

A diverse assemblage of *Ophiostoma* species, including two new taxa on eucalypt trees in South Africa

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Abstract Fungi in the Ophiostomatales include important pathogens of trees as well as agents of wood stain, reducing the economic value of timber. They rely on insects, such as bark beetles, for dispersal and are commonly associated with wounds on trees. Although *Ophiostoma* spp. have been reported from eucalypt wood chips in South Africa, very little is known about the diversity of the Ophiostomatales, or their insect associates, on plantation-grown *Eucalyptus* spp. The aim of this study was to consider the diversity and distribution of the Ophiostomatales infecting fresh wounds on *Eucalyptus* trees in the country. Additionally, knowledge regarding their association with nitidulid beetles, which have previously been shown to carry *Ophiostoma* spp., was sought. Surveys were conducted in five provinces where *Eucalyptus* spp. are commonly grown, and the fungi collected were identified using morphological comparisons and multigene sequence phylogenies. Of the 139 isolates collected, five *Ophiostoma* spp. were identified including *O. quercus*, *O. tsotsi* and *O. tasmaniense*. These were from cut stumps as well as from the nitidulid beetles *Brachypeplus depressus* and *Carpophilus* spp. In addition, two new taxa in the *O. stenoceras*–*Sporothrix schenckii* complex were identified from *Eucalyptus* trees infested by *Phoracantha semipunctata*. The two new taxa are described as *O. candidum* sp. nov., and *O. fumeum* sp. nov., respectively. The results of this study clearly show that the diversity and ecology of *Ophiostoma* spp. on *Eucalyptus*

trees in South Africa is poorly understood and that further studies are required to determine the possible economic relevance of these fungi.

Keywords Cerambycidae · Forestry · Nitidulidae · Ophiostomatales · *Sporothrix*

Introduction

The Ophiostomatales (Ascomycetes) includes the three fungal genera *Ceratocystiopsis* Upadhyay & Kendrick with *Hyalorhinocladiella* Upadhyay & Kendrick anamorphs, *Grosmannia* Goidanich with *Leptographium* Lagerberg & Melin anamorphs and *Ophiostoma* H. & P. Sydow with *Pesotum* Crane and *Sporothrix* Hektoen & Perkins anamorphs (Zipfel et al. 2006). The sexual states of these fungi are characterized by mostly black ascomata with long erect necks giving rise to sticky spore drops that facilitate dispersal by insects (Malloch and Blackwell 1993). In the anamorph states, fruiting structures including synnemata, such as are found in *Pesotum* spp., and mononematous conidiophores in *Leptographium* spp., *Hyalorhinocladiella* spp. and *Sporothrix* spp. are characteristic of the Ophiostomatales (Crane and Schoknecht 1973; Wingfield et al. 1991). Although more clearly defined than a decade ago, the taxonomy of fungi in the Ophiostomatales remains in a state of flux. For example, there is emerging evidence that the genus *Ophiostoma*, which comprises the largest number of species, is a generic aggregate including groups of species that represent distinct monophyletic lineages (De Beer and Wingfield 2006).

Species in the Ophiostomatales are often extremely variable in their culture morphology on artificial media, making their identification very difficult. Species in the *O.*

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piceae complex, for example, have been found in numerous studies to have variable culture morphology on artificial media, making morphological delineation of species in the complex subjective (Przybyl and De Hoog 1989; Brasier and Kirk 1993; Halmshlager et al. 1994; Kamgan Nkuekam et al. 2008a, b). Furthermore, there is substantial evidence that DNA-based studies using the ITS gene region are not sufficient to separate closely related species in the *O. piceae* complex (Chung et al. 2006; Kamgan Nkuekam et al. 2008a). Therefore, the trend in recent years has been to use sequence data for protein coding genes, such as the beta tubulin gene, which resolve species in the Ophiostomatales more appropriately (Chung et al. 2006; Kamgan Nkuekam et al. 2008a). In a recent study, Grobbelaar et al. (2009) used a multigene phylogeny including four gene regions to separate species of the *O. piceae* complex. The study led to the discovery and description of *O. tsotsi* Grobbelaar, Z.W. de Beer & M.J. Wingf., a cryptic species that was treated for many years collectively with *O. quercus* (Georgev.) Nannf. (Grobbelaar et al. 2010a).

The Ophiostomatales includes a number of important tree pathogens and agents of sapstain in lumber. Important pathogens include the Dutch elm disease fungi, *O. ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier (Gibbs 1978; Brasier 2000) and *Leptographium wageneri* (Goheen & F. W. Cobb) T.C. Harr. (Cobb 1988; Harrington 1993). Most species of *Ophiostoma*, however, result in staining of the sapwood, particularly in conifers, that can reduce its commercial value (Seifert 1993). For example, *O. minus* (Hedgcock) H. & P. Sydow, *O. pluriannulatum* (Hedgcock) H. & P. Sydow and *O. piceae* (Munch) Syd. & P. Syd. are among the most important sapstain species of lumber in the northern hemisphere (Seifert 1993).

Fungi in the Ophiostomatales require wounds to infect their hosts and are commonly vectored by arthropods that either visit wounds on trees or produce these wounds themselves. Of these insects, bark beetles are by far the best known for their mutualistic relationship with these fungi (Whitney 1982; Paine et al. 1997; Six 2003; Kirisits 2004). However, a wide variety of insects, such as cerambycid beetles (Mathiesen-Käärik 1953; Jacobs and Wingfield 2001; Jacobs and Kirisits 2003), weevils (Mathiesen-Käärik 1953; Jacobs and Wingfield 2001; Kirisits 2004) and phoretic mites carried by bark beetles (Bridges and Moser 1986; Moser 1997; Malloch and Blackwell 1993) are also associated with these fungi.

South Africa has limited natural timber resources. The country mostly has a grassland vegetation type with native forests covering about 0.56% of the total land area in the country (Lawes et al. 2004). This exerts great pressure on the available natural tree resources, as timber suitable for construction, firewood and other wood products are limited in the country. Plantations of non-native trees were

established in South Africa in the last century in an attempt to alleviate the demand for timber and wood products (Anonymous 2008). These plantation areas now comprise approximately 1.3 million ha (1.2% of the country surface area). The plantations include non-native *Eucalyptus* spp. that cover approximately 0.58 million ha, 39% of the total area planted (Anonymous 2008).

Reports of ophiostomatoid fungi occurring on *Eucalyptus* spp. are limited. *Ophiostoma quercus* (Georgev.) Nannf., has been reported from *E. grandis* trees in Uruguay (Harrington et al. 2001), and more recently, *O. tsotsi* was reported from *Eucalyptus* in Africa (Grobbelaar et al. 2009) and China (Grobbelaar et al. 2010b). There have been relatively few reports of *Ophiostoma* spp. on *Eucalyptus* from Africa, and these include *O. quercus* on *E. grandis* (De Beer et al. 2003a) and *O. stenoceras* (Robak) Nannf. on *Eucalyptus* spp. in South Africa and from soil in eucalypt plantations in Kenya (De Beer et al. 2003b). *Ophiostoma tsotsi* was found on *Eucalyptus* spp. in South Africa as well as in Malawi (Grobbelaar et al. 2009), and *Leptographium eucalyptophilum* K. Jacobs, M.J. Wingf. and J. Roux was described from *Eucalyptus* trees in the Republic of Congo (Jacobs et al. 1999). Other *Ophiostoma* spp. known from Africa are confined to reports from South Africa where they are known either from non-native bark beetles infesting *Pinus* spp. (Zhou et al. 2001, 2006) or from indigenous trees (De Beer et al. 2003a, b; Kamgan Nkuekam et al. 2008a; Roets et al. 2008, 2010).

The aim of this study was to increase the base of knowledge pertaining to fungi in the Ophiostomatales on plantation-grown *Eucalyptus* spp. in South Africa. Collections were focused on freshly made wounds on trees and on the nitidulid beetles (Coleoptera: Nitidulidae) that visit these wounds. Morphological characteristics, mating studies and phylogenetic inference based on DNA sequence data were used to identify these fungi.

Materials and methods

Fungal isolations from trees

To investigate the occurrence of Ophiostomatoid fungi on eucalypts in South Africa, collections were made from freshly cut stumps, since wounds are known to be infection sites for these fungi. Wounds are also commonly visited by nitidulid beetles, making it possible to collect both ophiostomatoid fungi and their possible nitidulid vectors. Collections were made between February 2007 and November 2008 in most of the commercial eucalypt-growing areas of the country. The overall aim was to collect material from as many different geographical and climatic areas of the country as possible. Samples were

mainly collected from harvesting wounds (stumps) of felled trees as well as from logs lying on the plantation floor. However, where damage to the stems of trees, caused by wood-boring beetles, was observed, samples were also collected. Areas and trees sampled included eucalypts in the Eastern Cape Province from Kumbu and Lotobeni, Limpopo Province from Tzaneen and Louis Trichard in the Soutpansberg area, KwaZulu-Natal Province from Kwambonambi, the Mpumalanga Province from Sabie, and the Western Cape Province from Cape Town, George and Stellenbosch. The climatic regions where samples were collected included Mediterranean (Cape Town, Stellenbosch), temperate (Lotobeni, Kumbu) and subtropical (KwaMbonambi, Tzaneen, Soutpansberg) regions and included winter (Cape Town, Stellenbosch), year-round (George) and summer (KwaZulu Natal, Mpumalanga, Limpopo Province) rainfall areas. For comparative studies, some *Ophiostoma* isolates collected from *Eucalyptus* trees infested by *Phoracantha* spp. were obtained from the culture collection of FABI.

