

# Multi-gene phylogenies and phenotypic characters distinguish two species within the *Colletogloeopsis zuluensis* complex associated with *Eucalyptus* stem cankers

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**Abstract:** *Colletogloeopsis zuluensis*, previously known as *Coniothyrium zuluense*, causes a serious stem canker disease on *Eucalyptus* spp. grown as non-natives in many tropical and sub-tropical countries. This stem canker disease was first reported from South Africa and it has subsequently been found on various species and hybrids of *Eucalyptus* in other African countries as well as in countries of South America and South-East Asia. In previous studies, phylogenetic analyses based on DNA sequence data of the ITS region suggested that all material of *C. zuluensis* was monophyletic. However, the occurrence of the fungus in a greater number of countries, and analyses of DNA sequences with additional isolates has challenged the notion that a single species is involved with Coniothyrium canker. The aim of this study was to consider the phylogenetic relationships amongst *C. zuluensis* isolates from all available locations and to support these analyses with phenotypic and morphological comparisons. Individual and combined phylogenies were constructed using DNA sequences from the ITS region, exons 3 through 6 of the  $\beta$ -tubulin gene, the intron of the translation elongation factor 1- $\alpha$  gene, and a partial sequence of the mitochondrial ATPase 6 gene. Both phylogenetic data and morphological characteristics showed clearly that isolates of *C. zuluensis* represent at least two taxa. One of these is *C. zuluensis* as it was originally described from South Africa, and we provide an epitype for it. The second species occurs in Argentina and Uruguay, and is newly described as *C. gauchensis*. Both fungi are serious pathogens resulting in identical symptoms. Recognising them as different species has important quarantine consequences.

**Taxonomic novelty:** *Colletogloeopsis gauchensis* M.-N. Cortinas, Crous & M.J. Wingf. sp. nov.

**Key words:** Bayesian inference, *Colletogloeopsis*, *Eucalyptus* canker, multilocus gene phylogeny, *Mycosphaerella*.

## INTRODUCTION

*Colletogloeopsis zuluensis* (M.J. Wingf., Crous & T.A. Cout.) M.-N. Cortinas, M.J. Wingf. & Crous (Cortinas *et al.* 2006) causes a serious stem canker disease on *Eucalyptus* species. The disease was first reported in 1987 in South Africa, and the pathogen was described as a species of *Coniothyrium*, namely *C. zuluense* M.J. Wingf., Crous & T.A. Cout. (Wingfield *et al.* 1997). The disease spread very rapidly through the country, initially occurring only on a single *Eucalyptus grandis* clone, but ultimately occurring in all parts of South Africa with a sub-tropical climate, and on a wide variety of *Eucalyptus* species and hybrids. Substantial research has thus been undertaken to better understand the disease and to develop disease-resistant planting stock through breeding and selection programmes (Van Zyl *et al.* 1997, 2002a).

Symptoms of *Colletogloeopsis* canker are very obvious, at least at the onset of disease. Initial infections include small, circular necrotic lesions on the green stem tissue in the upper parts of trees. These lesions expand, becoming elliptical, and the dead bark covering them typically cracks, giving a “cat-eye” appearance (Fig. 1). Lesions coalesce to form large cankers that girdle the stems, giving rise to the production of epicormic shoots and ultimately trees with malformed or dead tops. Infections occur annually on the new green tissue and they penetrate the cambium to form black kino-filled pockets. Thus kino pockets with irregular borders of infected tissue can be seen within the infected wood

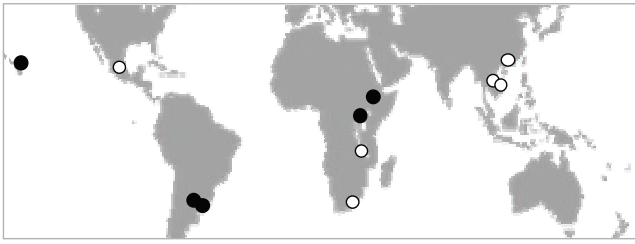
of trees coincident with the annual rings (Fig. 1). Small black pycnidia can be seen on the surface of dead bark tissue (Fig. 1), from where black conidial tendrils exude under moist conditions. Conidia are small, aseptate and dematiaceous, appearing black in colour when seen in mass on the host or agar media.

Subsequent to the discovery of Coniothyrium canker in South Africa, the disease has been found in many other countries. Its first discovery outside South Africa was in Thailand where it is associated with typical symptoms on *E. camaldulensis* (Van Zyl *et al.* 2002b). More recently, the disease has been found in other countries in Africa (Alemu *et al.* 2003, 2005), South and Central America (Roux *et al.* 2002, Alemu *et al.* 2004), as well as South-East Asia (Old *et al.* 2003, Cortinas *et al.* 2004, 2006) (Fig. 2). Interestingly, the disease remains unknown in the areas of origin of *Eucalyptus*, although it might occur there at very low and undetectable levels (Wingfield 2003, Slippers *et al.* 2005).

The first taxonomic treatment of *C. zuluensis* was based on morphological characteristics of the pathogen. The presence of pycnidia and pigmented aseptate, ellipsoidal conidia arising from percurrently proliferating conidiogenous cells were consistent with species placed in *Coniothyrium* Corda. DNA sequence comparisons have, however, made it possible to recognise that the fungus has a clear phylogenetic position in *Mycosphaerella* Johanson (Alemu *et al.* 2005). It is moreover not related to species of *Coniothyrium* s. str., which are anamorphs of



**Fig. 1.** External symptoms of the stem canker disease on *E. grandis* in Uruguay caused by *C. gauchensis*. A, B. Mature clones showing the typical lesions on the surface of the trunk. C. Distinctive black circular lesions on green twigs. D. Stem with typical cracked lesions. E. Stem showing internal symptoms below the bark lesions. F. Kino-pockets of infected tissue within the wood. G. Pycnidia on cracked lesions.



**Fig. 2.** Geographical range of the collection of isolates used in this study. The map includes isolates from South Africa, Malawi, Vietnam, Thailand and China, indicated with white dots (Group 1) and isolates from Uruguay, Argentina, Hawaii-U.S.A., Ethiopia and Uganda, indicated with black dots (Group 2).

*Leptosphaeria* spp. This realisation has led to the transfer of *Coniothyrium zuluense* to *Colletogloeopsis* Crous & M.J. Wingf. (Cortinas *et al.* 2006). *Colletogloeopsis* is a well-recognised *Mycosphaerella* anamorph and its circumscription was amended to include species with pycnidoid conidiomata. Within *Mycosphaerella*, *C. zuluensis* clusters with a group of well-known leaf and stem pathogens of *Eucalyptus* including *M. ambiphylla* A. Maxwell, *M. cryptica* (Cooke) Hansf., *M. molleriana* (Thüm.) Lindau, *M. nubilosa* (Cooke) Hansf., *M. vespa* Carnegie & Keane, *M. suttonii* Crous & M.J. Wingf., and *Phaeophleospora eucalypti* (Cooke & Masee) Crous, F.A. Ferreira & B. Sutton (Cortinas *et al.* 2006).

Different isolates of *C. zuluensis* have been found to be highly variable in morphology (Fig. 3) and pathogenicity to different *Eucalyptus* clones (Van Zyl 1997, Wingfield *et al.* 1997, Van Zyl 2002a). Nonetheless, previous phylogenetic analyses based on the nuclear ribosomal small subunit (18S) and internal transcribed spacer regions and the ribosomal 5.8 gene (ITS1, 5.8S, ITS2) had shown that *C. zuluensis* was monophyletic (Van Zyl 2002b, Alemu *et al.* 2005). As additional surveys of *Eucalyptus* plantations are undertaken, an understanding of the geographical range of *C. zuluensis* continues to expand. Additional isolates from new regions have thus become available for DNA sequence comparisons and these have provided the opportunity to re-consider the taxonomic status of *C. zuluensis*, and the variation observed in its morphology and pathogenicity.

The aim of this study was to consider whether the previously recognised *C. zuluensis* can be retained when applying multi-gene analyses using a large collection of isolates not previously available. To accomplish this objective, individual and combined phylogenetic analyses using the ITS region,  $\beta$ -tubulin gene (BT2), the elongation factor 1- $\alpha$  (EF1 $\alpha$ ) gene, and the mitochondrial ATPase 6 (ATP6) gene, were carried out. Morphological and other phenotypic characters were also considered.

## MATERIALS AND METHODS

### Isolates

A collection of 45 isolates was chosen to reflect the geographical distribution of *C. zuluensis*. In addition, several species of *Mycosphaerella* known to be closely

related to *C. zuluensis* were also included (Table 1). All these isolates were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), Pretoria, South Africa. Single-conidial cultures were established from mature pycnidia isolated from lesions taken from the stems of *Eucalyptus* trees in South Africa and Uruguay. The contents of single pycnidia were diluted in sterile distilled water, and spread on the surface of Petri dishes containing MEA (20 g/L Biolab malt extract, 15 g/L Biolab agar). After 24–36 h, germinating conidia were transferred to fresh MEA plates and incubated for 30 d at 25 °C. Reference strains are preserved in CMW, and have been deposited at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (Table 1). Nomenclature, descriptions and illustrations were deposited in MycoBank.

