CHAPTER 4

TWO SPECIES OF *MYCOSPHAERELLA* ARE ASSOCIATED WITH MYCOSPHAERELLA SPECKLE ON BANANA FOLIAGE IN SOUTH AFRICA

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ABSTRACT

Mycosphaerella musae causes Mycosphaerella leaf speckle on banana leaves. Surveys conducted in the five banana growing areas of South Africa during 1999–2001 resulted in the collection of various leaf specimens exhibiting speckle symptoms. Following morphological examination of the infected material, monoconidial and hyphal tip isolates of the causal organisms were established from each sample. Sequence data of the ITS region was obtained to compare the South African *Mycosphaerella* isolates from banana leaves with *M. musicola*, *M. fijiensis*, *M. musae* and *M. eumusae*. These results confirm that *M. musae* and a species closely related to *M. colombiensis*, a *Eucalyptus* leaf blotch pathogen reported from Colombia, are causing speckle disease. The latter two are responsible for speckle symptoms on banana leaves in South Africa.

Introduction

Four of the various fungal leaf diseases described on banana (*Musa* spp.) involve species in the genus *Mycosphaerella*, namely *Mycosphaerella musicola* R. Leach (yellow Sigatoka), *M. fijiensis* M. Morelet (black Sigatoka), *M. musae* (Speg.) Syd. & P. Syd. (Mycosphaerella speckle), and *M. eumusae* Crous & Mourichon (eumusae leaf spot) (Jones 2000, Crous & Mourichon 2002). *Mycosphaerella fijiensis* is considered to be the most severe pathogen of banana foliage, causing serious losses in tropical regions (Jones 2000). Since 1962, *M. musicola* has acquired a worldwide distribution, and now occurs in every banana growing country except Egypt, Israel and the Canary Islands (Carlier *et al.* 1994, Jones 2000). Although it is a less virulent pathogen than *M. fijiensis,* it is considered to be important in sub-tropical countries. *Mycosphaerella musae* has a worldwide distribution, but is only considered important in areas with sub-tropical climates (Jones 2000).

Very little research has been conducted on foliar diseases of banana in South Africa, with only two *Mycosphaerella* diseases being recorded. Van den Boom and Kuhne (1969) reported the presence of *M. musicola* in South Africa. This report, however, was based on field symptoms, and the local presence of this pathogen has only recently been confirmed via morphology, pathogenicity and DNA sequence data (Surridge *et al.* 2003a). Brodrick (1973) reported the presence of *M. musae* in South Africa, though this report was erroneously quoted as a first report of *Cladosporium musae* E.W. Mason in subsequent literature (Gorter 1977, Crous *et al.* 2000). Although this situation has been resolved by the confirmation of *C. musae* causing Cladosporium speckle on banana in South Africa (Surridge *et al.* 2003b), the etiology of *M. musae* remains unresolved. To elucidate the disease status, surveys of the five banana growing

areas in South Africa were conducted during 1999–2001, which resulted in the collection of various specimens exhibiting symptoms of Mycosphaerella speckle. The purpose of the present study was to establish the identity of the organism(s) responsible for causing Mycosphaerella speckle of banana in South Africa.

MATERIALS AND METHODS

Isolates

Between 1999 and 2001, 147 leaf samples exhibiting symptoms of Mycosphaerella speckle were randomly collected from Cavendish cultivars in the five banana growing areas of South Africa: Levubu, Tzaneen, Kiepersol, Komatipoort and southern KwaZulu-Natal. Samples were transported to the laboratory in paper envelopes and maintained at 5 °C until being processed (2–3 d).

A portion of a lesion on each leaf was excised and submersed in 2 % sodium hypochlorite for 30 sec., transferred to 70 % ethanol for 1 min, and rinsed twice in sterile distilled water. Sections (2 mm x 2 mm) were dissected from the lesion and plated on half-strength potato-dextrose agar (PDA) (Merck, Germiston, South Africa) supplemented with 0.2 g/l Novobiocin in 90-mm Petri-dishes. Plates were incubated for 3–7 d at 25 °C and fungi that developed were isolated by hyphal tip excision and cultured on PDA plates at 25 °C for 60 d until identification.