Pieces of bark and wood were collected from wounds, especially those with bark flaps, and stored in brown paper bags after they had been inspected with a $\times 20$ magnification hand lens for the presence of fruiting bodies (ascomata or conidiophores) resembling those of the ophiostomatoid fungi. All the samples were transported to the laboratory in plastic bags to retain moisture, and processed following the methods described by Kamgan Nkuekam et al. (2008a, 2010a). Replicates of each pure isolate obtained were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative specimens were also deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands. Dried specimens of representative isolates were deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

Collection of insects and fungal isolation

Nitidulidae were collected from beneath bark flaps on cut stumps and wounds on *Eucalyptus* spp. This was done using an aspirator pooter (Fergusson 1982). Living insects were transferred to cylindrical labeled glass containers containing a piece of tissue paper that served to reduce contact and conflict among the insects. In the laboratory, the collected insects were inactivated by cooling the glass containers in a box containing ice. The insects were grouped according to morphological characteristics using an Axiocam dissection microscope (Carl Zeiss, Germany). Representatives of each insect group were preserved in 70% ethanol prior to identification, by Dr. Andrew Cline, Senior Insect Biosystematist, Plant Pest Diagnostics Center, California Department of Food & Agriculture, USA.

Isolation of fungi from the insects was done by crushing individual specimens between two slices of carrot and incubating them for 5 days at 25°C (Moller and Devay 1968; Heath et al. 2009). Fungi were isolated by transferring fruiting bodies (mycelium, ascomata, ascospores) growing on the carrots to 2% malt extract agar (MEA: 20 g/L malt extract and 15 g/L agar, Biolab, Midrand, South Africa, and 1,000 mL sterile deionised water) containing 0.05 g/L of the antibiotic streptomycin sulphate (Sigma-Aldrich, Steinheim, Germany).

Morphological characterization

All isolates resembling species of Ophiostomatales were characterized based on commonly used characteristics for this group. Single drops of conidia, ascospores or small pieces of mycelium were transferred from pure cultures to Oatmeal agar media (OMA: 30 g Oats, 20 g Biolab agar and 1,000 mL deionised water) to promote sporulation. Cultures were incubated at 25°C until sporulation and then grouped into morphotypes based on color (Rayner 1970) and macro-morphology. Fruiting structures from selected isolates representing each morphotype were mounted in 80% lactic acid on microscope slides and studied using a Zeiss Axiocam light microscope (München-Hallbergmoos, Germany). Fifty measurements of all characteristic morphological features were made for isolates chosen as the types of new species and ten measurements were made for additional isolates. The means were then calculated for relevant morphological structures. Measurements were noted as (minimum–) mean minus st. dev. – mean plus st.dev. (–maximum).

Scanning electron microscopy was used to observe fruiting bodies (conidia, conidiophores) of the asexual states of the fungi. For this purpose, specimens were prepared as described by Grobbelaar et al. (2009). The specimens were critical point dried (Bio-Rad E3000, Watford, England), then mounted and coated with gold in a sputter coater (Emitech K550X, Ashford, England) and examined using a JEOL JSM-840 scanning electron microscope (JEOL, Tokyo, Japan).

Growth in culture

Three isolates of each new species identified in this study were used for growth studies in culture. A disk of agar (9 mm diam.) bearing mycelium of the test isolates was transferred from the actively growing margins of 7-day-old cultures and placed upside down at the centers of 90-mm Petri dishes containing 2% MEA. Five replicate plates were used for each isolate at each growth temperature considered. Two diameter measurements, perpendicular to each other, were taken daily for each colony and the averages of ten diameter measurements for each temperature were computed.

DNA sequence comparisons

Representative isolates of each morpho-group were selected for DNA sequence comparisons. For each isolate, a single drop of spores was collected from ascomata where available, or from synnemata of pure cultures and were transferred to 2% MEA and incubated for 7–10 days. Mycelium was scraped from the surface of the actively growing cultures and then transferred to 1.5-mL Eppendorf tubes using a sterile hypodermic needle. DNA was extracted using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, CA, USA) following the manufacturer's instructions.

The internal transcribed spacer regions (ITS1, ITS4) and 5.8S gene of the ribosomal RNA operon were amplified with an Eppendorf Mastercycler (Merck, Germany) using primers ITS1 and ITS4 (White et al. 1990). Part of the β -tubulin (BT) gene and the transcription elongation factor-1 α (TEF) gene were amplified using the primers T10 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995), and EF1F and EF2R (Jacobs et al. 2004), respectively.

The PCR reaction mixtures as well as the thermal cycling conditions were the same as those described previously (Kamgan Nkuekam et al. 2008a, b). An aliquot of 5 μ l of the PCR products were stained with GelRed™ Nucleic Acid Gel stain (Biotium, Hayward, USA), separated on a 1% agarose gel and visualized under UV light. PCR products were purified using Sephadex G-50 Gel (Sigma-Aldrich), following the manufacturer's instructions. Subsequently, the concentrations of the purified PCR products were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, USA). Sequencing reactions were performed using the Big Dye cycle sequencing kit with Amplitaq DNA polymerase, FS (Perkin-Elmer, Warrington, UK) following the manufacturer's protocols on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequencing PCRs were prepared as described by Kamgan Nkuekam et al. (2008a) and both DNA strands were sequenced.

Compilation of sequence datasets

Contigs of both sequenced strands for each isolate and each gene region were assembled using Sequence Navigator 1.01 (ABI PRISM; Perkin Elmer). Additional sequences of related species in the Ophiostomatales were obtained from the GenBank database (<http://www.ncbi.nlm.nih.gov>) for comparisons. Sequences were aligned using the E-INS-i option in the online version of MAFFT 6 (Katoh and Toh 2008). To avoid the inclusion of large numbers of identical sequences in the phylogenetic analyses, isolates collected were sequenced and grouped based on their BT haplotypes using MEGA 4.0.1 (Tamura et al. 2007). For each BT haplotype, only a few representatives were included in the analyses of the ITS and TEF datasets.

Phylogenetic analyses

Phylogenetic analyses of the ITS, TEF and BT datasets were performed independently of each other. This was done to avoid masking important information and because reference sequences are not available for the same sets of isolates for all gene regions. For each dataset, maximum parsimony (MP), Bayesian analyses (MB), and maximum likelihood (ML) analyses were done. However, since the sequence data for intron 5 of the BT gene varies greatly between different species groups, the dataset was treated in subsets in order to obtain improved alignments for species definition. MP analyses were performed in a similar manner for both BT datasets in PAUP 4.0b10 (Swofford 1998), using the following settings: 100 random sequence addition replicates, tree bisection-recognition (TBR) branch swapping, and 'multrees' option in effect. Confidence levels of the MP phylogenies were estimated with the bootstrap method (1,000 replications).

Bayesian analyses based on Markov Chain Monte Carlo (MCMC) were performed with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) as outlined previously (Kamgan Nkuekam et al. 2010a). Appropriate substitution models were determined using the Akaike Information Criterion (AIC) in MrModeltest 2.2 (<http://www.abc.se/~nylander/>). The model applied to both ITS and TEF was GTR+I+G, while for the two subsets of BT data it was HKY+G and HKY+G, respectively. Burn-in values were determined using Tracer 1.4 (<http://beast.bio.ed.ac.uk/Tracer>) to discard trees that formed before the point of convergence, and the posterior probability in the majority rule consensus trees were calculated by MCMC sampling in MrBayes v.3.1.2, using the best-fit model of evolution mentioned above.

Maximum likelihood (ML) analyses were conducted online using PhyML 3.0 (Guindon and Gascuel 2003). The AIC was used in Modeltest 3.7 (Posada and Crandall 1998) to select appropriate substitution models for the three datasets. For ITS, the selected model was GTR+I+G (gamma shape parameter = 1.0169; Pinvar = 0.2617), for the TEF, it was GTR+I+G (gamma shape parameter = 2.0444; Pinvar = 0.1411), while for the two subsets of BT it was TVM+G (gamma shape parameter = 0.1978) and HKY+G (gamma shape parameter = 0.1612), respectively.

Results

Fungal isolations from trees

A total of 111 isolates resembling species in the Ophiostomatales were obtained from eucalypt trees in this study. More than 300 trees were sampled in the process (Table 1). Samples were obtained from the stumps of recently felled

Table 1 Geographic distribution, host range and Genbank accession numbers for *Ophiostoma* isolates collected in this study, including BT haplotypes of isolates from the *O. piceae* complex

