MOLECULAR PLANT PATHOLOGY (2001) **2**(6), 319–325

Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt pathogen *Ceratocystis fimbriata*

IRENE BARNES*, ANUPAMA GAUR, TREENA BURGESS, JOLANDA ROUX, BRENDA D. WINGFIELD[1] AND MICHAEL J. WINGFIELD

Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa; [1]*Department of Genetics, FABI, University of Pretoria, Pretoria, 0002, South Africa*

SUMMARY

Ceratocystis fimbriata is a serious wilt and canker stain pathogen with a wide geographical distribution and host range that includes both woody and herbaceous plants. Previous studies using hybridization have shown that isolates of *C. fimbriata* from different hosts and origins differ in colony morphology, pathogenicity and growth rate, as well as conidial state. It has therefore been suggested that distinct strains, linked to host or geographical origin, are encompassed in *C. fimbriata*. The aim of this study was to develop PCR-based microsatellite markers for population studies on *C. fimbriata*. ISSR-PCR was used to target specific microsatellite regions of DNA from *C. fimbriata*. These amplified products were cloned and sequenced. Primer pairs were designed from these sequences to flank the microsatellite regions. From 24 primer pairs, 11 polymorphic primers were selected and tested on a number of *C. fimbriata* isolates representing a wide host and geographical range. Cluster analyses of the results indicate that these markers clearly distinguish between different geographical and host specific populations of *C. fimbriata*. The results are concordant with sequence data from the internal transcribed spacer (ITS) region of the rDNA operon of the same isolates. These markers will be useful in future studies of *C. fimbriata* population structure and diversity.

INTRODUCTION

The fungal pathogen, *Ceratocystis fimbriata* Ell. & Halst., causes serious wilt and canker-stain diseases on a wide range of plants world-wide. Some of the economically important agricultural and tree crops damaged by this pathogen include sweet potato, cacao, stone fruit trees, poplar, rubber, coffee and *Eucalyptus* spp. (Kile, 1993; Olsen and Martin, 1949; Pontis, 1951; Roux *et al*., 2000). Although *C. fimbriata* has predominantly been reported from Central and South America (McCracken and Burkhardt, 1977), its occurrence is geographically widespread in temperate and tropical regions of the world.

The identification of *C. fimbriata* in disease reports and general taxonomic treatments has been based, for the most part, on morphological and cultural characteristics (Upadhyay, 1981). Isolates from different hosts and geographical areas, however, have been shown to differ not only in colony morphology but also in growth rate and conidial state (Webster and Butler, 1967). Pathogenicity tests have shown that some isolates tend to be host specific. For example, inoculation studies carried out on cacao, coffee and sweet potato with an isolate of *C. fimbriata* from pimento gave negative results (Leather, 1966). Likewise, Pontis (1951), inoculated coffee trees with coffee and sweet potato isolates and showed that only the coffee isolate could infect coffee.

The name, *C. fimbriata* f.sp. *platani,* has been assigned to *C. fimbriata* isolates that specifically infect plane trees (*Platanus* spp.). Although morphologically indistinguishable from the type species of *C. fimbriata* from sweet potato, the plane fungus is specifically pathogenic to its hosts. Furthermore, Webster and Butler (1967), in hybridization studies, showed that differences exist in *C. fimbriata* isolates from different hosts and origins. Based on this finding, they suggested that *C. fimbriata* comprises distinct host-specific strains. Host specialization in morphologically indistinguishable isolates illustrates the taxonomic limits of morphology. It also emphasizes the advantages of contemporary molecular techniques that might facilitate differentiation between strains or cryptic forms within fungal species (Fisher *et al*., 2000).

Many different DNA fingerprinting techniques have been used to define fungal populations. There is, however, an increasing interest in using co-dominant markers in population studies due to their ability to detect and characterize multiple alleles at a given locus. Microsatellite regions provide an attractive source of polymorphisms between isolates, and many properties favour their use as genetic markers. They are abundant in Eucaryotic

* *Correspondence* : Irene Barnes, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, 74 Lunnon Road, Pretoria, South Africa, 0002. E-mail: irene.barnes@fabi.up.ac.za

Table 1 Isolates of *Ceratocystis fimbriata* used for the development and testing of microsatellite markers and their respective microsatellite profile.

