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Relationships of *Ceratocystis fimbriata* Isolates from Colombian Coffee-Growing Regions Based on Molecular Data and Pathogenicity

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Abstract

One of the most important diseases that reduce the profitability of coffee plantations in Colombia is coffee tree canker. This disease, caused by *Ceratocystis fimbriata*, has increased in severity and incidence in recent years, resulting in substantial losses to resource-poor farmers. The aim of this investigation was to consider the relatedness of *C. fimbriata* isolates from different Colombian coffee-growing regions, and thus to provide a foundation for future development of resistant coffee varieties. Fifty isolates from 11 provinces were characterized based on morphology and pathogenicity. In addition, isolates were compared using restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA (RAPD) markers. DNA sequence comparisons for the internal transcribed spacer (ITS) region, Mat-2 HMG Box and partial β-tubulin gene, were also made. The results of all comparisons clearly showed that Colombian isolates of *C. fimbriata* are highly variable. All molecular parameters used for these comparisons also showed that *C. fimbriata* isolates from Colombia reside in two major phylogenetic lineages. We believe that one of these lineages represent *C. fimbriata sensu stricto* and the other, a new taxon that needs further characterization. Isolates had different levels of pathogenicity on coffee, with some causing death of more that 90% and others killing <5% of inoculated plants. There was also a clear pattern of distribution of the most pathogenic strains within coffee-growing areas.

Introduction

Coffee (*Coffea arabica* L.) is the most important agricultural product of Colombia, where the income of approximately 300 000 families depends on this crop. Currently, there are close to 800 000 ha under coffee cultivation in Colombia (Federación Nacional de Cafeteros de Colombia, 2001) and maintaining the health of the trees is important. In this regard, several pests and pathogens limit coffee production in Colombia. Of these, coffee rust caused by *Hemileia vastatrix* Berk. & Br. and coffee canker caused by *Ceratocystis fimbriata* Ellis & Halsted are the most destructive. *Ceratocystis fimbriata* is an important pathogen in many parts of the world, where it causes wilt and canker-stain diseases on a wide range of plants. Hosts are mainly woody angiosperms including rubber [*Hevea brasiliensis* (Wiild ex Juss.) Mull-Arg.], mangoes (*Mangifera indica* L.), pear [*Prunus persica* (L.) Batsch.], plane trees (*Platanus* spp.), *Eucalyptus* spp. and coffee (Webster and Butler, 1967; Kile, 1993). Various annual crop plants such as sweet potato (*Ipomoea batatas* L.) and taro [*Colocasia esculenta* (L.) Schott] are also damaged by *C. fimbriata* (Halsted and Fairchild, 1891; Kile, 1993).

Coffee canker disease caused by *C. fimbriata* was first reported from Indonesia (Java) in 1900 (Pontis, 1951). It was subsequently found in Colombia in 1932, in the province of Quindio (Castaño, 1951). Currently, the disease is found in all the Colombian coffee-growing areas. Its severity and incidence has increased significantly in recent years, resulting in substantial losses for resource-poor farmers (Castro, 1998). Symptoms of plants affected by coffee canker include yellowing of foliage, dieback and wilt. When the outer surface of the bark on the trunks of trees is removed, dark lesions are obvious. Usually, these lesions extend downwards towards the roots and girdle the trunk, causing tree death (Pontis, 1951; Castro, 1991).

Mechanical injuries, especially pruning wounds, constitute the principal mode of ingress for *C. fimbriata* (Pontis, 1951). The fungus is easily transported on

agricultural tools or with soil particles on the shoes of farmers. Castro (1991) showed that the principal factor associated with the spread of coffee canker in Colombia is infection through damage to the stems of trees, made by the shoes of farmers needing to secure themselves on the steep slopes on which coffee is cultivated. Fifty-six per cent of the coffee plantations in Colombia are established on land with slopes steeper than 70° (Caballero and Baldion, 1993). Under these conditions, it is almost impossible for farmers to cultivate coffee without damaging the trees and spreading *C. fimbriata*-infested soil, widely in plantations.

Ceratocystis fimbriata is an ascomycete associated with insects that are attracted to the fruity aroma that it produces (Crone and Bachelder, 1961; Iton, 1966). The fungus is characterized by ascomata that have long necks with dark globose bases surrounded by a dense network of hyphae. Asci are evanescent and can only be seen in the very early stages of development. Ascospores are characteristically hat-shaped and exuded at the apex of the ascomatal necks, in sticky masses. *Ceratocystis fimbriata* produces chains of cylindrical conidia and oval thick-walled chlamydospores through long-tubular conidiophores and the anamorph is accommodated in *Chalara* (Hunt, 1956), more recently treated in *Thielaviopsis* (Paulin-Mahady et al., 2002).

Ceratocystis fimbriata has long been recognized as a variable fungus that is composed of distinct strains (Webster and Butler, 1967). Host specialization has been demonstrated in various strains of this fungus. The best studied of these host-specific strains is *C. fimbriata forma specialis platani*, which causes canker stain disease of plane trees in the Northern hemisphere (Walter et al., 1952; Santini and Capretti, 2000). Similarly, Leather (1966) reported a strain of *C. fimbriata* causing cankers in pimento (*Pimenta officinalis* Lindl.), that was unable to attack cacao, coffee and sweet potato. Host-specialized isolates could thus represent distinct but closely related species.

Studies using molecular markers have added support to the view that *C. fimbriata* encompasses host-specialized groups. Witthuhn et al. (1999) found that isolates of *C. fimbriata* from *Populus* spp., *Prunus* spp. and plane trees could be separated from each other based on restriction fragment length polymorphism (RFLP) analysis of internal transcribed spacer (ITS) regions. Santini and Capretti (2000) presented similar results using random amplified polymorphic DNA (RAPD) and minisatellite markers to analyse the Italian population of *C. fimbriata* f.sp. *platani*. This population had a high level of homogeneity. Isolates obtained from *Populus* spp., *Prunus* spp. and sweet potato grouped in separate clades in accordance with their hosts. Barnes et al. (2001b) developed a set of microsatellite markers to study the relationship between strains of *C. fimbriata* from different hosts and origins. These authors showed that isolates obtained from the same host clustered closely together in well-resolved groups. This provides further support for the view that host specificity represents an important factor in separating isolates of *C. fimbriata*.

Very little is known regarding the biology, ecology or genetic behaviour of *C. fimbriata* in Colombia. This is despite the fact that it is one the most important pathogens and occurs on a wide array of hosts including coffee, *Citrus* spp., cocoa (*Theobroma cacao* L.), rubber and native forest trees (Pardo-Cardona, 1995). Barnes et al. (2001b), showed, using ITS sequences and microsatellite markers, that isolates of *C. fimbriata* obtained from coffee and citrus in Colombia reside in two strongly resolved clades, which are genetically isolated from isolates of the fungus from other hosts. Although these data are reasonably unequivocal, they arose from a general study based on a relatively small collection of isolates. The discovery of two groups of *C. fimbriata* isolates in Colombia has prompted us to consider a wider collection of isolates, specifically collected from different coffee-growing regions. In this study, we thus consider 50 isolates from 11 provinces from Colombia. These are characterized based on morphology, pathogenicity, comparisons of RFLP, RAPD markers as well as sequences of the ITS regions of the ribosomal RNA operon, the Mat-2 HMG Box gene and partial sequences of β-tubulin gene.

