

Endophyte isolations from *Syzygium cordatum* and a *Eucalyptus* clone (Myrtaceae) reveal new host and geographical reports for the Mycosphaerellaceae and Teratosphaeriaceae

Angelica Marsberg · Bernard Slippers · Michael J. Wingfield · Mariëka Gryzenhout

Abstract Species of Mycosphaerellaceae and Teratosphaeriaceae (Ascomycetes) cause important leaf, shoot and canker diseases globally on a broad range of hosts, including *Eucalyptus* and other Myrtaceae. Recently, species of the Mycosphaerellaceae and Teratosphaeriaceae have been isolated as asymptomatic endophytes. In this study, endophytic species of Mycosphaerellaceae and Teratosphaeriaceae were isolated from samples taken from healthy native *Syzygium cordatum* (Myrtaceae) and related non-native *Eucalyptus grandis* x *E. camaldulensis* (hybrid clone) growing in Mtubatuba, KwaZulu Natal, South Africa. Multi-locus sequence analysis (MLSA) using the Internal Transcribed Spacer (ITS) region, the partial Large Subunit (LSU; 28S nrDNA) of the nuclear ribosomal DNA operon and Translation Elongation Factor-1 α (TEF-1 α) genes were used to correctly identify the 22 resulting isolates. The isolates grouped in five clades representing *Readeriella considenianae* that was isolated only from the *Eucalyptus* hybrid clone, *Mycosphaerella marksii* and *M. vietnamensis* from *S. cordatum* and *Pseudocercospora crystallina* from both *S. cordatum* and the *Eucalyptus* hybrid clone. Interestingly, the serious canker pathogen *T. zuluensis* was isolated from *Eucalyptus* leaves, although it is known only from stem and branch cankers. Of the species found, *R. considenianae* and *M. vietnamensis* were found in South Africa for the first time, while *M. marksii*, *M. vietnamensis* and *P. crystallina* were shown to naturally infect native *S. cordatum* for the first time. Despite the limited

number of trees sampled, the new host and distribution reports show that more intensive sampling, especially following an endophyte approach, will reveal more complete patterns of host preference and geographical distribution for these fungi.

Keywords Mycosphaerellaceae · Teratosphaeriaceae · *Eucalyptus grandis* · *Syzygium cordatum* · Native and non-native trees · South Africa

Introduction

Species of fungi in the Mycosphaerellaceae and Teratosphaeriaceae (Ascomycetes) previously resided in the single genus, loosely referred to as *Mycosphaerella sensu lato* (*Capnodiales*, *Dothideomycetes*). This group of fungi included approximately 3,000 species names with diverse ecological roles representing endophytes, pathogens and saprobes (Corlett 1995; Crous et al. 2000; Hunter et al. 2004, 2006; Crous et al. 2009c; Hunter et al. 2011). The pathogens in these genera include important leaf and shoot pathogens of a number of hosts, including *Eucalyptus* and other trees in the Myrtaceae. Collectively they cause the disease known as Mycosphaerella Diseases or Teratosphaeria Diseases (Hunter et al. 2011) in order to accommodate the groups that were previously defined solely as Mycosphaerella leaf blotch [MLB] or Mycosphaerella leaf diseases [MLD] (Crous et al. 1991; Hyde et al. 2007; Hunter et al. 2009; Sánchez Márquez et al. 2011). Globally, over 30 species of *Mycosphaerella* and *Teratosphaeria*, and their associated anamorph species, have been found to cause important leaf and canker diseases of *Eucalyptus* (Crous et al. 2000, 2004; Crous et al. 2006; Crous 2009; Crous et al. 2009a, b).

Morphological identification is difficult for species of the Mycosphaerellaceae and the Teratosphaeriaceae because they typically grow slowly in culture and many also sporulate only

A. Marsberg · B. Slippers · M. J. Wingfield
Department of Genetics, Forestry and Agricultural Biotechnology
Institute, University of Pretoria, Private Bag x20, Hatfield,
0028 Pretoria, South Africa

M. Gryzenhout (*)
Department of Plant Sciences, University of the Free State, PO Box
339, Bloemfontein 9300, South Africa
e-mail: GryzenhoutM@ufs.ac.za

on host tissue (Crous 1998; Crous et al. 2001a; Hunter et al. 2004, 2006). Where morphology can be used for identification, it is based on the associated but morphologically variable anamorph genera (Crous 1998; Crous et al. 2001a; Hunter et al. 2004). Thus, in recent years, DNA sequence data from the Internal Transcribed Spacer (ITS) ribosomal DNA region has most frequently been used to identify the species in these two groups (Crous et al. 2001a, b; Hunter et al. 2004, 2006). Application of multi-gene analyses using sequences for the ITS region, Large Subunit (LSU) of the nuclear ribosomal DNA operon, Translation Elongation Factor-1 α (TEF-1 α) and Actin (Act) genes showed that *Mycosphaerella* was polyphyletic (Crous et al. 2001b; Hunter et al. 2006; Crous 2009). Consequently, this led to the separation of the families to include the Cladosporiaceae (Schubert et al. 2007; Bensch et al. 2010), Dissoconiaceae (Crous et al. 2009a), Teratosphaeriaceae and the Mycosphaerellaceae (Crous et al. 2007, 2009d). Following this taxonomic rearrangement, many species of *Mycosphaerella* have been transferred to the closely related Teratosphaeriaceae, which include pathogens of *Eucalyptus* such as *Teratosphaeria cryptica*, *T. nubilosa*, *T. gauchensis* and *T. zuluensis* amongst others (Crous et al. 2007; Andjic et al. 2010).

In South Africa, numerous species of the Mycosphaerellaceae and Teratosphaeriaceae are known to infect *Eucalyptus* trees (Crous and Wingfield 1996; Crous 1998; Hunter et al. 2004, 2011). Fewer species including *M. syzygii*, *M. marasasii* and *P. syzygiiicola* have been found to be associated with the closely related native Myrtaceous tree, *Syzygium cordatum* (Sutton and Crous 1997; Crous 1999; Crous et al. 2001a). Previous studies identifying the Mycosphaerellaceae and Teratosphaeriaceae on *Eucalyptus* spp. and *S. cordatum* followed a broad approach where trees were sampled over a large geographic area. Furthermore, the samples were collected from diseased trees. In the present study, a more dense endophyte sampling approach was used to directly compare the Mycosphaerellaceae and Teratosphaeriaceae species endophyte assemblages from a single *Eucalyptus grandis* hybrid clone and *S. cordatum* tree. Samples were collected at the same time from the same geographical location and identified using the most recently available multi-locus sequence phylogenies.