Isolate no.	Host	Country	Collector	Microsatellite profile	ITS GenBank accession no.
CMW 1547	*Ipomoea batatas*	Papua New Guinea (PNG)	E.H.C. McKenzie	4 1 225306211	AF264904
CMW 1894	*Platanus* sp.	Switzerland	O. Petrini	4 7 217673513	AF395682
CMW 1896	*Platanus* sp.	Switzerland	O. Petrini	4 7 217673513	AF395681
CMW 2218	*Platanus* sp.	France	C. Grosclaude	4 8 217663513	AF395680
CMW 2219	*Platanus* sp.	France	C. Grosclaude	4 7 217673513	AF395679
CMW 2242	*Platanus* sp.	Italy	A. Panconesi	4 7 217673513	AF264903
CMW 2901	*Populus tremuloides*	Canada	G. Smalley	3 2 251101233	AF395696
CMW 3264	*Populus tremuloides*	Canada	G. Smalley	3 4 342401823	No sequence
CMW 2911	*Prunus* sp.	USA	R. Bostock	2 2 252201133	AF395693
CMW 2913	*Populus tremuloides*	USA	T. Hinds	3 2 252201233	AF395694
CMW 4791	*Eucalyptus* clone	Congo	J. Roux	2 5 213242312	AF395685
CMW 4793	*Eucalyptus* clone	Congo	J. Roux	3 2 216432513	AF395684
CMW 4829	*Citrus* sp.	Colombia	B.L. Castro	3 2 216432513	AF395688
CMW 4835	*Coffea* sp.	Colombia	B.L. Castro	2(10)239553724	AF395689
CMW 4824	*Coffea* sp.	Colombia	B.L. Castro	2(10)332553724	AF395692
CMW 4844	*Coffea* sp.	Colombia	B.L. Castro	1 9 216225312	AF395691
CMW 4845	*Coffea* sp.	Colombia	B.L. Castro	1 4 214274312	AF395690
CMW 4903	*Eucalyptus* sp.	Brazil	A. Alfenas	1 6 214215312	AF395683
CMW 5312	*Eucalyptus grandis*	Uganda	J. Roux	3 2 117332513	AF395687
CMW 5328	*Eucalyptus grandis*	Uganda	J. Roux	3 3 218432413	AF395686

All isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

genomes, have a co-dominant inheritance, are highly polymorphic, easy to use and score, and they are selectively neutral with a high mutation rate (Rafalski *et al.*, 1996; Tautz, 1989). In this study, we report on the development and characterization of PCR based microsatellite markers for *C. fimbriata.* These primers were tested on isolates collected from a wide geographical and host range (Table 1). Results based on microsatellites were also compared with those from sequence data obtained from the internal transcribed spacer region (ITS).

The isolates of *Ceratocystis fimbriata* were grown on potato dextrose agar (Biolab). Hyphal tips from germinating ascospores were isolated and incubated for 2 weeks at 25 °C in 50 mL malt extract broth (2%, Biolab). The mycelium was collected by filtration, lyophilized, and DNA was extracted for each isolate as described previously (Barnes *et al.*, 2001).

DNA from isolates CMW 4822 and CMW 4835 was randomly amplified using ISSR primers 5′DHB$(CGA)_5$, 5′HV$(GT)_5$G, 5′DDB$(CCA)_5$, 5′DBD$(CAC)_5$, 5′HVH$(GTG)_5$, 5′NDB$(CA)_7$C and 5′NDV$(CT)_8$. The polymerase chain reaction (PCR) mixture consisted of a final concentration of 2 ng DNA, 200 μM of each dNTP, 600 nM primer, 3.5 U *Taq* DNA polymerase and 1 × buffer with $MgCl_2$ (Roche Molecular Biochemicals, Alameda, CA). PCR conditions consisted of a 2 min denaturation step at 95 °C followed by 40 cycles of 30 s at 95 °C, 45 s at 48 °C and 2 min at 72 °C and a final step at 72 °C for 10 min. The ISSR primers produced between 7 and 10 bands each, ranging in size from 200 to 2600 bp. These products were purified using the Magic PCR Preps Purification System (Promega Corp., Madison, WI), and cloned using the pGEM®-T Easy Vector System (Promega Corp.) according to the manufacturer's instructions. Recombinant plasmids were selected and grown in 2 mL Luria-Bertani broth supplemented with 100 μg/mL ampicillin (Sigma Chemical Co., USA). The cells were incubated at 37 °C for 8 h, after which plasmid DNA was recovered using alkaline lysis (Sambrook *et al.*, 1989) and re-suspended in 20 μL water containing RNase (10 mg/mL, Roche Molecular Biochemicals). Restriction digests with *Eco*RI (Roche Molecular Biochemicals) were performed on the plasmid DNA. The sizes of the cloned fragments were determined using Agarose gel electrophoresis.