### DNA extraction and amplification

To extract DNA, mycelium was scraped from the surface of cultures grown in Petri dishes, freeze dried, frozen in liquid nitrogen and ground to a fine powder. The protocol followed by Cortinas *et al.* (2004) was simplified as follows: DBE extraction buffer (200 mM Tris-HCl pH 8, 150 mM NaCl, 25 mM EDTA pH 8, 0.5 % SDS) was added directly to the ground mycelium and incubated for 2 h at 80 °C (or until pigments changed colour from green to red). In the extraction-DNA enrichment procedure, one volume of phenol was used first and one volume of a 1:1 phenol-chloroform solution thereafter.

Four gene regions were amplified for all isolates included in this study (Fig. 4). The ITS region of the ribosomal DNA was targeted using the primers ITS1: 5' TCC GTA GGT GAA CCT GCG G and ITS4: 5' GCT GCG TTC TTC ATC GAT GC (White *et al.* 1990). Exons 3 to 6 and the respective introns (BT2) of the  $\beta$ -tubulin gene region were amplified using the primers BT2A: 5' GGT AAC CAA ATC GGT GCT GCT TTC and BT2B: 5' AAC CTC AGT GTA GTG ACC CTT GGC (Glass & Donaldson 1995). The intron sequence of the EF1- $\alpha$  gene was amplified using the primers EF1-728F: 5' CAT CGA GAA GTT CGA GAA GG and EF1-986R: 5' TAC TTG AAG GAA CCC TTA CC (Carbone & Kohn 1999) and intron 2 and exon 3 of the ATP6 gene was amplified using the set of primers 5'ATT AAT TSW CCW TTA GAW CAA TT and 5'TAA TTC TAN WGC ATC TTT AAT RTA developed by Kretzer & Bruns (1999).

PCR reactions were prepared in a total volume of 25  $\mu$ L including 1.5  $\mu$ L of genomic 1/10 dilution DNA, 1 U of *Taq* polymerase, 10  $\times$  *Taq* buffer, 10 pmol of each primer, 0.8 mM of each dNTPs, and 2.0 mM MgCl<sub>2</sub> (ITS) or 4.0 mM MgCl<sub>2</sub> (BT2, EF1- $\alpha$ , ATP6). PCR amplicons were visualised under UV light on 1 % or 2 % agarose gels. Different cycling conditions were used for the various gene regions. For the ITS region, 96 °C, 3 min initial denaturation and cycles of 95 °C, 30 s, 54 °C, 30 s, 72 °C, 1 min were repeated 10 times followed by 25 cycles of 95 °C, 30 s, 56 °C, 30 s, 72 °C, 1 min with 5 s extension after two cycles. A final elongation step of 7 min at 72 °C was also included. The same cycling conditions were used for ATP6 region changing the annealing temperature to 50 °C.

Table 1. Isolates of *Colletogloeopsis* and related species used in the phylogenetic studies.

Species	Strain numbers	Country	Host	Date	GenBank no.					
					ITS	BT2	EF1- $\alpha$	ATP6		
<i>Colletogloeopsis gauchensis</i>	CMW7302	Uruguay	<i>E. grandis</i>	2001	DQ240186	DQ240075	DQ240128	DQ240025		
	CMW7274;	Uruguay	<i>E. grandis</i>	2001	DQ240187	DQ240076	DQ240129	DQ240026		
	CMW7294;	Uruguay	<i>E. grandis</i>	2001	DQ240188	DQ240077	DQ240130	DQ240027		
	CMW7300;	Uruguay	<i>E. grandis</i>	2001	DQ240189	DQ240078	DQ240131	DQ240028		
	CMW7270	Uruguay	<i>E. grandis</i>	2001	-	-	-	DQ240068		
	CMW17328	Uruguay	<i>E. grandis</i>	2005	DQ240190	DQ240079	DQ240132	DQ240029		
	CMW17330	Uruguay	<i>E. grandis</i>	2005	DQ240191	DQ240080	DQ240133	DQ240030		
	CMW17323	Uruguay	<i>E. grandis</i>	2005	DQ240215	DQ240122	-	DQ240069		
	CMW17324	Uruguay	<i>E. grandis</i>	2005	DQ240216	DQ240123	-	DQ240070		
	CMW17326	Uruguay	<i>E. grandis</i>	2005	DQ240217	DQ240124	-	DQ240071		
	CMW17332;	Uruguay	<i>E. grandis</i>	2005	DQ240218	DQ240125	-	DQ240072		
	CMW10895;	Hawaii-US	<i>E. grandis</i>	2002	DQ240192	DQ240081	DQ240134	DQ240031		
	CMW10893;	Hawaii-US	<i>E. grandis</i>	2002	DQ240193	DQ240082	DQ240135	DQ240032		
	CMW10894	Hawaii-US	<i>E. grandis</i>	2002	DQ240194	DQ240083	DQ240136	DQ240033		
	CMW7331;	Argentina	<i>E. grandis</i>	2001	DQ240195	DQ240084	DQ240137	DQ240034		
	CMW7342	Argentina	<i>E. grandis</i>	2001	DQ240196	DQ240085	DQ240138	DQ240035		
	CMW7378	Argentina	<i>E. grandis</i>	2001	DQ240197	DQ240086	DQ240139	DQ240036		
	CMW14336;	Argentina	<i>E. grandis</i>	2003	DQ240198	DQ240087	DQ240140	DQ240037		
	CMW7137	Uganda	<i>E. grandis</i>	2001	DQ240199	DQ240088	DQ240141	DQ240038		
	CMW15835;	Uganda	<i>E. grandis</i>	1999	DQ240200	DQ240089	DQ240142	DQ240039		
CMW8991;	Ethiopia	<i>E. camaldulensis</i>	2001	DQ240201	DQ240090	DQ240143	DQ240040			
CMW8978	Ethiopia	<i>E. camaldulensis</i>	2001	DQ240202	DQ240091	DQ240144	DQ240041			
CMW19356	Ethiopia	<i>E. camaldulensis</i>	2000	-	-	DQ240181	-			
<i>Colletogloeopsis zuluensis</i>	CMW1772	South Africa	<i>E. grandis</i>	1989	DQ240203	DQ240092	DQ240145	DQ240042		
	CMW7426	South Africa	<i>E. grandis</i>	1997	DQ239979	-	DQ240182	-		
	CMW7459	South Africa	<i>E. grandis</i>	1997	DQ239981	-	DQ240183	-		
	CMW7488;	South Africa	<i>E. grandis</i>	1997	DQ239975	-	DQ240184	-		
	CMW7489	South Africa	<i>E. grandis</i>	1997	DQ239980	-	DQ240185	-		
	CMW17314	South Africa	<i>E. grandis</i>	2005	DQ240204	DQ240093	DQ240146	DQ240043		
	CMW17316	South Africa	<i>E. grandis</i>	2005	DQ240205	DQ240094	DQ240147	DQ240044		
	CMW17320	South Africa	<i>E. grandis</i>	2005	DQ240206	DQ240095	DQ240148	DQ240045		
	CMW17321	South Africa	<i>E. grandis</i>	2005	DQ240207	DQ240096	DQ240149	DQ240046		

Table 1. (Continued).

Species	Strain numbers	Country	Host	Date	GenBank no.					
					ITS	BT2	EF1- $\alpha$	ATP6		
	CMW13328;	South Africa	<i>E. grandis</i>	-	DQ239974	-	DQ240172	-		
	CMW13324;	South Africa	<i>E. grandis</i>	-	AY738214	-	DQ240173	-		
	CMW17318	South Africa	<i>E. grandis</i>	2005	DQ240213	DQ240126	DQ240174	DQ240073		
	CMW17322	South Africa	<i>E. grandis</i>	2005	DQ240214	DQ240127	DQ240175	DQ240074		
	CMW7449;	South Africa	<i>E. grandis</i>	1997	DQ239976	DQ240102	DQ240155	DQ240052		
	CMW7452	South Africa	<i>E. grandis</i>	1997	DQ239977	DQ240103	DQ240156	DQ240053		
	CMW7442	South Africa	<i>E. grandis</i>	1997	DQ239978	DQ240104	DQ240157	DQ240054		
	CMW7468	South Africa	<i>E. grandis</i>	1997	DQ239983	DQ240105	DQ240158	DQ240055		
	CMW15971	China	<i>E. urophylla</i>	2004	DQ240208	DQ240097	DQ240150	DQ240047		
	CMW15080	China	<i>E. urophylla</i>	2004	DQ240209	DQ240098	DQ240151	DQ240048		
	CMW15964	China	<i>E. urophylla</i>	2004	DQ240210	DQ240099	DQ240152	DQ240049		
	CMW17425	Malawi	<i>E. grandis</i>	2004	DQ240211	DQ240100	DQ240153	DQ240050		
	CMW17438	Malawi	<i>E. grandis</i>	2004	DQ240212	DQ240101	DQ240154	DQ240051		
	CMW17356	Malawi	<i>E. grandis</i>	2004	DQ240219	-	-	-		
	CMW6859	Vietnam	<i>E. urophylla</i>	2000	-	DQ240106	DQ240159	DQ240056		
	CMW6860	Vietnam	<i>E. urophylla</i>	2000	DQ239985	DQ240107	DQ240160	DQ240057		
	CMW6857;	Vietnam	<i>E. urophylla</i>	2000	DQ239986	-	DQ240171	-		
	CMW15834;	Mexico	<i>E. grandis</i>	2000	DQ239987	DQ240108	DQ240161	DQ240058		
	CMW15833;	Mexico	<i>E. grandis</i>	2000	DQ239988	DQ240109	DQ240162	DQ240059		
	CMW5235;	Thailand	<i>E. camaldulensis</i>	1997	DQ239990	DQ240110	DQ240163	DQ240060		
	CMW5236	Thailand	<i>E. camaldulensis</i>	1997	DQ239989	DQ240111	DQ240164	DQ240061		
<i>Mycosphaerella ambiphylia</i>	CMW13704;	Australia	<i>Eucalyptus</i>	-	DQ239970	DQ240116	DQ240169	DQ240066		
<i>Mycosphaerella colombiensis</i>	CMW4944;	Colombia	<i>Eucalyptus</i> sp.	-	DQ239993	DQ240112	DQ240165	DQ240062		
<i>Mycosphaerella molleriana</i>	CMW4940;	Portugal	<i>Eucalyptus</i> sp.	1995	DQ239969	DQ240115	DQ240168	DQ240065		
<i>Mycosphaerella nubilosa</i>	CMW6210;	Australia	<i>Eucalyptus</i> sp.	2000	DQ239999	DQ240113	DQ240166	DQ240063		
<i>Mycosphaerella suttonii</i>	CMW5348;	Indonesia	<i>Eucalyptus</i> sp.	1996	DQ239972	DQ240117	DQ240170	DQ240067		
<i>Mycosphaerella vespa</i>	CMW11588	Australia	<i>Eucalyptus</i> sp.	-	DQ239968	DQ240114	DQ240167	DQ240064		

