Biodiversity and biogeography of the cacao (*Theobroma cacao*) pathogen *Moniliophthora roreri* in tropical America

W. Phillips-Mora[a]*, M. C. Aime[b] and M. J. Wilkinson[c]

[a]*Tropical Agricultural Research and Higher Education Centre (CATIE), 7170 Turrialba, Costa Rica;* [b]*USDA-Agricultural Research Service, Systematic Botany and Mycology Laboratory, 10300 Baltimore Avenue, Beltsville, MD 20705, USA; and* [c]*Institute of Biological Sciences, Edward Llwyd Building. The University of Wales, Aberystwyth, Ceredigion SY23 3DA, UK*

Moniliophthora roreri, the cause of moniliasis or frosty pod rot, occurs on the neotropical rainforest genera *Theobroma* and *Herrania*. While this basidiomycete has had devastating effects on the cacao tree (*T. cacao*) in tropical America, where it is confined, little is known of its biogeography and intraspecific genetic variability. Here, AFLP and ISSR profiles of 94 isolates of *M. roreri* from across its geographic range in Central/South America were analyzed. The study provided limited evidence to support the hypothesis that *M. roreri* is capable of sexual reproduction. The highest levels of genetic diversity occurred in Colombia and not in Ecuador as originally believed. The fungus was broadly divided into five genetic groups. Two of these have a wide geographic range: Bolívar group (north of Santander in Colombia, eastern Venezuela, peripheral Ecuador, Peru), and Co-West group (western Colombia, central Ecuador, Central America). The other groups are all apparently endemic to Colombia (Co-East and Co-Central groups) or north-western Ecuador (*Gileri* group). We speculate that central/north-eastern Colombia may represent the centre of origin for *M. roreri*. Sequence data from the internal transcribed spacer region of the nuclear rDNA repeat were congruent with the AFLP/ISSR results, dividing *M. roreri* into two broad groups: the Orientalis group, comprising most isolates from the Co-East, Co-Central and Bolívar groups, and the Occidentalis group, comprising isolates from the Co-West and *Gileri* groups. The spread of *M. roreri* into new areas and countries mediated by human activity is discussed.

Keywords: AFLP, cocoa, frosty pod rot, ISSR, ITS, moniliasis

Introduction

Moniliophthora roreri is a parasitic basidiomycete belonging to the Marasmiaceae (Aime & Phillips-Mora, 2005) with a host range limited to apparently all species of the closely related genera *Herrania* and *Theobroma* (Evans, 1981). The fungus attacks only the fruits of these species causing internal and external pod damage that results in total pod loss. The presence of the disease on the cacao tree (*Theobroma cacao*) has had such devastating effects on yield that long-term economic viability of the crop has been compromised (Evans, 2002). In different Latin American countries losses attributable to the disease have been so severe that cacao cultivation has been abandoned in entire areas (Rorer, 1918; Parsons, 1949; Enriquez *et al*., 1981).

Shaded agro-ecosystems such as cacao provide a promising means of addressing the challenges of creating a forest-like habitat for tropical biodiversity in a rapidly deforested landscape, while simultaneously providing a lucrative crop for local agricultural communities (Perfecto *et al*., 1996). The conservation value of the crop is heavily dependent on its local economic viability. However, the economic and therefore ecological significance of this crop is currently in flux as *M. roreri* has progressively moved from its native range in N.E. South America to invade northwards through Central America and Mexico, and westwards across the Andes and towards the Amazonian forests.

The genetic diversity of fungi can affect all aspects of their biology including the relationship between a pathogen and its host. Genetic variation allows pathogens to adapt readily to changing environmental conditions and evolve new pathogenic types quickly in response to enhanced resistance in crops, such as those brought about by advances in crop breeding (Carlile & Watkinson, 1994; Wang & Szmidt, 1998). 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). 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. Many phytopathogenic fungi have no sexual stage or a sexual stage not yet found in nature.

Ciferri & Parodi (1933) originally classified M. roreri (as Monilia roreri) as an anamorphic ascomycete due to the apparent absence of any meiotic state or sexual structures and morphological similarities to other phytopathogens in the genus. However, the advent of means to examine ultrastructural characters allowed Evans et al. (1978) to conclude that M. roreri should be re-assigned to the Basidiomycota, where, until recently, it was classified as Incertae sedis (Aime & Phillips-Mora, 2005). Although M. roreri is most commonly believed to be an anamorphic fungus, Evans et al. (2002) report that there may be a cytological mechanism that enables the fungus to undergo sexual reproduction. These authors documented the occurrence of what appeared to be meiosis during the sporogenesis and germination of the 'conidia' of M. roreri. Additionally, hybridization between M. roreri and the L-biotype of the closely related species M. perniciosa (formerly Crinipellis perniciosa) may also be possible (Griffith et al., 2003).

Few studies have attempted to characterize the extent or pattern of genetic and physiological variability of M. roreri (Zadoks, 1997), and current knowledge of its variability are solely limited to morpho-physiological characters taken from a small number of isolates (Herrera et al., 1989; Ram, 1989). The origin and dispersal mechanisms of M. roreri have also remained obscure despite the dramatic impact that this pathogen has had on the economies of many tropical American countries and the real threat of further dispersal to other countries in America and other continents. Ecuador has been most widely considered the centre of origin of M. roreri since the beginning of the 20th century (Briton-Jones, 1934; Erneholm, 1948), when the pathogen caused a well documented outbreak on cacao (Rorer, 1918) in what was then the major cacao producer in the world. Rorer (1918) concluded that frosty pod rot was caused by an unknown fungal pathogen indigenous to Ecuador because the same disease was observed in the forest on pods of wild cacao species. Conversely, some references mention the appearance and devastating effects of a disease matching the symptoms of frosty pod rot in the lower Cauca Valley, Department of Antioquia, western Colombia during the second half of the 19th century (Aguirre, 1881; Carrasquilla, 1916; van Hall, 1932; Parsons, 1949; Brew, 1977). The effects of the disease first became evident in this area of Colombia in 1851, some 50 years before the Ecuadorian outbreak. Based on the presence of wild hosts infected with frosty pod, Holliday (1957) and Thorold