Materials and methods

Sample collection

A *S. cordatum* tree was sampled in a private nature reserve in the Mtubatuba area of KwaZulu Natal, South Africa (E32°9' 54.1; S28°29'53.0). An *E. grandis* x *E. camaldulensis* hybrid clone was selected in an adjacent plantation approximately 500 m from the *S. cordatum* tree. Four leaves from each of

four twigs that originated from four branches located in different positions, and a trunk increment core with the bark attached, were collected from each tree. Ten disks (approximately 5 mm in diameter) were cut from each leaf and 5 pieces (3 mm long) were taken from each twig and branch. Trunk increments were approximately 5 mm in diameter. The material was surface disinfected by first immersing them in 10 % hydrogen peroxide (H₂O₂) for 3–5 min, followed by two washes of 1 min each with sterilised distilled water.

After surface disinfection, the samples from the *Eucalyptus* and *S. cordatum* were plated onto 2 % Malt Extract Agar (MEA) (20 g malt extract, 20 g agar, 1 L distilled water; Biolab, Midrand, South Africa). These were incubated at 25 °C for a month to allow for slow growing endophytic fungi to emerge. The endophytic fungi resembling the Mycosphaerellaceae and Teratosphaeriaceae that emerged were purified by transferring them to new MEA plates and further incubated at 25 °C. Primary isolations were monitored for a month to ensure that these slow growing fungi were all captured. Approximately 50 isolates emerged of which only a subset of these were used in the study. These cultures are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), Pretoria, South Africa.

Multi-locus sequence analysis

Mycelium was scraped from the surface of cultures growing on 2 % MEA and freeze dried for 24 h. DNA was subsequently extracted using a modified CTAB method (Moller et al. 1992). After precipitating the DNA, the supernatant was discarded and the DNA pellet was washed twice with 100 μ l of 70 % ethanol and centrifuged at 10,000 rpm for 5 min for each wash step. The ethanol was removed and the pellet was air dried after the second wash until all the ethanol evaporated. The DNA pellet was re-suspended in 50 μ l nuclease-free water and allowed to incubate overnight at 4 °C (Moller et al. 1992). The DNA concentration of each sample was determined using a NanoDrop™ 1000 Spectrophotometer version 3.7 (Thermo Fisher Scientific Inc., Germiston, South Africa) and the working DNA stocks were diluted to 40 ng/ μ l with nuclease-free water.

The full-length ITS region of the ribosomal operon was amplified for each sample. PCR amplification was performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using the V9G (de Hoog and Gerrits van den Ende 1998) and ITS4 (White et al. 1990) primer pair. The volume for each reaction was 25 μ l with 40 ng of template DNA, 10 pmoles of each primer (Integrated DNA Technologies, Coralville, Iowa, USA), 1 \times NH₄ Reaction Buffer (Roche Products Pty Ltd, South Africa), 1.5 mM MgCl₂ (Roche Products Pty Ltd, South Africa), 75 μ M dNTPs (Roche

Products Pty Ltd, South Africa) and 5 units of Fast Start Taq (Roche Products Pty Ltd, South Africa). Nuclease-free water was added to obtain a final volume of 25 μ l. The cycle parameters were as follows: 94 °C denaturation for 5 min, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing at 50 °C for 30 s, and elongation at 72 °C for 45 s. A final extension step followed at 72 °C for 7 min. The primers used to amplify the TEF-1 α were EF1-728 F and EF1-986R (Carbone and Kohn 1990) while LROR (Moncalvo et al. 1995) and LR7 (Vilgalys and Hester 1990) were used to amplify the partial LSU (28S nrDNA) of the nuclear ribosomal DNA operon. The cycling conditions for the ITS and TEF-1 α were identical, while those for the LSU consisted of an initial denaturation step of 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min with a final extension step at 72 °C for 7 min.

The PCR products were cleaned with Sephadex G-50 columns (Steinheim, Germany) to enable direct DNA sequencing. The cleaned PCR product was used as a template for each sequencing reaction performed with the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems, Foster City, CA). Forward and reverse sequencing was performed with the same primers used for the PCR amplification. However, two additional internal primers, namely LR3R (Vilgalys and Hester 1990) and LR5 (Rehner and Samuels 1995) were required for the LSU region. The sequencing reactions were subsequently run on an ABI PRISM™ 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA) and the resultant sequences were manually analysed with CLC Main Workbench version 5.6 (CLC Bio A/S, Aarhus, Denmark) to assemble contigs with both the forward and reverse sequences. The sequences were identified by BLAST analysis on the GenBank database housed at the National Centre for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov).

In order to assign the isolates to genera, the LSU sequences were aligned with those for all species belonging to the Mycosphaerellaceae and Teratosphaeriaceae using MAFFT version 5.667 (Katoh et al. 2005) and a dataset with reference sequences provided by Dr E. W. Groenewald (CBS-KNAW Fungal Biodiversity Centre; Netherlands). The auto algorithm was employed in MAFFT as it automatically selects the appropriate strategy for the size of the dataset. A neighbour-joining tree, with complete deletion, was produced in MEGA version 4.0 (Tamura et al. 2007) to determine the genera in which the isolates could be placed. Due to the large size of the dataset (data not shown), only the species representing the nearest neighbours to the isolates of unknown identity were retained for further analyses.

The ITS and TEF-1 α sequences of these nearest neighbours were obtained from GenBank and included in the additional datasets. The ITS data set consisted of 33 taxa, which

included 22 sequences generated in this study and 11 reference sequences. The LSU dataset consisted of 34 taxa, of which 22 were generated sequences and 12 were reference sequences. The TEF-1 α dataset was comprised of 36 taxa, including 22 generated sequences and 14 reference sequences. The sequences for the three gene regions were aligned using the auto algorithm in MAFFT version 5.667 (Katoh et al. 2005). The combined ITS/LSU/TEF-1 α dataset consisted of 33 taxa, of which 22 sequences were generated and 11 were reference sequences.

