

## *Ophiostoma* species (Ophiostomatales, Ascomycota), including two new taxa on eucalypts in Australia

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**Abstract.** The genus *Ophiostoma* accommodates ascomycetes in the order Ophiostomatales, some of which are important pathogens of trees. Although these fungi are well known in the northern hemisphere, very little is known regarding their occurrence or importance in Australia. The aim of the present study was to collect *Ophiostoma* spp. infecting wounds on *Eucalyptus* spp. in Australia, where most of these trees are native. Collections were made in three states of Australia and the isolates were identified using morphological and multigene-sequence comparisons. Of the 76 isolates collected, two previously unknown species of *Ophiostoma* were found and these are described here as *O. tasmaniense* sp. nov. and *O. undulatum* sp. nov. In addition, *O. quercus* (Georgev.) Nannf. and *O. tsotsi* Grobbelaar, Z.W.de Beer & M.J.Wingf. are reported for the first time from eucalypts in Australia and the distribution of *Pesotum australiae* Kamgan Nkuekam, Jacobs & Wingfield is expanded to include eucalypts in Tasmania. In pathogenicity tests, very small lesions were observed in both the bark and xylem of *E. grandis* (Hill) Maiden trees, suggesting that none of the collected species is a pathogen of *Eucalyptus* spp.

### Introduction

The Ophiostomatales are ascomycetes that include *Ophiostoma* H. & P. Sydow with *Pesotum* J.L.Crane & Schokn. and *Sporothrix* Hektoen & C.F.Perkins anamorphs, *Ceratocystiopsis* H.P.Upadhyay & W.B.KenDr with *Hyalorhinocladia* H.P. Upadhyay & W.B.KenDr anamorphs and *Grosmannia* Goid. with *Leptographium* Lagerb. & Melin anamorphs (Upadhyay 1981; Zipfel *et al.* 2006). These genera are commonly referred to as ophiostomatoid fungi and they produce fruiting structures with similar morphologies adapted to insect dispersal (Wingfield *et al.* 1993). Sexual forms of these fungi are mostly ascomata with long necks, exuding sticky spores at their apices that attach easily to the bodies of passing insects, which transport them to new substrates (Upadhyay 1981; Wingfield *et al.* 1993; Jacobs and Wingfield 2001). Asexual forms are erect conidiophores with sticky spores at their apices (*Hyalorhinocladia*, *Pesotum* and *Leptographium*) or dry spores (*Sporothrix*) that can be wind-dispersed (Crane and Schoknecht 1973; Malloch and Blackwell 1993; Jacobs and Wingfield 2001).

*Ophiostoma* H. & P.Sydow species with their *Pesotum* and *Sporothrix* anamorphs include important pathogens of trees such as *O. ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, responsible for two Dutch elm disease pandemics that have

devastated elm trees in Europe and the United States of America (Sinclair and Lyon 2005). Numerous other *Ophiostoma* spp. are agents of sapstain, causing discoloration of timber, thus reducing its commercial value (Seifert 1993). A well known example is *O. quercus* (Georgev.) Nannf., causing sapstain on *E. grandis* (Hill) Maiden trees in South Africa (De Beer *et al.* 2003). However, blue stain is much better known where it occurs on conifers (Seifert 1993).

Ophiostomatoid fungi require wounds to infect trees and they are commonly vectored by arthropods. Most *Ophiostoma* spp. depend on bark beetles or weevils for their dispersal and typically have a mutualistic relationship with these insects (Mathiesen-Käärik 1953; Whitney 1982; Paine *et al.* 1997; Kirisits 2004). In this regard, they also have a close association with phoretic mites associated with these insects (Bridges and Moser 1986; Levieux *et al.* 1989; Malloch and Blackwell 1993; Moser 1997). This association with timber-infesting insects makes the ophiostomatoid fungi especially well adapted for accidental introduction into new environments.

The eucalypts include more than 700 tree species distributed in three genera, *Eucalyptus* L'Her., *Corymbia* K.D.Hill & L.A.S.Johnson and *Angophora* Cav. (Hill and Johnson 1995). Almost all eucalypts are endemic to Australia, with only a few

species that are restricted to some islands in southern Asia (Potts and Pederick 2000). In their native range, *Eucalyptus* spp. constitute ~78% of forests, whereas more than 85% of hardwood plantations comprise *Eucalyptus* spp. (Anonymous 2009). In the rest of the world, eucalypts have been planted for more than 200 years and, presently, numerous species are grown in plantations, covering more than 20 million hectares in more than 60 countries (Iglesias-Trabado and Wilstermann 2008). In this regard, they represent a significant element of the economies of countries where they provide fuel, paper pulp and construction timber.