(1975) postulated that western Colombia represents the possible place of origin of the pathogen. However, a group of largely overlooked historical references (Anonymous, 1832, 1850; Ancízar, 1956; Arenas, 1993) mentioned the devastating effects on cacao cultivation in north-eastern Colombia (Department of Norte de Santander and, later, in the Department of Santander) caused by a pod disease matching the symptoms of frosty pod rot as early as 1817. One of these reports (Anonymous, 1832) appears to represent the earliest reference to the disease, occurring 34 years before the outbreak in the lower Cauca Valley of Colombia and 100 years before the outbreak in Ecuador.

It is likely that infected pods from wild sources initiated the mentioned outbreaks of disease. However, more recent outbreaks in Peru (Evans et al., 1998), Central America and Mexico (Phillips-Mora et al., 2006a,b) are almost certainly the product of inadvertent human-mediated spread of the pathogen from infected cacao areas into countries and regions previously free of the pathogen. The reports of frosty pod rot in Venezuela, Panama, Costa Rica, Nicaragua, Peru, Honduras, Guatemala, Belize and Mexico are all very recent (from Venezuela in 1941 to Mexico in 2005).

The present study aims to measure the extent of genetic diversity within M. roreri across its geographic range, and to use this information to make inferences about its origin, biogeography, and the possible mechanisms of variation and dispersal of the pathogen. The following hypotheses are tested: (i) the centre of origin and genetic diversity of M. roreri occurs in Colombia; (ii) human activities have been the major force responsible for the dispersal of M. roreri into new areas and countries; (iii) sexual reproduction has played an important role on the origin of the observed genetic diversity. The information collected here will be of great utility in improving control strategies for frosty pod rot, establishing more effective quarantine measures, assisting and targeting the search for resistant germplasm, and predicting the results of plant breeding programmes.

Materials and methods

Isolates

The 94 isolates of M. roreri used in this study originated in all countries and most important areas affected by frosty pod rot during the collection period (Table 1). Samples were collected mainly in 1999–2000, and were obtained from infected fruits of T. cacao trees or other species of Theobroma and Herrania. A significant percentage of all isolates were collected in Ecuador, since at the time of collection this country was regarded by most researchers as the centre of origin of the pathogen. A considerable representation of isolates was also obtained from Colombia and Costa Rica to compare changes in genetic diversity of the fungus with distance from the presumed centre of origin. Samples were isolated from pods showing initial or intermediate stages of external necrosis and initially grown on V8 medium (20% w/v V8

Table 1 Geographic origin and host of 94 isolates of *Moniliophthora roreri*

Groups of isolates	Place of collection of the isolates[a]	Host
Colombia, Department Norte Santander	(Co1) Vereda Pedregales, Zulia; (Co2) Vereda Punta del Palo, Tibú	*T. cacao*
Colombia, Department Santander	(Co4, Co5) La Suiza Farm, Rio Negro; (Co6) Puente de Piedra, Bucaramanga (Co14); Santa Inés, San Vicente de Chucurí; (Co15, Co16) Barranco Amarillo/Rancho Grande, El Carmen de Chucurí	*T. cacao*
Colombia, Central Departments (Antioquia, Caldas & Huila)	(Co7) Támesis, Antioquia; (Co8) San Jerónimo, Antioquia; (Co13) Urabá, Antioquia; (Co9) Granja Lucker, Caldas; (Co10, Co11, Co12) Palestina, Caldas at 1520, 1350 and 1200 meters above sea level, respectively; (Co17) Rivera, Huila	*T. cacao*
Costa Rica, Atlantic Region	(C1, C21, C24) CATIE, Turrialba; (C2) La Lola Farm, Matina; (C6); Keköldi, Talamanca; (C11) Earth, Pocora; (C12) Peralta, Turrialba; (C17) Penshurt; (C26) Guayabo, Turrialba; (C27) Manzanillo, Talamanca; (C28) Siquirres; (C29) Cahuita. C1 was collected in 1987	*T. cacao*
Costa Rica, Pacific Region	(C3) San Isidro del General; (C5) Golfito; (C7) Buenos Aires, Osa; (C19) Punta Uvita, Osa	*T. cacao*
Costa Rica, Northern Region	(C9) Upala; (C13) La Fortuna; C15) Veracruz, San Carlos; C16) Puerto Viejo, Sarapiquí	*T. cacao*
Costa Rica, CATIE's International Germplasm Collection, Turrialba	*Theobroma* spp.: (C14) *T. grandiflorum*; (C20) *T. bicolor*; (C22) *T. speciosum*; (C25) *T. mammosum Herrania* spp.: (C18) *H. nitida*; (C23) *H. albiflora*	Different species
Ecuador, Province Guayas	(E1) Naranjal; (E2) Río Congo, Balzar; (E3) San Juan, El Empalme; (E32) Tenguel	*T. cacao*
Ecuador, Province Los Ríos	(E4, E5, E6, E7, E8) Mocache; (E9, E10, E11, E12, E18) Quevedo; (E13) Ventanas; (E14) Juana de Oro; (E15, E16) Vinces; (E17) Baba; (E20) Nestlé Farm, San Pablo; (E30) La Variante, Buena Fe	*T. cacao*
Ecuador, Province Manabí	(E21) Chone, Chone; (E22) Quiroga, Bolívar; (E23) Calceta, Bolívar	*T. cacao*
Ecuador, Province Esmeraldas	(E24) San Mateo; (E25) Chinca, Quinindé; (E26) Viche, Quinindé; (E27) El Consuelo, Quinindé	*T. cacao*
Ecuador, Province Pichincha	(E28, E29) Santo Domingo de los Colorados	*T. cacao*
Ecuador, Province Napo	(E36) Nueva Primavera, Río Napo	*T. bicolor*
	(E37) Anangu, Río Napo. Both collected by H. Evans in 1999	*T. cacao*
Ecuador, Province Unknown	(E38) and (E40) were re-isolated by H. Evans in 1977 from an artificially inoculated *Herrania* pod (CBS 199.77), and from an artificially inoculated *T. cacao* seedling (CBS 202.77), respectively	*T. cacao*
Ecuador, Province Imbabura	(E42) El Rocío, Guadual; (E43) Lita-Alto Tambó. Collected by H. Evans in 1999	*T. gileri*
Honduras, Department Gracias a Dios	(H1) Planyare, Wampusirpe, Mosquitia	*T. cacao*
Nicaragua, Región Atlántico Norte, and Departments Río San Juan & Matagalpa	(N1) Kosuly, Waslala, Region Atlántico Norte; (N4) Puerto Cabezas, Region Atlántico Norte; (N3) San Carlos, Río San Juan; (N5) Rio Blanco, Matagalpa	*T. cacao*
Peru, Departments Huánuco & San Martín	(P1) Tingo María, Huánuco; (P2) Tocache, San Martín. Collected by U. Krauss in 1997	*T. cacao*
Panamá, Provinces Chiriquí, Bocas del Toro & Colón	(Pa1) Paso Canoas, Chiriquí; (Pa5) Tibete, Bocas del Toro, (Pa6) Coclecito, Colón; (Pa7) Buena Vista, Colón; (Pa8) Escobal, Colón	*T. cacao*
Venezuela, States Mérida, Zulia & Táchira	(V2) La Rocolita, Mérida; (V3) Playa Grande, Zulia; (V4) Colón, Zulia; (V5) Colón, Táchira	*T. cacao*