Parsimony trees were constructed for the individual and combined datasets using PAUP version 4.0 b10 (Swofford 2003). The heuristic search function with random sequence additions (100) and the Tree Bisection Reconnection (TBR) algorithm was used to obtain the equally most parsimonious trees. All uninformative characters, including gaps and missing data, were excluded and characters were reweighted according to the consistency index (CI). Bootstrap values were calculated after 1,000 replicates to determine the level of branch support (Felsenstein 1985). Tree length (TL), consistency index (CI), retention index (RI) and the homoplasy index (HI) were all calculated to assess the trees for signal, noise and reliability (Hillis and Huelsenbeck 1992). A partition homogeneity test (PHT) consisting of 1,000 replicates was used to determine whether the ITS, LSU and TEF-1 α datasets could be combined (Farris et al. 1994). The trees were rooted to *Neofusicoccum ribis* and *Phaeobotryosphaeria visci* as they are sister taxa to the Mycosphaerellaceae and Teratosphaeriaceae in the Dothiodesmycetes (Maxwell et al. 2005).

MrBayes version 3 was used for Bayesian analyses of the various datasets (Ronquist and Huelsenbeck 2003). MrModeltest version 2.2 was first used to determine the nucleotide substitution model that would best fit the individual datasets (Nylander 2004). The Markov Chain Monte Carlo (MCMC) analyses of six chains from random tree topology were made twice on 1,000,000 generations and trees were saved every 1,000 generations. Tracer version 1.4 was used to determine the posterior probabilities indicating the level of branch support (Rambaut and Drummond 2007).

Results

Multi-locus sequence analysis

Sequence data were obtained for 22 isolates, of which nine were from the *S. cordatum* tree and 13 from the *E. grandis* x *E. camaldulensis* clone (Table 1). Maximum parsimony and Bayesian analyses were done on the individual and combined datasets (Figs. 1, 2, 3, and 4) with appropriate models as determined by MrModeltest version 2.2 (Table 2). The maximum parsimony and Bayesian trees for the individual and combined datasets had similar topologies, although clades

Table 1 Details of the fungal species and reference sequences used in the phylogenetic analyses

Species	Accession		Host	Country	Genbank accession		
	CMW ^a	CBS ^b			ITS	LSU	TEF-1 α
<i>Mycosphaerella ellipsoidea</i>	–	CBS 110843	<i>Eucalyptus</i> sp.	South Africa	AY725545	–	–
	4934	–	<i>Eucalyptus</i> sp.	South Africa	–	DQ246253	DQ235129
	–	CBS 110843	<i>Eucalyptus</i> sp.	South Africa	–	GQ852602	–
	5166	–	<i>Eucalyptus</i> sp.	South Africa	–	–	DQ235127
<i>Mycosphaerella endophytica</i>	–	CBS 114662	<i>Eucalyptus</i> sp.	South Africa	DQ302953	GU214435	–
	5225	–	<i>Eucalyptus</i> sp.	South Africa	–	–	DQ235128
	14912	CBS 111519	<i>Eucalyptus</i> sp.	South Africa	DQ267579	–	DQ235131
<i>Mycosphaerella marksii</i>	14781	CBS 682.95	<i>E. grandis</i>	South Africa	DQ267587	DQ246249	DQ235133
	5230	–	<i>E. botryoides</i>	Australia	DQ267588	DQ246246	DQ235135
	37697	–	<i>Syzygium cordatum</i>	South Africa	JQ732925	JQ732974	JQ733021
<i>Mycosphaerella marksii</i>	33962	–	<i>S. cordatum</i>	South Africa	JQ732926	JQ732975	JQ733022
	37682	–	<i>S. cordatum</i>	South Africa	JQ732929	JQ732978	JQ733025
	37694	–	<i>S. cordatum</i>	South Africa	JQ732930	JQ732979	JQ733026
	38277	–	<i>S. cordatum</i>	South Africa	JQ732931	JQ732980	JQ733026
<i>Mycosphaerella vietnamensis</i>	23442	–	<i>E. camaldulensis</i>	Vietnam	DQ632678	EU882135	–
	23441	CBS 119974	<i>E. grandis</i>	Vietnam	DQ632675	JF700944	–
	37695	–	<i>S. cordatum</i>	South Africa	JQ732923	JQ732972	JQ733019
	37696	–	<i>S. cordatum</i>	South Africa	JQ732924	JQ732973	JQ733020
<i>Pseudocercospora crystallina</i>	3042	–	<i>E. bicostata</i>	South Africa	DQ267578	DQ204746	DQ211662
	3033	CBS 681.95	<i>E. bicostata</i>	South Africa	DQ632681	DQ204747	DQ211663
	38912	–	<i>Eucalyptus grandis</i> x <i>E. camaldulensis</i>	South Africa	JQ732911	JQ732960	JQ733008
	38913	–	<i>S. cordatum</i>	South Africa	JQ732927	JQ732976	JQ733023
	37698	–	<i>S. cordatum</i>	South Africa	JQ732932	JQ732981	JQ733028
<i>Pseudocercospora crystallina</i>	37698	–	<i>S. cordatum</i>	South Africa	JQ732932	JQ732981	JQ733028
<i>Readeriella considianae</i>	–	–	<i>E. stellulata</i>	Australia	GQ852792	GQ852681	–
	–	–	<i>E. stellulata</i>	Australia	GQ852791	GQ852680	–
	37671	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	JQ732893	JQ732942	JQ732990
	37674	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	JQ732896	JQ732945	JQ732994
	37675	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	JQ732897	JQ732946	JQ732995
	37676	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	JQ732899	JQ732948	JQ732997
	37678	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	JQ732901	JQ732950	JQ732999
	37680	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	JQ732903	JQ732952	JQ733001
	4940	CBS 111164	<i>E. globulus</i>	Portugal	AF309620	DQ246220	DQ235104
2734	CBS 111132	<i>E. globulus</i>	U.S.A.	AF309619	DQ246223	DQ235105	
<i>Teratosphaeria zuluensis</i>	17320;	–	<i>E. grandis</i>	Zambia	DQ240148	EU019296	DQ240206
	15833	–	<i>Eucalyptus</i> sp.	Mexico	–	–	DQ240162
<i>Teratosphaeria zuluensis</i>	37669	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	JQ732891	JQ732940	JQ732988
	37670	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	JQ732892	JQ732941	JQ732989
	37672	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	JQ732894	JQ732943	JQ732992
	37679	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	JQ732902	JQ732951	JQ733000
	37690	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	JQ732915	JQ732964	JQ733012
	37693	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	JQ732920	JQ732969	JQ733016
	7773	–	<i>Ribes</i> sp.	U.S.A.	DQ246604	DQ246263	DQ235142
<i>Phaeobotryosphaeria visci</i>	–	CBS 100163	<i>Sphaeropsis visci</i>	Germany	EU673324	DQ377870	EU673292