Materials and Methods

Fungal isolation

Ceratocystis fimbriata isolates were obtained from soil samples randomly collected in coffee plantations affected by coffee canker. They were also collected from stems on symptomatic coffee plants growing at 13 research stations across 11 provinces from Colombia (Fig. 1). For isolation from soil samples, 5-cm long sticks, freshly cut from young coffee plants, had their bark removed and were, thereafter, pressed into moistened soil samples in plastic containers. This technique was shown to be effective for baiting *C. fimbriata* from soil samples in Colombia (Castro, 1994). In the case of isolation from symptomatic plants, small pieces (approximately 1 cm) of discoloured wood from diseased stems were placed between carrot discs, under conditions of high humidity, to stimulate ascomatal production (Moller and DeVay, 1968). Once ascomata had developed on coffee-stick baits or carrots, ascospore masses were transferred to V8-juice agar medium containing thiamine (100 μg/l) and antibiotics (100 mg/l of tetracycline; 200 mg/l of chloramphenicol and 200 mg/l of penicillin). Isolates were transferred several times to new agar in Petri dishes for purification. Fifty isolates representing the range of collection areas were then selected for further study (Table 1).

Colony and morphological characterization

Single ascospore masses were transferred to the centres of Petri dishes containing approximately 25 ml V8-juice agar medium (pH 5.5) supplemented with thiamine (100 μg/l). Dishes were then incubated in the dark for 16 days at 24°C and 90% relative humidity. Each isolate was grown on five Petri dishes. Colony

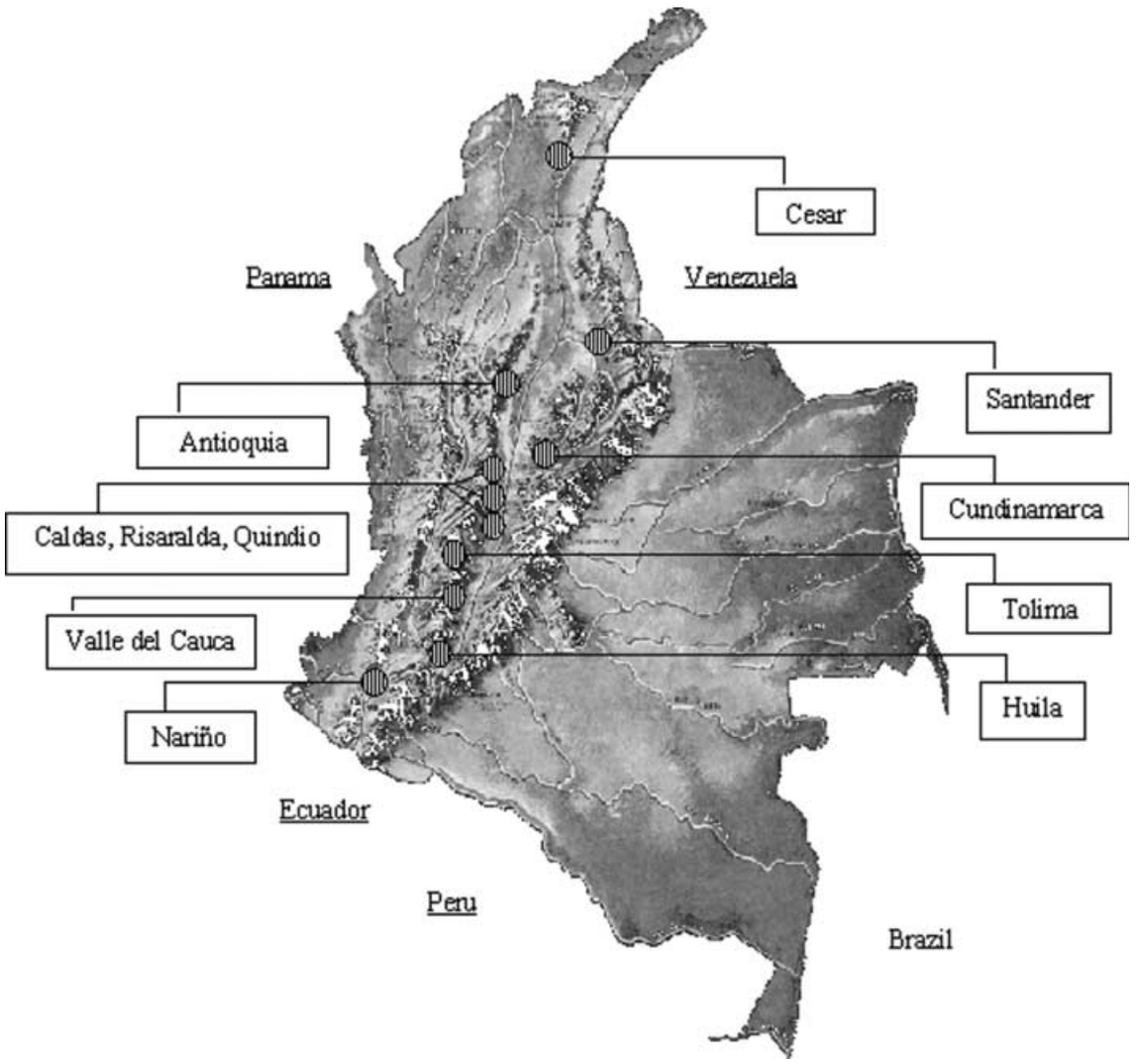

Fig. 1 Map of Colombia showing the provinces where isolates of *Ceratocystis fimbriata* were collected

diameter was measured every 24 h and growth rate was determined using regression analysis. Differences in growth rate were analysed statistically using one-way ANOVA and Duncan's mean test ($P = 0.05$) (SAS Statistical software, 1989). Additionally, characterization of colony colour (Maerz and Paul, 1930) and ascomatal distribution on plates (Webster and Butler, 1967) was also made.

Detailed morphological characteristics were determined for 10 isolates (Table 1). Characteristics considered included perithecial base diameter, neck length, length and width of ascospores, endoconidia and chlamydospores (Upadhyay, 1981). Measurements were taken for each character, for 50 randomly selected structures, using Zeiss Axioplan 2 light microscope (Carl Zeiss, Heidenheim, Germany).