CMW= Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

CBS= Culture collection of the Centraalbureau voor Schimmelcultures, Uppsalaalaan, Utrecht, The Netherlands. CPC= Culture collection of Pedro Crous housed at CBS.

For  $\beta$ -tubulin, 96 °C, 3 min initial denaturation and cycles of 95 °C, 30 s, 57 °C, 45 s, 72 °C, 45 s were repeated 40 times. For EF1- $\alpha$ , 96 °C, 3 min and cycles of 95 °C, 30 s, 54 °C, 45 s, 72 °C, 45 s were repeated 40 times with 5 s extension after two cycles. A final elongation step of 7 min at 72 °C was included.

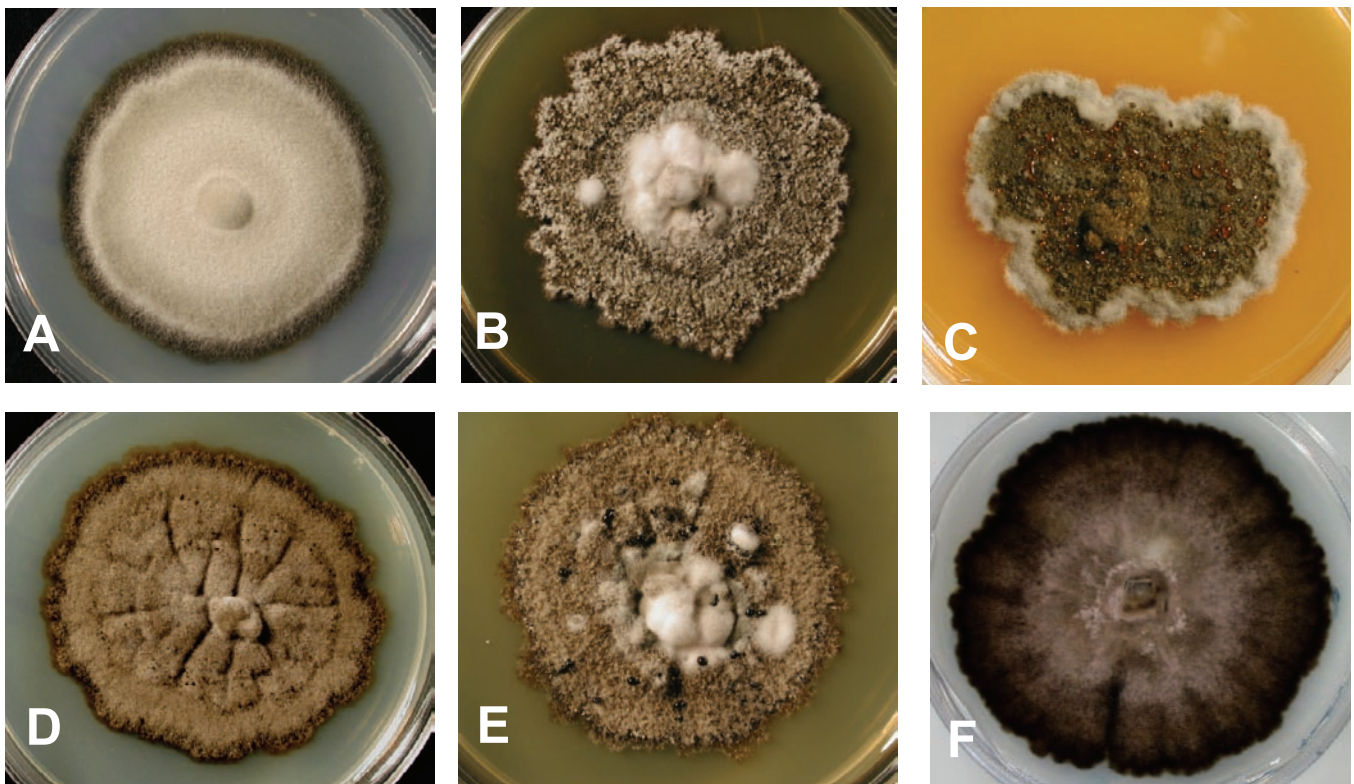
PCR amplification products were purified using Sephadex G-50 columns (Sigma- Aldrich, Steinheim, Germany) or treated with a mix of Exonuclease III and Shrimp alkaline phosphatase (Exo-Sap); 0.7 U of each enzyme per PCR reaction were incubated at 37 °C for 15 min followed by 80 °C for 15 min before sequencing. Sequencing reactions were prepared in 10  $\mu$ L with 2  $\mu$ L of purified PCR product, 10 pmol of the same primers used for the first PCR amplifications, 2  $\mu$ L 5 $\times$  dilution buffer and ABI Prism Big Dye Terminator mix, v. 3.1 (Applied Biosystems Inc., Foster City, California).

Sequencing PCR cycles consisted of 25 repetitions at 96 °C, 10 s; 50 °C, 4 s; 60 °C, 4 min. Sequencing reactions were cleaned using Sephadex G-50 or precipitated using EDTA, Sodium Acetate and Ethanol according to the protocol supplied by Applied Biosystems (Applied Biosystems Inc., Foster City, California).

### Phylogenetic analyses

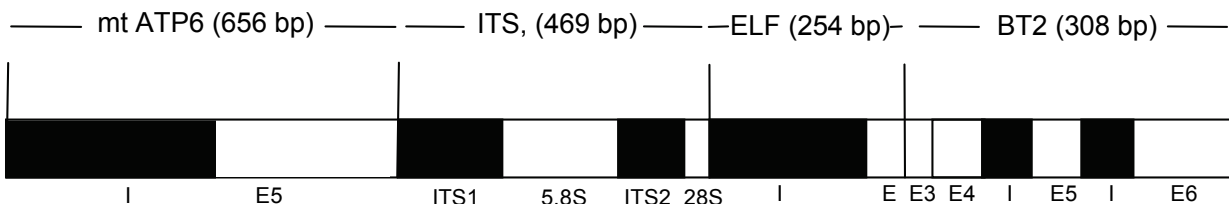
Alignments of sequence data were made using Clustal W under MEGA 3.0 (Kumar *et al.* 2004) and manually adjusted. All sequences generated in this study were deposited in GenBank (Table 1). Alignments were deposited in TreeBASE.

Maximum parsimony and distance analyses were conducted considering the individual and combined partitions. Most parsimonious (MP) trees were generated using PAUP v. 4.0b10 (Swofford 2002).



**Fig. 3.** Characteristics of isolates of Group 1 (*C. zuluensis*), and isolates of Group 2 (*C. gauchensis*). Columns A–C show three different colony morphologies belonging to the Group 2 isolates: CMW 7272, CMW 7269, CMW 7293. Columns D–F show three different colony morphologies that belong to the Group 1 isolates: CMW 7488, CMW 5236, CMW 7479.

### Individual datasets



### Combined dataset

(1670 bp)

**Fig. 4.** Schematic structural organization of the genomic regions used in this study. ITS regions and intron sequences are represented in solid black. Letters "I" indicate introns and letters "E" indicate exons. Sizes of the individual and combined partition alignments are given in brackets. Note that intron between E3 and E4 in the BT2 region is not present.

**Table 2.** Summary of the shared fixed positions found in the DNA regions of ITS, BT2, EF1- $\alpha$  and ATP6 among *Colletogloeopsis* isolates associated with *Eucalyptus* stem cankers. The total number of fixed shared positions between the two groups is given in the last column.

Locus	Locations according to the alignments* and the nucleotide fixed state found in group 1 <sup>a</sup> and group 2 <sup>b</sup>											No. of fixed positions
ITS	89*	107	116	396	436	-	-	-	-	-	-	5
	T <sup>a</sup> /C <sup>b</sup>	T/C	T or C/del	C/T	C/T							
BT2	8	28	29	35	38	41	46	50	174	261	300	11
	T/C	A/G	G/A	G/A	G/A	T/G	T or G/A	A/G	T/C	G/C	T/C	
EF1- $\alpha$	114	122	137	143	153 to 172	175	183	195	196	-	-	9
	C/T	del/ C	C/A	C/T	in /del <sup>c</sup>	G/A	C/T	G/A	A/G			
ATP6	644	-	-	-	-	-	-	-	-	-	-	1
	A/G											

\*Location of the fixed shared polymorphisms. The number in this cell and in all the other cells represent the location of fixed shared polymorphisms. They are defined in base pairs counting from the beginning of the alignment

<sup>a</sup>The first letter before the slash bar represents the state character shared by isolates of the group 1, *C. zuluensis*.