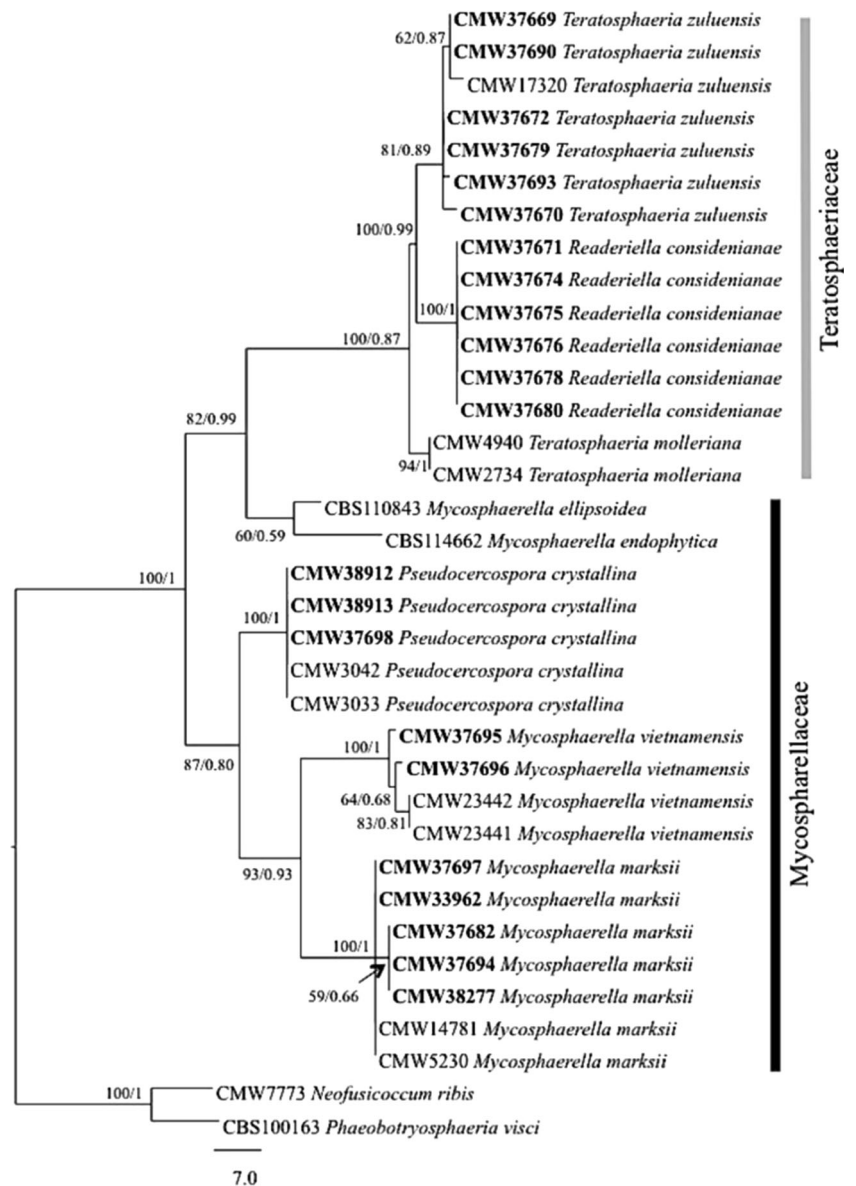
Genbank accessions in bold were used in the combined analyses

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

^b CBS = CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands

– = not available

Fig. 1 One of the equally most parsimonious trees obtained from a heuristic search of the ITS dataset. Bootstrap values obtained from 1,000 replicates along with posterior probabilities are shown at the nodes. The tree was rooted with *Neofusicoccum ribis* and *Phaeobotryosphaeria visci*. Isolates identified in this study are indicated in **bold**. Tree length = 209, CI = 0.785, RI = 0.944 and HI = 0.215



often collapsed for the more conserved gene regions. The PHT value for the combined datasets was $p < 0.001$, which indicated that the datasets were not congruent. However, the data were combined since no inconsistencies in the major clades were observed, other than those mentioned above, to strengthen the support for the congruent clades.

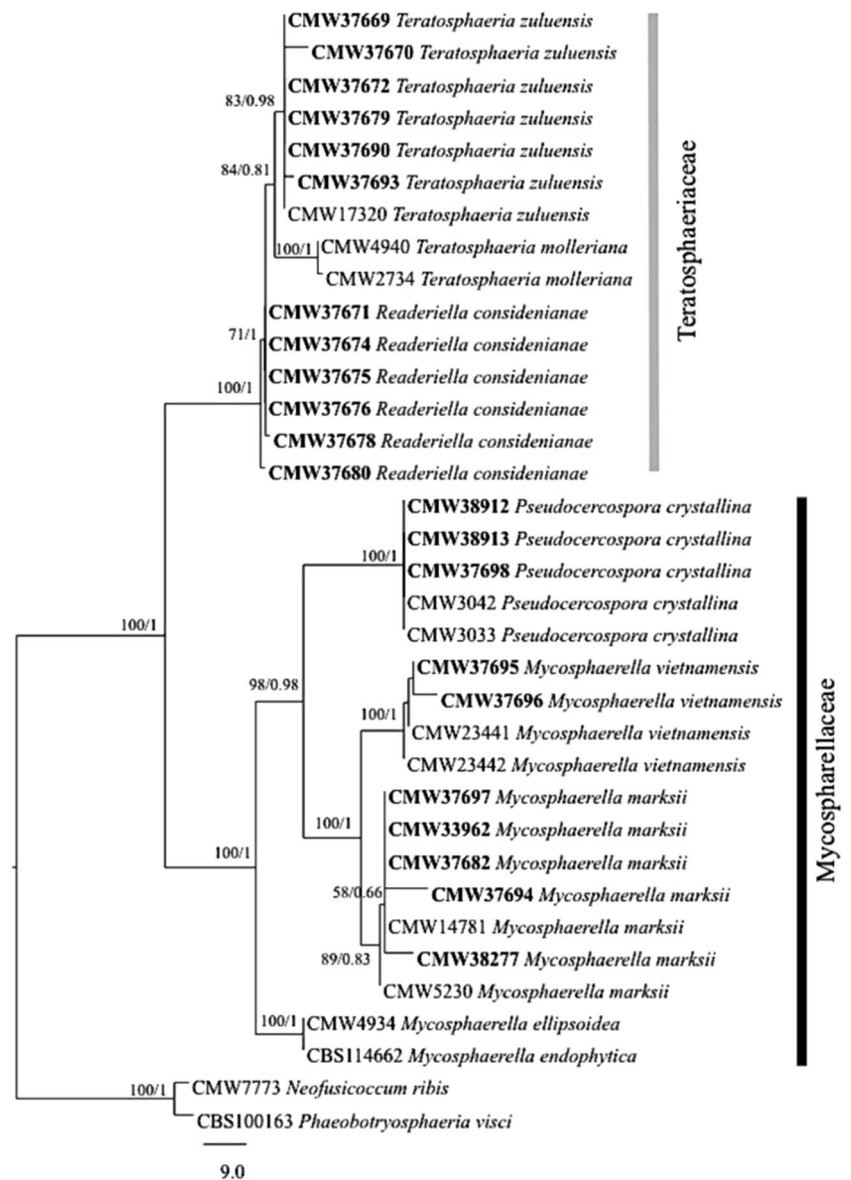
One of the 160 equally most parsimonious trees obtained from a heuristic search of the combined ITS, LSU and TEF-1 α datasets was chosen for presentation (Fig. 4). Two distinct groups of isolates were observed representing species in the Mycosphaerellaceae (100 % bootstrap support) and Teratosphaeriaceae (100 % bootstrap support). The isolates grouped into five clades with strong bootstrap support, representing *M. marksii* (5 isolates), *M. vietnamensis* (2 isolates), *P. crystallina* (3 isolates), *R. considianae* (6 isolates) and *T. zuluensis* (6 isolates). Of these species, *P. crystallina* was