All cultures are maintained on PDA slants and under mineral oil at 4 °C at the Forestry and Agricultural Biotechnology Institute (FABI) in Pretoria, South Africa. Representative strains have been deposited at the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, The Netherlands.

DNA amplification and phylogeny

Molecular diagnosis of the *Mycosphaerella* species associated with symptoms on banana foliage in South Africa was achieved by PCR and sequencing of the ITS region of the DNA isolated from cultures obtained above. DNA was isolated using the method of Surridge *et al.* (2003a).

Randomly selected Mycosphaerella speckle isolates and *M. musicola* isolates were subjected to an ITS-PCR with primers ITS1 and ITS4 (White *et al.,* 1990). Each PCR tube contained a total volume of 25 µl: 18.7 µl sterile distilled Sabax water, 2.5 µl PCR buffer with $MgCl_2$ (10x), 2 µl dNTPs (2.5 µM), 0.5 µl primer 1 (50 µM), 0.5 µl primer 2 (50 µM), 0.5 µl DNA (27 ng/µl), and 0.3 µl Expand Taq (5U/µl). DNA amplification was performed in a PCR thermal cycler using the following programme: 10 min at 95 °C, 35 cycles of 30 sec at 95 °C, 45 sec at 55 °C and 2 min at 72 °C, followed by 7 min at 72 °C, and then held at 4 °C. The PCR product was analysed on a 1 % agarose gel. The ITS PCR products were purified with a "High pure PCR product purification kit" (Roche, Germany). DNA sequences were determined using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Applied Biosystems, UK). ITS2 and ITS3 (White *et al.* 1990) were included as internal primers to confirm the sequence data obtained.

ITS sequences for *M. musicola, M. fijiensis, M. musae* (Table 1) and *M. eumusae* were obtained from GenBank. Representative DNA sequences were manually aligned by inserting gaps. Ambiguously aligned regions were excluded from the data set before analysis and gaps treated as missing data. Phylogenetic analysis was based on parsimony using PAUP 4.0b8 (Phylogenetic Analysis Using Parsimony) (Swofford, 2000). Heuristic searches were done with random addition of sequences (1000 replicates), tree bisection-reconnection (TBR), branch swapping and MULPAR effective and MaxTrees set to auto-increase. Phylogenetic signal in the data sets was accessed by evaluating tree length distributions over 100 randomly generated trees. The consistency (CI) and retention (RI) indices were determined for all data sets. Phylogenetic trees were rooted with *Mycocentrospora acerina* (R. Hartig) Deighton (Stewart *et al.* 1999) and *Fusarium circinatum* Nirenberg & O'Donnell as a monophyletic sister outgroups to the remaining taxa. Bootstrap analyses were conducted to determine confidence in branching points (1000 replicates) for the most parsimonious trees generated.

Discovery of ITS sequence homology between several Mycosphaerella speckle isolates and *M. colombiensis* Crous & M.J. Wingf., using GenBank nucleotide blast searching, lead to the sequencing of the α-elongation factor region of both *M. colombiensis* and the Mycosphaerella speckle isolates. Sequencing was performed using primers EF1 and EF2 (Glass & Donaldson, 1995). Sequences were analysed as above.