<sup>b</sup>Character state shared by isolates of the group 2, *C. gauchensis*.

<sup>c</sup>The grey box in the EF1- $\alpha$  line indicates the position of the 20 bp in/del that could be used for diagnostic purposes.

For parsimony analyses, heuristic searches were used with the steepest descent option and the TBR swapping algorithm. The characters were equally weighted and treated as unordered. Statistical support of the nodes in the trees was tested with 1000 bootstrap replicates. Distance analyses were conducted using MEGA 3.0 (Kumar *et al.* 2004). Pairwise distances were estimated using the Kimura with two parameters model (Kimura 1980). A gamma distribution  $\alpha = 0.5$  was used to take into account the differences in mutation rate among sites, due to the mix of coding and non-coding sequences present in the analysed fragments. The individual gene reconstructions were performed with Minimum Evolution (Rzhetsky & Nei 1993). Gaps generated in the alignment were treated as missing data. One thousand bootstrap replicates were made to assess the statistical support of the nodes in the phylogenetic trees. Trees were rooted to midpoint.

Partitions were considered together using Bayesian analyses (Ronquist & Huelsenbeck 2003). It has recently been shown that the Bayesian method is more sensitive to under-specification than over-specification of the evolutionary model (Huelsenbeck & Rannala 2004) when calculating the posterior probabilities. Consequently, a time-reversible complex model with gamma-distributed rate variation (GTR + I + G) was selected to combine the data sets. This model of DNA substitution allows the consideration of different rates of substitutions among sites, different nucleotide frequencies, and differences in the rate of substitutions among nucleotides. Therefore, four sets of analyses were run in MrBayes 3.1.1 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) calculating

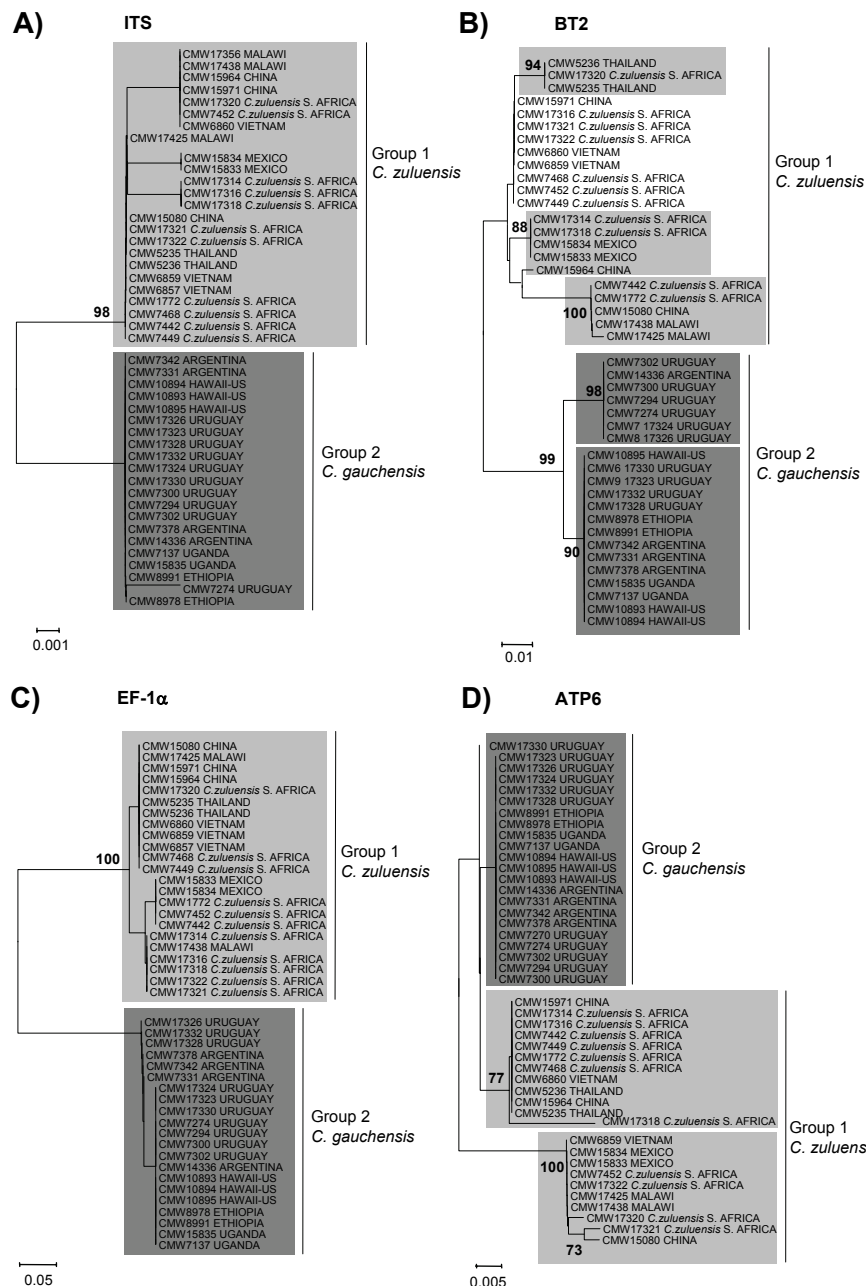
marginal posterior probabilities using the selected time reversal GTR + I + G model of nucleotide substitution (Tavaré 1986, Yang 1993, 1994) and default values for the prior settings. Four Monte Carlo Markov chains were run for 3 million generations. Trees and parameters were recorded every 100 generations. Likelihood stability was reached at 30 000 generations. This number of generations was then established as the “burn-in” period (represented by 3001 trees). A half compatible consensus tree was recovered from the remaining sampled trees. The Bayesian procedure was repeated four times. The posterior probabilities are indicated close to the respective nodes on the tree and the sequences of *Mycosphaerella colombiensis* Crous & M.J. Wingf. and *M. suttonii* Crous & M.J. Wingf. were used as outgroups.

#### Temperature sensitivity studies

Plugs (3 mm diam) of colonised agar were cut from actively growing cultures and placed at the centres of Petri dishes containing MEA. Isolates tested for growth characteristics at different temperatures included those from South Africa (CMW 7442, CMW 7449, CMW 7479, CMW 7488), and others from Uruguay (CMW 7269, CMW 7274, CMW 7279, CMW 7300). Three plates were prepared for each isolate and these were incubated at temperatures between 5 °C and 35 °C at 5 ° intervals, for 6 wk. A second set of isolates from Ethiopia (CMW 8282, CMW 8292) and from China (CMW 15966, CMW 15971) were tested in a similar manner but for an incubation period of 8 wk. Growth was recorded weekly by measuring average colony diameter.

<i>M. molleriana</i>	C-AGCAGC-A	TCTTCGCA--	-----GA	ATCGCAATTA	CTACTAGCGG
<i>M. ambiphylla</i>	C-AGCAGC-A	TCTTCGCA--	-----GA	ATCGCAATTA	CTACTAGCGG
SOUTH AFRICA 1	ATACCACCGT	TCGCTGCAAT	ACATCGCCAG	GCAGCATCCG	CTATTATTGA
SOUTH AFRICA 2	ATACCACCGT	TCGCTGCAAT	ACATCGCCAG	GCAGCATCCG	CTATTATTGA
THAILAND 1	ATACCACCGT	TCGCTGCAAT	ACATCGCCAG	GCAGCATCCG	CTATTATTGA
MEXICO 1	ATACCACCGT	TCGCTGCAAT	ACATCGCCAG	GCAGCATCCG	CTATTATTGA
MEXICO 2	ATACCACCGT	TCGCTGCAAT	ACATCGCCAG	GCAGCATCCG	CTATTATTGA
THAILAND 2	ATACCACCGT	TCGCTGCAAT	ACATCGCCAG	GCAGCATCCG	CTATTATTGA
VIETNAM 1	CTACCACCGT	TCGCTGCAAT	ACACCGCCAG	GCAGCATCCG	CCATCCTTGA
VIETNAM 2	CTACCACCGT	TCGCTGCAAT	ACACCGCCAG	GCAGCATCCG	CCATCCTTGA
ARGENTINA 1	CTACCACC--	-----	-----AG	ACAGCATCTG	CCATCATTGA
URUGUAY 1	CTACCACC--	-----	-----AG	ACAGCATCTG	CCATCATTGA
HAWAII 1	CTACCACC--	-----	-----AG	ACAGCATCTG	CCATCATTGA
ETHIOPIA 1	CTACCACC--	-----	-----AG	ACAGCATCTG	CCATCATTGA
URUGUAY 2	CTACCACC--	-----	-----AG	ACAGCATCTG	CCATCATTGA
UGANDA 1	CTACCACC--	-----	-----AG	ACAGCATCTG	CCATCATTGA

**Fig. 5.** Partial alignment of isolates showing the characteristic 20 bp elongation factor 1- $\alpha$  in/del. The presence of the in/del identifies the Group 1 isolates (light grey) from Group 2 (dark grey) isolates. All isolates in Table 1 can be assigned correctly into Groups 1 or 2 according to the presence/absence of this fragment.