Pathogenicity tests

Pathogenicity tests were conducted under greenhouse conditions at Chinchiná, Colombia, using the coffee variety Caturra. One 30-μl drop from a suspension of approximately 19 500 ascospores/ml was transferred into an inverted U-shaped wound approximately 2 cm in diameter, made at the mid-point of the stems of 9-month-old plants grown in plastic bags. This technique was similar to that used effectively by Castro (1994).

Twenty-five plants were inoculated for each of the 50 isolates and an equal number of plants were inoculated with 30 μl of sterile water as controls. Plants were maintained, fully randomized in a greenhouse at approximately 23°C daytime and 18°C night-time temperature and disease development was recorded every week for six and a half months. Every week, recently dead plants were enumerated and phloem lesion lengths on the stems of these trees were measured. At the time of terminating the experiment, lesion lengths were measured on all remaining plants that had not died. Phloem lesion length data were statically analysed using one-way ANOVA and Duncan's mean test ($P = 0.05$) (SAS Statistical software, 1989).

Table 1
Isolates of *Ceratocystis fimbriata* from Colombia, used in this study

Isolate[a]	Substrate	Origin (experimental station)	Province	Test[b]
CMW5732	Plant	Marquetalia	Caldas	RAPD, M
CMW5728	Plant	Marquetalia	Caldas	
CMW5729	Plant	La Catalina	Risaralda	M
CMW5740	Plant	La Catalina	Risaralda	M
CMW5730	Plant	Consacá	Nariño	RAPD
CMW10876	Plant	Consacá	Nariño	RAPD
CMW5731	Plant	Naranjal	Caldas	RAPD
CMW5738	Plant	Naranjal	Caldas	
CMW5733	Plant	El Rosario	Antioquia	RAPD
CMW5735	Plant	El Rosario	Antioquia	RAPD
CMW10844	Plant	Guayacanes	Antioquia	RAPD
CMW5748	Plant	Guayacanes	Antioquia	RAPD
CMW5734	Plant	Gigante	Huila	RAPD
CMW5739	Plant	Gigante	Huila	RAPD
CMW5736	Plant	Paraguaicito	Quindio	RAPD
CMW5745	Plant	Paraguaicito	Quindio	RAPD
CMW5737	Plant	Valle	Valle	RAPD
CMW5741	Plant	La Sirena	Valle	RAPD
CMW5742	Plant	La Sirena	Valle	RAPD
CMW5743	Plant	Pueblo Bello	Cesar	RAPD
CMW5744	Plant	Pueblo Bello	Cesar	RAPD
CMW5746	Plant	Líbano	Tolima	RAPD, M
CMW5747	Plant	Santa Barbará	Cundinamarca	RAPD
CMW11137	Plant	Santander	Santander	RAPD
CMW5749	Plant	Santander	Santander	RAPD, M
CMW5750	Soil	El Rosario	Antioquia	RAPD
CMW10877	Soil	El Rosario	Antioquia	
CMW5751	Soil	Valle	Valle	RAPD
CMW5761	Soil	Valle	Valle	RAPD
CMW5752	Soil	Gigante	Huila	M
CMW5762	Soil	Gigante	Huila	RAPD
CMW5753	Soil	La Catalina	Risaralda	RAPD
CMW10878	Soil	La Catalina	Risaralda	RAPD
CMW5757	Soil	Consacá	Nariño	RAPD
CMW5764	Soil	Consacá	Nariño	
CMW5755	Soil	Naranjal	Caldas	
CMW5754	Soil	Naranjal	Caldas	RAPD, M
CMW10875	Soil	Paraguaicito	Quindio	RAPD, M
CMW9555	Soil	Paraguaicito	Quindio	RAPD
CMW5756	Soil	Marquetalia	Caldas	RAPD
CMW11138	Soil	Marquetalia	Caldas	RAPD
CMW5758	Soil	La Sirena	Valle	RAPD
CMW5765	Soil	La Sirena	Valle	
CMW5759	Soil	Santa Barbará	Cundinamarca	RAPD
CMW5763	Soil	Santa Barbará	Cundinamarca	RAPD
CMW5760	Soil	Líbano	Tolima	RAPD
CMW5766	Soil	Líbano	Tolima	RAPD
CMW5767	Soil	Santander	Santander	RAPD, M
CMW11139	Soil	Santander	Santander	M
CMW5768	Soil	Pueblo Bello	Cesar	RAPD

[a]All the isolates are mantained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
[b]All the isolates were used for pathogenicity tests, colony growth studies and RFLP analysis. Isolates used for RAPD analysis (RAPD) and morphology measurements (M) are indicated.

Molecular characterization of isolates

Restriction fragment length polymorphisms Fifty isolates were grown for 15 days on V8-juice agar medium at room temperature. Mycelia, including perithecia and spores, were scraped from the agar surface using a sterile spatula and transferred to 1.5 ml Eppendorf tubes. DNA extraction was performed as described by Lee and Taylor (1990). The ITS regions and the 5.8S rDNA were amplified for all the isolates using the primers ITS1 (5′ TCC GTA GGT GAA CCT GCG G 3′) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) (White et al., 1990).

Polymerase chain reaction (PCR) consisted of 2 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.4 μM of each primer, 1 × PCR buffer, 1.25 U of *Taq* DNA Polymerase (Gibco BRL, Carlsbad, CA, USA) and 25 ng of DNA template. Amplification reactions were performed using an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 1.5 min at 94°C, 1 min at 55°C and 2 min at 72°C. A final elongation step was undertaken at 72°C for 5 min. PCR products were visualized using UV light and separated on a 1.5% agarose gel with 2 μl ethidium bromide (10 mg/ml).

PCR amplicons were digested with the restriction enzymes *Hae*III, *Alu*I, *Cla*I, *Msp*I, *Eco*RI and *Sau*3AI using the buffers and conditions recommended by the manufacturer (Gibco BRL). Restriction fragments were separated on 12% polyacrylamide gels (Sambrook et al., 1989) and fragment sizes were determined by comparison with the molecular marker ϕX174 DNA-*Hae*III (Gibco BRL) using the software IMAGE MASTER VDS version 3.0 (Amersham Pharmacia Biotech, NJ, USA).

RFLP banding profiles for each restriction enzyme were scored for the presence (1) or absence (0) of bands. A binary matrix was constructed and an unrooted UPGMA dendrogram was generated using the Jackard distance index with the program NTSYS version 2.1 (Rohlf, 2000).

Random amplified polymorphic DNA marker analyses Genetic variability in a set of 40 isolates (Table 1) of *C. fimbriata* was considered using RAPD markers. Primers OPK-08 (5′ GAACACTGGG 3′), OPD-20 (5′ GGTCTACACC 3′), OPK-13 (5′ GGTTGTACCC 3′), OPK-17 (5′ CCCAGCTGTG 3′) and OPK-12 (5′ TGGCCCTCAC 3′) (Operon Technologies, Alameda, CA, USA) were selected from an initial screening of 20 primers. Each 25 μl PCR reaction included 3 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.8 μM of primer, 1 × PCR buffer, 1 U of Taq DNA Polymerase (Gibco BRL) and 25 ng of DNA template. Amplification reactions were performed as described for RFLPs above, but using an annealing temperature of 36°C.