Pathogenicity trials

Selected cultures of *M. colombiensis* (STE-U 1104–1106, ex-type) and Mycosphaerella speckle strains (CBS 110967, CBS 110968, CBS 110969 and CMW 10901, CMW 10902, CMW

10903, CMW 10904, CMW 10905, CMW 10906, CMW 10907, CMW 10908) were inoculated onto young banana (cv. Cavendish) and young *Eucalyptus urophylla* S.T. Blake plants in a greenhouse maintained at 27 °C, and monitored over a 4-month-period. Plants used for inoculation were approximately 1 m high and growing in 5 l bags filled with potting soil. Plants were moved into the greenhouse 30 d prior to inoculation to allow for acclimatisation. Plants were watered every third day and exposed to normal sunlight hours. The adaxial surface of the leaves was lightly abraised with a syringe needle to remove a portion of the waxy cuticle. An agar plug overgrown with mycelium was then placed onto the epidermis, covered with clear strip of laboratory film, and marked with the isolate number. Each isolate was inoculated onto two leaves on each of three plants. Sterile agar plugs served as controls. Plants were inspected regularly for symptom development and isolations were made from lesions as described above after 90–120 d.

RESULTS

DNA amplification and phylogeny

Parsimony analysis of the ITS-1 and ITS-2 regions of the rDNA operon was conducted to determine the phylogenetic placement of South African Mycosphaerella speckle isolates in relation to other *Mycosphaerella* species known from banana. Alignment by inserting gaps resulted in a total of 555 characters used in the comparison of the different species. A total of 359 constant characters, 113 parsimony-uninformative characters and 83 parsimony-informative characters were obtained. Heuristic searches on the data generated 100 most parsimonious trees, of which tree number 1 is presented (Fig. 1).

Phylogenetic analysis of the sequence data delineated two species of *Mycosphaerella* to be associated with Mycosphaerella speckle symptoms. One species conformed to *M. musae*, while the other proved to be similar to *M. colombiensis*. Based on ITS sequence data, only one base pair difference (AF309612) was observed between the latter isolates and *M. colombiensis*. The clade containing these isolates and *M. colombiensis* could be differentiated by 33 steps from the clades containing *M. fijiensis, M. musicola, M. eumusae* and *M. musae*. Parsimony analysis of the α-elongation factor region of the genome was conducted to determine the phylogenetic placement of the unknown Mycosphaerella speckle isolates in relation to *M. colombiensis*, and other *Cercospora* and *Mycosphaerella* species. Alignment by inserting gaps resulted in a total of 350 characters. Inserted gaps were treated as "missing" data. A total of 43 constant characters, 37 parsimony-uninformative characters and 270 parsimony-informative characters were obtained. Heuristic searches on the data generated 100 most parsimonious trees, of which tree number 1 is presented (Fig. 2). The unknown Mycosphaerella speckle isolates differed from *M. colombiensis* by 6 base pairs according to α-elongation factor, which may suggest that the banana isolates represent a sister taxon to *M. colombiensis*. Sequence data were deposited in GenBank (Table 1).

Pathogenicity tests

Banana plants inoculated with the South African isolate of *M. musae*, the verified isolate of *M. colombiensis*, as well as the unknown Mycosphaerella speckle isolates, exhibited typical speckle symptoms (Fig. 3A) within 90–120 d of inoculation, from which the fungus could be re-isolated. Known symptoms of *M. colombiensis* on *E. urophylla* in Colombia include amphigenous, light brown leaf spots. *Eucalyptus urophylla* leaves inoculated with the South African isolate of *M. musae*, the verified isolate of *M. colombiensis*, as well as the unknown

Mycosphaerella speckle isolates developed leaf blotch symptoms (Fig. 3B) from which the respective fungi could be re-isolated, confirming Koch's postulates.

Discussion

Comparison of the ITS region of the South African Mycosphaerella speckle isolates delineated them in two clearly differentiated clades, supported by a 100 % bootstrap value. From the sequence data, it appears that the unknown Mycosphaerella speckle isolates could represent *M. colombiensis*. This is further supported by results of the pathogenicity study, where isolates of the unknown speckle pathogen incited spots on leaves of *E. urophylla*, which were indistinguishable from those caused by *M. colombiensis*. Likewise, *M. colombiensis* isolates from *E. urophylla* caused lesions similar to those of *M. musae* and the unknown speckle pathogen on banana leaves. Results also indicated that *M. musae* is capable of inducing leaf spots on *E. urophylla*. Besides endorsing the similarity between the unknown speckle pathogen and *M. colombiensis,* cross-pathogenicity of the two taxa, and of *M. musae*, obviously holds implications for the epidemiology of Mycosphaerella speckle on banana and *M. colombiensis* leaf spot on *E. urophylla*.