Species	CMW	Host/insect	Origin	ITS	BT		EF		
				Acc. no.	Haplotypes	Acc. no.	Acc. no.		
<i>O. candidum</i> sp. nov.	26482	<i>E. cloeziana</i>	Soutpansberg, LIM	NA	X	NA	NA		
	26483			HM051408	X	HM041873	NA		
	26484			HM051409	X	HM041874	NA		
	26485			HM051410	X	HM041871	NA		
	26486			HM051411	X	HM041872	NA		
	26487			NA	X	NA	NA		
	26488			NA	X	NA	NA		
	26489			NA	X	NA	NA		
	26817			NA	X	NA	NA		
<i>O. fumeum</i> sp. nov.	26813	<i>Eucalyptus</i> sp.	Soutpansberg, LIM	HM051412	Y	HM041878	NA		
	26814			HM051413	Y	HM041875	NA		
	26815			NA	Y	NA	NA		
	26816			HM051414	Y	HM041876	NA		
	26818		Zambia	HM051415	Y	HM041877	NA		
	26819			NA	Y	NA	NA		
	26820			NA	Y	NA	NA		
	26822			NA	Y	NA	NA		
	27057			<i>E. grandis</i>	Tzaneen, LIM	NA	A	NA	NA
<i>O. quercus</i>	27057	<i>Eucalyptus</i> sp.	George, WC			NA	F	NA	NA
	27058					HM051398	J	HM041859	NA
	27059					NA	B	NA	NA
	27060					NA	H	NA	NA
	27062					NA	J	NA	NA
	27064					NA	F	NA	NA
	27071					NA	F	NA	NA
	27072					NA	H	NA	NA
27073	NA	G	NA	NA					
27074	NA	H	NA	NA					
27075	NA	F	NA	NA					
27076	NA	I	NA	NA					
27077	NA	I	NA	NA					
27078	<i>E. diversicolor</i>	Cape Town, WC	NA	A	NA	NA			
27089			NA	A	NA	NA			
27145	<i>E. saligna</i>	Cape Town, WC	NA	B	NA	NA			
27147			<i>Eucalyptus</i> sp.	George, WC	NA	F	NA	NA	
27150	HM051405	G			HM041865	NA			
27152	HM051404	C	HM041860	HM041895					
27164	<i>Eucalyptus</i> logs	Stellenbosch, WC	HM051403	F	HM041864	HM041894			
27165			NA	A	NA	NA			
27168	<i>E. grandis</i>	Kumbu, EC	NA	A	NA	NA			
27169			NA	B	NA	NA			
27170			NA	A	NA	NA			
27171			NA	B	NA	NA			
27172			NA	B	NA	NA			
27173			HM051397	H	HM041856	HM041897			
27174			NA	B	NA	NA			

Table 1 (continued)

Species	CMW	Host/insect	Origin	ITS	BT		EF
				Acc. no.	Haplotypes	Acc. no.	Acc. no.
	27191	<i>E. diversicolor</i>	Cape Town, WC	NA	A	NA	NA
	27192			HM051399	A	HM041858	HM041893
	28196	<i>Eucalyptus</i> sp.	George, WC	NA	H	NA	NA
	28201	<i>Eucalyptus</i> logs	Cape Town, WC	HM051400	H	HM041857	HM041892
	28212	<i>E. grandis</i>	Lotebeni, EC	NA	B	NA	NA
	28213			NA	B	NA	NA
	28214			NA	A	NA	NA
	28215			HM051402	E	HM041863	HM041891
	28216			NA	B	NA	NA
	29693	<i>Carpophilus</i> sp.	Cape Town, WC	NA	A	NA	NA
	29717	<i>B. depressus</i>	Cape Town, WC	NA	A	NA	NA
	29734		Kumbu, EC	HM051407	D	HM041862	HM041896
	29736			NA	A	NA	NA
	29738			NA	A	NA	NA
	29824			NA	B	NA	NA
	29827		Stellenbosch, WC	NA	F	NA	NA
	29832			NA	A	NA	NA
	30863	<i>E. saligna</i>	Soutpansberg, LIM	NA	B	NA	NA
	30864			HM051406	B	HM041861	HM041890
	30865			NA	B	NA	NA
	30868	<i>E. grandis</i>	Sabie, MP	NA	A	NA	NA
	30869			NA	A	NA	NA
<i>O. tasmaniense</i>	27090	<i>E. diversicolor</i>	Cape Town, WC	NA	L	NA	NA
	27146	<i>E. saligna</i>	Cape Town, WC	HM051391	M	HM041849	NA
	27161	<i>E. diversicolor</i>	Cape Town, WC	HM051392	K	HM041850	HM041886
	27189			NA	K	NA	NA
	27190			NA	K	NA	NA
	27193			HM051396	L	HM041853	NA
	27195			HM051393	K	HM041851	HM041887
	29685	<i>Carpophilus</i> sp.	Cape Town, WC	NA	K	NA	NA
	29688			HM051395	K	HM041852	HM041888
	29691			NA	K	NA	NA
	29712	<i>B. depressus</i>	Cape Town, WC	HM051401	L	HM041855	HM041889
	29831		Stellenbosch, WC	HM051394	L	HM041854	HM041885
<i>O. tsotsi</i>	24954	<i>Eucalyptus</i> sp.	Pietermaritzburg, KZN	NA	2	NA	NA
	24956			NA	1	NA	NA
	24959			NA	1	NA	NA
	24964			NA	5	NA	NA
	24966			NA	1	NA	NA
	24968			NA	1	NA	NA
	24971			NA	1	NA	NA
	24977		Kwambonambi, KZN	NA	1	NA	NA
	24978			NA	5	NA	NA
	24981			NA	6	NA	NA
	24982			HM051390	3	HM041845	HM041882
	24983			HM051388	5	HM041847	HM041880
	24985			NA	1	NA	NA

Table 1 (continued)

Species	CMW	Host/insect	Origin	ITS Acc. no.	BT		EF Acc. no.
					Haplotypes	Acc. no.	
	24986	<i>E. grandis</i>	Tzaneen, LIM	HM051385	4	HM041848	HM041879
	24987			NA	2	NA	NA
	24988			HM051387	2	HM041846	HM041883
	24990			NA	5	NA	NA
	24992			NA	1	NA	NA
	24993			NA	1	NA	NA
	24996			NA	1	NA	NA
	24997			NA	5	NA	NA
	24999			NA	1	NA	NA
	25000			NA	2	NA	NA
	25002			NA	1	NA	NA
	25003			NA	1	NA	NA
	25004			NA	1	NA	NA
	25005			NA	2	NA	NA
	25006			NA	1	NA	NA
	25007			NA	1	NA	NA
	25009			NA	1	NA	NA
	25010			NA	2	NA	NA
	25011			NA	1	NA	NA
	25013			NA	1	NA	NA
	25014			NA	1	NA	NA
	25016			NA	5	NA	NA
	25018			NA	1	NA	NA
	25022			NA	1	NA	NA
	25024			NA	1	NA	NA
	25036	<i>B. depressus</i>	Kwambonambi, KZN	NA	1	NA	NA
	25038			HM051389	1	HM041843	HM041881
	25042			NA	5	NA	NA
	25048	<i>Ca. humeralis</i>	Kwambonambi, KZN	NA	5	NA	NA
	25055			NA	2	NA	NA
	25058			NA	5	NA	NA
	25066			NA	1	NA	NA
	25068			NA	1	NA	NA
	25071			NA	1	NA	NA
	25072			HM051386	6	HM041844	HM041884
	26328	<i>B. depressus</i>	Soutpansberg, LIM	NA	2	NA	NA
	26459	<i>E. cloeziana</i>	Soutpansberg, LIM	NA	1	NA	NA
	26460			NA	1	NA	NA
	26461			NA	1	NA	NA
	26462			NA	1	NA	NA
	26463			NA	1	NA	NA
	26464			NA	1	NA	NA
	26465			NA	5	NA	NA
	26467	<i>E. maculata</i>	Soutpansberg, LIM	NA	1	NA	NA
	26468			NA	1	NA	NA
	26469			NA	1	NA	NA

EC Eastern Cape, WC Western Cape, LIM Limpopo, KZN KwaZulu Natal, MP Mpumalanga, NA Not available

trees, fresh logs lying in the field and from the tunnels of *Phoracantha* spp. in standing trees. Four more *Ophiostoma* isolates were obtained from the culture collection of FABI. These isolates originated from Zambia where they were collected from *Eucalyptus* trees infested by *Phoracantha* spp. (Table 1) during surveys of diseases in that country.

Collection of insects and fungal isolation

Nitidulid beetles were found in most of the areas sampled. A total of 271 insects were collected from stumps of various *Eucalyptus* trees and they could be broadly divided into two groups based on morphology. These were *Brachypeplus* sp. (140 specimens), identified as *B. depressus* Erichson, and a *Carpophilus* sp. identified as *C. humeralis* Fabricius (120). Eleven specimens were recognized as a *Carpophilus* sp. but could not be identified to species level. Only 24 *Ophiostoma* isolates were obtained from the insects: 13 were from *B. depressus*, while 11 were from *Carpophilus* spp. (Table 1).

Morphological characterization

Three morphological groups were found among the ophiostomatoid fungi collected. Morpho-group A, comprising 122 isolates, produced *Pesotum* anamorphs and *Sporothrix* synanamorphs on OMA. They also produced limited numbers of ascomata with long erect necks exuding slimy masses of spores, scattered over the colony surface. Morpho-group B, consisted of nine isolates with white cultures and ascomata, produced abundantly and embedded within the mycelium mat. Morpho-group C consisted of eight isolates with gray-colored cultures and ascomata with very short necks deprived of ostiolar hyphae, produced in a thick mycelial mat by some strains.

Compilation of sequence data and phylogenetic analyses

All isolates sequenced produced fragments of approximately 400 bp for the BT gene region. Blast searches in the GenBank data base showed that all isolates of morpho-group A were members of the *O. piceae* complex while isolates of morpho-group B and morpho-group C belonged to the *S. schenckii*–*O. stenoceras* complex. Furthermore, the morpho-group A isolates all had intron 4 and lacked intron 5, while isolates from morpho-groups B and C lacked intron 4 but presented intron 5. The BT data for the three groups of isolates were thus analyzed separately in two subsets to obtain improved alignments for species definition.

Comparisons of the BT sequence data for the *O. piceae* subset of isolates from South Africa with those of related *Ophiostoma* spp. from GenBank resulted in a total of 238

characters including gaps, with 142 constant characters, 11 parsimony uninformative characters and 85 parsimony informative characters. Comparisons of the BT sequence data for the *O. stenoceras* subset of isolates with those of related *Ophiostoma* spp. from GenBank resulted in a total of 269 characters including gaps, with 145 constant characters, 3 parsimony uninformative characters and 121 parsimony informative characters.