RAPD banding profiles for each primer were scored for the presence (1) or absence (0) of bands. A binary matrix was constructed and an unrooted UPGMA dendrogram was generated using the Jackard distance index with the program NTSYS version 2.1. Nei's gene diversity (Nei, 1973) was calculated for the data using the program POPGENE version 1.32 (Yeh et al., 1999).

DNA sequence comparisons Eight isolates representing each of the two major clades obtained from the RFLP analysis were selected for DNA sequence comparisons (Table 2). Isolates were grown in 2% malt extract broth for 15 days at room temperature. Mycelium was harvested and lyophilized. DNA extraction was performed as described by Barnes et al. (2001a). The ITS regions, a portion of the β-tubulin gene and the

Table 2
Isolates of *Ceratocystis fimbriata* from coffee plantations in Colombia for which the internal transcribed spacer (ITS) regions and the 5.8S rDNA, a portion of the β-tubulin gene and the MAT-2 HMG box sequence data were generated. Sequences from isolates obtained from other hosts were taken from the GenBank

Isolates	Host/substrate	Country	Collector	GenBank accession number		
				ITS	β-tubulin	MAT-2 HMG box
CMW10844	Coffee	Colombia	M. Marin	AY177238	AY177229	AY177242
CMW5748	Coffee	Colombia	M. Marin	AY177237	AY177227	AY177246
CMW5751	Soil from coffee plantation	Colombia	M. Marin	AY177233	AY177225	AY177239
CMW5761	Soil from coffee plantation	Colombia	M. Marin	AY177234	AY177224	AY177240
CMW5752	Soil from coffee plantation	Colombia	M. Marin	AY177231	AY177223	AY177245
CMW10875	Soil from coffee plantation	Colombia	M. Marin	AY177236	AY177228	AY177241
CMW9555	Soil from coffee plantation	Colombia	M. Marin	AY177232	AY177226	AY177243
CMW5768	Soil from coffee plantation	Colombia	M. Marin	AY177235	AY177222	AY177244
CMW3205	*Populus Tremuloides*	USA	T. Harrington	AF395695		
CMW2901	*Populus Tremuloides*	Canada	G. Samalley	AF395696		
CMW1547	*Ipomoea batatas*	Papua New Guinea	E. McKenzie	AF264904		AF164169
CMW1896	*Platanus* sp.	Switzerland	O. Petrini	AF395681		
CMW5312	*Eucalyptus grandis*	Uganda	J. Roux	AF395687		
CMW4903	*Eucalyptus* sp.	Brazil	A. Alfenas	AF395683		
CMW2911	*Prunus sp.*	USA	R. Bostock	AF395693	AY177230	

MAT-2 HMG box, were amplified using the primers ITS1 and ITS4, Bt1-a (5′TTC CCC CGT CTC CAC TTC TTC ATG 3′)/Bt1-b (5′ GAC GAG ATC GTT CAT GTT GAA CTC 3′) (Glass and Donaldson, 1995) and CFM2-1 (5′GCT ACA TTT TGT ATC GCA AAG AC 3′)/CFM2-2 (5′TAG TGG GGA TAT GTC AAC ATG 3′) (Witthuhn et al., 2000), respectively.

The 25 μl PCR mixture included 0.2 mM of each dNTP; 0.4 μM of each primer (ITS1/ITS4; Bt1-a/Bt1-b; CFM2-1/CFM2-2); 1× Expand HF buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme), 1.25 U of Expand High Fidelity PCR system enzyme mix (Roche Molecular Biochemicals, Mannheim, Germany) and 5 ng of DNA template. The PCR programme consisted of an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 30 s at 94°C, 50 s at 58°C and 2 min at 72°C. Final chain elongation took place at 72°C for 5 min.

PCR products were visualized using UV light and separated on a 1.5% agarose gel with 2 μl ethidium bromide (10 mg/ml). The products were then purified using the High Pure PCR Product Purification Kit (Roche Molecular Biochemicals) for direct sequencing, using an ABI PRISM Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and were analysed on an ABI Prism 377 DNA sequencer.

Sequences were aligned using the program SEQUENCE NAVIGATOR version 1.0.1 (Applied Biosystems) and analysis was achieved using Phylogenetic Analysis Using Parsimony (PAUP) software version 3.1.1. (Swofford, 1998). The heuristic search option based on parsimony with random stepwise addition and tree bisection reconnection was used. Gaps were treated as fifth character and confidence intervals using 1000 bootstrap replicates were calculated. Rooted trees, with sequences from *C. fimbriata* isolates from *Populus tremuloides*, *Prunus spp*. and *I. batatas* as outgroups, were constructed for ITS, β-tubulin and MAT-2 regions, respectively. Additionally, ITS sequences of isolates from plane tree, sweet potato and *Eucalyptus* were obtained from the GenBank and included in the analysis (Table 2). A partition-homogeneity test was conducted to evaluate the possibility of combining the data partitions. A mid-point rooted multilocus phylogenetic tree was generated following the same PAUP options described earlier.

Results

Fungal isolation

Ceratocystis fimbriata was isolated from all the localities sampled, including all the provinces considered in this study. In total, 79 soil samples and 53 stems with coffee canker were collected. *Ceratocystis fimbriata* was successfully isolated from 84% of the soil samples and 86% of the plant samples collected.

Colony and morphological characterization

Perithecial necks ranged from 408 to 627 (−689) μm long and their base diameters ranged between 138 and 185 (−192) μm. Hat-shaped ascospores were 2.4–2.7 (−3.4) μm long and 5.7–6.5 (−7.0) μm wide. Endoconidia were abundantly produced in all the isolates [16.4–19.4 (−20.2) × 2.4–2.7 (−2.8) μm] and chlamydospores were usually produced in chains in old cultures [12.5–13.4 (−13.6) × 10–10.9 (11.2) μm]. These measurements are similar to those previously described for *C. fimbriata* (Castaño, 1951; Hunt, 1956; Webster and Butler, 1967; Upadhyay, 1981; Prieto et al., 1987).

Isolates could be divided into four groups based on colony colour and there were three groups based on the distribution of perithecia on the medium. Most of the isolates (35) had colonies that were olive green (Plate 15 L4, Maerz and Paul, 1930), 11 isolates were stone grey (Plate 31 A2), two isolates were bronze (Plate 14 L7) and two were greyish white (Plate 35 B1). Twenty-two isolates produced perithecia in concentric rings; 14 isolates had perithecia grouped in a

central clump and 14 other isolates produced these structures evenly over the entire surface of the medium. All bronze-coloured isolates produced perithecia in concentric rings and the stone grey-coloured isolates produced perithecia only in central clumps. However, the olive green-coloured isolates included all three forms of perithecial distribution.

