinctive morpho-physiological traits. In this respect, Cuatrecasas (1958) has highlighted the particularly propitious climatic and topographical conditions existing in Colombia for the morphological diversification and evolution of the species.

Under the premise that genetic groups arose within or close to the areas where the isolates were collected in Colombia, and that current environments resembled the original environments, it is possible to hypothesise that the following contrasting conditions influenced the evolution of the genetic groups: Co-East, close to the hypothetical ancestral group, probably evolved in the Middle Magdalena area at intermediate altitudes (approximately 500-800 m.a.s.l.) and under favourable environmental conditions in terms of temperature and precipitation. From there, the fungus spread to the area now occupied by Co-Central group and this would have required the ability to withstand lower temperatures. Movement of *M. roreri* into the north-eastern flanks of the Cordillera Oriental of Colombia needed the ability to survive in conditions with limited precipitation. Interestingly, there are distinctive characters of Bolívar group that are suggestive of adaptation for low precipitation (e.g. larger spores). This area is characterised by low altitudes, high temperatures, moderate to low precipitation and a marked drought period of 3-4 consecutive months (<100 mm precipitation $month^{-1}$). Finally, Co-West and Gileri groups possibly differentiated, respectively, in western Colombia and north-western Ecuador, where the predominating conditions are very favourable for the pathogen and the hosts: low to moderate altitudes, relatively high temperatures, high precipitation and the

absence of a marked dry season. It is therefore questionable whether these groups were subjected to any identifiable strong selective pressure.

Perhaps the most significant divergence within the species arose when *M. roreri* dispersed from the hypothetical place of origin in the Middle Magdalena area at over 460 m.a.s.l. into the lowlands of western Colombia, the wettest zone of the country where annual precipitation and average temperature in certain areas can be as high as 5.000 mm y^{-1} and 26°C, respectively. This thesis is supported by the findings of the AFLP/ISSR and ITS analyses that a strong separation exists between genetic groups originated in Eastern Colombia (Co-East, Co-Central and Bolívar) and those originated in Western Colombia/north-western Ecuador (Co-West and Gileri, respectively).

9.7 RECENT AND FUTURE MOVEMENTS OF *M. RORERI*

The historical review presented in Chapter 3 clearly establishes that modern dispersal of *M. roreri* occurred throughout Latin America and was probably mediated by the active participation of human beings. In some cases, molecular evidence presented here corroborated previous reports indicating or suggesting this participation, and in others, this is the only explanation of the presence of isolates of a specific genetic group in areas far away of the centre of differentiation of that group. Perhaps, the most illustrative case refer to Bolívar group, which is present in the two extreme sides of north South America, eastwards Colombia and Venezuela and westwards in Ecuador and Peru. It is difficult to conceive how this distribution may have arisen other than through human-assisted dispersal.

Human beings are considered as one of the most effective dispersal agents of moniliasis because the fungus is admirably suited to this indirect method of dissemination. It follows that major efforts to avoid disease dispersal into new areas and countries should emphasise the education of the population over the risks of moving pods or other cacao materials (even apparently healthy) from infected to areas that are free of the disease. This approach is nevertheless subject to weaknesses. For example, in the face of the appearance of the disease into Costa Rica in 1978, the government ran an intensive and costly campaign to prevent further dispersal of the disease within the country. In spite of these efforts, the

fungus moved (with the probable human intervention) from the Atlantic to the Pacific coastlines (approximately 130 km east to westwards) and from the Panamanian to the Nicaraguan border (approximately 300 km south to northwards) within only two years. This experience emphasises that there are possibly other social and cultural factors that need to be considered when establishing effective educational and quarantine measures.

The possibility of the coexistence of two or more genetic groups within one area warrants consideration as it may have important biological consequences and will impact on the disease control strategies applied. Brasier (1995) argued that through geographical transposition, previously allopatrically (i.e. geographically) isolated fungal species may suddenly be brought into contact and sometimes into direct competition. In these circumstances there may be an added potential for evolutionary development through recombination between immigrant and resident taxa. Given the fact that *M. roreri* possess the capacity for sexual recombination, the potential for epidemiological change of the disease in the event of co-existence is thus made more severe. For this reason, it is probably prudent now to establish quarantine barriers between areas infected with different major groups of *M. roreri*.