[a]Most isolates were collected by the first author from fruits of *Theobroma cacao* in 1999–2000. Exceptions are indicated in the table.

juice, 0·1% w/v asparagine, 2·0% w/v maltose, 1·8% w/v agar). Then, mycelia of *M. roreri* were obtained by growing the isolates in mineral liquid medium for 8 days according to the method of Weising *et al.* (1995). Genomic DNA was extracted from dry mycelium using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

AFLP and ISSR profiling

DNA samples were processed using the GIBCO-BRL/ AFLP Analysis System II kit (Life Technologies) according to the manufacturer's protocol except that volumes of reaction mixtures and $|y^{33}P|$-ATP were halved. Four combinations of primers (M-CAA/E-AT, M-CAA/E-TG, M-CAG/E-TG and M-CAG/E-AT, where E = *EcoRI* and M = *MseI* restriction enzymes) were selected for application across the complete set of isolates after a preliminary screen of 20 combinations on three *M. roreri* isolates (Co-2, E-6 and N4) representing geographically extreme points in the distribution of the pathogen. AFLP products were separated by electrophoresis through a denaturing 7% polyacrylamide gel before it was transferred onto a rigid support, covered with cellophane and dried. Dried gels were exposed to X-rays films (Kodak X-OMAT AR) for 2–7 days to visualize the AFLP fingerprints. Films were developed and the restriction fragments scored by visual examination and recorded as present or absent.

ISSR analyses were performed using the method reported by Charters *et al.* (1996). The following seven primers from Set #9 of the University of British Columbia were used: #823 (5′-TCTCTCTCTCTCTCTCTCC-3′), #874 (5′-CCCTCCCTCCCTCCCT-3′), #880 (5′-GGA-GACGACGACAGGAGA-3′), #885 (5′-BHBGAGAGAGAGA-GAGA-3′), #890 (5′-VHVGTGTGTGTGTGTGT-3′), #891 (5′-HVHTGTGTGTGTGTGTG-3′) and #816 (5′-CACACACACACACACAT-3′). These primers were selected on the basis of reliable amplification following a pre-screen of 20 primers against a representative group of four geographically distant isolates (Co-16, E-6, Co-17 and N3). The restriction fragments were scored by visual examination and recorded as present or absent.

Repeatability of banding patterns for both AFLP and ISSR gels was confirmed by adding 3–5 representative samples in each gel.

Sequence analyses of the internal transcribed spacers (ITS)

A segment of the ribosomal RNA genes in nuclear DNA comprising the ITS one and two (ITS-1, and ITS-2) and the 5·8S ribosomal subunit (collectively known as the ITS region) was amplified using the primers ITS4 (5′-TCC-TCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAG-TAAAAGTCGTAACAAGG-3′) and conditions described by White *et al.* (1990). PCR reaction mixtures (25 μL) consisted of 1 × *Taq* polymerase buffer (Bioline), 1·5 mM MgCl$_2$, 0·5 μM each primer, 0·2 mM dNTPs (Bioline),

0·02 U/μl *Taq* polymerase (Bioline, UK) and 0·4 ng/μL genomic DNA. Thermal cycling conditions involved an initial cycle at 94°C for 1 min; 30 cycles of 94°C for 1 min, 52°C for 30 s and 72°C for 1 min (extension), and a final cycle at 72°C for 7 min.