Regression analysis showed that *C. fimbriata* colonies had a lineal growth rate (*r* values between 0.94 and 0.99) that differed significantly among isolates ($F = 60.83$; df = 249; $P < 0.001$). *Ceratocystis fimbriata* isolates had an average growth rate of 0.527 cm/day and a range of values between 0.647 and 0.407 cm/day was found (C.V. = 2.29).

Pathogenicity tests

Two weeks after inoculation, dark lesions were observed associated with wounds on inoculated plants, showing that the fungus had colonized the stem tissue. Yellowing of foliage appeared on plants 45 days after inoculation and death of plants began 66 days later. Cankers associated with the inoculations developed both above and below the inoculation points. Epicormic shoots were produced close to the cankers in many cases but these died some weeks later as the plant died. Plants that had died usually had deep girdling cankers close to the inoculation points.

Significant differences in lesion length were observed for the different isolates used in the inoculations. Lesion lengths ranged between 2.6 and 13.6 cm, with an average of 5.4 cm among all the isolates (Fig. 2). The most virulent isolates killed more than 90% of the inoculated plants and the least virulent isolates killed <5% (Fig. 3). One isolate used in this experiment did not cause death of any plants, by the end of the 196-day duration of the experiment (Fig. 3). No symptoms were observed on plants inoculated as controls. The pathogen was consistently reisolated from inoculated plants, but was absent from the control treatment.

Isolates from both within and between provinces of Colombia had distinctly different levels of pathogenicity on coffee plants. However, most of the strains with the highest levels of pathogenicity were concentrated in the north-west region of the country (Province of Santander) (Figs 1–3; Table 1).

Molecular characterization of isolates

Restriction fragment length polymorphisms Four restriction enzymes (*Eco*RI, *Cla*I, *Hae*III and *Sau*3AI) had cutting sites in the ITS region amplified for the *C. fimbriata* isolates, whereas *Msp*I and *Alu*I did not cut the PCR products in any of the isolates. The UPGMA dendrogram generated for the binary matrix yielded two clades that were related by a similarity index of 0.43. One of the clades grouped nine isolates that shared the same RFLP profile. The other group included several subclades related by similarity indices higher than 0.65 (Fig. 4).

Random amplified polymorphic DNA marker analyses RAPD profiles consisted of six (OPK-20 and OPK-08) to 16 (OPK-13) PCR products ranging in size from 495 to 3106 bp. Sixty-eight loci were observed from all the isolates and 67 of these were polymorphic.

The UPGMA dendrogram generated for the RAPD data showed two general groups related by a similarity index of 0.45 (Fig. 5). However, four isolates grouped outside these clades. One of the two groups consisted

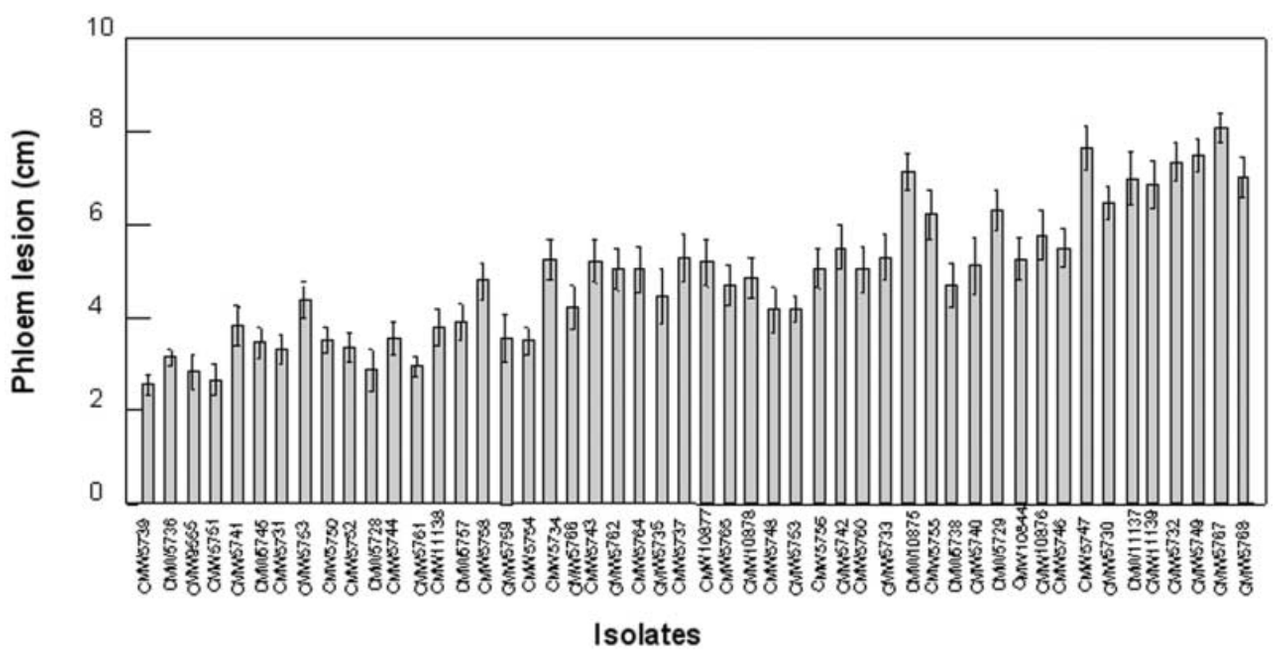

Fig. 2 Mean phloem lesion length (±SEM) after inoculation with 50 isolates of *Ceratocystis fimbriata* on 25 plants of coffee variety Caturra. Lesion lengths differ significantly from each other ($F = 2{,}62$; df = 249; $P > 0.001$)