isolated from both trees, whilst *M. marksii* and *M. vietnamensis* were isolated solely from *S. cordatum* and *R. considianae* and *T. zuluensis* were isolated from the *E. grandis* clone. All the identified species were isolated from the leaves of the two trees sampled. No isolates were isolated from the twigs or the trunk increments. The sequence alignments and phylogenetic trees for each gene region as well as the combined analysis were all deposited in TreeBASE (<http://purl.org/phylo/treebase/phyloWS/study/TB2:S14876?x-access-code=ec92eb8a6492c73537799c2fe03b8a0b&format=html>).

Discussion

Five species of Mycosphaerellaceae and Teratosphaeriaceae were isolated as endophytes in this study. These fungi were

Fig. 2 One of the equally most parsimonious trees obtained from a heuristic search of the LSU dataset. Bootstrap values obtained from 1,000 replicates along with posterior probabilities are shown at the nodes. The tree was rooted with *Neofusicoccum ribis* and *Phaeobotryosphaeria visci*. Isolates identified in this study are indicated in **bold**. Tree length = 229, CI = 0.782, RI = 0.947 and HI = 0.218



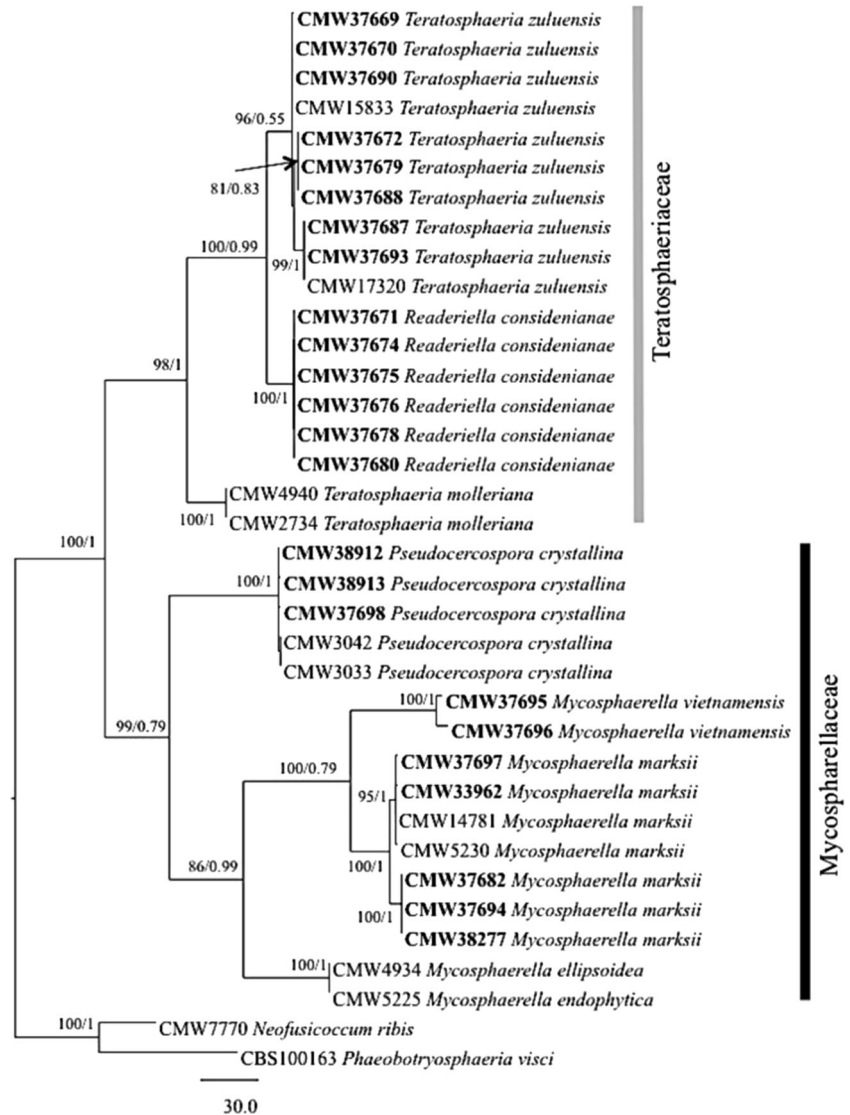
obtained from healthy *S. cordatum* and *E. grandis* x *E. camaldulensis* trees growing in close proximity to each other, in South Africa. These included a number of pathogens known to occur in South Africa and also fungi that have not previously been recorded in the country.