For each ISSR reaction, only the clones containing different sized inserts with lengths smaller than 1000 bp were sequenced using an ABI PRISM™ 377 Autosequencer (Perkin-Elmer Applied Biosystems Inc., Forster City, CA). Sequence reactions were carried out with universal plasmid primers T7 and SP6 using an ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems). Sequence electropherograms were analysed using Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems).

A total of 52 cloned fragments were sequenced to screen for microsatellite regions. From these, 18 fragments were found to contain microsatellite regions. Primer pairs were designed to flank these regions. Six of the cloned fragments contained microsatellite regions at the beginning of the sequence. Genome walking was performed as described by Burgess *et al.* (2001) using

DNA from isolate CMW 4829 digested with the restriction enzymes *Eco*RV, *Sca*I, *Hae*III and *Rsa*I (Roche Molecular Biochemicals). Primers were designed to flank the full microsatellite sequence.

A total of 26 primers were designed from the cloned fragments. These primers were further tested for polymorphisms using DNA from five isolates (CMW2218, CMW2242, CMW4791, CMW4829 and CMW5328) from different geographical locations. PCR reactions were performed in 25 μL reaction volumes, consisting of 1 ng DNA, 200 μM of each dNTP, 300 nM of each primer, 0.35 U Expand High Fidelity enzyme (Roche Molecular Biochemicals) and 1.2 × Expand HF buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme). The PCR programme consisted of a 2 min denaturation step at 96 °C followed by 10 cycles of 20 s at 94 °C, 48 s at the specified annealing temperature for each primer and 45 s at 72 °C. A further 25 cycles were carried out with a 5 s extension after each cycle with the annealing time altered to 40 s. A final elongation step was carried out for 10 min at 72 °C. Four primer pairs failed to amplify in PCR reactions at different annealing temperatures and one primer pair produced multiple bands. These five primers were discarded. Fractionation of the rest of the samples on PAGE gels (6% acrylamide in 50 mM Tris-borate-EDTA buffer; 7 h at 140 V), revealed that of the 19 remaining primer pairs, eight were monomorphic and 11 polymorphic.

One primer from each polymorphic primer pair, was resynthesized and labelled with either a TET or FAM phosphoramidite fluorescent dye (MWG, Ebersberg, Germany) (Table 2). The labelled primers were then used in subsequent PCR reactions to amplify all *C. fimbriata* isolates in this study (Table 1). Fluorescent-labelled PCR products (0.5 μL containing 1.5 ng DNA) were combined with 0.5 μL of the internal standard GENESCAN-TAMRA (Perkin-Elmer Corp.) and 1.5 μL of loading buffer. The samples were fractionated by PAGE (4.25%) on an ABI Prism 377 DNA sequencer. The size of the DNA fragments was determined using a combination of the GeneScan® 2.1 analysis software (Perkin Elmer Corp.) and Genotyper® 3.0 (Perkin Elmer Corp.). Genescan analysis showed that the polymorphic markers produced a total of 65 alleles across the 11 loci analysed (Table 2). The smallest and largest number of alleles per locus was 3 and 10, respectively. Alleles ranged from 154 to 415 bp in length.

Of the 11 loci, two or more alleles from 6 loci were sequenced to determine the source of polymorphisms. Most of the polymorphisms observed between the alleles were due to mutations within the repetitive microsatellite regions (Fig. 1). Indels of varying lengths also contributed to the polymorphisms. For example, the sequence data generated from the PCR amplicon using primer CF15/16 in isolate CMW 2901 from Canada, showed there was an insertion of 12 bp which was absent in all the other alleles sequenced (Fig. 1). However, elsewhere in the allele sequence of this isolate, there was a deletion of 13 bp. Thus, a size difference of only one base pair was observed, despite the fact that the polymorphism was considerably larger. This indicates that the microsatellite data might underestimate the genetic diversity.

Primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), were used to sequence the ITS1, 5.8S and ITS4 ribosomal RNA operon regions. The PCR reaction mix consisted of 2–10 ng DNA, Expand High Fidelity PCR System enzyme mix (1.75 U) (Roche Molecular Biochemicals), 1 × Expand HF buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme), 200 nM of each primer and 200 μM of each dNTP. The same PCR programme was used as described above for the microsatellite primers. The annealing temperature was set to 55 °C. PCR amplicons were purified using the Magic PCR Preps Purification System (Promega, Madison, WI) and sequenced in both directions.