9.8 PHYSIOLOGICAL EVIDENCE OF ADAPTATION

Results from the morpho-physiological study suggest that *M. roreri* can exhibit a significant degree of phenotypic plasticity. Temporary and permanent (genetic) morpho-physiological modifications were observed in this study and have been proposed as possible adaptations of the fungus to specific environmental conditions. For instance, when the fungus was grown at 18 ºC, all isolates increased the size of the spores, probably as a mechanism of survival under this sub-optimal temperature (Chapter 7). A similar phenomenon has been reported in old spores of *M. roreri*, and this related with the formation of resistant structures through the enlargement of the spore walls (Campuzano, 1981). This recurrent mechanism could explain the capability of *M. roreri* to survive in the field during periods of adverse conditions or between crop cycles since larger thick-walled spores not only protect them against adverse temperatures but also, against water stress (Carlile & Watkinson, 1994).

Other morpho-physiological observations revealed differences between genetic groups of *M. roreri* that may have adaptive significance. For instance, Co-Central isolates grew faster than all other groups at 24°C and were fostered in its growth and sporulation at 30°C, a sub-optimal temperature for most isolates. Since these isolates were collected in Central Colombia at altitudes higher than 800 m.a.s.l. where low temperatures are possibly the most limiting climatic factor, this adaptation may be interpreted as a genetic mechanism to readily produce and liberate the spores when conditions are propitious, which possibly occur in this area even at high temperatures. Conversely, Co-West and Gileri isolates developed the capability to growth and sporulate less intensely than other groups perhaps as a mechanism of metabolic economy under the favourable conditions in the areas where they originated. Apparently, in environments where humidity is not a limiting factor, growth and sporulation of *M. roreri* tends to increase at high altitudes and to decrease at low altitudes.

There is also some evidence that variation in spore size may have adaptive importance in *M. roreri*. Moreover, it is noteworthy that isolates possessing larger spores (as observed in Bolívar group) occur primarily in areas with significant dry periods. It is certainly plausible that large spore size restricts desiccation during dry periods and so aids survival in these regions. In contrast, isolates with small spores (as observed in Co-West and Gileri groups) occur in wetter environments, where desiccation is possibly less of an issue but where a small spore could improve dispersal by the wind.

The significant plasticity observed for *M. roreri* helps to explain reports mentioned the ability of the fungus to thrive under a wide range of environmental conditions, from sea level to over 1000 m and from very dry (irrigated) to very humid zones (Evans, 1981). This result also suggests that *M. roreri* could be capable of surviving in areas beyond its current geographic range and is sufficiently robust to accommodate for climatic change.

In the present research, samples of *M. roreri* were collected in very diverse environments. This has permitted a more precise delimitation of the ecological range of the fungus. *M. roreri* was collected from 0 to 1520 m.a.s.l. from places where annual precipitation is in the range 780- 5500 mm and mean annual temperature falls between 18.6 °C and 28 °C.

The disease currently extends from the Honduras/Guatemala border in the North (approximately 15° 22` N) to southern Peru in the South (approximately 13° 00` S).

9.9 VIRULENCE OF REPRESENTATIVE ISOLATES OF *M. RORERI*

The ability of *M. roreri* to infect its hosts over such diverse environments may be partly attributed to the generalised high virulence of many of its isolates as determined in Chapter 6. When seven isolates of *M. roreri* representing four of the five genetic groups were inoculated onto several divergent cacao clones, the genetic variation encompassed by the isolates was not matched by similar diversity in the extent of their virulence. Indeed, there was apparently only limited variation in virulence between isolates, with all isolates showing high levels of virulence against most cacao genotypes and only two isolates evoking slightly reduced virulence. It follows that, under appropriate conditions, all isolates are capable of inflicting similarly high levels of damage to cacao pods. The identification of one cultivated cacao clone (ICS-95) that exhibited a significant resistant reaction against all *M. roreri* isolates is highly relevant because it opens the enticing possibility that it may be possible to identify cacao genotypes that contain a durable resistance against the disease.

9.10 AFFINITIES BETWEEN *M. RORERI* AND THE BASIDIOMYCETES

Molecular evidence presented in Chapter 5 strongly supports the hypothesis that *M. roreri* is a Basidiomycete that belongs to the order Agaricales and possibly to the family Tricholomataceae. DNA sequence analysis suggests that *M. roreri* is closely related to *C. perniciosa* and is consistent with both species sharing a recent common ancestor. This information could benefit efforts to control the disease by enabling a rational transfer of genetic, pathological or biological information from a better-studied but closely related fungal taxon (e.g. *C. perniciosa)* (Berbee & Taylor, 1999), and may facilitate the effective exploitation of the large genomic and transgenomic data sets that are being produced for these diseases.

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