Even though the sampling in this study was limited, it revealed two new geographical records for the Mycosphaerellaceae. These include *R. considenianae* and *M. vietnamensis* that are recorded from South Africa for the first time. *R. considenianae* has recently been found to occur on *E. considenianae* in Australia, but is not an important pathogen (Summerell et al. 2006; Taylor et al. 2012). *M. vietnamensis* is known to occur as a weak pathogen on *E. camaldulensis* and *E. grandis* in Vietnam (Burgess et al. 2007). In the present study, it was not only recorded in South Africa for the first time,

but it was also found on a non-native *Eucalyptus* host for the first time.

This study revealed that some fungal species of Mycosphaerellaceae and Teratosphaeriaceae co-occur on *S. cordatum* and the sampled *Eucalyptus* clone. Based on their identity in previous publications, other species have the potential to do so. For example, *P. crystallina* was found in the leaves of both *S. cordatum* and the *Eucalyptus* clone. This fungus is known from leaf spots on *E. bicostata* as well as on *E. grandis* x *E. camaldulensis* in South Africa (Crous and Wingfield 1996). The fungus was previously known only from South Africa, but has since been found to occur on *Eucalyptus* in China (Burgess et al. 2007). Its native host is unknown and it could have originated on *Eucalyptus* and moved to native *S. cordatum* or vice versa. *M. marksii* is not considered an important pathogen. However, it has been

Fig. 3 One of the equally most parsimonious trees obtained from a heuristic search of the TEF-1 α dataset. Bootstrap values obtained from 1,000 replicates along with posterior probabilities are shown at the nodes. The tree was rooted with *Neofusicoccum ribis* and *Phaeobotryosphaeria visci*. Isolates identified in this study are indicated in **bold**. Tree length = 642, CI = 0.783, RI = 0.945 and HI = 0.217



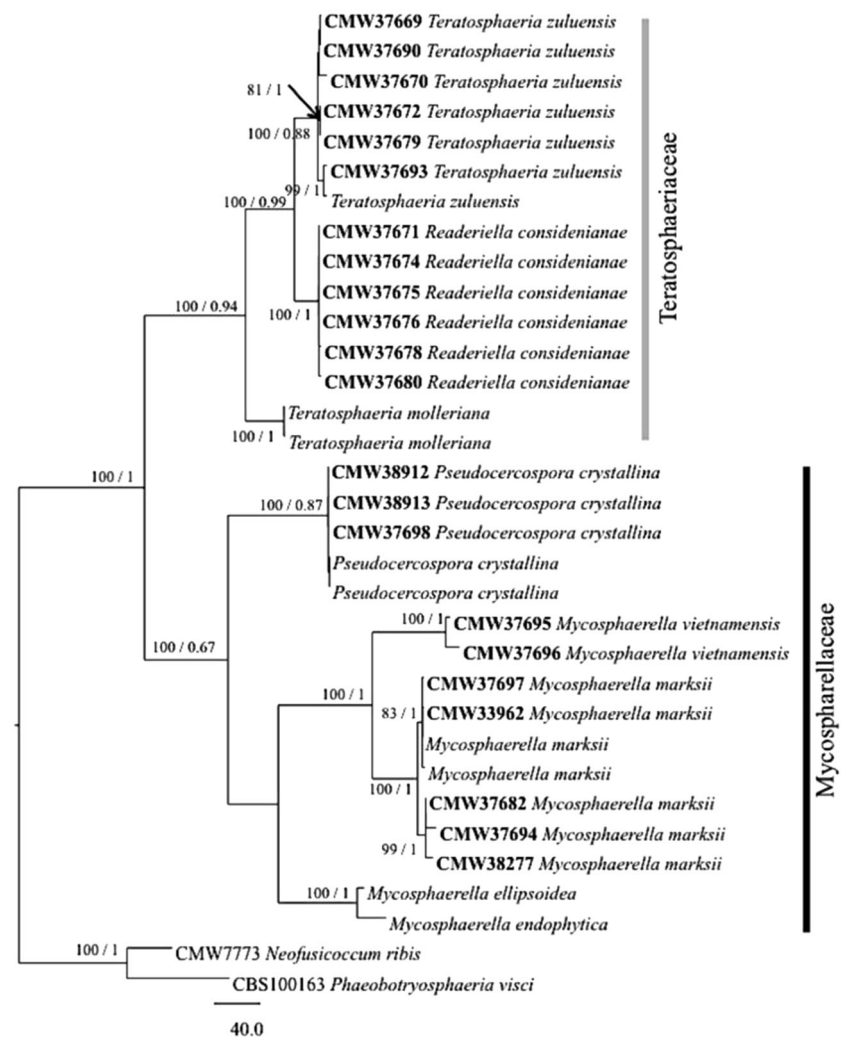
found to constitutively infect the leaves of *Eucalyptus* in Australia, China, Ethiopia, Indonesia, Madagascar, Portugal, South Africa and Uruguay (Crous and Wingfield 1996; Crous 1998; Hunter et al. 2004; Burgess et al. 2007; Hunter et al. 2011). In this study it was identified from the leaves of *S. cordatum*, but not from the leaves of the *Eucalyptus* clone sampled. It seems likely that this fungus has been introduced into South Africa and colonised *S. cordatum*. *M. vietnamensis* has been shown to colonise the leaves of *S. cordatum* in this study but is also known to occur in other *Eucalyptus* spp. as a weak pathogen in South East Vietnam (Burgess et al. 2007).

The ability of species in the Mycosphaerellaceae and Teratosphaeriaceae to co-occur on closely related hosts, such as *Eucalyptus* and *S. cordatum*, indicates that they have the capacity to jump to either commercially important trees or, alternatively, they could potentially threaten native trees. As *Eucalyptus* spp. are not native to South Africa, pathogens may have been introduced that have the potential to colonise native

Myrtaceae spp., such as *S. cordatum*. However, the opposite may also be true where native trees serve as inoculum sources for fungi that are pathogenic to *Eucalyptus* (Pavlic et al. 2007; Rodas et al. 2008). This study highlights that the latter may be occurring for leaf infecting fungi but remains largely unrecognised. The results in this study showing evidence of possible cross infections are surprising and the consequence of such cross infections deserves further attention. Crous and Groenewald (2005) suggested that the phenomenon whereby some fungal species move from one host to another in search of their preferred host on which they cause disease, be termed the ‘‘pogo-stick hypothesis’’ (Crous and Groenewald 2005).