**Fig. 6.** Phylograms generated using Minimum Evolution and K2P with gamma distribution,  $\alpha = 1$ . A. ITS. B.  $\beta$ -tubulin. C. EF1- $\alpha$ . D. ATP6. Values on branches are bootstrap support (1000 replicas).



## Morphology

Descriptions are based on sporulation *in vivo*. Wherever possible, 30 measurements ( $\times 1000$  magnification) were made of structures mounted in lactic acid, the 95 % deviation determined, and the extremes of spore measurements given in parentheses. Colony colours (surface and reverse) were assessed after 25 d on MEA at 25 °C in the dark, using the colour charts of Rayner (1970).

## RESULTS

### PCR and sequence analyses

Sequenced amplicons obtained from *C. zuluensis* isolates for the four different gene regions were aligned to study fixed polymorphisms. Alignments of 469 bp (ITS), 308 bp (BT2), 254 bp (EF1- $\alpha$ ) and 656 bp

(ATP6) were generated. The intron between the exons 3 and 4 of the  $\beta$ -tubulin gene was missing in all isolates studied. Visual analyses of the characters defined two groups among the isolates based on the fixed, shared polymorphisms. The first group included isolates from South Africa, China, Thailand, Vietnam and Malawi and a second group comprised isolates from Uruguay, Argentina, Hawaii, Uganda and Ethiopia. Positions in base pairs of the different fixed characters in the alignments for the various isolates are shown in Table 2. Five fixed characters were found at the ITS region, eleven were found in the BT2 dataset, eight were found at the EF1- $\alpha$  intron where a 20-base-pair indel was also found (Fig. 5). One fixed position was found in the ATP6 region.

### Phylogenetic analyses

Individual phylograms were obtained for each gene region and parsimony data produced very similar

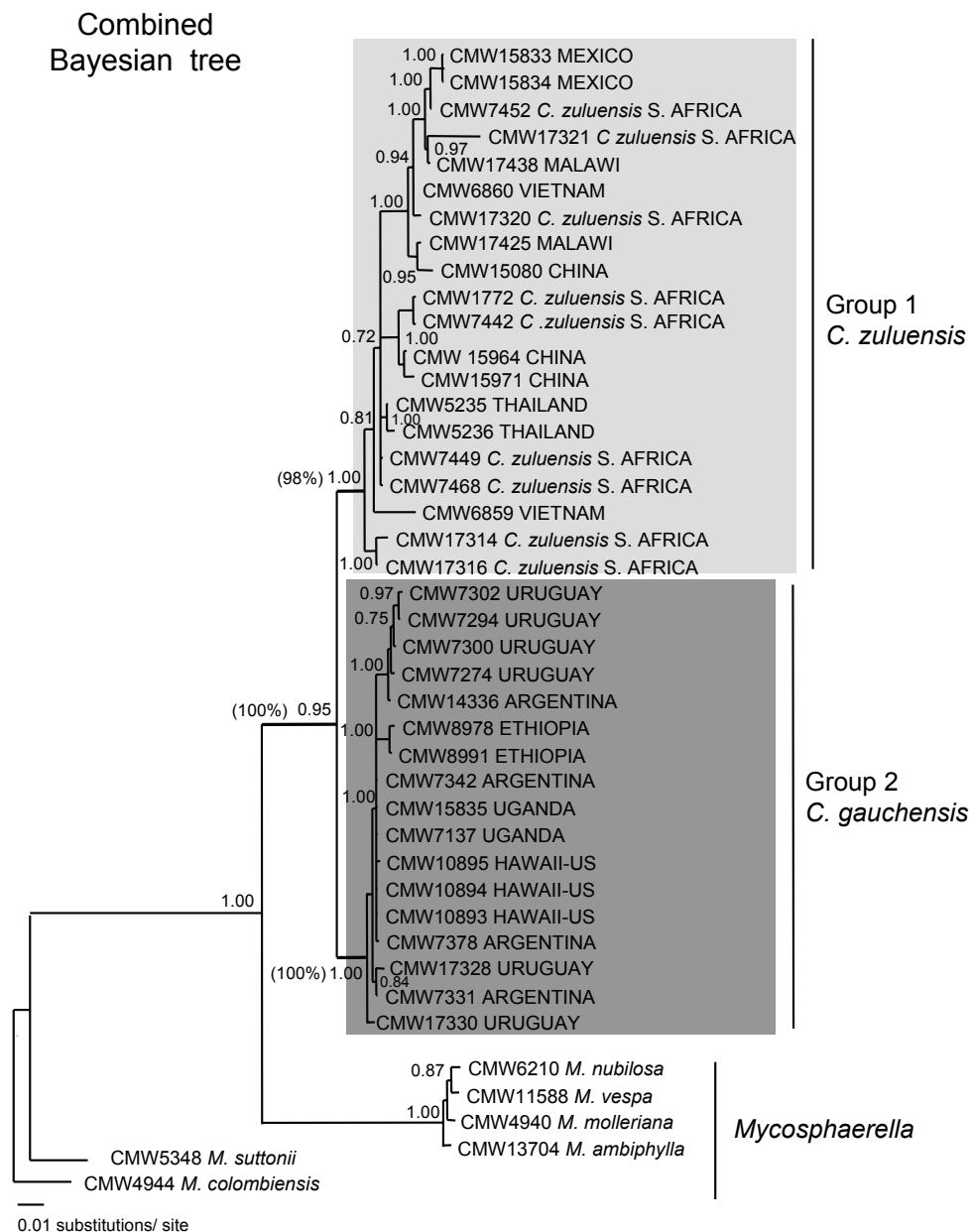


Fig. 7. Bayesian combined tree using a GTR+G+I model of substitutions. Posterior probabilities are shown on the branches. Parsimony bootstrap values are shown in brackets.

topologies to those of the distance trees. Therefore, only distance trees are presented (Fig. 6). In all cases the Bootstrap cut-off of 70 % was established.

Analyses of sequence data for the ITS region resolved two coherent clusters for the *Colletogloeopsis* isolates considered. These groups represented isolates from South Africa, Malawi, Mexico, Thailand, Vietnam and China (Group 1) and those from Uruguay, Argentina, Hawaii, Ethiopia and Uganda (Group 2). The separation of these two groups had 98 % bootstrap support in the ITS tree. In the BT2 and EF-1 $\alpha$  trees, these two groups had 99 % and 100 % support, respectively. For the ATP6 tree, three groups could be distinguished although only one of these had strong support (100 %). The group having reasonable support included isolates from Vietnam, Mexico, Malawi, China and South Africa. Internal sub-clusters could be distinguished within the Group 1 and Group 2 clusters in the ITS, BT2 and EF1- $\alpha$  trees. These sub-clusters had greater than 70 % bootstrap support only in the BT2 tree. The assortment of isolates within the sub-clusters was different in different trees.

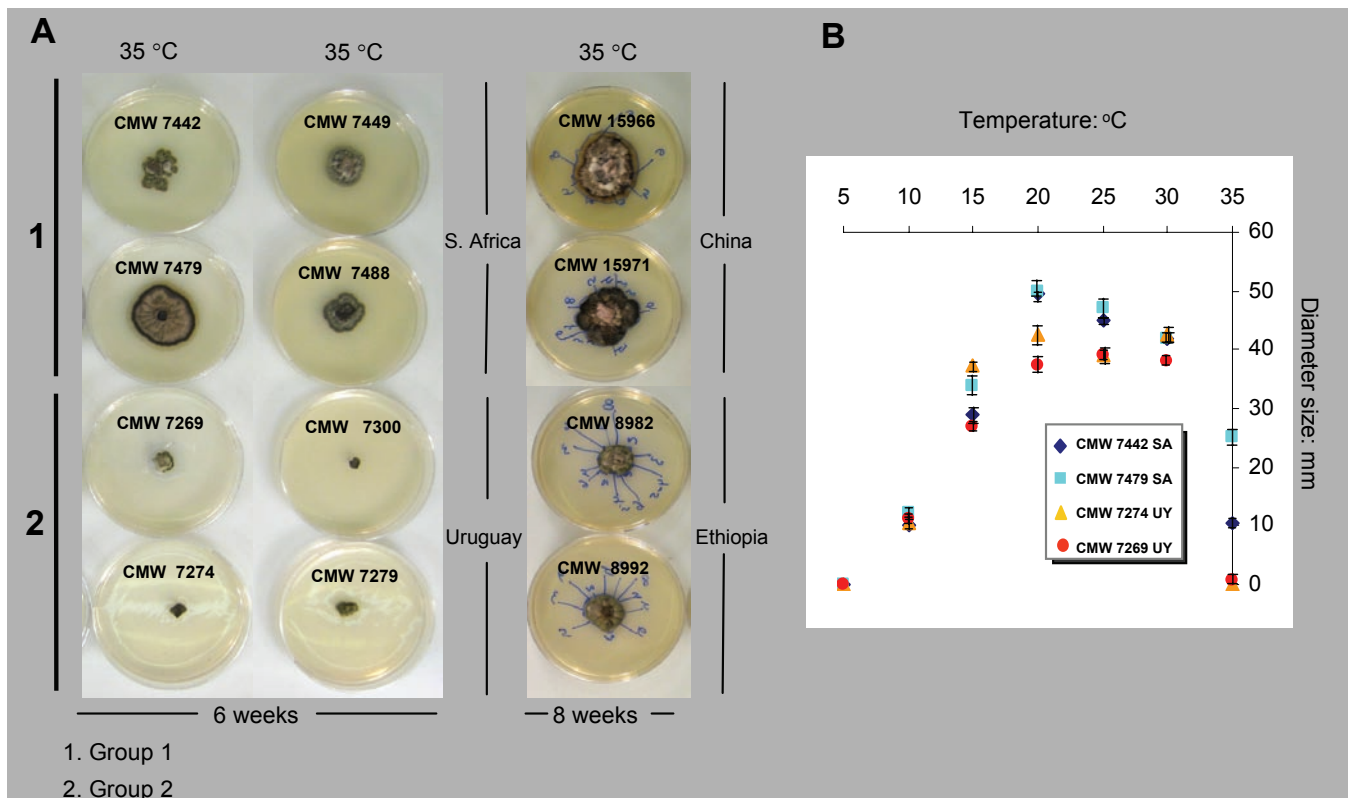
The level of polymorphism observed in the datasets was different for each individual analysed region. The  $\beta$ -tubulin data set presented the highest level of variation followed by the EF1- $\alpha$ , ITS and ATP6 data sets, respectively. A close inspection of the ATP6 data matrix showed few polymorphisms explaining the poor resolution obtained in the tree.