Maximum parsimony analyses of the BT data set of the *O. piceae* subset of isolates generated 308 trees. The consistency indices (CI) and retention index (RI) values were 0.617 and 0.912, respectively. Analyses of the BT dataset of the *O. stenoceras* subset of isolates generated 648 trees, with CI and RI values of 0.494 and 0.874, respectively. For each dataset, a 50% majority rule tree obtained from Bayesian analyses and a bootstrap tree was also obtained from a MP as well as from maximum likelihood analyses (ML). For each dataset, a ML tree is presented (Figs. 1 and 2) indicating at the relevant node, MP bootstrap values, Bayesian posterior probabilities, and ML bootstrap values respectively.

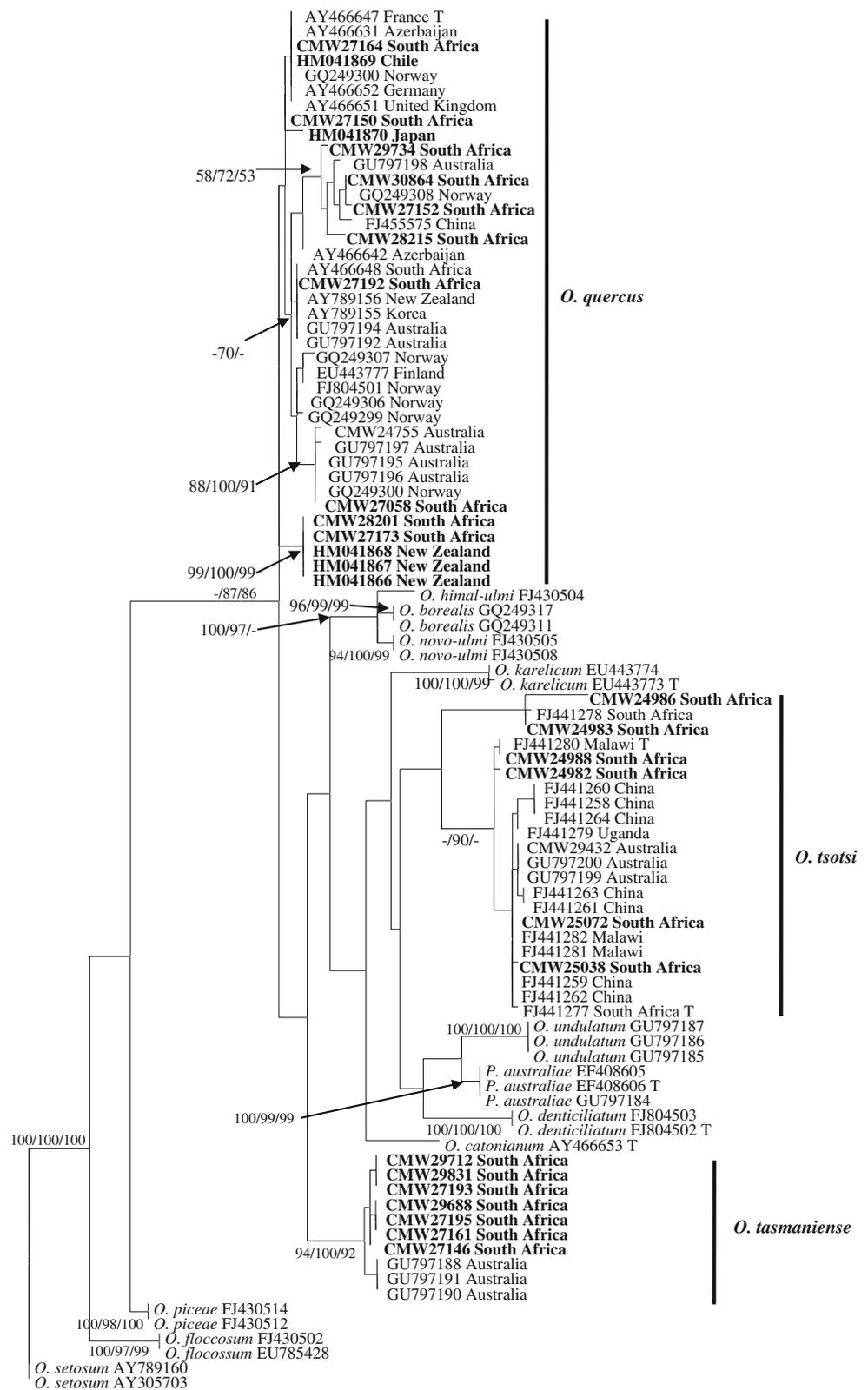
In the BT tree (Fig. 1), isolates representing the *O. piceae* subset were resolved into three groups. The first set of isolates grouped with strains of *O. quercus*. The second set of isolates grouped with strains of *O. tsotsi*, while the third set of isolates grouped with strains of *O. tasmaniense* (Fig. 1). In the BT tree representing the *O. stenoceras* dataset (Fig. 2), isolates from South Africa could be resolved into two clades, well supported at the nodes and clearly distinct from other *Ophiostoma* reference strains. Isolates representing morpho-groups B and C resided in well-resolved clades with good statistical support.

Representatives of each haplotype found in the BT dataset of the *O. piceae* subset of isolates were selected for phylogenetic analyses of the TEF dataset. Comparisons of the TEF dataset of the *O. piceae* subset of isolates with those of related *Ophiostoma* spp. from GenBank resulted in a total of 520 characters including gaps, with 95 constant characters, 18 parsimony uninformative characters and 407 parsimony informative characters. Maximum parsimony analyses of the TEF dataset generated 1,122 best trees, with CI and RI values of 0.661 and 0.932, respectively. For the TEF dataset, a 50% majority rule tree was obtained from Bayesian analyses and a bootstrap tree was also obtained from a MP as well as a ML (Fig. 3) analyses.

Results of analyses of the TEF data (Fig. 3) were similar to those of the BT dataset. Representatives of the *O. piceae* subset of isolates were again resolved into three clades. One set of isolates grouped with strains of *O. quercus*, the second set grouped with strains of *O. tsotsi*, while the third set of isolates grouped with strains of *O. tasmaniense*.

For the ITS dataset, representatives of each morpho-group found in the survey were selected for phylogenetic

Fig. 1 Phylogram obtained from maximum likelihood analyses of DNA sequence data from part of the BT gene region, showing the relationship between members of the *Ophiostoma piceae* complex from eucalypt trees in South Africa and reference strains of other *Ophiostoma* spp. Isolates sequenced in this study are in *bold*. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values are indicated at each relevant node. *T* Ex-type isolates



analyses. Comparisons of the ITS dataset with those of related *Ophiostoma* spp. from GenBank resulted in a total of 799 characters including gaps, with 216 constant

characters, 80 parsimony uninformative characters and 503 parsimony informative characters. Maximum parsimony analyses of the ITS dataset generated 2,069 best trees,

Fig. 2 Phylogram obtained from maximum likelihood analyses of DNA sequence data from part of the BT gene region, showing the relationship between *O. fumeum* and *O. candidum* from eucalypt trees in South Africa and other members of the *Sporothrix schenckii*-*Ophiostoma stenoceras* complex. Isolates sequenced in this study are in *bold*. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values are indicated at each relevant node