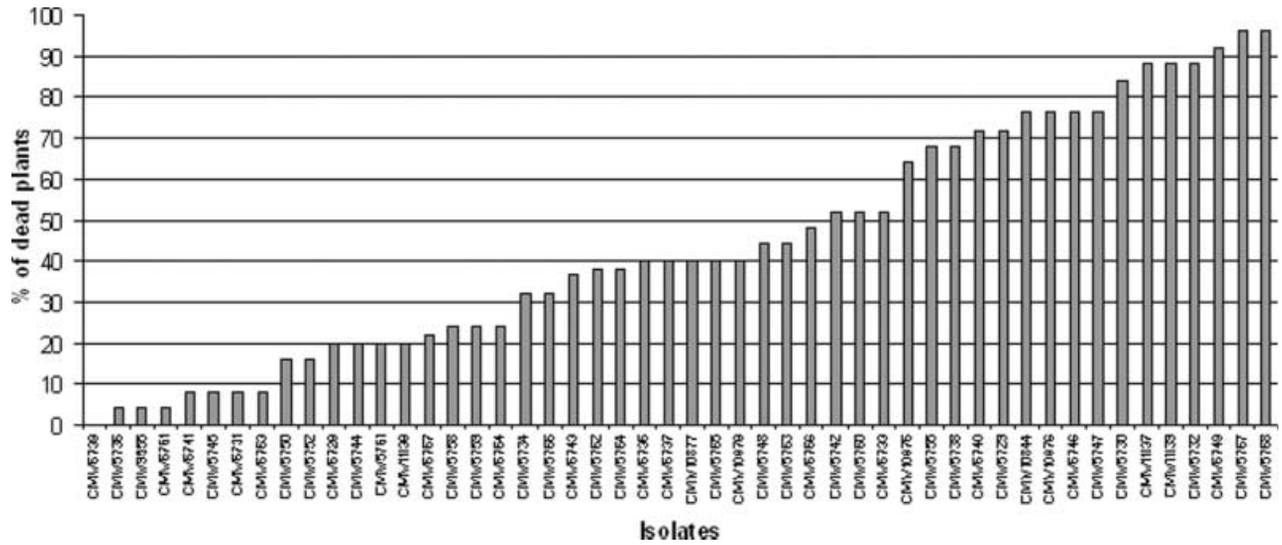

Fig. 3 Percentage of dead coffee plants, 196 days after inoculation with 50 isolates of *Ceratocystis fimbriata* from Colombia. Each isolate was inoculated on 25 plants of coffee variety Caturra

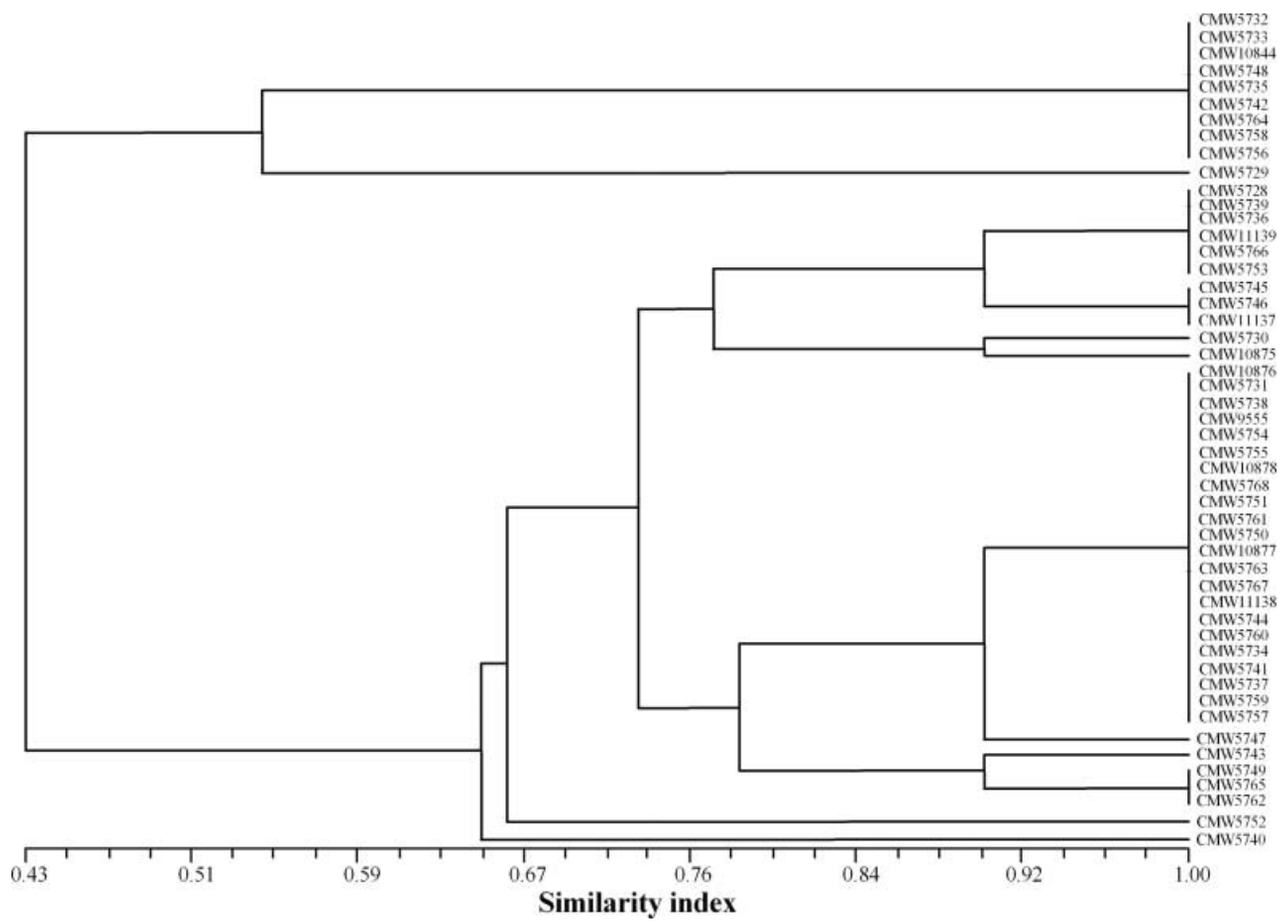

Fig. 4 Dendrogram generated from restriction fragment length polymorphisms of the ITS region using the enzymes *Eco*RI, *Cla*I, *Hae*III and *Sau*3AI, for 50 isolates of *Ceratocystis fimbriata* from Colombia. Un-rooted UPGMA tree produced from Jackard distance matrix using the software NTSYS version 2.1

of seven isolates (group I) that formed part of a nine-isolate clade of the RFLP dendrogram. Only two isolates shared the same RAPD banding profile, thus many different genotypes (38) was found across the population. This high level of genetic variability was confirmed by the Nei's gene diversity index ($H = 0.506$).