Notwithstanding their relatedness at molecular level, the unknown speckle isolates and *M. colombiensis* differ morphologically. Ascospores of the latter are obovoid, 1-septate, not constricted at the septum, 11–15 x 3–4 µm. It has a *Pseudocercospora* anamorph with narrowly obclavate or subcylindrical conidia, 1–5 septate, 25–60 x 2.5–3.5 µm, and olivaceous black

colonies (Crous 1998). Colonies of the unknown speckle isolates were olivaceous grey in colour, and did not produce an anamorph. When sporulating on 2 % water agar, the isolates produced obovoid, unconstricted, 1 septate ascospores, 6–10 x 2–2.5 µm, notably smaller than those of *M. colombiensis* and also somewhat shorter than those of *M. musae*. The disinclination of the unknown speckle pathogen to produce an anamorph is reminiscent of *M. musae*, though anamorphs of the latter species have been observed. Stover (1994) reported that *M. musae* existed endophytically, producing brown, verruculose conidia, 55–200 x 2.5–3 µm, with a prominent basal scar. Based on current concepts of cercosporoid fungi (Crous *et al.* 2001), this conforms to the mitosporic genus *Stenella*. Some isolates, however (Stover 1994), also produced conidia that were smooth-walled with faint (*Passalora*) or no (*Pseudocercospora*) scars. These findings suggest that, besides *M. musae* and *M.* cf. *colombiensis*, more *Mycosphaerella* species may be associated with Mycosphaerella speckle on banana. For the time being, however, it is concluded that *Mycosphaerella* causing speckle symptoms on banana in South Africa is paraphyletic.

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Table 1: Collection and sequence details of the *Mycosphaerella* isolates included in the phylogenetic analysis.

Mycosphaerella sp.	Isolate number	Locality	Date	Collector	Host/Cultivar	GenBank accession number
M. musicola	CMW 6375	Komatipoort, SA	19-May-00	A. Viljoen	Williams	AF509728
M. musicola	CMW 6373	Kiepersol, SA	17-Mar-00	A. Viljoen	Williams	AF509729
M. musicola	CMW 6314	Kiepersol, SA	24-Jun-00	A. Viljoen	Grande Nain	AF509730
M. musicola	CMW 6325	Komatipoort, SA	16-Mar-00	A. Viljoen	Grande Nain	AF509731
M. musicola	CMW 6368	KwaZulu Natal, SA	24-Jun-00	A. Viljoen	Grande Nain	AF509732
M. musicola	CMW 6340	Kiepersol, SA	14-Jun-00	A. Viljoen	Williams	AF509733
M. musicola	CMW 6346	Kiepersol, SA	14-Jun-00	A. Viljoen	Williams	AF509734
M. musicola	CMW 6365	Tzaneen, SA	24-Jun-00	A. Viljoen	Chinese Cavendish	AF509735
M. musicola	ATCC 22115	Philippines	-	D.S. Meredith	Lacatan	AF181706
M. colombiensis	CBS 110967	-	-	P.W. Crous	-	AF309612
M. colombiensis	CBS 110968	-	-	P.W. Crous	-	AF222838
M. cf. *colombiensis*	CMW 10901	Natal, SA	5-Jun-00	A.K.J. Surridge	Williams	ITS: AY217105
M. cf. *colombiensis*	CMW 10902	Mpumalanga, SA	25-Jul-00	A.K.J. Surridge	Williams	ITS: AY217106 EF: AY217112
M. cf. *colombiensis*	CMW 10903	Mpumalanga, SA	25-Jul-00	A.K.J. Surridge	Grande Naine	ITS: AY217107 EF: AY217113
M. cf. *colombiensis*	CMW 10904	Mpumalanga, SA	25-Jul-00	A.K.J. Surridge	Grande Naine	ITS: AY217108 EF: AY217114