PCR products were purified using the NucleoSpin Extract Kit (Macherey-Nagel) according to the manufacturer's instructions. The sequencing reactions were performed using the ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction using the same primers that were used for amplification. The reaction mixture was prepared with 0·4 μL of primer (4 μM), 4 μL of Kit Mix and 5·6 μL of cleaned PCR-product. Cycle sequencing (Sanger *et al.*, 1977) was done with the following programme: 30 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. The samples were cleaned using Edge Gel Filtration Cartridges (Edge BioSystems) and dried in a Labcoco dryer at 60°C for 10 min. Sequencing was performed on a Perkin-Elmer ABI-373XL with the following options: Power setting 35, Collection time 14·5 and Basecaller ABI50. The polyacrylamide concentration used was 5·25%. The sequences were processed using the Lasergene software (DNASTar Inc.). Each sequence was assembled and checked for inaccurate base calling using SeqMan II (DNASTar Inc.). Consensus sequences were then aligned manually using MegAlign (DNASTar Inc.). Regions of ambiguous alignment and incomplete data at the termini of sequences were excluded from analyses.

Data analysis

Patterns of AFLP and ISSR bands were analysed both separately and jointly as a combined data set using the MVSP software (Kovach Computing). Similarity matrices were compiled using Jaccard coefficient and subjected to the unweighted pair-group method (UPGMA) to generate dendrograms. To estimate support of the various groupings, 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 with bootstrap values.

The software InfoGen version 1·0 (Grupo InfoStat: Universidad Nacional de Córdoba, Argentina) was employed to perform a principal coordinates analysis (PCO) and a minimum spanning tree (MST) using Jaccard distances with the AFLP/ISSR bands. MST was superimposed on the PCO ordinations. The MST is the minimum-distance tree that connects all vertices in a graph using the smallest possible distance between samples. Analyses of molecular variance (AMOVA) were performed using InfoGen to test for statistical differences between genetic groupings and two apparent genetic associations.

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. 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 performed using either the set of 94 isolates or a subset of 16 Colombian isolates and two Ecuadorian isolates (E42 and E43).

Shannon's diversity index (Shannon & Weaver, 1949) and the percentage of polymorphic loci were calculated for all isolates and, separately, for isolates from Ecuador, Colombia and Central America using the POPGENE software (Yeh *et al*., 1997).

Results

AFLP and ISSR profiling

AFLP profiles of the 94 isolates collectively yielded 163 bands, with an average of 40·8 amplicons per combination. There was a high level of polymorphism between profiles of isolates considering the presumed asexual mode of reproduction of this basidiomycete. Indeed, 65% of the products (106 bands) generated were polymorphic between at least some of the isolates. Variable markers that were present or absent only in one or two genotypes (31% of the total) were deemed rare alleles. 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.

There was a similarly high level of polymorphism observed between ISSR profiles, with some 75 of the 105 amplicons generated (i.e. 71%) yielding polymorphic bands. Twenty-three amplicons (30·7%) were designated as rare alleles. Rare alleles were most frequently observed in E42/E43 with 13 such alleles and Co5/Co6 with 3. Profiles of the following isolates yielded a single rare allele: Co14, Co14/Co5, Co15, Co15/Co16, E32 and Pa8.

Combined genetic distance analysis

UPGMA dendrograms generated by the AFLP and the ISSR band profiles were highly conserved (Mantel test, Pearson correlation coefficient $r = 0·95$, $P < 0·001$). For this reason, a combined data set comprising 268 loci and 94 isolates was used to create a consensus UPGMA dendrogram. This analysis increased resolution and degree of support of the groups identified, with four discrete groups of isolates supported by bootstrap values > 95% and AMOVA ($P < 0·01$), and a poorly supported fifth group of six diverse isolates (indicated by III) from the same area in Colombia (Fig. 1).

From the different *Theobroma* and *Herrania* species analysed, only the two isolates from *Theobroma gileri* plants growing in Northwest Ecuador (E42, E43) diverged from isolates taken from *T. cacao* (coefficient of similarity of 0·63 and 79% bootstrap support). These two isolates are well supported (100% bootstrap support) and comprise Group I (Gileri). The remaining isolates subdivide into two major clusters at a coefficient of similarity of 0·67 (84% bootstrap support), with each containing two discrete genetic groups. The first major cluster is heterogeneous and includes all Colombian isolates except Co8 and Co13 from the department of Antioquia, Colombia and Co1 and Co2 from the Department of Norte de Santander, Colombia. It contains two distinct groups. Group II (Co-Central) is well supported (100% bootstrap) and includes 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. Group III (Co-East) is less well supported (bootstrap 51%) and includes all isolates from the Department of Santander, Eastern Colombia (Fig. 1).

The second major cluster contains the remaining two, well-supported groups that include isolates from all countries under study. Group IV (Co-West) comprises isolates from Central Ecuador, two isolates from western Antioquia in Colombia (Co13 and Co8) and all Central American isolates. Two subgroups are supported by high bootstrap values: one embracing isolates from Central Ecuador collected along an imaginary line from Esmeraldas in the north to Naranjal in the south, and the other comprising the isolates from western Antioquia and Central America. Group V (Bolívar) comprises two isolates from the 'Norte de Santander' Department in Colombia (Co1, Co2), all Peruvian and Venezuelan isolates, and 10 isolates from the periphery of Ecuador.

The geographic range occupied by each of the groups is shown in Fig. 2. Names assigned to the five groups reflect their geographic origin, the host plant (*Gileri* group) or in one case (Bolívar group) is assigned in recognition of Simon Bolívar who played a leading role in establishing the independence of the South American countries containing these isolates.

Genetic diversity in Colombia, Ecuador and Central America

To test the hypothesis regarding the centre of diversity of *M. roreri*, separate UPGMA dendrograms were produced for isolates from each of Colombia, Ecuador and Central America (Fig. 3). Colombian isolates included the greatest richness (i.e. spread) of genetic diversity, and the highest levels of genetic diversity as measured using Shannon's diversity index ($0·20 \pm 0·06$) and on the basis of the percentage of polymorphic loci (46·6%) across samples.