For each isolate, the presence or absence of an allele at a specific locus was scored for each microsatellite primer and the data compiled in a matrix. Both the microsatellite matrix and the ITS sequences were analysed using PAUP (Phylogenetic Analysis Using Parsimony [*and other methods]) (Swofford, 1998). The heuristic search option (based on parsimony) with random step-wise addition and tree bisection reconnection (TBR) was used. All characters were given equal weight and the Mulpar option was in effect. The length distribution of 1000 trees and confidence intervals using 1000 bootstrap replicates were calculated. *Ceratocystis albofundus* de Beer, Wingfield & Morris (CMW 2475, accession no. AF043605), was used as the outgroup for the ITS sequence data set and treated as a monophyletic sister group with respect to the ingroup. All trees generated from the microsatellite matrix were rooted using the Midpoint rooting option. The microsatellite data matrix was also analysed using cluster analysis of the Bray–Curtis coefficient of similarity and Euclidean distance using the program PRIMER (Clarke and Warwick, 1994). Four parsimonious trees for both the microsatellite and the ITS data were generated, with tree lengths of 96 and 389, respectively. One tree from the ITS data (Fig. 2), with similar topology and minor differences in branch lengths from the other three trees was chosen for presentation. The dendograms produced from the analysis of the microsatellite data using the Bray–Curtis coefficient of similarity and the Euclidean distance were similar to the phylogenetic trees produced using PAUP. The dendogram based on similarity analysis was selected for presentation (Fig. 2), and bootstrap values based on parsimony are included.

The phylogenetic and similarity trees generated from the microsatellite and ITS data clearly resolved isolates of *C. fimbriata* into distinct subgroups based on hosts. All the *Platanus* isolates from France, Switzerland and Italy grouped together, forming a European subgroup with strong bootstrap values (100%) in both the microsatellite and the ITS tree. Within some of the other host-based subgroups, it was also possible to recognize further groupings linked to the geographical origin of isolates. For example,

Table 2 Core sequences and allelic properties of polymorphic primers designed for *C. fimbriata.*

Primer	Primer sequence	Ta*	Fluorescent label	Core sequence	Allelic range	No. of alleles	Alleles	GenBank acc. no.
AG 1 AG 2	CGG GGA TGC TGT TGT CTC G GTT TCT CGA CTT CCA GGC CC	58	FAM	(T)7C(T)2CGC(T)4(CTTT)2GC(T)4C(T)3C(T)2G(T)4(CTT)2	255–266	4	255,263,265,266	AY055016
AG 7 AG 8	GGG GCG GTG GTG CAA TTG TC CGA GAC AGC AAC ACA AGC CC	56	TET	(TC)21(TTC)2	284–304	10	284,285,286,288,289,290,298,299,301,304	AY055017
AG 15 AG 16	GGA TAG CAG CGA CAA GGA CC CTT GAC CGA CCT GCC GAT TG	62	FAM	Regions rich in A interrupted by C and G	272–276	3	272,274,276	AY055018
AG 17 AG 18	CGG CCC TGC CAA CGG ATG GTC GGT GGT GGA GAC GGT C	62	FAM	(T)5(C)2(CT)2T(CTT)6(T)2(C)3TC(T)3	304–309	5	304,305,307,308,309	AY055019
CF 5 CF 6	CAT GGG CAT GCC TAG CCT TG GAC CAA AGA TGG TGG CGA GC	62	TET	(TGC)11	359–385	9	359,365,366,368,369,371,377,380,385	AY055020
CF 11 CF 12	GGG ACG AAA CTG GAG CGT CT CTC CCA AAC TCC ATG CTC TTG	60	TET	CA(AC)7GC(AC)2(N)*x*(G)8	216–230	6	216,217,218,219,222,230	AY055021
CF 13 CF 14	GAT CGA TCG GCA GAC CGA TAC GGA ACT CTG ATG CCT CCA GTG	64	TET	(T)5(N)*x*(A)7(N)*x*(C)11(N)*x*(AGCAC)5	402–415	7	402,403,405,406,410,414,415	AY055022
CF 15 CF 16	CAG GGA CTA GGG TCT GCC AG CGT TTG CAA GGC AAG GCA GC	60	FAM	(CT)5(N)*x*(CT)3(N)*x*(CT)3 sequence rich in T	218–267	6	218,240,248,250,254,267	AY055023
CF 17 CF 18	CGA GCC AAG ACG TTC ATT GAA G GAA ACC GAG AGT CAT CGT CC	64	TET	(CA)15 sequence rich in GT and T	266–292	8	266,267,268,271,272,277,279,292	AY055024
CF 21 CF 22	GCA CTA CGA GAA TAG AAT GCA G GCG TTG AAA GAT GTG GCG TG	60	TET	(T)8(N)*x*(T)6(N)*x*(C)2(T)3C(CT)2(CCTT)2C(T)3C(T)2C(T)4	250–259	3	250,255,259	AY055025
CF 23 CF 24	CAG GGA ATT CCC GAT GGC AG CAT GAT CGA CAA GGG CGC TG	60	TET	TGCA(TG)15	154–168	4	154,156,160,168	AY055026

