

# 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 $\alpha$  (TEF-1 $\alpha$ ) genes were used to correctly identify the 22 resulting isolates. The isolates grouped in five clades representing *Readeriella consideniana* 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. consideniana* 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

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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 $\alpha$  (TEF-1 $\alpha$ ) 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 (H<sub>2</sub>O<sub>2</sub>) 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  $\mu$ 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  $\mu$ 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/ $\mu$ 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  $\mu$ l with 40 ng of template DNA, 10 pmoles of each primer (Integrated DNA Technologies, Coralville, Iowa, USA), 1 $\times$  NH<sub>4</sub> Reaction Buffer (Roche Products Pty Ltd, South Africa), 1.5 mM MgCl<sub>2</sub> (Roche Products Pty Ltd, South Africa), 75  $\mu$ 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  $\mu$ 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 $\alpha$  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 $\alpha$  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](http://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 $\alpha$  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 $\alpha$  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 $\alpha$  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 $\alpha$  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 <sup>a</sup>	CBS <sup>b</sup>			ITS	LSU	TEF-1 $\alpha$
<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	<b>DQ267587</b>	<b>DQ246249</b>	<b>DQ235133</b>
	5230	–	<i>E. botryoides</i>	Australia	<b>DQ267588</b>	<b>DQ246246</b>	<b>DQ235135</b>
	37697	–	<i>Syzygium cordatum</i>	South Africa	<b>JQ732925</b>	<b>JQ732974</b>	<b>JQ733021</b>
<i>Mycosphaerella marksii</i>	33962	–	<i>S. cordatum</i>	South Africa	<b>JQ732926</b>	<b>JQ732975</b>	<b>JQ733022</b>
	37682	–	<i>S. cordatum</i>	South Africa	<b>JQ732929</b>	<b>JQ732978</b>	<b>JQ733025</b>
	37694	–	<i>S. cordatum</i>	South Africa	<b>JQ732930</b>	<b>JQ732979</b>	<b>JQ733026</b>
	38277	–	<i>S. cordatum</i>	South Africa	<b>JQ732931</b>	<b>JQ732980</b>	<b>JQ733026</b>
<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	<b>JQ732923</b>	<b>JQ732972</b>	<b>JQ733019</b>
	37696	–	<i>S. cordatum</i>	South Africa	<b>JQ732924</b>	<b>JQ732973</b>	<b>JQ733020</b>
<i>Pseudocercospora crystallina</i>	3042	–	<i>E. bicostata</i>	South Africa	<b>DQ267578</b>	<b>DQ204746</b>	<b>DQ211662</b>
	3033	CBS 681.95	<i>E. bicostata</i>	South Africa	<b>DQ632681</b>	<b>DQ204747</b>	<b>DQ211663</b>
	38912	–	<i>Eucalyptus grandis</i> x <i>E. camaldulensis</i>	South Africa	<b>JQ732911</b>	<b>JQ732960</b>	<b>JQ733008</b>
	38913	–	<i>S. cordatum</i>	South Africa	<b>JQ732927</b>	<b>JQ732976</b>	<b>JQ733023</b>
	37698	–	<i>S. cordatum</i>	South Africa	<b>JQ732932</b>	<b>JQ732981</b>	<b>JQ733028</b>
<i>Pseudocercospora crystallina</i>	37698	–	<i>S. cordatum</i>	South Africa	<b>JQ732932</b>	<b>JQ732981</b>	<b>JQ733028</b>
<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	<b>JQ732893</b>	<b>JQ732942</b>	<b>JQ732990</b>
	37674	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	<b>JQ732896</b>	<b>JQ732945</b>	<b>JQ732994</b>
	37675	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	<b>JQ732897</b>	<b>JQ732946</b>	<b>JQ732995</b>
	37676	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	<b>JQ732899</b>	<b>JQ732948</b>	<b>JQ732997</b>
	37678	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	<b>JQ732901</b>	<b>JQ732950</b>	<b>JQ732999</b>
	37680	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	<b>JQ732903</b>	<b>JQ732952</b>	<b>JQ733001</b>
	4940	CBS 111164	<i>E. globulus</i>	Portugal	<b>AF309620</b>	<b>DQ246220</b>	<b>DQ235104</b>
2734	CBS 111132	<i>E. globulus</i>	U.S.A.	<b>AF309619</b>	<b>DQ246223</b>	<b>DQ235105</b>	
<i>Teratosphaeria zuluensis</i>	17320;	–	<i>E. grandis</i>	Zambia	<b>DQ240148</b>	<b>EU019296</b>	<b>DQ240206</b>
	15833	–	<i>Eucalyptus</i> sp.	Mexico	–	–	DQ240162
<i>Teratosphaeria zuluensis</i>	37669	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	<b>JQ732891</b>	<b>JQ732940</b>	<b>JQ732988</b>
	37670	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	<b>JQ732892</b>	<b>JQ732941</b>	<b>JQ732989</b>
	37672	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	<b>JQ732894</b>	<b>JQ732943</b>	<b>JQ732992</b>
	37679	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	<b>JQ732902</b>	<b>JQ732951</b>	<b>JQ733000</b>
	37690	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	<b>JQ732915</b>	<b>JQ732964</b>	<b>JQ733012</b>
	37693	–	<i>E. grandis</i> x <i>E. camaldulensis</i>	South Africa	<b>JQ732920</b>	<b>JQ732969</b>	<b>JQ733016</b>
<i>Neofusicoccum ribis</i>	7773	–	<i>Ribes</i> sp.	U.S.A.	<b>DQ246604</b>	<b>DQ246263</b>	<b>DQ235142</b>
<i>Phaeobotryosphaeria visci</i>	–	CBS 100163	<i>Sphaeropsis visci</i>	Germany	<b>EU673324</b>	<b>DQ377870</b>	<b>EU673292</b>