At least 10 fungal species residing in the Ophiostomatales are known from trees in Australia, including the human pathogen *Sporothrix schenckii* Hektoen & C.F.Perkins (Conias and Wilson 1998), and two species isolated from mouldy hay, *Ophiostoma stenoceras* (Robak) Nannf. and *O. nigrocarpum* (R.W.Davidson) de Hoog (O'Reilly and Altman 2006). Almost all the other species have been isolated from stained and/or bark beetle-infested non-native *Pinus* spp. These include *Ophiostoma ips* (Rumb.) Nannf. (Stone and Simpson 1987, 1989, 1991), *Ceratocystiopsis minuta* (Siem.) Upadh. & Kendrick (Stone and Simpson 1989), *Grosmannia huntii* (R.C.Rob.-Jeffr.) Zipfel, Z.W.de Beer & M.J.Wingf. (Jacobs *et al.* 1998), *O. quercus*, *O. floccosum* Mathiesen, *Pesotum fragrans* (Mathiesen) Okada & Seifert (Harrington *et al.* 2001), an unidentified species of *Leptographium* (Vaartaja 1967) and *Pesotum* (reported as *Graphilbum*) (Stone and Simpson 1991). To the best of our knowledge, the only member of the Ophiostomatales reported from a host tree native to Australia is *Pesotum australiae* Kamgan Nkuekam, Jacobs & M.J.Wingf. that occurs on wounds on native *Acacia mearnsii* de Wild (as '*P. australi*'); Kamgan Nkuekam *et al.* 2008a). On the basis of the recent descriptions of numerous previously undescribed species of *Ophiostoma* from hardwoods elsewhere in the world, it is highly likely that numerous species await discovery in Australia and especially on eucalypts that dominate the natural landscape. Knowledge of these species on eucalypts in their native range would be valuable in elucidating the origin and movement of wood-inhabiting pathogens in this genus.

This investigation represents a collaborative effort between researchers from Universities and Forestry Departments in Australia and South Africa. The aim was to increase available knowledge pertaining to the Ophiostomatales occurring in Australia. Wounds on eucalypt trees in the eastern part of Australia were thus inspected for the presence of these fungi. All isolates were identified using a combination of morphological and DNA-sequence data and their potential pathogenicity to these trees was tested in greenhouse inoculation studies.

## Materials and methods

### Collection of samples

Surveys for Ophiostomatales infecting *Eucalyptus* and *Corymbia* spp. in Australia were conducted between March and April in 2008. Samples were collected from stumps as well as from wounds made by stem-boring insects (e.g. *Phoracantha* spp.). Sampling was undertaken in the states of Tasmania, New South Wales and the southern part of Queensland. In Tasmania,

areas sampled included numerous localities near Burnie, Tarraleah and Geevestown. In New South Wales (NSW), commercial plantations and native eucalypt forests between Sydney and up to the border with Queensland were visited. Some key areas sampled in NSW included Pine Creek State Forest, Wattagan State Forest, Wedding Bells State Forest, Crab-Tree plantation, Ingalba State Forest and other localities around these main areas. In the state of Queensland, samples were collected from *Eucalyptus* trees grown close to south-eastern Queensland.

After determining the presence of ophiostomatalean fruiting structures (mycelium, ascomata, synnemata) with a  $\times 20$  magnification lens, pieces of wood were cut from trees and placed in separate brown paper bags for each tree sampled. All the samples in bags were placed in larger plastic bags to prevent desiccation and to promote sporulation, and were transported to the laboratory.

Isolations and purification of fungi from wood samples were carried out as described by Kamgan Nkuekam *et al.* (2008a, 2008b). Ten additional isolates collected in Australia during previous surveys (2005) were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI, www.fabinet.up.ac.za) at the University of Pretoria. These isolates originated from felled *Eucalyptus grandis* logs infested by cerambycid beetles near Brisbane. All isolates used in the study were deposited in the culture collection (CMW) of the Tree Pathology Co-operative Program (TPCP), South Africa, and representative cultures have also been 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. Representative isolates of species closely related to the Australian species isolated in the present study, and for which insufficient DNA-sequence data were available from GenBank, were obtained for reference purposes from CMW (Table 1). These included those for *O. borealis* G.Kamgan Nkuekam, H.Solheim & Z.W.de Beer, *O. denticiliatum* Linnakoski, Z.W.de Beer & M.J.Wingf., *O. karelicum* Linnakoski, Z.W.de Beer & M.J.Wingf. and *P. australiae*.