*The annealing temperature is represented by Ta (°C).


```
PRIMER AG 7/8
CMW4829  *A*T*TAAAT*CCGGTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTTCTTCCC--TCCCG*
CMW1896  *A*G*TAAAC*CCGGTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC--------TTCTTCCCCCTCCCG*
CMW5312  *G*G*TAAAT*CCGGTCTCTCTCTCTCTCTCTC----------------------TTCTTCCCCCTCCCG*
CMW5328  *G*G*TAAAT*CCGGTCTCTCTCTCTCTCTCTC----------------------TTCTTCCCCCTCCCG*

PRIMER AG 17/18
CMW4829  *GTTTTTCCCTCTTCTTCTTCTTCTTCTTTTCCCTCTTTAAAA*G*T*
CMW4835  *GTTTTTCCCTCTTCTTCTTCTTCTTCTTTTCCCTCTTTAAAA*G*T*
CMW5312  *GTTTTTCCCTCTTCTTCTTCTTCTT---TTCCCTCTTTAAAA*A*T*
CMW1894  *GTTTTTCCCTCTTCTTCTTCTTCTT---TTCCCTCTTTAAAA*G*T*
CMW4903  *GTTTTTCCCTCTTCTTCTTCTTCTT---TTCCCTCTTTAAAA*G*A*

PRIMER CF 5/6
CMW4903  *TTTT-*G*G*GTGCTGCTGCTGCTG------------------*A*CCTTCTTC------TTTC*G*
CMW5383  *TTTT-*A*A*GTGCTGCTGCTGCTGCTG---------------*A*TCGGCCCTCTTATTTTTC*G*
CMW1896  *TTTTT*A*G*GTGCTGCTGCTGCTGCTGCTGCTG---------*G*TCGGCCCTCTTATTTTTC*C*
CMW4829  *TTTTC*A*G*GTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG*A*TCGGCCCTCTTATTTTTC*C*

PRIMER CF 15/16
CMW4829  *C*------------*T*A*A*CC*CTCGCTCTTG*TCTCTCTCTCT------CCCA*T*T*CCCAGTCTCTCTCG*CTCTCCCCACCTA*T*A*T*
CMW2901  *C*CCGTGAGTATTT*C*G*A*AC*----------*TCTCTCTCTCTC---------*-*G*C------------A*-------------*C*G*C*
CMW2219  *A*------------*T*G*G*CT*CTCGCTCTTG*TCTCTCTCTCTCT----CCCA*T*T*CCCAGTCTCTCTCG*CTCTCCCCACCTA*T*A*C*
CMW4795  *C*------------*T*G*G*CC*CTCGCTCTTG*TCTCTCTCTCTCTCTCTCCCA*T*T*C------------G*CTCTCCCCACCTA*T*A*T*
CMW5312  *C*------------*T*G*G*CC*CTCGCTCTTG*TCTCTCTCTCTCTCTCTCCCA*T*T*C------------G*CTCTCCCCACCTA*T*A*T*

PRIMER CF 17/18
CMW5328  *C*T*GAAACACACACACACACA------------T*A*A*A*
CMW4793  *C*C*CACACACACACACACACA------------T*A*A*A*
CMW1896  *C*C*CACACACACACACACACA------------T*A*G*A*
CMW2911  *A*C*CACACACACACACACACA------------T*A*A*A*
CMW4829  *C*C*CACACACACACACACACACACACACACACAT*A*G*G*

PRIMER CF 21/22
CMW4822  *A*C*G*G*TAAAAG*T*A*TCCCT*GA*G*AATTAAA------------GCCTTTTTTTT-GTCCC--*TTC*C*T*G*A*G*CCT*C*C-*CT*T*T*A*A*C*
CMW4791  *A*C*G*G*TAAAAG*T*A*TCCCT*GA*G*AATCAAA------------GCCTTTT-----GTCCC--*TTC*T*C*G*A*G*CCT*C*TC*CT*T*T*A*G*C*
CMW4903  *A*C*G*G*TAAAAT*T*A*TCCCT*GA*G*AATCAAA------------GCCTTTT-----GTCCC--*TTC*T*C*G*A*G*CCT*C*CC*CT*T*T*A*G*C*
CMW4835  *A*C*G*G*TAAAAG*T*A*TCCCT*GA*G*AATCAAA------------GCCTTTTTTTTTGTCCC--*TTC*T*T*G*A*G*CCT*C*CC*CT*T*T*A*A*C*
CMW2901  *G*T*C*A*AGAAGG*C*G*TCC-T*AG*A*TATAAAGAAAAGAAAAAAAGCCTTTT-----G-CCCTG*TTC*T*C*A*G*A*C--*T*CC*GC*G*C*G*A*T*
```