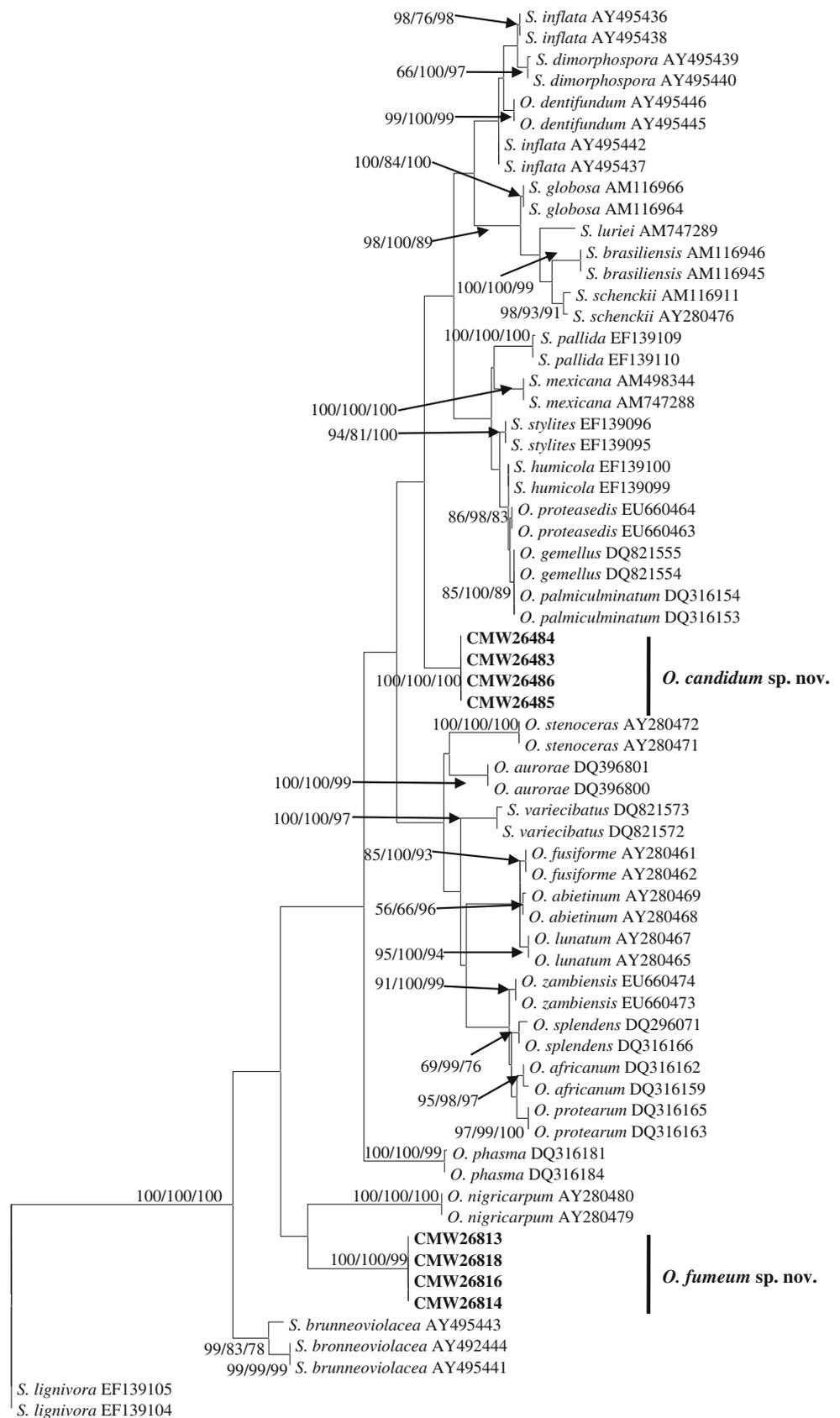
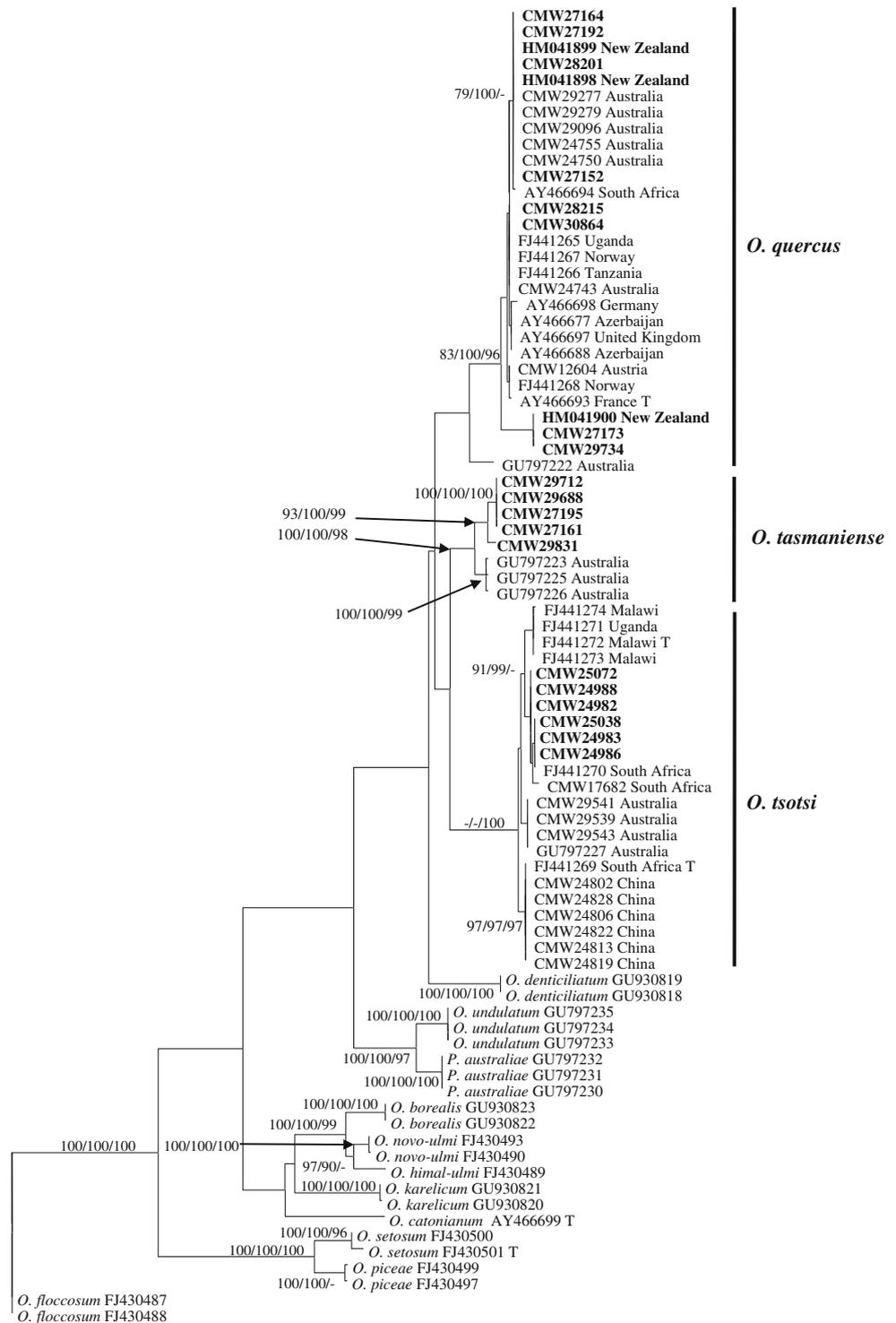


Fig. 3 Phylogram obtained from maximum likelihood analyses of DNA sequences of the TEF gene region, showing the relationship between members of the *Ophiostoma piceae* complex from eucalypt trees in South Africa and reference strains of other *Ophiostoma* spp. Isolates sequenced in this study are in **bold**. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values are indicated at each relevant node. T Ex-type isolates

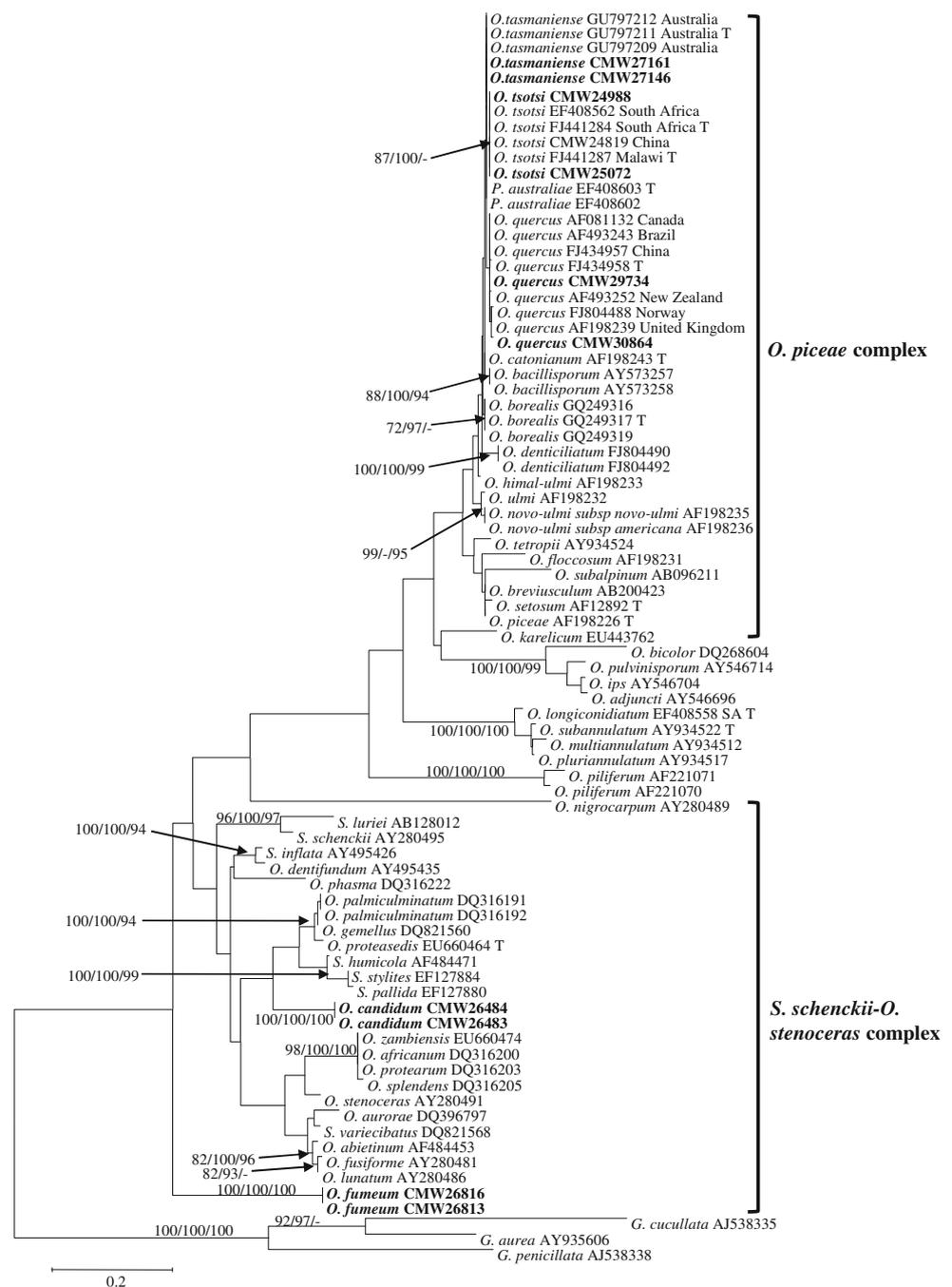


with CI and RI values of 0.566 and 0.865, respectively. For the ITS dataset, a 50% majority rule tree obtained from Bayesian analyses and a bootstrap tree was obtained from MP as well as ML analyses (Fig. 4).