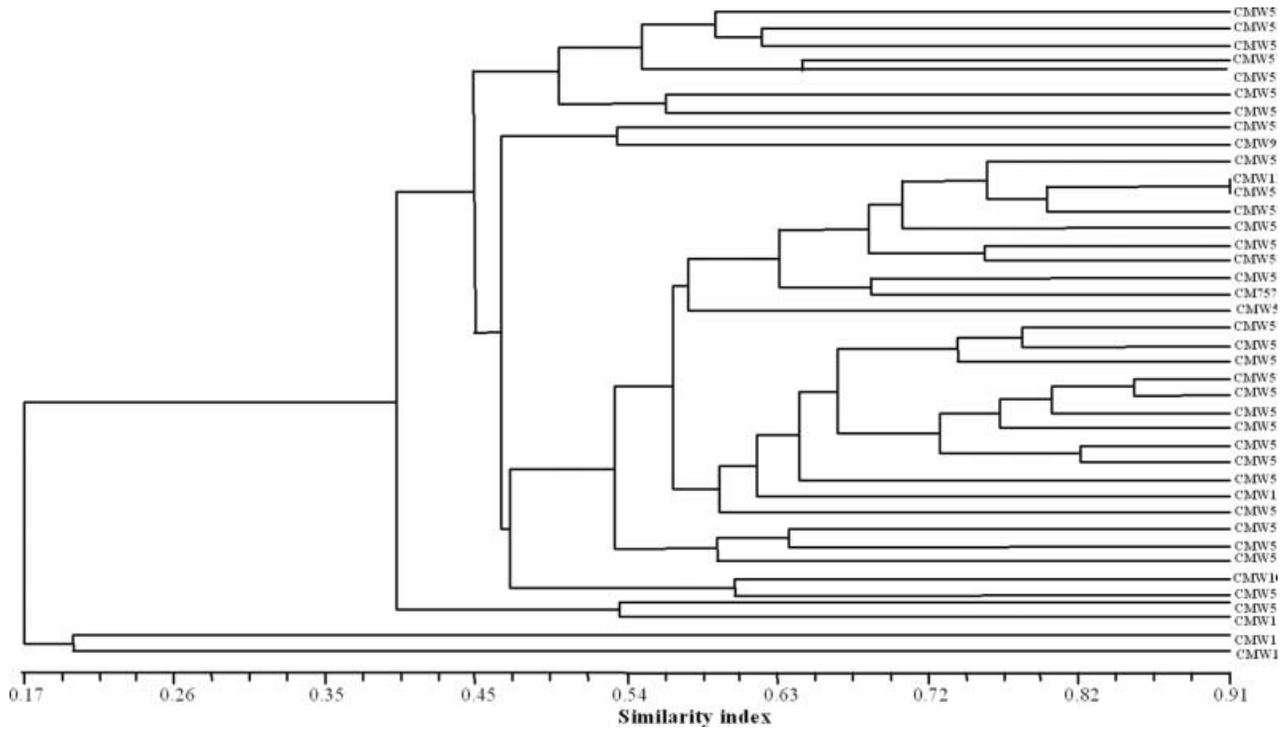

Fig. 5 Dendrogram generated from random amplified polymorphic DNA data for 40 isolates of *Ceratocystis fimbriata* from Colombia. Un-rooted UPGMA tree produced from Jackard distance matrix using the software NTSYS version 2.1.

DNA sequence comparisons Parsimony analysis of the ITS sequences for a data set of 660 characters showed that 491 characters were constant and 157 were parsimony-informative. The most parsimonious tree (215 steps) is shown in Fig. 6. This tree consisted of two major clades that differed in more than 21 nucleotide changes. The first group (I) included five *C. fimbriata* isolates from Colombia that had nearly the same ITS sequences and was strongly supported by a 100% bootstrap value. The second clade (II) was more diverse and consisted of isolates on coffee from Colombia as well as isolates from other hosts and countries. The three isolates from Colombia in this clade grouped together with a 100% bootstrap value. This tree had a consistency index (CI) of 0.87 and a retention index (RI) of 0.9.

Phylogenetic trees produced using β-tubulin gene and the MAT-2 HMG box sequences also gave rise to two different clades supported by high bootstrap values. A small number of nucleotide changes were found between these clades: four changes for the β-tubulin gene and one base changed in the MAT-2 HMG box sequence (trees not shown). However, the clades contained the same isolates that grouped together in the ITS phylogenetic tree, as well as in the RFLP's UPGMA tree.

P-value of 1.0 was obtained from the partition-homogeneity test, indicating that the three genes studied could be combined. A total of 1403 characters were included in the data set and 40 of these were parsimony-informative. This tree (length: 48; CI: 1; RI: 1) had the same topology as the individual trees, with a 100% bootstrap value supporting the separation of the isolates into two distinct different phylogenetic lineages (Fig. 7).

Discussion

In this study, we were able to isolate *C. fimbriata* from a large number of soil and plant samples collected from coffee plantations in 11 provinces of Colombia. Recovery rates were high from both soil and plant samples and our consistent isolations confirm that the pathogen is widely distributed in these coffee plantations. *Ceratocystis fimbriata* appears to be a natural inhabitant of the soils in the Andean mountains of Colombia, where coffee is most commonly cultivated. These results suggest that disease levels are most likely to worsen in the future, unless effective control measures can be found.

Comparisons of colony morphology showed that *C. fimbriata* from Colombia has the typical characteristics described for the fungus (Webster and Butler, 1967). These authors noted that isolates from coffee tend to have olive green colonies with perithecia distributed across the entire agar surface. Our observations using a much higher number of isolates showed that coffee isolates from Colombia have a much greater diversity of cultural morphology than has been reported previously. Despite differences in culture morphology, we were not able to distinguish any particular feature of fruiting structure morphology that reflected groupings of isolates. In general, the morphology of the fungus was consistent with that previously reported for isolates from coffee (Pontis, 1951; Prieto et al., 1987).