M. musae	CMW 10905	Mpumalanga, SA	25-Jul-00	A.K.J. Surridge	Grande Naine	ITS: AY217104
M. fijiensis	ATCC 36054	Honduras	-	R.H. Stover	*Musa* AAA	AF297225
M. fijiensis	ATCC 22117	Hawaii	-	D.S. Meredith	Gros Michel	AF297234
M. fijiensis	ATCC 22116	Philippines	-	D.S. Meredith	Giant Cavendish	AF181705
Mycocentrospora acerina	ATCC 34539	Norway	-	K. Arsvol	Carrot	-
M. colombiensis	CBS 110967	-	-	P.W. Crous	-	EF: AY217109
M. colombiensis	CBS 110968	-	-	P.W. Crous	-	EF: AY217110
M. colombiensis	CBS 110969	-	-	P.W. Crous	-	EF: AY217111
Cercospora piaropi	-	-	-	-	-	EF: AF146149
Cercospora piaropi	-	-	-	-	-	EF: AF146146
Cercospora beticola	-	-	-	-	-	EF: AF146140
M. graminicola	-	-	-	-	-	EF: AW180959
M. graminicola	-	-	-	-	-	EF: AW181005
Fusarium circinatum	MRC 7541	USA	-	T. Gordon	*Pinus radiata*	EF: AF160295