### Culture characteristics and morphology

Isolates resembling fungi in the Ophiostomatales were grouped into morphotypes on the basis of features of their cultures on oatmeal agar media (OMA: 30 g oats, 20 g Biolab agar and 1000 mL deionised water). Single drops of conidia, ascospores or small pieces of mycelium were transferred from pure cultures to OMA to promote sporulation for morphological comparisons. Cultures were incubated at 25°C until sporulation and then grouped into morphotypes on the basis of colour (Rayner 1970) and macro-morphology. Fruiting structures (ascomata and ascospores; synnemata and conidia) of selected isolates representing each of the morphotypes were mounted in 80% lactic acid on microscope slides and studied under a Zeiss Axiocam light microscope (Zeiss, München-Hallbergmoos, Germany). Fifty measurements of all characteristic morphological features were made for isolates chosen as the types of new species and 10 measurements were made for

**Table 1. List of *Ophiostoma* isolates and their morphogroups collected during the surveys in Australia and sequenced in the study**  
Reference species for which TEF was sequenced for comparative studies are listed below the morphogroups. T = ex-type isolate

Morphogroup	ID	Host	CMW	Accession number			Collector	Country
				ITS	BT	TEF		
A1	<i>O. quercus</i>	<i>Corymbia variegata</i>	29552	NA	NA	NA	G. Kamgan Nkuekam	Australia
			<sup>B</sup> 29279	NA	NA	NA	G. Kamgan Nkuekam	Australia
		<i>Eucalyptus dunii</i>	29277	NA	NA	NA	G. Kamgan Nkuekam	Australia
		<i>E. agglomerata</i>	29299	GU797206	GU797194	GU797224	G. Kamgan Nkuekam	Australia
		<i>E. grandis</i>	29091	GU797207	GU797192	NA	G. Kamgan Nkuekam	Australia
		<i>E. nitens</i>	<sup>B</sup> 29096	NA	NA	NA	G. Kamgan Nkuekam	Australia
			29103	GU797203	GU797193	GU797221	G. Kamgan Nkuekam	Australia
			29117	NA	NA	NA	G. Kamgan Nkuekam	Australia
			29118	GU797204	GU797198	GU797222	G. Kamgan Nkuekam	Australia
			29356	NA	NA	NA	G. Kamgan Nkuekam	Australia
			24755	NA	NA	NA	M.J. Wingfield	Australia
			24750	GU797205	GU797195	NA	M.J. Wingfield	Australia
		24743	NA	NA	NA	M.J. Wingfield	Australia	
		24745	GU797208	GU797197	NA	M.J. Wingfield	Australia	
		24758	GU797202	GU797196	NA	M.J. Wingfield	Australia	
A2	<i>O. tasmaniense</i>	<i>E. globulus</i>	<sup>A,B,C</sup> 29115	GU797209	GU797190	GU797225	G. Kamgan Nkuekam	Australia
			<sup>A,C</sup> 29116	GU797212	GU797191	GU797226	G. Kamgan Nkuekam	Australia
			<sup>C</sup> 29086	NA	NA	NA	G. Kamgan Nkuekam	Australia
		<i>E. nitens</i>	<sup>T,A,B,C</sup> 29088	GU797211	GU797188	GU797223	G. Kamgan Nkuekam	Australia
		<sup>C</sup> 29099	GU797210	GU797189	GU797220	G. Kamgan Nkuekam	Australia	
		<i>E. saligna</i>	29272	NA	NA	NA	G. Kamgan Nkuekam	Australia
A3	<i>P. australiae</i>	<i>E. nitens</i>	<sup>C</sup> 29101	GU797213	GU797184	GU797232	G. Kamgan Nkuekam	Australia
A4	<i>O. tsotsi</i>	<i>E. grandis</i>	29293	GU797216	GU797199	GU797229	G. Kamgan Nkuekam	Australia
		<i>E. pilularis</i>	29294	GU797214	GU797200	GU797228	G. Kamgan Nkuekam	Australia
		29432	NA	NA	NA	G. Kamgan Nkuekam	Australia	
		29433	NA	‘	‘	G. Kamgan Nkuekam	Australia	
		<sup>B</sup> 29539	NA	‘	‘	G. Kamgan Nkuekam	Australia	
		29540	GU797215	GU797201	GU797227	G. Kamgan Nkuekam	Australia	
		<sup>B</sup> 29541	NA	NA	NA	G. Kamgan Nkuekam	Australia	
		29543	NA	NA	NA	G. Kamgan Nkuekam	Australia	
		29548	NA	NA	NA	G. Kamgan Nkuekam	Australia	
		B	<i>O. undulatum</i>	<i>E. grandis</i>	<sup>C</sup> 19392	NA	NA	NA
<sup>A,C</sup> 19394	GU797219				GU797185	GU797235	M.J. Wingfield	Australia
<sup>T,A,B,C</sup> 19396	GU797218				GU797186	GU797233	M.J. Wingfield	Australia
<sup>A,B,C</sup> 19397	GU797217				GU797187	GU797234	M.J. Wingfield	Australia
19402	NA				NA	NA	M.J. Wingfield	Australia
Reference strains	<i>O. borealis</i>	<i>Tilia cordata</i>	17860	EF408594	GQ249311	GU930823	T. Kirisits	Austria
		<i>Betula pubescens</i>	<sup>T</sup> 18966	EF408593	GQ249317	GU930822	G. Kamgan Nkuekam	Norway
	<i>O. denticiliatum</i>	<i>Scolytus ratzeburgi</i> from <i>Betula</i> sp.	<sup>T</sup> 29493	FJ804490	FJ804502	GU930818	R. Linnakoski	Norway
		‘	<sup>T</sup> 29494	FJ804491	FJ804503	GU930819	R. Linnakoski	Norway
	<i>O. karelicum</i>	‘	<sup>T</sup> 23099	EU443762	EU443773	GU930821	R. Linnakoski	Russia
		‘	23101	EU443763	EU443774	GU930820	R. Linnakoski	Finland
	<i>P. australiae</i>	<i>Acacia mearnsii</i>	6589	EF408602	EF408605	GU797231	M.J. Wingfield	Australia
‘		<sup>T</sup> 6606	EF408603	EF408606	GU797230	M.J. Wingfield	Australia	