Ecuadorian isolates exhibited lower overall genetic diversity as measured by Shannon's diversity index ($0·15 \pm 0·04$) and the percentage of polymorphic loci (38·1%). Interestingly, when the Gileri isolates were excluded from the analysis, these values reduced further to $0·11 \pm 0·04$ and 21·3% respectively. Indeed, the Gileri isolates possessed the highest abundance of rare alleles for both AFLP and ISSR analyses.
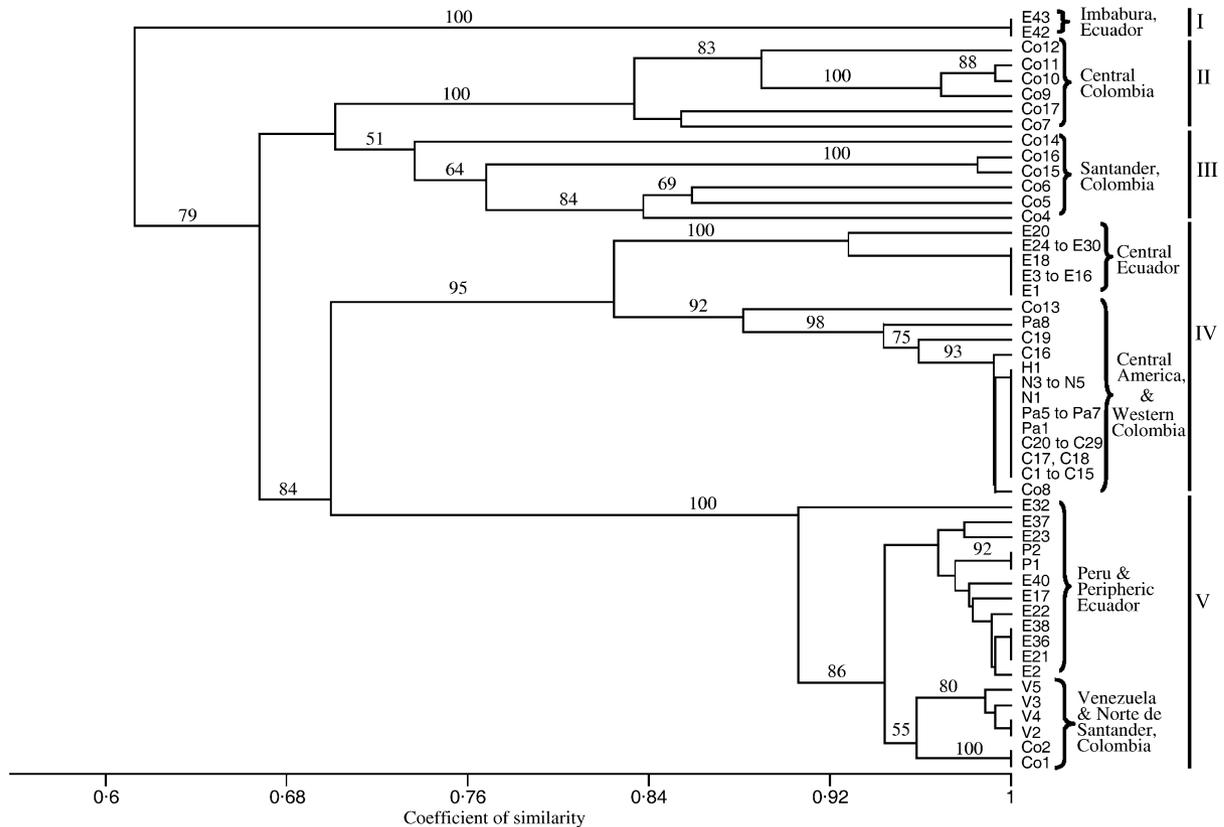
Figure 1 UPGMA dendrogram generated from combined AFLP and ISSR data sets of 94 isolates of *Moniliophthora roreri*. Bootstrap values above 50% (based on 1000 bootstrap re-samples) are indicated above the branches. Major genetic groups and isolates are: I = Gileri (E43, E42); II = Co-Central (Co12, Co11, Co10, Co9, Co17, Co7); III = Co-East (Co14, Co16, Co15, Co6, Co5, Co4); IV = Co-West (E20, E30, E29, E28, E27, E26, E25, E24, E18, E16, E15, E14, E13, E12, E11, E10, E9, E8, E7, E6, E5, E4, E3, E1, Co13, Pa8, C19, C16, H1, N5, N4, N3, N1, Pa7, Pa6, Pa5, Pa1, C29, C28, C27, C26, C25, C24, C23, C22, C21, C20, C18, C17, C15, C14, C13, C12, C11, C9, C7, C6, C5, C3, C2, C1, Co8), and V = Bolívar (E32, E37, E23, P2, P1, E40, E17, E22, E38, E36, E21, E2, V5, V3, V4, V2, Co2, Co1).

The Central American isolates exhibited the lowest diversity, with all 36 isolates showing a high level of similarity (> 0·94), 33 of which were identical across all loci. The isolates from this region all belonged to the Co-West group. Accordingly, this region generated the lowest estimates of diversity using Shannon's diversity index and the percentage of polymorphic loci (0·006 ± 0·005 and 5·6% respectively).

Principal coordinates analysis (PCO) and the minimum spanning tree

The first three axes in the PCO plot (Fig. 4) collectively accounted for 80% of the total variation, with PCO1, PCO2 and PCO3 comprising 45%, 20% and 15% respectively. The plot of PCO1 against PCO2 clearly differentiated three of the five groups (Gileri, Co-West, and Bolívar) and provided only very weak separation of Co-East and Co-Central groups (Fig. 4). Interestingly, isolates E20, E32 and Co13 occupied somewhat intermediate position between genetic groups: E20 and E32 between Co-West and Bolívar, and Co13 between Co-West and Gileri. The minimum spanning tree (MST)

shown linked the Bolívar group and the *Gileri* group to the Co-West group rather than the more diverse Co-East + Co-Central cluster. The Co-West group was then itself linked to the Co-East + Co-Central Cluster via Co15 and Co16 (Co-East group).