Teratosphaeria zuluensis is a well-known and serious pathogen associated with *E. grandis* clones in South Africa, which causes severe cankers on the stems and branches of *Eucalyptus* trees (Wingfield et al. 1996; Cortinas et al. 2010). In this study, the fungus was isolated from the leaves of the *Eucalyptus* clone. This was a most unexpected result as

Fig. 4 One of the equally most parsimonious trees obtained from a heuristic search of the combined ITS, LSU and TEF-1 α datasets. Bootstrap values obtained from 1,000 replicates along with posterior probabilities are shown at the nodes. The tree was rooted with *Neofusicoccum ribis* and *Phaeobotryosphaeria visci*. Isolates identified in this study are indicated in *bold*. Tree length = 1091, CI = 0.775, RI = 0.941 and HI = 0.225



symptoms of infection by the fungus have never been seen on leaves and is found only on green stem and branch tissues. Whether the fungus can act as a pathogen of leaves is unknown and this deserves further study. The presence of this serious pathogen within leaves, however, represents a

previously overlooked niche that could represent possible opportunities for it to be moved between areas, countries and continents. This may indeed represent a means by which it was introduced into South Africa, potentially from China where it is thought to be native (Chen et al. 2011).

Table 2 Statistical results for the phylogenetic analysis

Maximum parsimony									
Dataset	Number of taxa	Excluded characters	Included characters	Tree number	Tree length	CI	RI	HI	
ITS	35	400	96	51	196	0.760	0.944	0.240	
LSU	35	531	136	21	223	0.794	0.953	0.206	
TEF-1 α	36	40	232	16	642	0.783	0.945	0.217	
Combined	33	1023	470	160	1091	0.775	0.941	0.225	
MrBayes									
Dataset	Model	Preset state freqpr		NST	Rates		Burnin		
ITS	GTR+G	(1, 1, 1, 1)		6	gamma		1,000		
LSU	GTR+G	(1, 1, 1, 1)		6	gamma		1,000		
TEF-1 α	GTR+G	(1, 1, 1, 1)		6	gamma		1,000		
Combined	GTR+G	(1, 1, 1, 1)		6	gamma		1,000		

The isolates characterised in this study were derived from single *S. cordatum* and *Eucalyptus* trees. This limited sampling in terms of the host, however, revealed three new host and two new geographical reports. It also exposed examples of possible cross infections by potentially pathogenic fungi on these two tree species. This suggests that although *Eucalyptus* represents a well-studied tree in South Africa, the patterns of occurrence, host preference and ecological role of the Mycosphaerellaceae and the Teratosphaeriaceae remain poorly understood.

Acknowledgments We thank the Thutuka Funding programme of the National Research Foundation (NRF), members of the Tree Protection Co-operative Programme (TPCP) and the Department of Science and Technology (DST)/NRF Centre of Excellence in Tree Health Biotechnology (CTHB), South Africa for financial support. We are also grateful to Dr Ewald Groenewald (CBS-KNAW Fungal Biodiversity Centre; Netherlands), for his valuable assistance with the data analysis and for helpful suggestions regarding the manuscript. We also thank Miss Kerry-Anne Pillay and Mr Jan Nagel for technical assistance.