The two subsets of *Ophiostoma* spp., identified based on BT sequences and the presence and/or absence of introns 4 and 5, could clearly be recognized within the ITS tree. The

O. piceae complex subset resided in the top half of the tree, while the *S. schenckii*–*O. stenoceras* complex was confined to the lower part of the tree (Fig. 4). Isolates representing morpho-group A clustered with those of *O. quercus*, *O. tsotsi* and *O. tasmaniense*, but the clades representing these species were not well resolved. Isolates representing morpho-group B and morpho-group C formed two different

Fig. 4 Phylogram obtained from maximum likelihood analyses of DNA sequences of the ITS gene region, showing the relationship between *Ophiostoma* species from *Eucalyptus* trees in South Africa and reference strains of other *Ophiostoma* spp. Isolates sequenced in this study are in **bold**. MP bootstrap values, Bayesian posterior probabilities and ML bootstrap values are indicated at each relevant node. *T* Ex-type isolates



clades within the *S. schenckii*–*O. stenoceras* complex. These clades were clearly separated from other *Ophiostoma* reference isolates and they were statistically well supported (Fig. 4).

Taxonomy

Based on morphological examination and phylogenetic analyses using the ITS and BT gene regions, two *Ophiostoma* spp. from South Africa emerged as distinct and previously undescribed species within the *S. schenckii*–

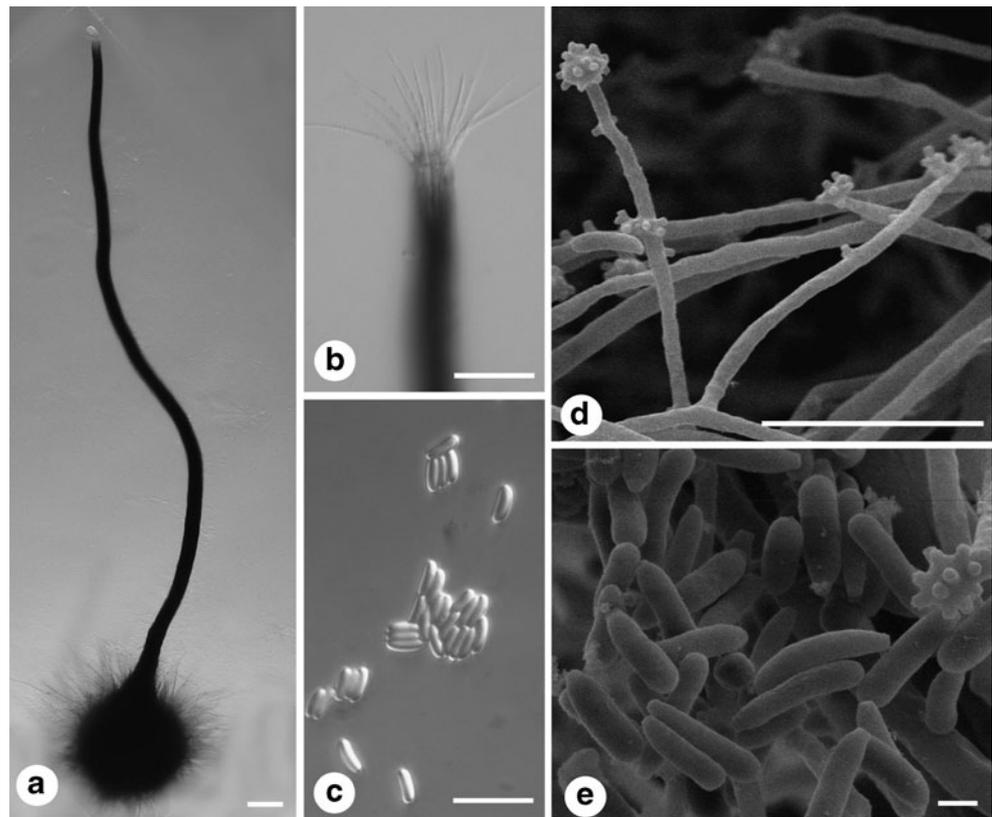
O. stenoceras complex of the Ophiostomatales. The following descriptions are provided for them.

Ophiostoma candidum Kamgan-Nkuek., Jol. Roux & Z. W. de Beer sp. nov. (Fig. 5). MB519314

Etymology: The name refers to the dazzling white colony color of the fungus on artificial media.

Coloniae albae tegetibus mycelii crassis, in MEA ut videtur madidae; usque ad 11.5 mm diametro in 10 diebus in MEA in 25°C. Crescit optime in 25°C; non crescit in 10°C nec 30°C

Fig. 5 Morphological characteristics of *Ophiostoma candidum* sp. nov. **a** Globose ascomatal base (scale bar 50 μ m), **b** divergent ostiolar hyphae (scale bar 20 μ m), **c** reniform ascospores (scale bar 10 μ m), **d** *Sporothrix* conidiogenous cell with denticles visible on tip of conidiogenous cell (scale bar 10 μ m), **e** oblong to curved conidia (scale bar 1 μ m)



nec 35°C. *Ascomata* in superficie coloniae dispersa et in mycelio inclusa, guttas sporarum albocreneas facientes. *Colla* ascomatum nigra *hyphis* ostiolaribus divergentibus. Bases ascomatum nigrae globosae pilis hypharum. Bases collorum laeves. *Ascosporae* reniformes hyalinae non septatae (3.5–) 4.0–4.5 (–5.0) × (1.0–) 1.0–1.5 (–1.7) μ m. Anamorphia *Sporothrix* conidiophoris hyalinis cylindricis apicem versus contractis, cum denticulis prominentibus. Conidia non septata, hyalina oblonga interdum acerosa nonnulla valde curvata (1.5–) 1.5–2.0 (–2.5) × (0.3–) 0.5–0.5 (–1.0) μ m.

Colonies white, with thick mycelial mats, with a wet appearance on MEA. Reverse Buff (19”d). Colony diameters reaching 11.5 mm in 10 days on MEA at 25°C. Optimal growth at 25°C. No growth at 10°C and at 30°C or 35°C. *Ascomata* scattered over the colony surface and embedded within mycelium on agar plates, producing white, creamy spore drops at the apices of the necks. *Ascomatal* necks black (270.5–) 506.0–875.0 (–970.5) μ m long. *Ascomatal* bases black, globose (84.5–) 124.5–184.5 (–206.0) μ m long and (90–) 119–182 (–233) μ m wide, with hyphal hairs. Neck bases smooth (24.0–) 29.5–41.0 (–51.0) μ m wide, middle of necks (16.5–) 19.0–25.0 (–27.5) μ m wide, tips of necks (9.0–) 11.5–16.0 (–22.0) μ m wide. *Ostiolar hyphae* divergent (16.0–) 22.5–34.0 (–39.0). *Asci* not seen, evanescent, deliquescing early in the development. *Ascospores* reniform, aseptate, hyaline (3.5–) 4.0–4.5 (–5.0) × (1.0–) 1.0–1.5 (–1.7) μ m.

Anamorph: *Sporothrix*, conidiophores hyaline, cylindrical, tapering towards the apex, (12.5–) 17.5–43.5 (–61.0) × (1.5–) 1.9–2.5 (–3.0) μ m, prominent denticles present. Conidia aseptate, hyaline, oblong, occasionally acerosa, distinctly curved in some cases (1.5–) 1.5–2.0 (–2.5) × (0.5–) 0.5–0.5 (–1.0) μ m.

Specimens examined: South Africa, Limpopo Province, Goedehoop area of the Soutpansberg, isolated from wound on *Eucalyptus cloeziana* tree struck by lightning, 21/06/2007, G. Kamgan Nkuekam and Jolanda Roux, holotype PREM60470, living culture CMW26484, CBS129713.

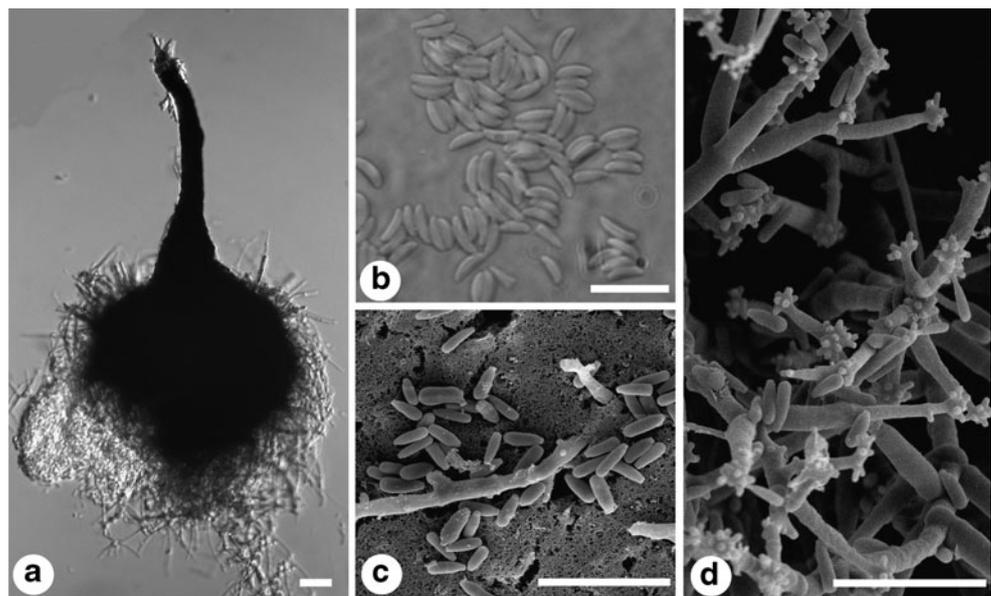
Additional specimens: South Africa, Limpopo Province, Goedehoop area of the Soutpansberg, isolated from wound on *Eucalyptus cloeziana* tree struck by lightning, 21/06/2007, G. Kamgan Nkuekam & J. Roux, holotype PREM60471, living culture CMW26483, CBS129714; isolated from wound on *Eucalyptus cloeziana* tree struck by lightning, 21/06/2007, G. Kamgan Nkuekam & J. Roux, holotype PREM60472, living culture CMW26485, CBS129711.