* Indicates varying lengths of homologous sequence between isolates
\- Indicates absence of nucleotides within a sequence.

Fig. 1 Sequences of *Ceratocystis fimbriata* isolates produced from primers AG 7/8, AG 17/18, CF 5/6, CF 15/16, CF 17/18 and CF 21/22.

all the isolates collected from diseased *Eucalyptus* species grouped collectively in a Central African clade, but this could be further subdivided to separate isolates from Congo and Uganda. *C. fimbriata* isolates from Congo and Uganda grouped together with 62% confidence in the microsatellite and 51% in the ITS tree. The same was true for the isolates from North America, where those infecting *Populus* spp. were distinct from those from *Prunus*. Within the *Populus* subgroup, isolates from Canada and the USA were distinct. An intriguing observation was that isolates from Colombia were resident in two strongly resolved clades, even though the isolates were taken from the same host. We believe that the Colombian groups are genetically isolated and further studies using the microsatellite primers and a greater number of isolates are needed to test this hypothesis.

Santini and Capretti (2000), used RAPD and direct amplification of minisatellite-region DNA (DAMD) PCR to examine the genetic variability in a population of *C. fimbriata* f.sp. *platani* from Italy and some isolates of *C. fimbriata* from North America. Their study showed that all Italian isolates have the same genotype, thus representing a clone. Although they suggested that the entire European population might be clonal and the result of a single introduction, the results of our study based on microsatellites show otherwise. Isolates from Switzerland, France and Italy had the same genotype, but differed from a second isolate from France. Although the numbers of isolates from Europe were few in our study, there is evidence for at least two different genotypes in Europe. We believe that this difference is due to the fact that Santini *et al.* (2000) used dominant markers, which have lower resolution than the co-dominant markers used in the present study.

We have shown that isolates from *Populus* in North America reside in a group that is distinct from the *Prunus* isolate. These results are similar to those of Santini *et al.* (2000), and strongly support the view that isolates of *C. fimbriata* can be divided into groups based on host. These data also support those emerging from hybridization experiments (Webster and Butler, 1967), host specificity (Kojima *et al.*, 1982), and pathogenicity assays (Leather, 1966; Pontis, 1951; Walter *et al.*, 1952), suggesting that the host represents an important factor separating isolates of *C. fimbriata.* Furthermore, the hypothesis that host range is an