After the individual analyses, combined parsimony and Bayesian analysis were carried out (Fig. 7). The reconstructed trees included the collection of

*Colletogloeopsis* isolates together with *Mycosphaerella* spp. A posterior probability of 1 and a 100 % bootstrap value separated the *Colletogloeopsis* isolates from the rest of *Mycosphaerella* spp. The parsimony and Bayesian half-compatible trees showed two major groups representing isolates from South Africa, Malawi, Mexico, Thailand, Vietnam and China (Group 1) and those from Uruguay, Argentina, Hawaii, Ethiopia and Uganda (Group 2) supported by posterior probabilities of 1 and 0.95 and 98 % and 100 % bootstrap values, respectively. A rich internal topology was found within these two groups. Numerous sub-clusters were supported with high probabilities and bootstrap values. A number of these included more than one isolate from the same locality. Nevertheless, location was not sufficient to explain how the sub-clusters were formed.

### Temperature sensitivity studies

Average colony diameter for the isolates from South Africa and from Uruguay was different at some of the tested temperatures after 6 wk (Fig. 8). No measurable growth was found at 5 °C, optimal growth occurred between 20 and 25 °C, and the diameters of colonies decreased when they were incubated at temperatures of 30 °C and above. Differences between isolates from the two regions were seen at 10 °C where the Uruguayan isolates grew more rapidly than isolates from South Africa. Between 20 °C and 25 °C both groups of isolates achieved their maximum diameter. Nevertheless, these maximum diameters were smaller for the Uruguayan isolates. The most obvious difference between South African and Uruguayan isolates was



**Fig. 8.** Results of culture growth studies at different temperatures. A. Isolates from South Africa and Uruguay were tested for a period of 6 weeks and those from China and Ethiopia for a period of 8 weeks. Each point on the graph represents the average of 6 measurements taken at each temperature.

observed at 35 °C. At this temperature, the Uruguayan isolates hardly displayed growth whereas South African isolates reached between 10 and 20 mm diam.

The results obtained in a second experiment including isolates from China and Ethiopia, were very similar to those comparing isolates from South Africa and Uruguay. After 8 wk, the differences in growth of the isolates from both origins were obvious at 35 °C (Fig. 8). This is consistent with the fact that isolates from China are phylogenetically related to those from South Africa and those from Ethiopia are related to those from Uruguay.

### Morphology

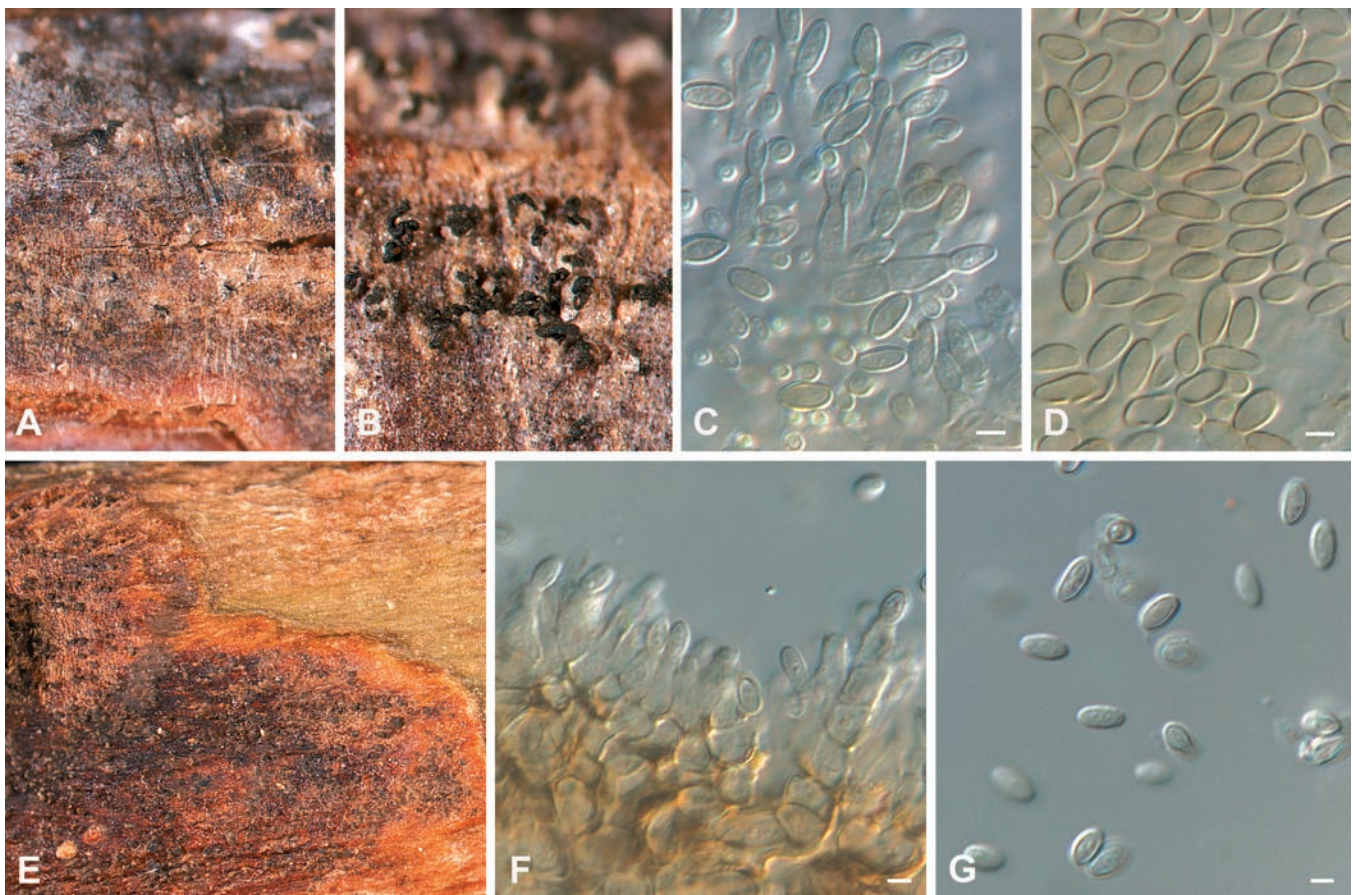
Isolates of *Colletogloeopsis* included in this study were morphologically variable in culture. Colony characteristics overlapped for isolates from South Africa and Uruguay, but it was possible to recognise some characteristics apparently exclusive to the Uruguayan isolates. Likewise, distinctly different conidial and conidiogenous cell characteristics were found when isolates from Uruguay were compared with those of *C. zuluensis* from South Africa (Fig. 9). The range of conidial lengths overlapped almost entirely between *C. zuluensis* [conidia (4–)4.5–5(–6) × 2–2.5(–3.5) μm] and the isolates from Uruguay [conidia (4–)5–6(–7.5) × (2–)2.5(–3) μm]. The Uruguayan conidia, however, had a larger maximum length, reaching 7.5 μm (6 μm for *C. zuluensis*). Conidia of *C. zuluensis* were slightly wider

(3.5 μm) as opposed to those from Uruguay, which were an average of 3 μm. Another distinctive characteristic of the fungus from Uruguay is that it has sympodial polyphialidic conidiogenous cells, which is different to *C. zuluensis*, which has percurrently proliferating monophialidic conidiogenous cells.

### Taxonomy

Phylogenetic analyses in this study supported two distinct groups of isolates, encompassed within the fungus currently treated as *C. zuluensis*. One of these groups of isolates is from South Africa, Malawi, Thailand, Vietnam, China and Mexico. The other group includes isolates from Uruguay, Argentina, Hawaii-U.S.A., Ethiopia and Uganda. These fungi can also be separated by characteristics of growth in culture, morphology and growth at different temperatures. Clearly, the South African fungus must retain the name *C. zuluensis*. At the time of describing this fungus, no ex-type cultures were deposited. We have thus provided a suite of isolates for which DNA sequence data are available, and that are tied to herbarium specimens to serve as epitypes. The fungus occurring in Uruguay and other countries represents a distinct taxon that is described below.