Ophiostoma fumeum Kamgan-Nkuek., Jol. Roux & Z. W. de Beer sp. nov. (Fig. 6). MB519315

Etymology: The name refers to the smoky gray colony color of the fungus on artificial media.

Coloniae fumaeae tegetibus mycelii crassis in MEA; usque ad 16.5 mm diametro in 10 diebus in MEA in 25°C.

Fig. 6 Morphological characteristics of *Ophiostoma fumeum* sp. nov. **a** Globose ascumatal base (scale bar 20 μ m), **b** allantoid ascospores (scale bar 10 μ m), **c** oblong conidia (scale bar 10 μ m), **d** *Sporothrix* conidiogenous cell with denticles visible on tip of conidiogenous cell (scale bar 10 μ m)



Crescit optime in 30°C; non crescit in 10°C. *Ascomata* in cultura serotina in mycelio inclusa, guttas sporarum albocreneas facientes. *Colla* ascomatum nigra sine *hyphis* ostiolaribus. Bases ascomatum nigrae globosae pilis hypharum. Bases collorum laeves. *Ascosporae* allantoidae hyalinae non septatae (4.0–) 4.5–5.5 (–6.0) × (1.3–) 1.4–1.7 (–1.9) μ m. Anamorpha *Sporothrix* conidiophoris hyalinis cylindricis cum denticulis prominentibus. Conidia non septata, hyalina oblonga vel cylindrica (1.0–) 1.5–2.0 (–2.5) × (0.4–) 0.5–0.6 (–0.8) μ m.

Colonies smoke gray (21^{***}d), with thick mycelium mat on MEA. Reverse smoke gray (21^{***}d). Colony diameters reaching 16.5 mm in 10 days on MEA at 25°C. Optimal growth at 30°C. No growth at 10°C. *Ascomata* produced late in culture and embedded within mycelium, producing white, creamy spore drops at the neck apices. *Ascomatal* necks black (50.5–) 77–119.7 (–145) μ m long. *Ascomatal* bases black, globose (81.0–) 103.0–144.5 (–165.0) μ m long and (97.0–) 116.0–164.0 (–186.5) μ m wide, with hyphal hairs. Neck base smooth, (31–) 30–43 (–50) μ m wide. *Ostiolar hyphae* absent. *Asci* not seen, evanescent, deliquescing early in the development. *Ascospores* allantoid, aseptate, hyaline (4.0–) 4.5–5.5 (–6.0) × (1.3–) 1.4–1.7 (–1.9) μ m.

Anamorph: *Sporothrix*, conidiophores hyaline, cylindrical, (2.2–) 4–7 (–10.5) × (0.5–) 0.5–0.5 (–1) μ m, prominent denticles present. Conidia aseptate, hyaline, oblong, to cylindrical (1.0–) 1.5–2.0 (–2.5) × (0.5–) 0.5–0.5 (–1.0) μ m.

Specimens examined: South Africa, Limpopo Province, Soutpansberg area, isolated from *Eucalyptus cloeziana* infested by *Phoracantha* beetles, 21/06/2007, G. Kamgan Nkuekam and J. Roux, holotype PREM60473, living culture CMW26813, CBS129712.

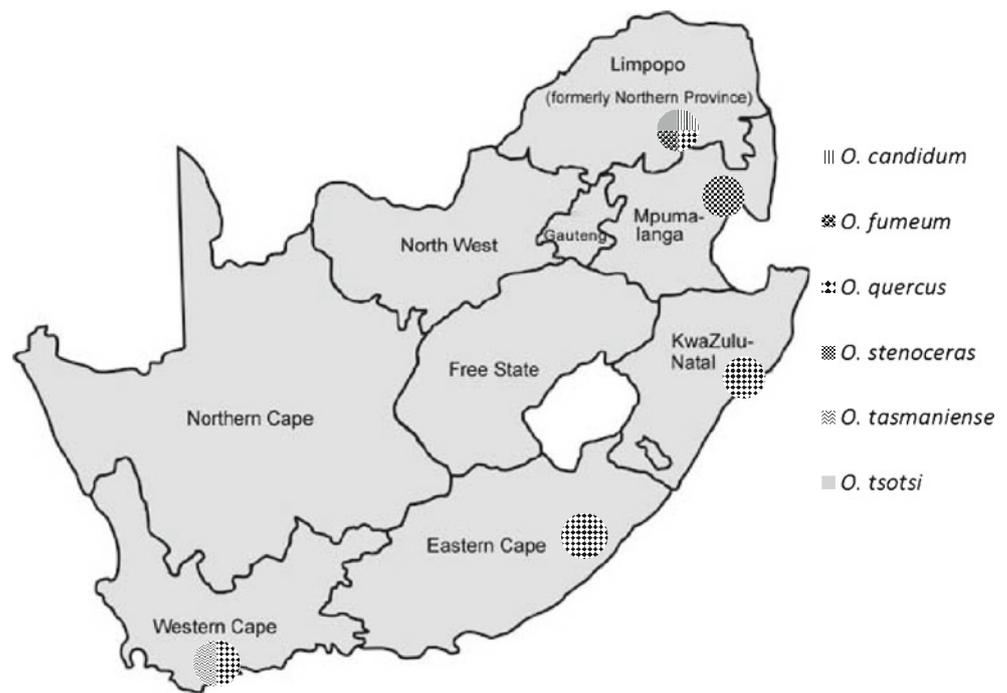
Additional specimens: South Africa, Limpopo province, Soutpansberg area, isolated from *Eucalyptus cloeziana* infested by *Phoracantha* beetles, 21/06/2007, G. Kamgan Nkuekam and J. Roux, paratype, living culture CMW26816/PREM60474, isolated from *Eucalyptus* spp. infested by *Phoracantha* beetles in Zambia, Copperbelt Province, J. Roux, paratype, living culture CMW26818/PREM60475.

The geographic distribution of the Ophiostomatales collected from eucalypts in this study is varied. *Ophiostoma quercus* is the most widely spread species, occurring in all five Provinces sampled (Fig. 7). It was obtained from three *Eucalyptus* spp. (*E. diversicolor*, *E. saligna*, *E. grandis*) and is associated with two nitidulid species (Table 1). *Ophiostoma tsotsi* was collected in two of the five Provinces sampled (Fig. 7), infecting three *Eucalyptus* spp. (*E. grandis*, *E. maculata*, *E. cloeziana*). It was also associated with two nitidulid species (Table 1). The remaining *Ophiostoma* species found in this study are currently known from only one Province each. *Ophiostoma candidum* and *O. fumeum* was recovered only from the Limpopo Province on *E. cloeziana*, while *O. tasmaniense* was obtained from two *Eucalyptus* spp. (*E. diversicolor*, *E. saligna*) and from two nitidulid species in the Western Cape Province (Fig. 7, Table 1).

Discussion

Results of this study have shown that *Ophiostoma* spp. occur widely on *Eucalyptus* trees and logs in South Africa. Five *Ophiostoma* spp. were identified. Two of these were previously undescribed species in the *S. schenckii*–*O. stenoceras* complex for which the names *O. candidum* and *O. fumeum* were provided. *O. tasmaniense* was found

Fig. 7 The current geographic distribution of Ophiostomatalean fungi on eucalypts in South Africa



for the first time outside Australia, while the geographic and host ranges for *O. quercus* and *O. tsotsi* were expanded substantially.

Ophiostoma fumeum and *O. candidum*, the two new taxa described in this study are phylogenetically related, and morphologically similar to species in the *S. schenckii*–*O. stenoceras* complex. Most members of this complex have white or gray cultures, *Sporothrix* anamorphs and allantoid to reniform to orange section-shaped ascospores (De Hoog 1974; De Beer et al. 2003b). *O. candidum* and *O. fumeum* both share these morphological characteristics.