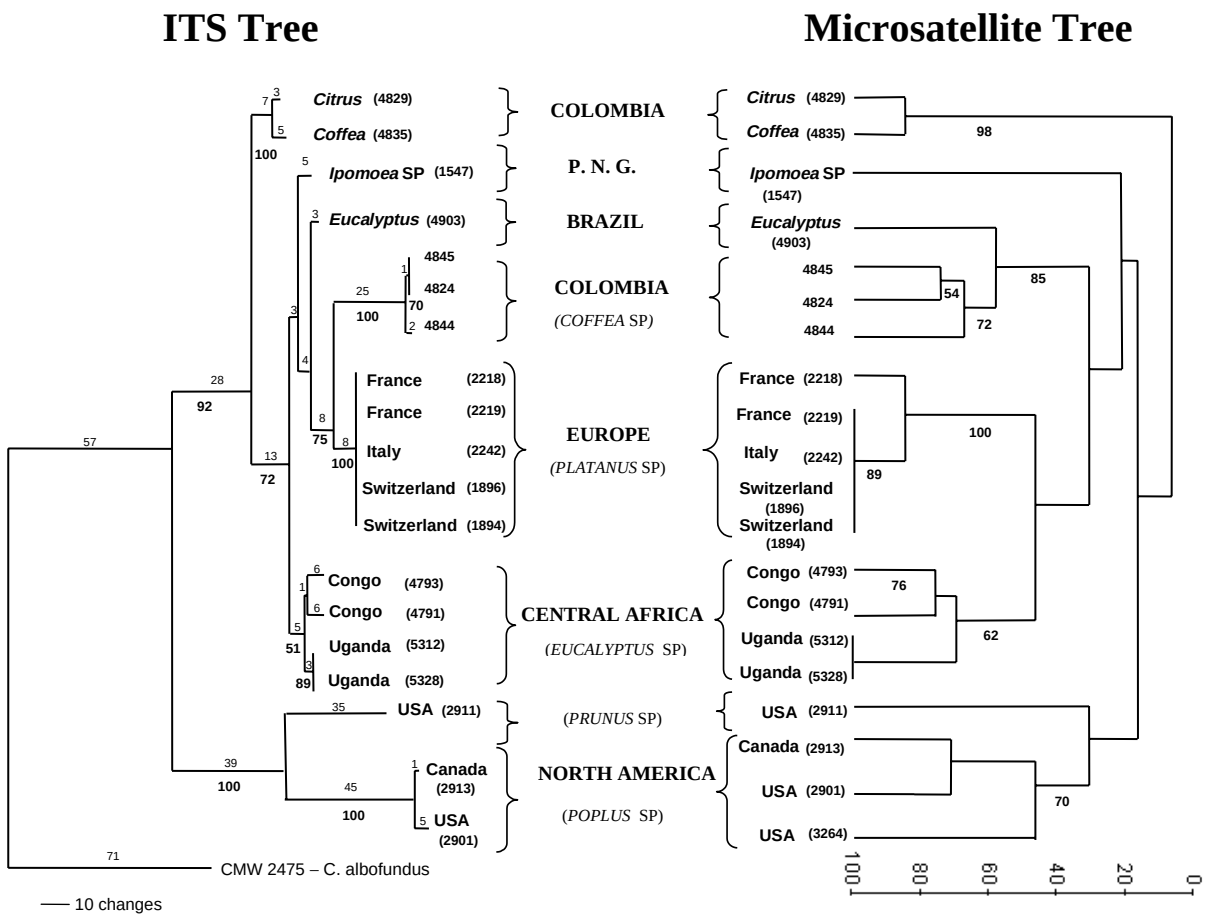

Fig. 2 Comparison of a dendogram for *Ceratocystis fimbriata* isolates produced using 11 polymorphic microsatellite loci with a phylogram produced from the ITS sequences of the same isolates. The dendogram was produced using cluster analysis of Bray–Curtis coefficient of similarity in PRIMER and the phylogram using the Heuristic search option with parsimony in PAUP 4. Bootstrap values are indicated below the branches while the number of base substitutions is indicated above the branches.

important factor in the evolution of *Ceratocystis* spp. (Witthuhn *et al.*, 1998) is supported by the results of this study.

Some alleles present in the 11 loci were unique to certain isolates or groups of isolates (Table 1). Goodwin *et al.* (1992), termed these 'private alleles'. In the North American subgroup for example, three alleles in different loci were unique to this group and not found in any other isolate. The same was true with the Central African subgroup that had one, and of the Colombian subgroups that had five unique alleles, not shared by any other isolates. Private alleles provide evidence for reproductive isolation over a long period (Fisher *et al.*, 2000), and the fact that these groups had unique alleles further defines the subgroups.

Detailed studies of the frequencies of specific alleles, particularly private alleles, in populations of *C. fimbriata* from different geographically diverse origins will make it possible to determine gene flow between populations, as well as random mating mechanisms within the populations. Allelic differences at these polymorphic loci can also be used to determine evolutionary history and relationships between populations of *C. fimbriata* from different countries. Determining the geographical origin of *C. fimbriata* will also aid in the development of management and quarantine strategies against certain strains of *C. fimbriata.*

In this study, 11 microsatellite primers that display strong polymorphisms in a wide variety of *C. fimbriata* isolates were produced. The markers clearly elucidate relationships between the different isolates based on host and geographical origin. These relationships were supported by an analysis of sequence data from the ITS region of the rDNA operon. The results also support those of previous studies based on hybridization (Webster and Butler, 1967) and pathogenicity (Leather, 1966; Pontis, 1951; Walter *et al.*, 1952), showing that *C. fimbriata* encompasses a wide variety of strains.