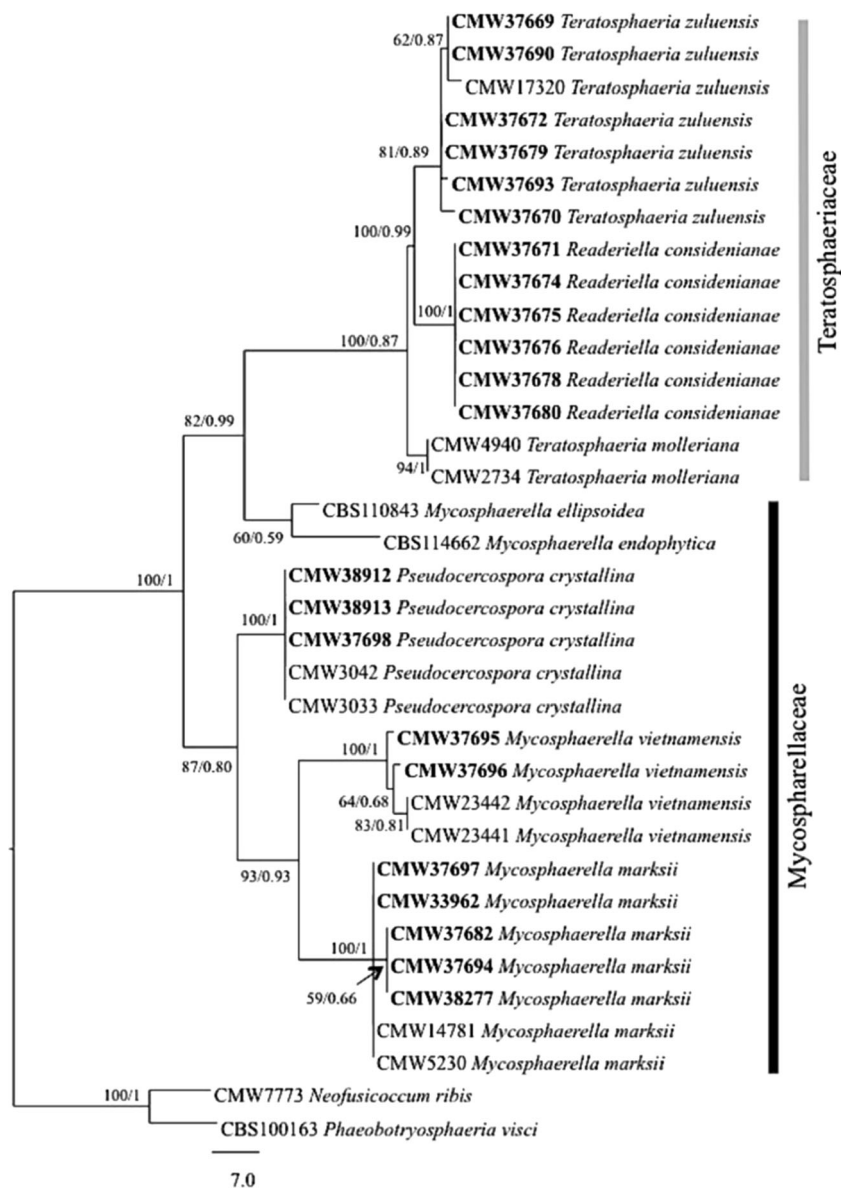
Genbank accessions in bold were used in the combined analyses