Mantel test to compare the molecular (AFLP + ISSR) and geographic dissimilarity matrices

The possible relationship between isolation by geographic distance and genetic distance was investigated using the Mantel test to compare the dissimilarity matrices obtained from the combined molecular data set and those that were generated using geographic distances between the isolate collection sites. This yielded a Pearson correlation coefficient of only $r = 0·4$, but with a probability of independence of $P < 0·001$. Given the recent expansion of the fungus' range out of Colombia, the analysis was repeated using only the set of 16 Colombian isolates and two Ecuadorian isolates from wild trees of *T. gileri* (E42, E43), where natural dispersal was considered likely to be far more prominent. When this was done, the correlation increased markedly ($r = 0·62$, $P < 0·001$).

Figure 2 Map indicating the geographic distribution of isolates of *Moniliophthora roreri* and the corresponding genetic groups (I = Gileri; II = Co-Central; III = Co-East; IV = Co-West; V = Bolívar).

ITS analysis

ITS sequences of approximately 741 bp in size were obtained for all 94 *M. roreri* isolates. Ten (1·4%) point mutations involving single base pair changes were detected among the isolates (Table 2). Overall, ITS sequence was less informative than AFLP/ISSR analyses. Most of the 94 isolates fell into two distinct groups (AMOVA, $P < 0·01$). Group I (herein termed Orientalis) comprises the Bolívar (except E32), Co-Central and Co-East isolates (except Co15 and Co16). Consequently, all Peruvian, Venezuelan

and peripheral Ecuadorian isolates and most of the Colombian isolates were contained in this group. Group II (herein termed Occidentalis) comprises all Co-West and Gileri isolates, one Bolívar isolate from Tenguel, Ecuador (E32) and the two Co-East isolates (Co15 and Co16) from El Carmen de Chucurí, Santander Department, Colombia. Thus, four Colombian isolates, all Central American and most Central Ecuadorian isolates belong to this association. The remaining isolates were defined by rare mutations, with two rare mutations observed in Co5 and Co6 (Co-East), two in E42 and E43 isolates (Gileri) and one each in Co-13 and E16 isolates (Co-West). Both groups contain isolates from a range of *Theobroma* and *Herrania* species.

Discussion

Congruence between the AFLP and ISSR matrices permitted the construction of a consensus UPGMA tree and PCO plot. On the basis of the combined UPGMA tree there were four main genetic groups of *M. roreri* (Co-Central, Co-West, Bolívar and Gileri) with > 95% support by bootstrap analysis and AMOVA. A fifth genetic group (Co-Eastern) was only modestly supported by bootstrap analysis. Principal component analyses also supported the presence of the four distinct groups, again providing weak evidence for separation of a fifth, Co-East group (Fig. 4). As expected, the ITS sequence data yielded a lower number of informative characters than either fingerprinting method. ITS data assigned most of the isolates into two large groups that were largely congruent with those identified from the AFLP/ISSR analyses. The Orientalis group contained almost all members of Co-East, Co-Central and Bolívar, and is apparently centred in north-east Colombia, whereas the Occidentalis group is comprised almost entirely of Co-West and Gileri isolates, and appears centred in western Colombia. The minimum spanning tree suggests ancient spread from Central and Eastern Colombia into Western Colombia (Fig. 4).

Distribution and variability of the genetic groups has some relevance for the designation of centres of diversity and origin for the species. Four of the genetic groups were

Table 2 Polymorphic nucleotides detected for the nuclear ribosomal DNA (partial 18S, ITS1, 5·8S, ITS2, partial 25S) of *Moniliophthora roreri* isolates belonging to the Orientalis and Occidentalis groups. Isolates Co5 and Co6 (Orientalis) and Co13, E16, E42 and E43 (Occidentalis) are placed separately since they have a different nucleotide pattern in relation to their respective group