ACKNOWLEDGEMENTS

We thank the National Research Foundation (NRF), members of the Tree Pathology Co-operative Program (TPCP), the THRIP

programme of the Department of Trade and Industry, and the University of Pretoria, South Africa, for financial support. We are also most grateful to colleagues in various parts of the world, and to culture collections for providing cultures without which this study could not have been undertaken.

REFERENCES

Barnes, I., Roux, J., Coetzee, M.P.A., Wingfield, B.D. and Wingfield, M.J. (2001) Characterisation of *Seiridium* spp. associated with Cypress Canker based on B-tubulin and histone sequences. *Plant Dis.* **85**, 317–321.

Burgess, T., Wingfield, M.J. and Wingfield, B.D. (2001) Simple sequence repeat markers distinguish among morphotypes of *Sphaeropsis sapinea*. *Appl. Environ. Microbiol.* **67**, 354–362.

Clarke, K.R. and Warwick, R.M. (1994) Measures of similarity of species abundance/biomass between samples. In: *Change in Marine Communities: an Approach to Statistical Analysis and Interpretation*, Chapter 2. Plymouth, UK: Plymouth Marine Laboratory, Natural Environment Council, pp. 1–8.

Fisher, M.C., Koenig, G., White, T.J. and Taylor, J.W. (2000) A test for concordance between the multilocus genealogies of genes and microsatellite in the pathogenic fungus *Coccidioides immitis*. *Mol. Biol. Evol.* **17**, 1164–1174.

Goodwin, S.B., Spielman, L.J., Matuszak, J.M., Bergeron, S.N. and Fry, W.E. (1992) Clonal diversity and genetic differentiation of *Phytophthora infestans* populations in northern and central Mexico. *Phytopathology*, **82**, 955–961.

Kile, G.A. (1993) Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (Wingfield, M.J., Seifert, K.A. and Webber, J.A., eds). St. Paul, Minnesota: APS Press, pp. 173–183.

Kojima, M., Kawakita, K. and Uritani, I. (1982) Studies on a factor in sweet potato root which agglutinates spores of *Ceratocystis fimbriata*, Black Rot Fungus. *Plant Physiol.* **69**, 474–478.

Leather, R.I. (1966) A canker and wilt disease of pimento (*Pimenta officinalis*) caused by *Ceratocystis fimbriata* in Jamaica. *Trans. Br. Soc.* **42**, 213–218.

McCracken, F.I. and Burkhardt, E.C. (1977) Destruction of sycamore by canker stain in the midsouth. *Plant Dis. Rep.* **61**, 984–986.

Olsen, E.O. and Martin, W.J. (1949) Relationship of *Ceratostomella fimbriata* from *Hevea* rubber tree and sweet potato. *Phytopathology*, **39**, 17.

Pontis, R.E. (1951) A canker disease of the coffee tree in Colombia and Venezuela. *Phytopathology*, **41**, 179–184.

Rafalski, J.A., Vogel, J.M., Morgante, M., Powell, W., Andre, C. and Tingey, S.V. (1996) Generating and using DNA markers in plants. In: *Nonmammaliean Genomic Analysis: a Practical Guide*. Academic Press, pp. 75–133.

Roux, J., Wingfield, M.J., Bouillette, J.P., Wingfield, B.D. and Alfenas, A.C. (2000) A serious new wilt disease of *Eucalyptus* caused by *Ceratocystis fimbriata* in Central Africa. *For. Pathol.* **30**, 175–184.

Sambrook, J., Fritsch, T. and Maniatis, T. (1989) *Molecular Cloning—a Laboratory Manual*. New York: Cold Spring Harbour Laboratory Press.

Santini, A. and Capretti, P. (2000) Analysis of the Italian population of *Ceratocystis fimbriata* f.sp. *platani* using RAPD and minisatellite markers. *Plant Pathol.* **49**, 461–467.

Swofford, D.L. (1998) *PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods)*, Version 4. Sunderland, MA: Sinaur Associates.

Tautz, D. (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucl. Acids Res.* **17**, 6463–6471.

Upadhyay, H.P. (1981) A monograph of *Ceratocystis* and *Ceratocystiopsis*. Athens, GA: University of Georgia Press.

Walter, J.M., Rex, E.G. and Schreiber, R. (1952) The rate of progress and destructiveness of canker stain of plane trees. *Phytopathology*, **42**, 236–239.

Webster, R.K. and Butler, E.E. (1967) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Can. J. Bot.* **45**, 1457–1468.

Witthuhn, R.C., Wingfield, B.D., Wingfield, M.J., Wolfaardt, M. and Harrington, T.C. (1998) Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. *Mycologia*, **90**, 96–101.