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

<sup>b</sup> 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.

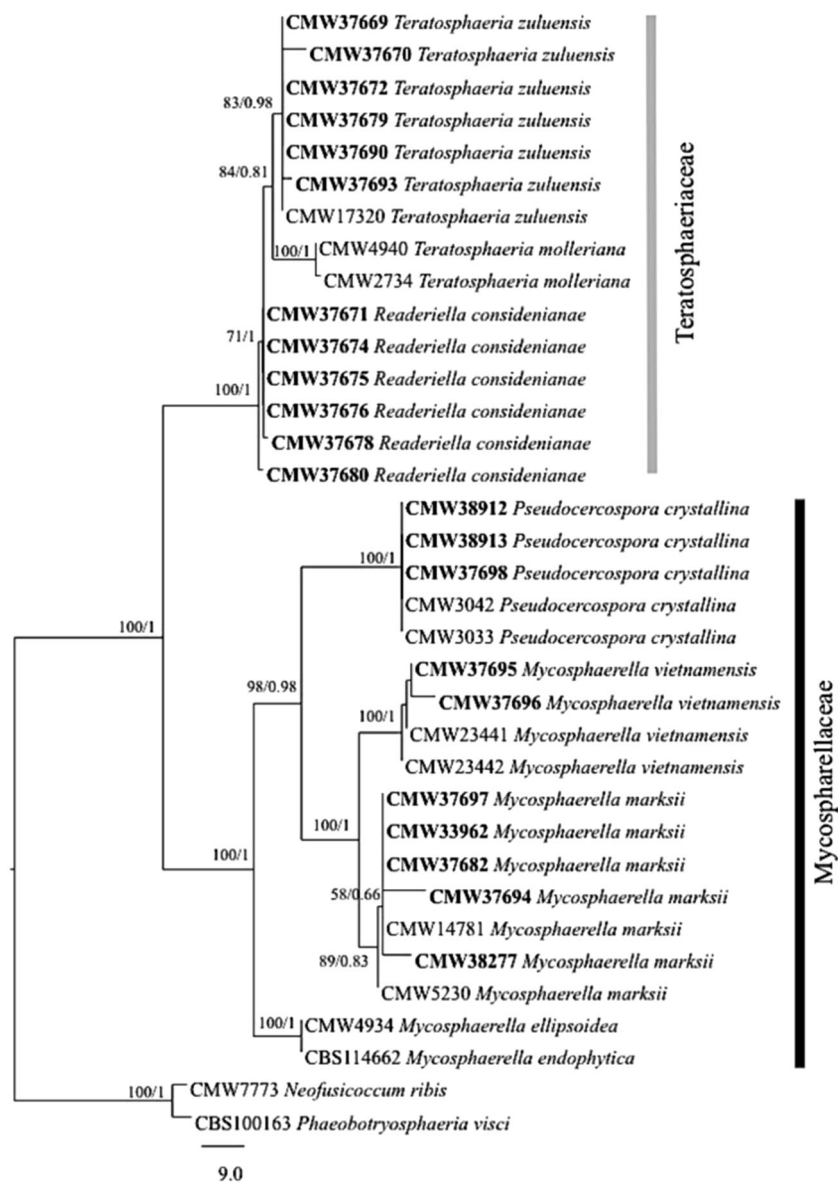
One of the 160 equally most parsimonious trees obtained from a heuristic search of the combined ITS, LSU and TEF-1 $\alpha$  datasets was chosen for presentation (Fig. 4). Two distinct groups of isolates were observed representing species in the Mycosphaerellaceae (100 % bootstrap support) and Teratosphaeriaceae (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.