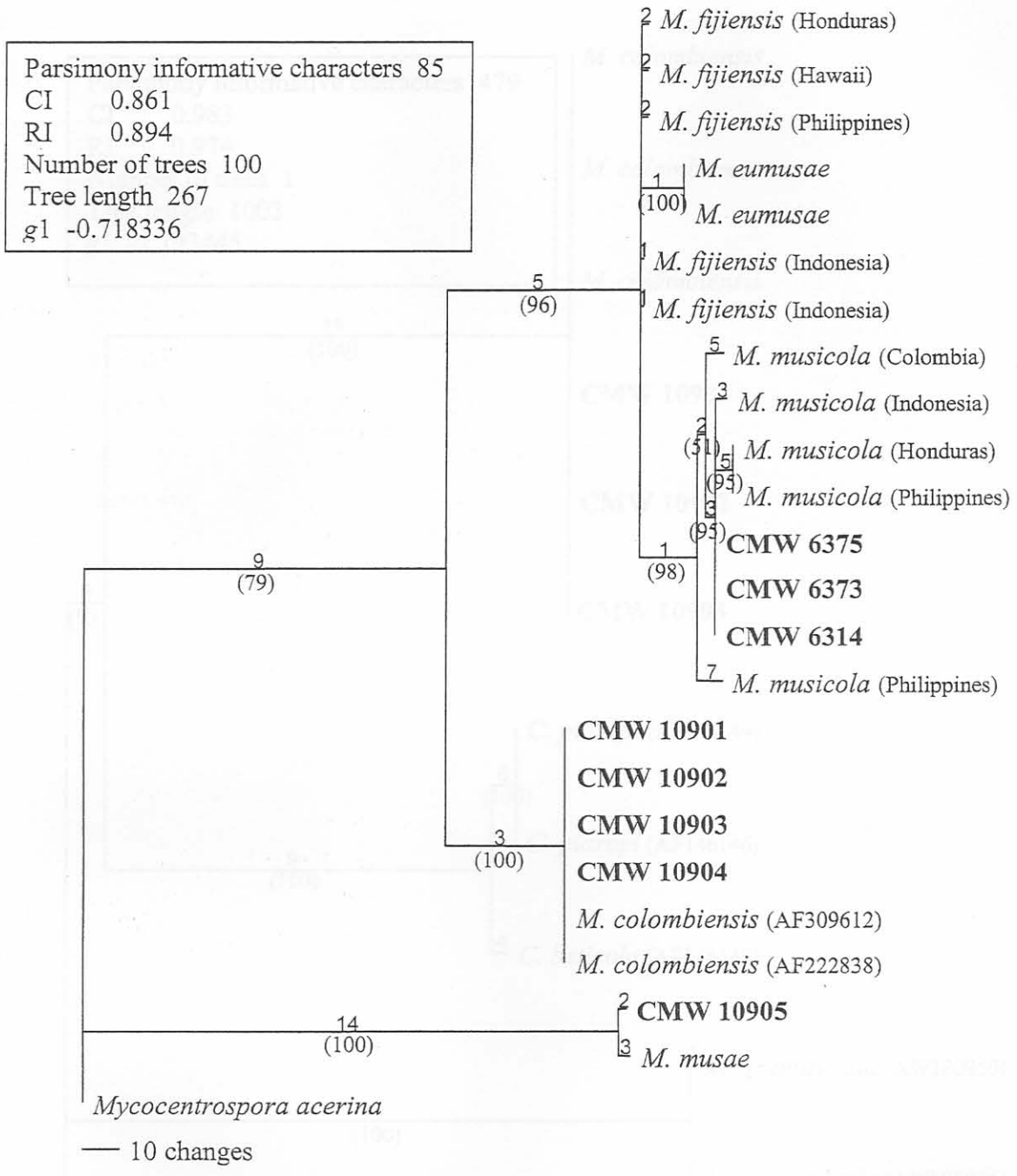

Figure 1: Phylogeny of the internal transcribed spacer sequence of *Mycosphaerella fijiensis*, *M. eumusae*, *M. musicola*, *M. musae*, *M. colombiensis* and South African isolates of *Mycosphaerella* causing Sigatoka and speckle leaf disease of bananas (tree number 1 of 100 trees is presented).