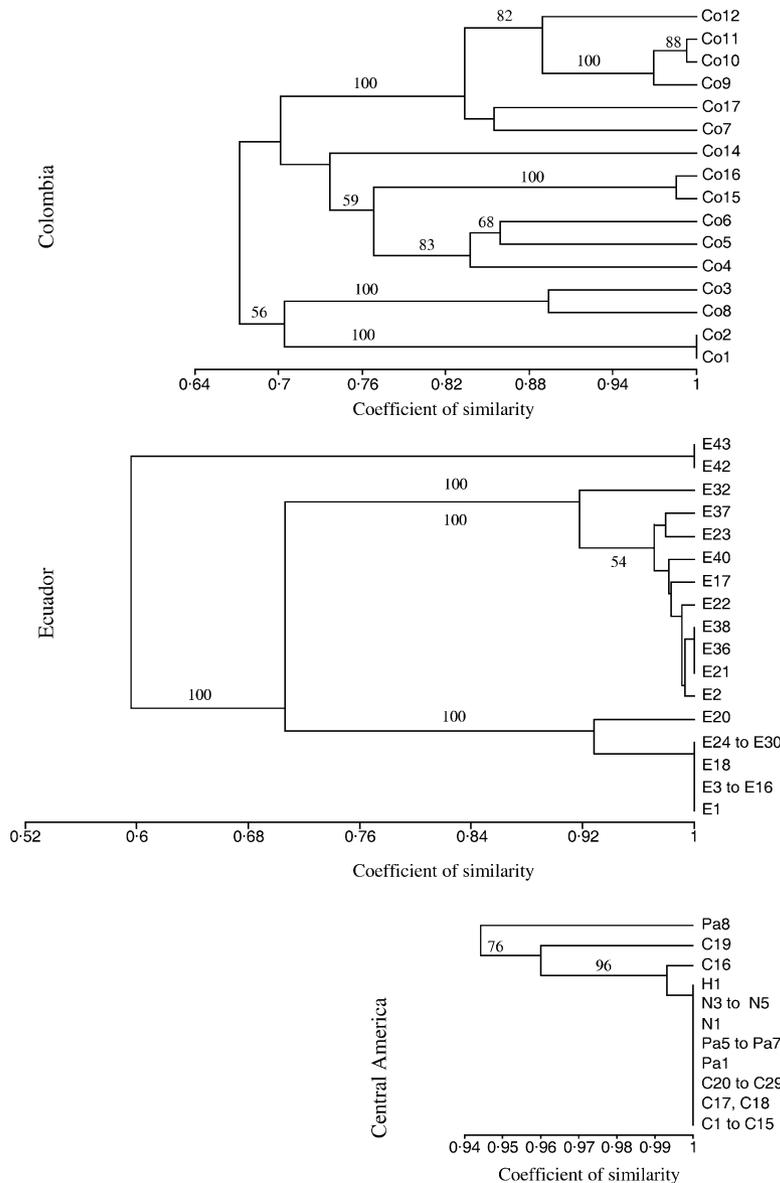| Group/isolates	Number of isolates	Location of the polymorphic nucleotides									
		66	133	134	420	428	429	449	471	547	604
Orientalis	28										
Co5/Co6	2	G	G	G	A	–	C	G	–	C	–
All others	26	G	G	–	A	–	C	–	–	C	–
Occidentalis	66										
Co13	1	A	G	–	–	–	C	–	T	T	G
E16	1	G	–	–	–	–	C	–	–	T	G
E42/E43	2	G	–	–	–	T	T	–	T	T	G
All others	62	G	–	–	–	–	C	–	T	T	G

Figure 3 UPGMA dendrograms of AFLP + ISSR band profile for isolates of *Moniliophthora roreri* from Colombia, Ecuador and Central America. Bootstrap values above 50% (based on 1000 bootstrap re-samples) are given.

found in Colombia, with the most variable Co-East and Co-Central groups apparently being endemic to this country. It is perhaps also worth noting that whilst the *Gileri* group is recorded exclusively from Ecuador, both isolates of this group were collected from sites close to the Colombian/Ecuadorian border (Fig. 2). Consequently, it is entirely plausible that representatives of the group could be eventually identified within Colombia. These observations therefore collectively point towards a centre of genetic diversity within Colombia. This assertion is supported by observations that the highest values of Shannon's diversity index and the highest percentage of polymorphic loci lies within Colombia. The thesis is further supported by the detection of more ITS variation among Colombian isolates than were noted elsewhere, even though there were considerably fewer samples

analysed from Colombia than from Ecuador. Thus, it is concluded that the centre of diversity of *M. roreri* lies in Colombia. This contradicts the most common hypothesis that the pathogen originated in Ecuador (Rorer, 1918; Briton-Jones, 1934; Erneholm, 1948). However, careful re-examination of the historical literature provides clear evidence of outbreaks in Colombia prior to the Ecuadorian outbreak. Indeed, several authors have recorded both the appearance and devastating effects of a cacao disease matching the symptoms of frosty pod in eastern Colombia as early as 1817 (Anonymous, 1832, 1850; Ancízar, 1956; Arenas, 1993), and afterwards in western Colombia during the second half of the 19th century (Aguirre, 1881; Carrasquilla, 1916; van Hall, 1932; Parsons, 1949; Brew, 1977).

The highest levels of genetic diversity within Colombia, as revealed using both AFLP and ISSR data sets, were

Figure 4 Principal coordinates analysis (PCO1 and PCO2) showing output of minimum spanning tree (MST) from Jaccard matrix of distances using 268 AFLP/ISSR bands of 94 isolates of *Moniliophthora roreri*. The MST appears superimposed on the PCO ordinations and was obtained by calculating the minimum-distance tree that connects all vertices in the graph using the smallest possible genetic distance between samples.
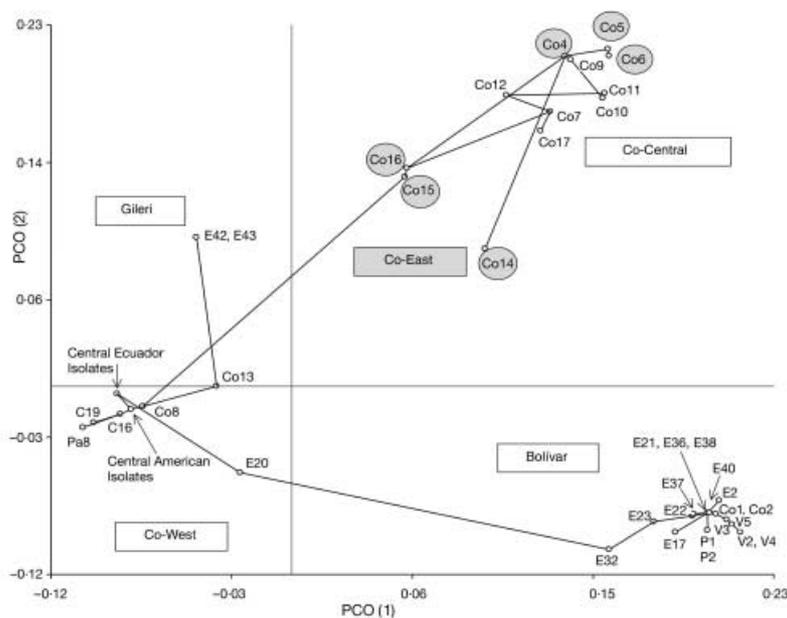
found to reside in central/north-eastern Colombia. This is consistent with this area containing the centre of origin of *M. roreri*. This thesis is supported by the decline in diversity with distance from the area, with a reasonably high correlation coefficient being obtained between the geographic and genetic matrices of distance when Colombian and the two Gileri isolates were compared using the Mantel test. However, it could be argued that the presence of seemingly ancient populations of wild species of *Theobroma* and *Herrania* in the Middle Magdalena area of Colombia (Galán, 1947) suggests a possible origin of *M. roreri* as a disease of cacao in the north-east part of the country, since the stable presence of a host is essential for the survival of this obligate pathogen. However, careful consideration should be given to the genetic distinctiveness of isolates taken from *T. gileri* plants. The PCO plot indicates that genetic affinity of these two isolates from Ecuador closely coincide with their geographical separation between the Eastern/Central and Western Colombian groupings, perhaps indicating that divergence of these wild types from those infecting cultivated hosts is due to ancient isolation rather than recent spread of the disease from cultivated stands of cacao. If this were so, then it is most plausible that *M. roreri* originated as a disease of a wild host species, probably belonging to *Theobroma* or *Herrania*, although insufficient sampling from wild material means that it would be premature to infer where or when this event occurred.