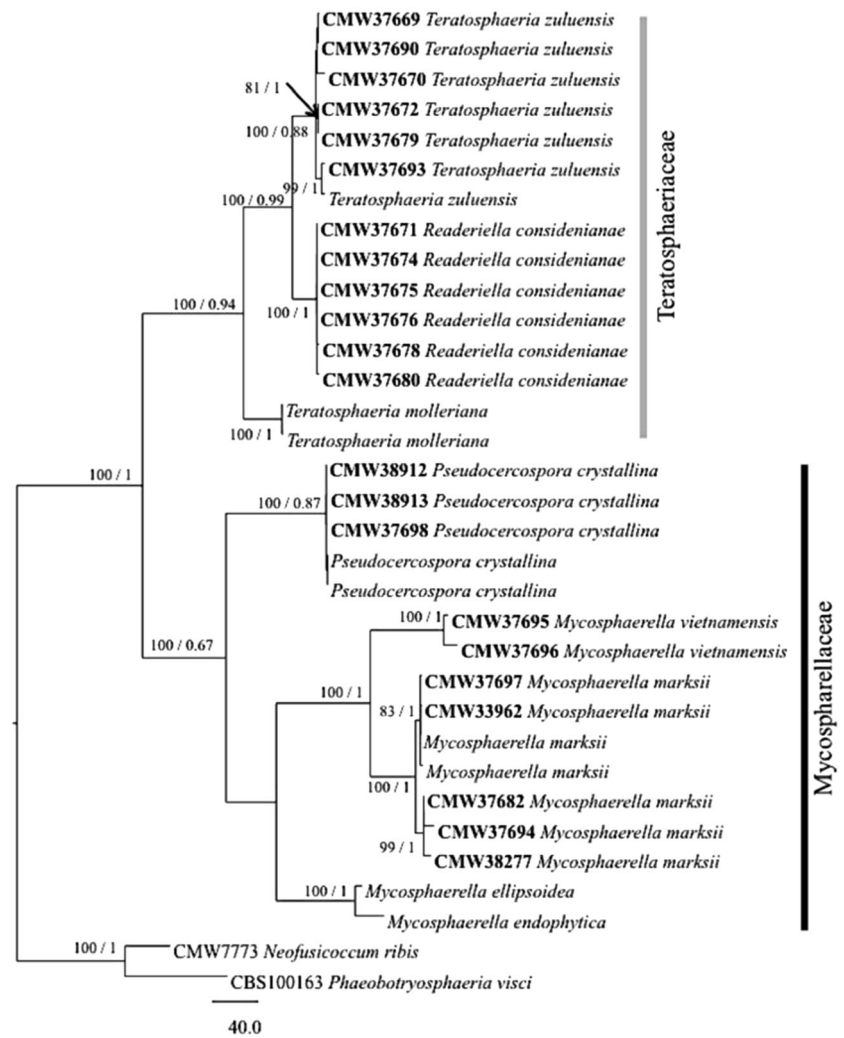
Even though the sampling in this study was limited, it revealed two new geographical records for the Mycosphaerellaceae. These include *R. considenianae* and *M. vietnamensis* that are recorded from South Africa for the first time. *R. considenianae* has recently been found to occur on *E. considenianae* 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. 4** One of the equally most parsimonious trees obtained from a heuristic search of the combined ITS, LSU and TEF-1 $\alpha$  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 $\alpha$	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 $\alpha$	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.

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