***Colletogloeopsis gauchensis*** M.-N. Cortinas, Crous & M.J. Wingf., **sp. nov.** MycoBank MB500854. Figs 9–10.



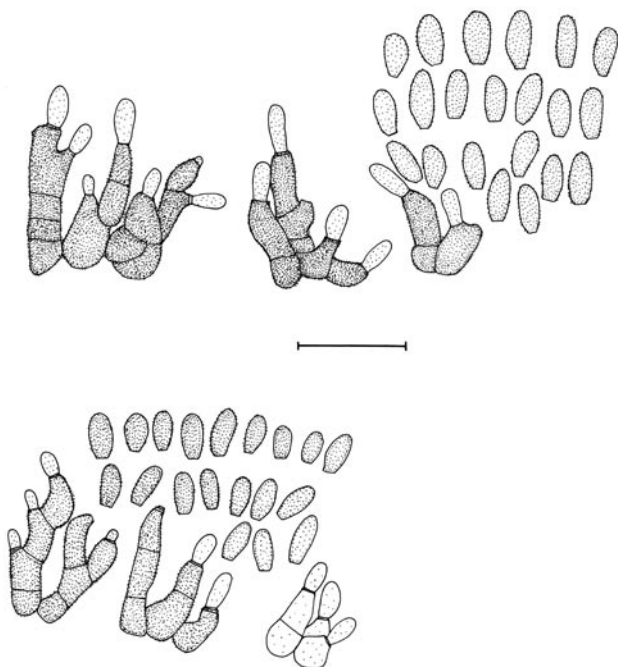
**Fig. 9.** *Colletogloeopsis* spp. sporulating on *E. grandis* stems. A–D. *Colletogloeopsis gauchensis* (holotype). A–B. Pycnidia with black cirri. C. Conidiogenous cells. D. Conidia. E–G. *Colletogloeopsis zuluensis* (epitype). E. Pycnidia. F. Conidiogenous cells. G. Conidia. Scale bars = 2.5 μm.

**Etymology:** Named after the gauchos people of South America that live in the same area where this species is distributed and where it was first collected. In the same genus, *C. zuluensis* is named after the KwaZulu-Natal Province and the “Zulu” people of South Africa.

*Colletogloeopsis zuluensis* similis, sed conidiis angustioribus, (4–)5–6(–7.5) × (2–)2.5(–3) µm et phialidibus nonnumquam sympodialiter proliferantibus distincta.

Lesions caulicolous, subcircular to irregular, dark brown, 2–10 mm diam, with a raised, red-brown border. *Conidiomata* pycnidial to somewhat acervular, subepidermal, single, rarely aggregated, occurring in necrotic tissue, globose to slightly depressed, becoming erumpent, up to 120 µm diam, exuding conidia in a long cirrus; conidiomatal walls composed of 2–3 layers of medium brown *textura angularis*; opening by a central ostiole or irregular rupture; ostiolar region lined with thick-walled, brown, smooth, septate hyphae that are sometimes branched below, 3–4 µm wide, with obtuse ends that flare apart (upper 1–6 cells). *Conidiophores* subcylindrical, subhyaline to medium brown, smooth to finely verruculose, 0–3-septate, unbranched or branched below, 10–20 × 3–6 µm. *Conidiogenous cells* subhyaline to medium brown, doliform to subcylindrical, smooth to finely verruculose, mono- to polyphialidic, proliferating percurrently, with several percurrent proliferations near the apex. *Conidia* medium brown, thick-walled, finely verruculose, broadly ellipsoidal, apex obtuse to subobtuse, base subtruncate to bluntly rounded, (4–)5–6(–7.5) × (2–)2.5(–3) µm; base frequently with a minute marginal frill.

**Specimens examined:** Uruguay, El Tarugo, bark of 1-yr-old *E. grandis* tree, Feb. 2005, M.J. Wingfield, CBS H-19724 **holotype**, cultures ex-holotype CMW 17331–17332; La Herradura, CBS H-19722, cultures CBS 119467–119466 = CMW 17542–17543; *ibid.*,



**Fig. 10.** *Colletogloeopsis* spp. sporulating on *E. grandis* stems. Conidiogenous cells and conidia of *Colletogloeopsis gauchensis* (holotype) (top). Conidiogenous cells and conidia of *Colletogloeopsis zuluensis* (epitype) (bottom). Scale bar = 10 µm.

CBS H-19723, cultures CBS 119465 = CMW 17545, CMW 17544; La Juanita, CBS H-19725, cultures CBS 119468 = CMW 17558, CMW 17559; *ibid.*, CBS H-19726, cultures = CMW 17560–17561.

**Cultural characteristics:** Colony characteristics on MEA at 25 °C are variable. Colony colours were similar to those of *C. zuluensis* (Van Zyl *et al.* 1997, 2002). Surface colours range from greyish yellow-green, dull green, isabelline, greenish olivaceous to grey-olivaceous; colonies in reverse range from dark grey, dark olive-grey to dark green (Rayner 1970); margins are smooth, regular or irregular. Some cultures develop a characteristic white outer zone of aerial mycelium (Fig. 3). Paler colonies develop smoother surfaces with white aerial mycelium; some strains produce a diffuse yellow pigment in MEA.

**Notes:** *Colletogloeopsis gauchensis* [conidia (4–)5–6(–7.5) × (2–)2.5(–3) µm] can readily be distinguished from *C. zuluensis* [conidia (4–)4.5–5(–6) × 2–2.5(–3.5) µm] by its slightly longer conidia, and the presence of sympodial polyphialidic conidiogenous cells (Figs 9–10). Furthermore, it grows readily at 10 °C, with hardly any to no growth at 35 °C. In contrast, *C. zuluensis* grows more slowly at 10 °C, and faster at 35 °C than *C. gauchensis*, and strains of *C. gauchensis* do not form conidiomata in culture.

***Colletogloeopsis zuluensis*** (M.J. Wingf., Crous & T.A. Cout.) M.-N. Cortinas, M.J. Wingf. & Crous, *Mycol. Res.* 110: 235. 2006. Figs 9–10. [as *zuluense*].

**Basionym:** *Coniothyrium zuluense* M.J. Wingf., Crous & T.A. Cout., *Mycopathologia* 136: 142. 1997.

**Specimens examined:** South Africa, KwaZulu-Natal, Kwambonambi, Teza nursery, bark of 1-yr-old *E. grandis* tree, Jan. 1996, M.J. Wingfield, IMI 370886 **holotype**; KwaZulu-Natal, Kwambonambi, *E. grandis*, Feb. 2005, M.J. Wingfield, CBS H-19721 **epitype here designated**, culture ex-epitype CMW 17321–17322; CBS H-19717, culture CBS 119427 = CMW 17531, CMW 17530; CBS H-19720, culture CBS 119471 = CMW 17528, CMW 17529; CBS H-19719, culture CBS 119470 = CMW 17320, CMW 17319; CBS H-19718, culture CBS 119469 = CMW 17526, CMW 17527.

## DISCUSSION

Phylogenetic analyses for a large number of *C. zuluensis* isolates from different parts of the world and based on multiple gene regions have shown clearly that this material represents at least two discrete taxa. These species are described based on material from South Africa and Uruguay, but both taxa include collections from many different countries. Thus *C. zuluensis* is now known from South Africa, Malawi, Thailand, Vietnam, China and Mexico. Likewise, *C. gauchensis* described in this study occurs not only in Uruguay but also in Argentina, Hawaii-U.S.A., Ethiopia and Uganda. The two fungi thus represent distinct phylogenetic species but they can clearly be distinguished from each other based on morphological characteristics and growth characteristics in culture.

Twenty-six fixed nucleotide positions allowed us to separate the collection of *C. zuluensis* *s. lat.* isolates used in this study into two distinctive groups. One of

these fixed polymorphisms found in the EF1- $\alpha$  intron can easily be used to discriminate between *C. zuluensis* and *C. gauchensis*. This 20 bp fragment between positions 153 to 172 in *C. zuluensis* is absent in *C. gauchensis*. The p-distance among the *Colletogloeopsis* isolates considered in this study displayed a range of 0 to 1 % divergence in ITS sequences, 0–8 % for BT2 sequences, 0–24 % for EF1- $\alpha$  sequences and 0–4 % for ATP6 data-matrices respectively. These ranges showed that there was sufficient variation within *Colletogloeopsis* to suspect that more than one taxon was represented in the collection of isolates. The distances are also consistent with values used in previous studies (Couch & Kohn 2002, Barnes *et al.* 2005) to separate taxa.

Very few morphological differences were found between isolates of *C. zuluensis* from South Africa and isolates of *C. gauchensis* from Uruguay. These differences include the fact that Uruguayan isolates have polyphialidic, sympodially and percurrently proliferating conidiogenous cells as opposed to the monophialidic, percurrently proliferating conidiogenous cells in *C. zuluensis*. The conidia of *C. gauchensis* are also consistently longer than those of *C. zuluensis* (Figs 9–10). Furthermore, *C. gauchensis* is adapted to cooler climates than *C. zuluensis*. On the contrary, isolates of *C. zuluensis* grow well at 35 °C, whereas those of *C. gauchensis* barely grow at this temperature.

Results of this study provide added support for the view that *C. zuluensis* and *C. gauchensis* are anamorphs of *Mycosphaerella*. They have an allopatric distribution and are considered sibling species only in terms of the fact that they are ecologically and morphologically very similar. The extent to which cryptic and sibling species occur in taxonomic groups varies depending on the group of fungi studied. However, the discovery of cryptic species such as *C. gauchensis* in this study is becoming a commonplace when DNA studies are implemented (see Crous *et al.* 2006). Results of such studies reveal that these species reflect collections of morphologically similar taxa that can only be discriminated based on minute morphological details or characteristics in pure culture. A further example of such a species complex in *Mycosphaerella* concerns “*Coniothyrium*” *ovatum* H.J. Swart (Crous *et al.* 2004a, b, 2006 – this volume), which will be treated elsewhere (Cortinas *et al.* in prep.).