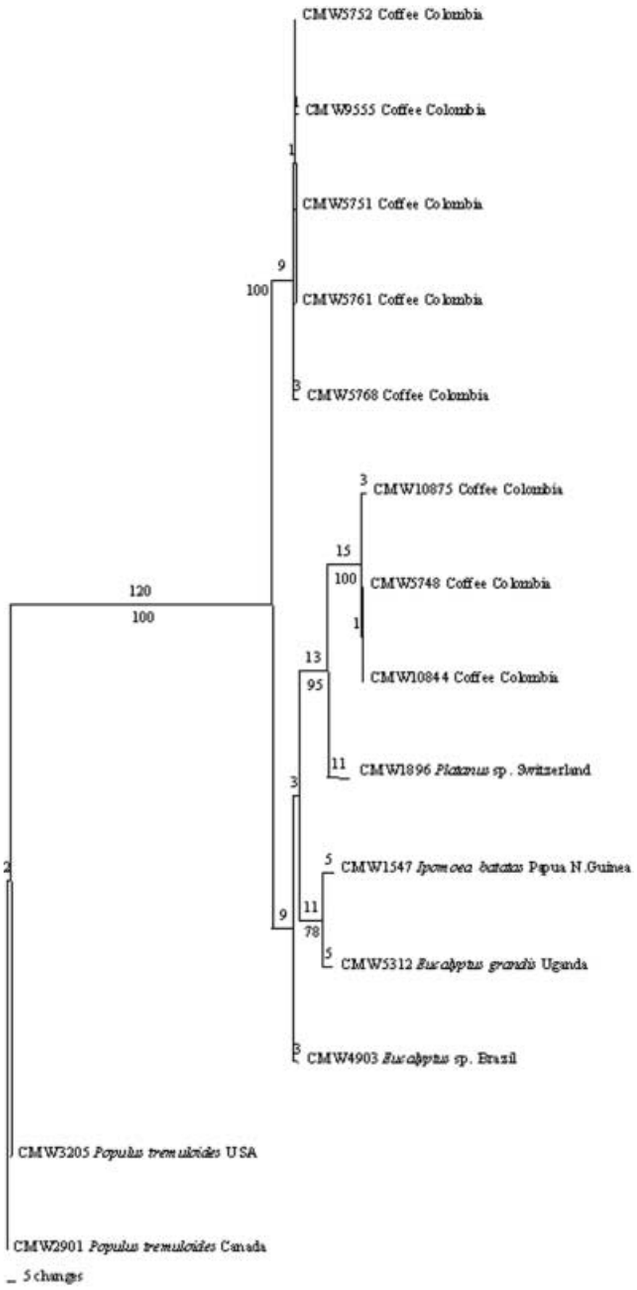

Fig. 6 The most parsimonious tree generated from DNA sequence data of ITS region of the rDNA operon for isolates of *Ceratocystis fimbriata*. Branch lengths are shown above and bootstrap values below the branches. The tree was rooted to the *C. Fimbriata* isolates CMW3205 and CMW2901 from *Populus tremuloides*. CI = 0.870; RI = 0.903; Tree length = 215

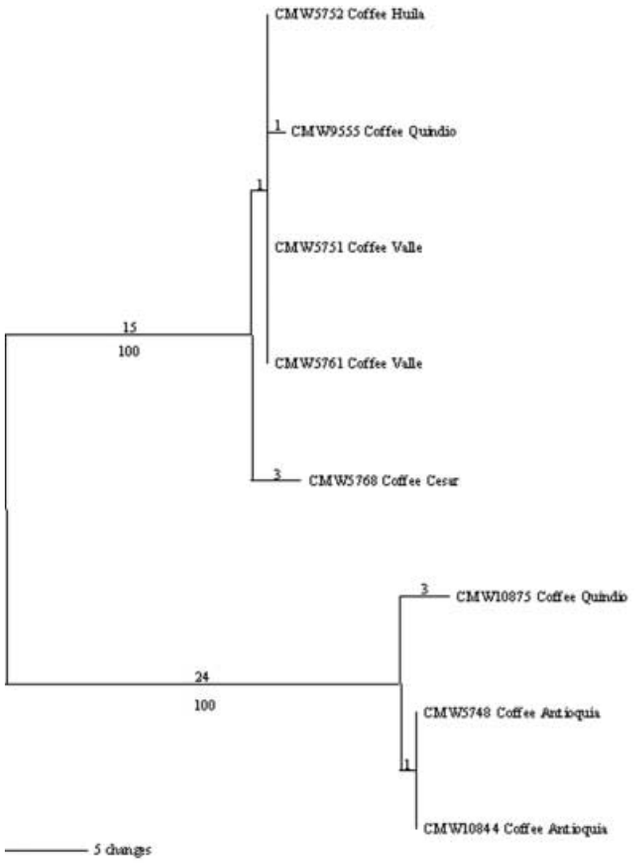

Fig. 7 One the four most parsimonious trees generated by the combined ITS, β-tubulin and MAT-2 HMG box DNA data sets for isolates of *Ceratocystis fimbriata* from Colombia. Branch lengths are shown above and bootstrap values below the branches. Midpoint rooting, CI = 1, RI = 1, Tree length = 48, P-value = 1

Pathogenicity tests showed a substantial variation in the virulence of isolates of *C. fimbriata* on coffee. Some isolates were able to kill 9-month-old coffee plants in only 66 days after inoculation, and others caused death of plants, only after 4 months. There are many possible explanations for the variation in pathogenicity of isolates. For example, differences could reflect selective pathogenicity to plants other than coffee, but the lack of inclusion of other plants, commonly known as hosts of *C. fimbriata* in Colombia, precludes us from assessing this question. What is clear is that there is a high level of variability in pathogenicity of isolates on a single coffee variety. This indicates a high level of genetic variability and possibly endemism of the fungus in Colombia.

There was a clear pattern of distribution linked to the pathogenicity of isolates. Thus, the most pathogenic strains originated in the north-west region of Colombia, where other important hosts of *C. fimbriata* (cocoa and citrus) are cultivated alongside coffee or in the case of native trees, that are used to provide shade for the plantations. We believe that these hosts play an important role in the epidemiology of *C. fimbriata*. Owing to different host–pathogen interactions some might also have provided new sources of virulence to the fungus as a result of different host–pathogen interactions. Based on our results, we recommended that agricultural authorities avoid the movement of virulent strains from the north-west region of Colombia to other coffee-growing regions, where this virulent strains appear not to be present.

Characterization of *C. fimbriata* isolates from Colombia using RFLPs showed that the isolates resided in two discrete groups. The same groups emerged from analysis of RAPD markers and sequence data for three genes. This result is consistent with that of

Barnes (2001b), who also showed that there are two discrete groups for *C. fimbriata* isolates in Colombia. The latter study was, however, based on a small number of isolates. The results of the present study provide robust evidence supporting the existence of two discrete phylogenetic lineages for *C. fimbriata* in Colombia.

It is most likely that the two phylogenetic lineages reflected in isolates of *C. fimbriata* in Colombia represent discrete taxa. *Ceratocystis fimbriata* was first described from sweet potato in 1890 in USA (Halsted, 1890). We believe that our isolates in the sequence group II are most closely related to *C. fimbriata sensu stricto*. This view is based on the fact that these isolates grouped together with several isolates obtained from different hosts and countries including sweet potato. Isolates residing in sequence group I would then represent a new taxon. However, further morphological comparisons will be necessary in order to describe it as a sibling species of *C. fimbriata*.

RAPD markers reflected high gene diversity within the isolates of *C. fimbriata* from Colombia. Such variability is comparable with values obtained for fungal species with predominant sexual reproduction, such as *Mycosphaerella fijiensis* ($H = 0.59$) (Brygoo et al., 1998) or *Venturia inaequalis* ($H = 0.46$) (Tenzer et al., 1999). However, this high value is probably due to an overestimation of genetic diversity associated with the presence of two discrete taxonomic entities that have emerged from our sequence data. Therefore, future population studies of *C. fimbriata* in Colombia should ensure that single populations consist of isolates belonging to the same phylogenetic lineage.

Our results represent the first intensive study of *C. fimbriata* in Colombia. The high variability found among isolates and distinct grouping of isolates was unexpected. Breeding and selection procedures aimed at developing planting stock resistant to *Ceratocystis* canker will need to include the knowledge that the fungus includes two distinct groups. This is particularly because planting stock resistant to one entity of the fungus need not necessarily be resistant to the other. Pathogenicity tests using selected isolates from the two phylogenetic lineages of the fungus in Colombia on a wide array of hosts are currently underway and should provide valuable information leading to the reduction of *Ceratocystis* canker in the future.

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