References

- Andjic V, Whyte G, Hardy G, Burgess T (2010) New Teratosphaeria species occurring on eucalypts in Australia. *Fungal Divers* 43:27–38
- Bensch K, Groenewald JZ, Dijksterhuis J, Starink-Willemsse M, Andersen B, Summerell BA, Shin HD, Dugan FM, Schroers HJ, Braun U (2010) Species and ecological diversity within the *Cladosporium cladosporioides* complex (Davidiellaceae, Capnodiales). *Stud Mycol* 67:1–94
- Burgess TI, Barber PA, Sufaati S, Xu D, Hardy G, JDB (2007) *Mycosphaerella* spp. on *Eucalyptus* in Asia; new species, new hosts and new records. *Fungal Divers* 24:135–157
- Carbone I, Kohn LM (1990) A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91:553–556
- Chen SF, Barnes I, Chungu D, Roux J, Wingfield MJ, Xie YJ, Zhou XD (2011) High population diversity and increasing importance of the *Eucalyptus* stem canker pathogen, *Teratosphaeria zuluensis*, in South China. *Australas Plant Pathol* 40:407–415
- Corlett M (1995) An annotated list of the published names in *Mycosphaerella* and *Sphaerella*: corrections and additions. *Mycotaxon* 53:37–56
- Cortinas MN, Barnes I, Wingfield MJ, Wingfield BD (2010) Genetic diversity in the *Eucalyptus* stem pathogen *Teratosphaeria zuluensis*. *Australas Plant Pathol* 39:383–393
- Crous PW (1998) *Mycosphaerella* spp. and their anamorphs: associated with leaf spot diseases of *Eucalyptus*. APS Press, St Paul
- Crous PW (1999) Species of *Mycosphaerella* and related anamorphs occurring on Myrtaceae (excluding *Eucalyptus*). *Mycol Res* 103:607–621
- Crous PW (2009) Taxonomy and phylogeny of the genus *Mycosphaerella* and its anamorphs. *Fungal Divers* 38:1–24
- Crous PW, Groenewald JZ (2005) Hosts, species and genotypes: opinions versus data. *Australas Plant Pathol* 34:463–470
- Crous PW, Wingfield MJ (1996) Species of *Mycosphaerella* and their anamorphs associated with leaf blotch disease of *Eucalyptus* in South Africa. *Mycologia* 88:441–458
- Crous PW, Wingfield MJ, Park RF (1991) *Mycosphaerella nubilosa*, a synonym of *M. molleriana*. *Mycol Res* 95:628–632
- Crous PW, Aptroot A, Kang JC, Braun U, Wingfield MJ (2000) The genus *Mycosphaerella* and its anamorphs. *Stud Mycol* 45:107–121
- Crous PW, Hong L, Wingfield BD, Wingfield MJ (2001a) ITS rDNA phylogeny of selected *Mycosphaerella* species and their anamorphs occurring on Myrtaceae. *Mycol Res* 105:425–431
- Crous PW, Kang JC, Braun U (2001b) A phylogenetic redefinition of anamorph genera in *Mycosphaerella* based on ITS rDNA sequence and morphology. *Mycologia* 93:1081–1101
- Crous PW, Groenewald JZ, Mansilla JP, Hunter GC, Wingfield MJ (2004) Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. *Stud Mycol* 50:195–214
- Crous PW, Wingfield MJ, Mansilla JP, Alfenas AC, Groenewald JZ (2006) Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. II. *Stud Mycol* 55:99–131
- Crous PW, Braun U, Groenewald JZ (2007) *Mycosphaerella* is polyphyletic. *Stud Mycol* 58:1–32
- Crous PW, Groenewald JZ, Summerell BA, Wingfield BD, Wingfield MJ (2009a) Co-occurring species of *Teratosphaeria* on *Eucalyptus*. *Persoonia* 22:38–48
- Crous PW, Schoch CL, Hyde KD, Wood AR, Gueidan C, De Hoog GS, Groenewald JZ (2009b) Phylogenetic lineages in the Capnodiales. *Stud Mycol* 64:17–47
- Crous PW, Summerell BA, Carnegie AJ, Wingfield MJ, Hunter GC, Burgess TI, Andjic V, Barber PA, Groenewald JZ (2009c) Unravelling *Mycosphaerella*: do you believe in genera? *Persoonia* 23:99–118
- Crous PW, Wingfield MJ, Groenewald JZ (2009d) Niche sharing reflects a poorly understood biodiversity phenomenon. *Persoonia* 22:83–94
- de Hoog GS, Gerrits van den Ende AHG (1998) Molecular diagnostics of clinical strains of filamentous *Basidiomycetes*. *Mycoses* 41:183–189
- Farris JS, Kallersjo M, Kluge AG, Bult C (1994) Testing significance of incongruence. *Cladistics* 10:315–319
- Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791
- Hillis DM, Huelsenbeck JP (1992) Signal, noise, and reliability in molecular phylogenetic analyses. *J Hered* 83:189–195
- Hunter GC, Roux J, Wingfield BD, Crous PW, Wingfield MJ (2004) *Mycosphaerella* species causing leaf disease in South African *Eucalyptus* plantations. *Mycol Res* 108:672–681
- Hunter GC, Wingfield BD, Crous PW, Wingfield MJ (2006) A multi-gene phylogeny for species of *Mycosphaerella* occurring on *Eucalyptus* leaves. *Stud Mycol* 55:147–161
- Hunter GC, Crous PW, Carnegie AJ, Wingfield MJ (2009) *Teratosphaeria nubilosa*, a serious leaf disease pathogen of *Eucalyptus* spp. in native and introduced areas. *Mol Plant Pathol* 10:1–14
- Hunter GC, Crous PW, Carnegie AJ, Burgess TI, Wingfield MJ (2011) *Mycosphaerella* and *Teratosphaeria* diseases of *Eucalyptus*; easily confused and with serious consequences. *Fungal Divers* 50:145–166
- Hyde KD, Bussaban B, Paulus B, Crous PW, Lee S, McKenzie EHC, Photita W, Lumyong S (2007) Diversity of saprobic microfungi. *Biodivers Conserv* 16:7–35
- Katoh K, Kuma K, Toh H, Miyata T (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment; <http://mafft.cbrc.jp/alignment/software/>. *Nucleic Acids Res* 33:511–518
- Maxwell A, Jackson SL, Dell B, Hardy G (2005) PCR-identification of *Mycosphaerella* species associated with leaf diseases of *Eucalyptus*. *Mycol Res* 109:992–1004
- Moller EM, Bahnweg G, Sandermann H, Geiger HH (1992) A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Res* 20:6115–6116
- Moncalvo JM, Wang HH, Hseu RS (1995) Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacers and 25S ribosomal DNA sequences. *Mycologia* 87:223–238

- Nylander JAA (2004) MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University; <https://github.com/nylander/MrModeltest2>
- Pavlic D, Slippers B, Coutinho TA, Wingfield MJ (2007) Botryosphaeriaceae occurring on native *Syzygium cordatum* in South Africa and their potential threat to *Eucalyptus*. *Plant Pathol* 56:624–636
- Rambaut A, Drummond AJ (2007) Tracer v1. 4; <http://tree.bio.ed.ac.uk/software/tracer/>
- Rehner SA, Samuels GJ (1995) Molecular systematics of Hypocreales: a teleomorph gene phylogeny and the status of their anamorphs. *Can J Bot* 73:816–823
- Rodas CA, Roux RJ, van Wyk M, Wingfield BD, Wingfield MJ (2008) *Ceratocystis neglecta* sp. nov., infecting *Eucalyptus* trees in Colombia. *Fungal Divers* 28:73–84
- Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–1574
- Sánchez Márquez S, Bills GF, Zabalgoitia I (2011) Fungal species diversity in juvenile and adult leaves of *Eucalyptus globulus* from plantations affected by *Mycosphaerella* leaf disease. *Ann Appl Biol* 158:177–187
- Schubert K, Groenewald JZ, Braun U, Dijksterhuis J, Starink M, Hill CF, Zalar P, De Hoog GS, Crous PW (2007) Biodiversity in the *Cladosporium herbarum* complex (Davidiellaceae, Capnodiales), with standardisation of methods for *Cladosporium* taxonomy and diagnostics. *Stud Mycol* 58:105–156
- Summerell BA, Groenewald JZ, Carnegie AJ, Summerbell RC, Crous PW (2006) *Eucalyptus* microfungi known from culture. 2. *Alysiidiella*, *Fusculina* and *Phlogicylindrium* genera nova, with notes on some other poorly known taxa. *Fungal Divers* 23:323–350
- Sutton BC, Crous PW (1997) *Lecanostictopsis* gen. nov., and related leaf-spotting fungi on *Syzygium* species. *Mycol Res* 101:215–225
- Swofford DL (2003) {PAUP*. Phylogenetic analysis using parsimony (* and other methods). Version 4.}; <http://paup.csit.fsu.edu/downl.html>
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 24:1596–1599
- Taylor K, Andjic V, Barber PA, Hardy GEJ, Burgess TI (2012) New species of *Teratosphaeria* associated with leaf diseases on *Corymbia calophylla* (Marri). *Mycol Prog* 11:159–169
- Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J Bacteriol* 172:4238–4246
- White TJ, Bruns T, Lee S and Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols a guide to methods and applications*, pp. 315–322. Academic Press, New York
- Wingfield MJ, Crous PW, Coutinho TA (1996) A serious canker disease of *Eucalyptus* in South Africa caused by a new species of *Coniothyrium*. *Mycopathologia* 136:139–145