Ophiostoma candidum and *O. fumeum* described in this study formed distinct clades within the *S. schenckii*–*O. stenoceras* complex. The complex includes 9 species described from arthropod-infested *Protea* infructescences (Roets et al. 2006, 2008, 2010), 11 species associated with soil (Zhou et al. 2006; Marimon et al. 2007; De Meyer et al. 2008; Madrid et al. 2010), 5 from hardwoods (De Beer et al. 2003b; Aghayeva et al. 2004, 2005; De Meyer et al. 2008), 4 from conifers (Aghayeva et al. 2004; Zhou et al. 2006; De Meyer et al. 2008), and 4 human pathogens (Marimon et al. 2007, 2008). Fifteen of these species, including all those from the Proteaceae are known only from Southern Africa. Four of the South African species have some form of association with *Eucalyptus* spp. *O. stenoceras* was previously isolated from *E. smithii* R.T. Baker, *E. grandis* and *E. fastigata* Deane & Maiden in South Africa, and from soil from a *Eucalyptus* plantation in Kenya (De Beer et al. 2003b). *Sporothrix lignivora* de Meyer, Z.W. de Beer & M.J. Wingf. was first described

from *Eucalyptus* utility poles (De Meyer et al. 2008), *S. variecibatus* Roets, Z.W. de Beer & P.W. Crous from decaying *Eucalyptus* leaves and mites (Roets et al. 2008), and *S. schenckii* from *Eucalyptus* wood stacks used to strengthen tunnels in gold mines (Findlay 1970). *O. candidum* was isolated from an *E. cloeziana* tree struck by lightning in the Limpopo Province, and *O. fumeum* from *Eucalyptus* trees infested with the cerambycid beetle *Phoracantha semipunctata* in the Limpopo Province and Zambia.

It has been suggested that possibly all the species in the *S. schenckii*–*O. stenoceras* complex from *Protea* infructescences are vectored by, and have some form of association with, arthropods, especially mites (Roets et al. 2007, 2009). The only other species in this complex that was isolated from an arthropod, in this case a root-infesting bark beetle, is *O. aurorae* X.D. Zhou & M.J. Wingf. (Zhou et al. 2006). However, this species was found with the beetle only in a single location during an extensive survey (Zhou et al. 2001), suggesting that the association with the beetle is not fixed. Another species, *S. variecibatus*, was isolated from a mite from *Protea* (Roets et al. 2008). The fact that *O. fumeum* was isolated in the present study from the same host and *Phoracantha* galleries a few thousand kilometers apart suggests that there could be some form of association between the host tree, fungus and insect. Given their close association with Ophiostomatoid fungi (Malloch and Blackwell 1993; Roets et al. 2008), it is probable that mites are involved in this association. Considering the geographical distribution, hosts and possible arthropod associations, it

appears that southern Africa could represent a center of diversity for species in the *S. schenckii*–*O. stenoceras* complex.

The three known species of *Ophiostoma*, *O. quercus*, *O. tsotsi* and *O. tasmaniense*, found in the present study are members of the *O. piceae* complex. The so-called *O. piceae* complex was first recognized to comprise nine species of fungi infecting either hardwood or coniferous trees (Harrington et al. 2001). In recent years, the number of species in the complex has expanded substantially, and it is now recognized to consist of at least two major lineages, one of which includes only hardwood-infesting species (Harrington et al. 2001; Grobbelaar et al. 2009, 2010a; Linnakoski et al. 2009; Kamgan Nkuekam et al. 2008b, 2010a). All three species found on *Eucalyptus* in this study form part of this hardwood lineage.

It was not surprising to find *O. quercus* on *Eucalyptus* trees in this study. The fungus was first reported from South Africa on native *Olinia* sp. and on *E. grandis* and *Quercus robur* L. (De Beer et al. 2003a). This was followed by reports of the fungus from non-native bark beetles infesting *Pinus* trees (Zhou et al. 2006) and more recently from wounds on native South African trees (Kamgan Nkuekam et al. 2008a). It has also been reported from Uganda (Kamgan Nkuekam et al. 2008b) and Tanzania (Grobbelaar et al. 2009). In this study, we collected *O. quercus* from three *Eucalyptus* spp. (*E. diversicolor*, *E. saligna*, *E. grandis*) and from logs of various *Eucalyptus* trees grown in the Soutpansberg and Tzaneen areas (Limpopo Province), Cape Town, George, Stellenbosch (Western Cape Province), Kumbu and Lotebeni (Eastern Cape Province) (Table 1). This finding has expanded the host and geographic ranges of *O. quercus* in South Africa substantially. It further supports the view that *O. quercus* has a wide host and geographic distribution (Brasier and Kirk 1993; Harrington et al. 2001; De Beer et al. 2003a; Kamgan Nkuekam et al. 2008b). It has, however, been suggested that it represents a species complex (Grobbelaar et al. 2008), a fact that was also clear in the phylogenetic analyses conducted for the current study. This could change views regarding the host and geographic range of the fungus in the future.

Isolates of *O. quercus* collected from eucalypt trees in this study were found to be genetically diverse, especially in the BT sequence data, where nine haplotypes of the fungus were identified. The ITS and TEF data were more conserved. This trend has emerged in the past from numerous studies considering *O. quercus* (Grobbelaar et al. 2008, 2009; Kamgan Nkuekam et al. 2010a). It provides further evidence of the fact that this heterothallic fungus reproduces sexually in Southern Africa, and if it is not native to the area, it has been present in the country for a very long period of time.

O. quercus was collected from a wide variety of sites in this study. The fungus was found in the Western Cape Province, which has a Mediterranean climate with hot sunny summers and cold, rainy winters, in Limpopo with a tropical climate, Tzaneen and Mpumalanga with subtropical climates, and in the Eastern Cape Province with a temperate climate with year-round rainfall (Fig. 7). The fungus thus seems to be highly adaptive to various climatic conditions, which explains why it has a worldwide distribution (Morelet 1992; Brasier and Kirk 1993; Harrington et al. 2001; De Beer et al. 2003a; Kamgan Nkuekam et al. 2008a, b; Paciura et al. 2010).

Ophiostoma tsotsi is a recently erected member of the *O. piceae* complex, closely related to *O. quercus*. It occurs throughout southern and eastern Africa on hardwood trees in countries such as Malawi, Uganda and South Africa (Grobbelaar et al. 2009). In Malawi, *O. tsotsi* occurs on *E. grandis* and native *Julbenardia globiflora* (Benth.) Troupin, in Uganda it is known from non-native *Acacia mearnsii*, while in South Africa it occurs on various *Eucalyptus* species (Grobbelaar et al. 2009). It has recently also been reported from *Eucalyptus* pulpwood chips in China (Grobbelaar et al. 2010b). In this study, *O. tsotsi* was found on three *Eucalyptus* spp. (*E. grandis*, *E. maculata*, *E. cloeziana*) and is reported for the first time from nitidulid beetles. *O. tsotsi* was for many years confused with *O. quercus* based on their cultural resemblance and similar morphology. However, it was recently recognized as a distinct taxon based on genetic studies using polymorphic sequence repeats (Grobbelaar et al. 2008) and multigene DNA sequence phylogenies (Grobbelaar et al. 2009). The presence of many haplotypes within the BT and TEF datasets shows that the species is highly variable.

Similar to *O. quercus*, *O. tsotsi* was collected from a wide variety of sites in this study. It was found in most areas where *O. quercus*, a closely related species, was collected (Fig. 7). Recent reports of *O. tsotsi* from *Eucalyptus* trees in Australia and China, and from agar wood in Vietnam, clearly show that the distribution and host range of the fungus is poorly understood (Paciura et al. 2010; Kamgan Nkuekam et al. 2010a; Grobbelaar et al. 2010b). It has also been shown that, like *O. quercus*, *O. tsotsi* could have a global distribution and can survive under wide varieties of climatic conditions.

This study represents the first report of *O. tasmaniense* on *Eucalyptus* trees in South Africa. The fungus was first described from eucalypts in Australia (Kamgan Nkuekam et al. 2010b). *O. tasmaniense* appears to prefer a Mediterranean climate. It was found exclusively in the Western Cape Province (Cape Town and Stellenbosch) in South Africa, while in Australia, it originated from numerous localities in Tasmania, which resides in a temperate climatic zone. In Australia, *O. tasmaniense* occurs on *E. nitens*, *E.*

globulus and *E. saligna* (Kamgan Nkuekam et al. 2010b), while in South Africa it was found on *E. saligna*, *E. diversicola*, and it was isolated from nitidulid beetles. It is possible that this fungus is native to Australia where eucalypts are endemic.

This study represents the first reports of *O. quercus*, *O. tsotsi* and *O. tasmaniense* from nitidulid beetles. The three fungi were isolated from *Brachypeplus depressus*, from an unknown *Carpophilus* species and from *Carpophilus humeralis*. *O. quercus* in the northern hemisphere is vectored by bark beetles in what is considered to be a loose association (Kirisits 2004; Romón et al. 2007). Most recently, it was also reported from a *Scolytus* sp. infesting birch trees in Norway (Linnakoski et al. 2009). The only other report of an *Ophiostoma* spp. associated with nitidulid beetles is *O. piceae* which was isolated from the surfaces of seven free-flying nitidulid beetles in the United States of America (Juzwik and French 1983). In the southern hemisphere, the only report of *O. quercus* from insects is from non-native bark beetles infesting pine trees in South Africa (Zhou et al. 2001). Our study strongly suggests that *O. quercus* and other closely related *Ophiostoma* spp. can be vectored by nitidulid beetles and that these insects spread these fungi in this country.

This study represents the most comprehensive consideration of *Ophiostoma* and nitidulid beetles infecting *Eucalyptus* trees in South Africa to have been undertaken. The results show that *Ophiostoma* spp. are diverse on wounds on *Eucalyptus* spp. in South Africa, and they highlight the need for additional studies of this type where other *Ophiostoma* species are likely to be discovered. Isolating *O. quercus*, *O. tasmaniense* and *O. tsotsi* from nitidulid beetles collected during the surveys suggests that nitidulid beetles are probably the vectors of these fungi, and it emphasizes the value of monitoring these insects and their fungal associates. Of the fungi collected in this study, *O. quercus*, *O. tasmaniense* and *O. tsotsi* have been reported from trees in countries other than South Africa. This suggests intercontinental movement of these fungi, most likely through infected plant material and insects often associated with them.

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