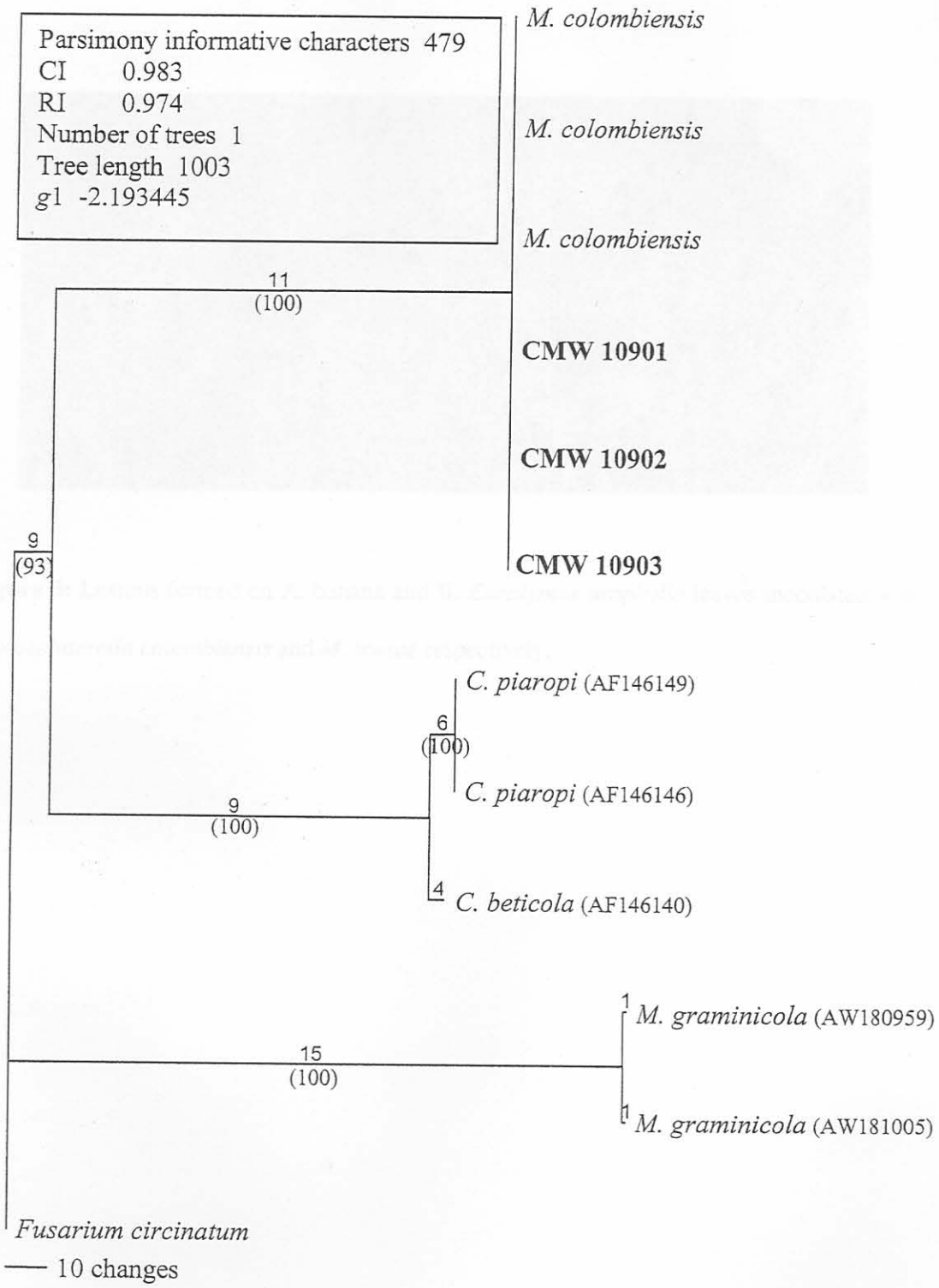

Figure 2: Phylogenetic tree of the alpha-elongation factor gene of *Mycosphaerella colombiensis* and South African isolates of *Mycosphaerella* causing speckle leaf disease of bananas (tree number 1 of 100 trees is presented).

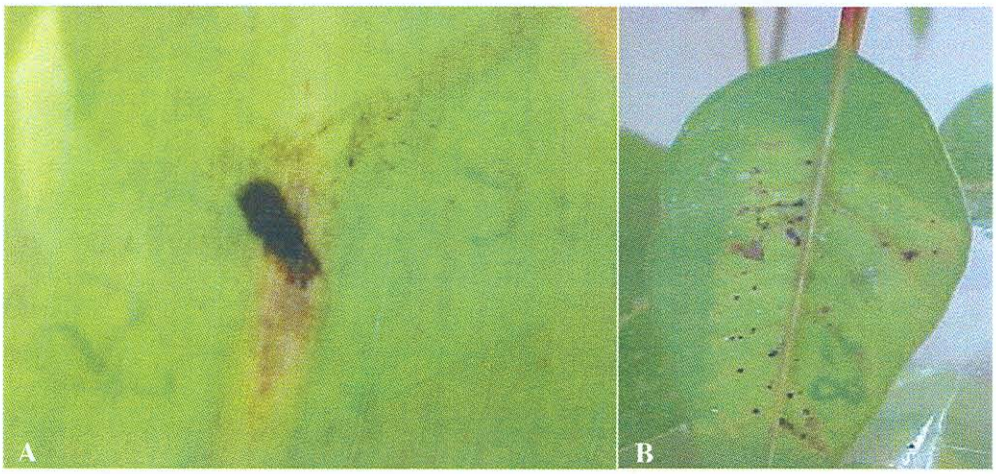

Figure 3: Lesions formed on A. banana and B. *Eucalyptus urophylla* leaves inoculated with *Mycosphaerella colombiensis* and *M. musae* respectively.