Intraspecific variation detected amongst isolates of *C. zuluensis* and to a lesser extent *C. gauchensis* showed internal structure in the individual and combined trees. Such intraspecific structure was only well-supported in the BT2, ATP6 and combined trees. Based solely upon the phylogenetic species concept, it would be possible to recognise additional species especially in this complex. For the present, however, we choose to not provide additional names before robust population biology studies are available.

Coniothyrium canker is one of the most important diseases of *Eucalyptus* worldwide (Old *et al.* 2003). In South Africa, it appeared relatively suddenly in a very limited location and spread rapidly, resulting in very substantial losses to the local forestry industry. The disease has also caused substantial damage to plantations in other countries such as Argentina and

Uruguay. It is thus intriguing that there are two distinct fungi associated with indistinguishable symptoms. The origin of the fungus is unknown and it is not known to occur in the native range of *Eucalyptus*. The evidence from this study shows that the two fungi are closely related and have adapted differently based on some ecological factor. Like most *Mycosphaerella* spp. they are highly host-specific to certain species of *Eucalyptus*, grow poorly in culture, and thus it seems reasonable to expect that their origin would be on *Eucalyptus* or a host closely related to it. A similar situation has emerged for species of *Chrysosporthe* Gryzenh. & M.J. Wing. (Gryzenhout *et al.* 2004). that are well-known pathogens of *Eucalyptus* but that appear to have originated on a wide variety of woody plants in the order *Myrtales* (Wingfield 2003, Gryzenhout *et al.* 2004, Seixas *et al.* 2004).

Recognition of two species within a collection of isolates that have previously been recognised as belonging to the single taxon has important consequences for disease control and quarantine. In the past, it has been suggested that the fungus originated in South Africa, and that it was restricted to that country (Wingfield *et al.* 1997). Thus, the appearance of the disease in other countries has often been linked to the movement of plant material and particularly seed to other countries. Although it has not been shown experimentally that *C. zuluensis* is moved on seed, this appears to be a likely mode of global distribution. There is a large international trade in *Eucalyptus* seed, which is variably controlled and monitored. Both *C. zuluensis* and *C. gauchensis* have now wide geographic distributions and this implies that they have been spread from one or a number of sources. Every effort should now be made to restrict them from further movement to new countries and areas.

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## REFERENCES

- Alemu G, Cortinas MN, Wingfield MJ, Roux J (2005). Characterisation of the *Coniothyrium* stem canker pathogen on *Eucalyptus camaldulensis* in Ethiopia. *Australasian Plant Pathology* **34**: 1–6.
- Alemu G, Roux J, Thu PQ, Wingfield MJ (2004). Coniothyrium stem canker of *Eucalyptus*, new to Argentina and Vietnam. *South African Journal of Science* **99**: 587–588.
- Alemu G, Roux J, Wingfield MJ (2003). Diseases of exotic plantation *Eucalyptus* and *Pinus* species in Ethiopia. *South African Journal of Science* **99**: 29–33.
- Barnes I, Crous PW, Wingfield BD, Wingfield MJ (2004). Multigene phylogenies reveal that red band needle blight of *Pinus* is caused by two distinct species of *Dothiostroma*, *D. septosporum* and *D.*

- pini*. *Studies in Mycology* **50**: 551–565.
- Carbone I, Kohn LM (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* **91**: 553–556.
- Cortinas MN, Burgess T, Dell B, Xu D, Crous PW, Wingfield BD, Wingfield MJ (2006). First record of *Colletogloeopsis zuluensis* comb. nov., causing stem canker of *Eucalyptus* in China. *Mycological Research* **110**: 229–236.
- Cortinas MN, Koch N, Thain J, Wingfield BD, Wingfield MJ (2004). First record of the *Eucalyptus* stem canker pathogen, *Coniothyrium zuluense* from Hawaii. *Australasian Plant Pathology* **33**: 309–312.
- Couch BC, Kohn LM (2002). A multilocus gene genealogy concordant with host preferences indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. *Mycologia* **94**: 683–693.
- Crous PW, Groenewald JZ, Mansilla JP, Hunter GC, Wingfield MJ (2004a). Phylogenetic reassessment of *Mycosphaerella* species and their anamorphs occurring on *Eucalyptus*. *Studies in Mycology* **50**: 195–214.
- Crous PW, Groenewald JZ, Pongpanich K, Himaman W, Arzanlou M, Wingfield MJ (2004b). Cryptic speciation and host specificity among *Mycosphaerella* species occurring on Australian *Acacia* species grown as exotics in the tropics. *Studies in Mycology* **50**: 457–469.
- Crous PW, Wingfield MJ, Mansilla JP, Alfenas AC, Groenewald JZ (2006). Phylogenetic reassessment of *Mycosphaerella* species and their anamorphs occurring on *Eucalyptus*. II. *Studies in Mycology* **55**: 99–131.
- Glass NL, Donaldson GC (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61**: 1323–1330.
- Gryzenhout M, Myburg H, Merwe NA van der, Wingfield BD, Wingfield MJ (2004). *Chrysoporthe*, a new genus to accommodate *Cryphonectria cubensis*. *Studies in Mycology* **50**: 119–142.
- Huelsbeck JP, Ronquist F (2001). MrBayes: Bayesian inference of phylogeny. *Bioinformatics* **17**: 754–755.
- Huelsbeck JP, Rannala B (2004). Frequentist properties of Bayesian posterior probabilities of phylogenetic trees under simple and complex substitution models. *Systematic Biology* **53**: 904–913.
- Kimura M (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**: 111–120.
- Kretzer A, Bruns TD (1999). Use of ATP6 in fungal phylogenetics: An example from *Boletales*. *Molecular Phylogenetics and Evolution* **13**: 483–492.
- Kumar S, Tamura K, Nei M (2004). MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* **5**: 150–163.
- Old KM, Wingfield MJ, Yuan ZQ (2003). *A manual of diseases of eucalypts in South-East Asia*, Center for International Forestry Research, Bogor, Indonesia.
- Rayner, R. W. (1970). *A mycological colour chart*. Commonwealth Mycological Institute, Kew.
- Roux J, Wingfield MJ, Cibrián D (2002). First report of *Coniothyrium* canker in Mexico. *Plant Pathology* **51**: 382.
- Ronquist F, Huelsenbeck JP (2003). MrBayes 3.0: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.
- Rzhetsky A, Nei M (1993). Theoretical foundation of the minimum-evolution method of phylogenetic inference. *Molecular Biology and Evolution* **10**: 1073–1095.
- Seixas CDS, Barreto RW, Alfenas AC, Ferreira FA (2004). *Cryphonectria cubensis* on an indigenous host in Brazil: a possible origin for eucalyptus canker disease? *Mycologist* **18**: 18–24.
- Slippers B, Stenlid J, Wingfield MJ (2005). Emerging pathogens: fungal host jumps following anthropogenic evolution. *Trends in Ecology and Evolution* **20**: 420–421.
- Swofford DL (2002). *PAUP\*: Phylogenetic Analysis using Parsimony (\*and other methods)*. Version 4. Sinauer Associates, Sunderland, Massachusetts, U.S.A.
- Tavaré S (1986). Some probabilistic and statistical problems on the analysis of DNA sequences. *Lectures on Mathematics in the Life Sciences* **17**: 57–86.
- Van Zyl LM, Coutinho TA, Wingfield MJ (2002a). Morphological, cultural and pathogenic characteristics of *Coniothyrium zuluense* isolates from different plantation regions in South Africa. *Mycopathologia* **155**: 149–153.
- Van Zyl LM, Coutinho TA, Wingfield MJ, Pongpanich K, Wingfield BD (2002b). Morphological and molecular relatedness of geographically diverse isolates of *Coniothyrium zuluense* from South Africa and Thailand. *Mycological Research* **106**: 51–59.
- Van Zyl LM, Wingfield MJ, Coutinho TA (1997). Diversity among isolates of *Coniothyrium zuluense*, a newly recorded *Eucalyptus* stem canker pathogen in South Africa. In: *Proceedings of the IUFRO Conference on Silviculture and Improvement of Eucalyptus* (EMBRAPA/CNPF). Salvador, 24–29 August 1997, Brazil. *Anais, Colombo*. **3**: 135–141.
- White TJ, Bruns T, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *A Guide to Molecular Methods and Applications* (Innis MA, Gelfand DH, Sninsky JJ, White JW, eds). Academic Press, New York: 315–322.
- Wingfield MJ (2003). Increasing threat of diseases to exotic plantation forests in the southern hemisphere: Lessons from *Cryphonectria* canker. *Australasian Plant Pathology* **32**: 133–139.
- Wingfield MJ, Crous PW, Coutinho TA (1997). A serious new canker disease of *Eucalyptus* in South Africa caused by a new species of *Coniothyrium*. *Mycopathologia* **136**: 139–145.
- Yang Z (1993). Maximum likelihood estimation of phylogeny from DNA sequences when substitution rates differ over sites. *Molecular Biology and Evolution* **10**: 1396–1401.
- Yang Z (1994). Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: Approximate methods. *Journal of Molecular Evolution* **39**: 306–314.