The UPGMA dendrograms compiled using only isolates from Colombia, Ecuador or Central America have value in suggesting historical patterns of spread of the disease. The tree generated by Ecuadorian and Central American isolates is strongly structured, with the Central American tree dominated by a single clone and those from Ecuador comprised of three distinct lineages. These

patterns are most consistent with secondary spread of the disease from Colombia into Ecuador from three source populations or regions, and of movement into Central America originating from a single region in Colombia.

After a historical dispersal phase from north-eastern Colombia, *M. roreri* was probably restricted to wild populations of the hosts and small cultivated stands of cacao in Colombia, Ecuador and perhaps Venezuela until more recent historical times when an explosion of cacao cultivation in South America towards the end of the 18th century provided the opportunity for rapid range expansion. Initial dispersal of *M. roreri* in cacao from central and eastern Colombia to other areas in Colombia, and thence to other countries probably occurred over a long period of time. This hypothesis is certainly concordant with the views of several authors who suggested that ancient and perhaps natural spread of the pathogen started amongst *Theobroma* and *Herrania* wild hosts (Holliday, 1957; Thorold, 1975; 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) further support this hypothesis. However, mediation by 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 three chains in Colombia, each of which represents significant barriers to natural dispersal.

The low genetic diversity of *M. roreri* within isolates from Venezuela, Panama, Costa Rica, Nicaragua, Honduras and Peru is consistent with a rapid, recent spread of the fungus from a common founder population. Evidence suggests that human activities have been the major force responsible for the dispersal of *M. roreri* into the new

areas and countries affected. 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). Conversely, the data here contradict Holliday (1957) who suggested that wild trees of *T. gileri* were responsible for the natural spread of the disease into Panama and furthermore indicate that there have been just one or very few introductions of *M. roreri* into Central America with most of the spread of the disease attributed to the rapid expansion of a single clonal isolate. The few polymorphisms observed in Central America possibly originated from recent mutations since they are rare and 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 over long distances (e.g. Central America) or periods of time, can be taken as a strong indication of clonal reproduction. An interesting example occurs with C1, a Costa Rican isolate collected in 1987 that shows the same multilocus genotype as Central American isolates collected in 1999. A similar situation occurs with E38, an Ecuadorian isolate obtained from an artificially inoculated pod of *Herrania* in 1977, which shows 100% sequence similarity with isolates E36 and E21 collected in the Napo and Manabí provinces in 1999. Spread of *M. roreri* from Manabí into the Amazonian 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, and cacao cultivars were imported from western Ecuador (Evans, 2002). However, the fact that isolates from Napo and Peru 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.

Historically, *M. roreri* has been considered an anamorphic fungus. Recently, however, Evans *et al.* (2002) found nuclear division patterns in the 'conidia' of *M. roreri* that could be indicative of meiosis. Furthermore, *M. roreri* may be capable of hybridization with *M. perniciosa* (Griffith *et al.*, 2003). However, the scarcity of evidence for recombination in the present research may indicate that sexual reproduction in *M. roreri* probably only occurs under very particular conditions. In fact, in fungi having both an asexual stage and a sexual stage, the latter may often only occur infrequently (Hawksworth *et al.*, 1996). Whether a particular pathogen reproduces clonally or by recombination depends on factors relating to its biology and its distribution in space and time (Taylor *et al.*, 1999). There are some data in the present study that are at least consistent with recombination. E20 and E32 showed some AFLP and ISSR bands typically present in other groups and so these isolates may represent recombinants between two groups, which are visualized in intermediate positions in the PCO plot (Fig. 4). 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 Province into the area in central Ecuador where E20 was collected. However, far more research is necessary in this field to demonstrate the role of sexual recombination in *M. roreri*.

The pattern of genetic variability reported here is consistent with the centre of diversity and origin of *M. roreri* on cacao being located within Colombia. The loss of diversity with distance from this country is suggestive of ancient migration to neighbouring Ecuador, followed by later spread to Venezuela and recent invasions into Peru and Central America. The study provided limited evidence to support the hypothesis that *M. roreri* is capable of sexual reproduction. The high speed of recent invasions in Central America and Peru has probably resulted from the rapid dispersal of infected cacao pods by man rather than natural modes of dispersal. The designation of a centre of diversity and origin for *M. roreri* has direct relevance for research efforts aiming to control the disease in cacao. The region containing the centre of origin and diversity of *M. roreri* in cacao is most likely to contain host trees with the highest diversity of resistance genes to the disease and more effective antagonist agents for biocontrol. This means that future germplasm collection expeditions for *Theobroma* cacao seeking to broaden resistance against *M. roreri* should focus on targeting wild populations of cacao in Colombia.

Acknowledgements

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