<sup>A</sup>Isolate used for growth studies.

<sup>B</sup>Isolate used in pathogenicity trials.

<sup>C</sup>Isolate used for mating compatibility trials.

NA, not available.

additional isolates. The means were then calculated for relevant morphological structures. Measurements were noted as (minimum–) mean minus s.d. – mean plus s.d. (–maximum).

Scanning electron microscopy (SEM) 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.* (2010). 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 with a JEOL JSM-840 scanning electron microscope (JEOL, Tokyo, Japan).

ITS



### Growth in culture

Three isolates of each undescribed species (Table 1) identified in the present study were used for growth studies in culture. This was carried out on malt extract agar (MEA) at seven different temperatures (5–35°C), following the technique described by Kamgan Nkuekam *et al.* (2008a).

### DNA-sequence comparisons

Representative isolates of each morphological group of fungi were selected for DNA-sequence comparisons. Single spore drops collected from the apices of ascomata or conidiophores in pure cultures were grown on 2% MEA for 7–10 days. Mycelium was scraped from the surfaces of the actively growing cultures and then transferred into 1.5-mL Eppendorf tubes with a sterile hypodermic needle. DNA was extracted using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions.

The internal transcribed spacer regions (ITS1, ITS4) and 5.8S gene of the rRNA operon were amplified on an Eppendorf Mastercycler (Merck, Hamburg, Germany) with primers ITS1 and ITS4 (White *et al.* 1990). Part of the  $\beta$ -tubulin gene (BT) and the transcription elongation factor-1 $\alpha$  gene (TEF) were also amplified with 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 and thermal cycling conditions were the same as those described previously (Kamgan Nkuekam *et al.* 2008a, 2008b). An aliquot of 5  $\mu$ L of the PCR products were stained with GelRed™ nucleic acid gel stain (Biotium, Hayward, CA, USA), separated on a 1% agarose gel and visualised under UV light. PCR products were purified using Sephadex G-50 Gel (Sigma-Aldrich, Steinheim, Germany), following the manufacturer's instructions. Subsequently, the concentrations of the purified PCR products were determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE, 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.* (2008b) and both DNA strands were sequenced for each gene region. Sequences for both strands of each isolate were examined visually and consensus sequences were assembled using Sequence Navigator (Applied Biosystems Division, Perkin-Elmer, Foster City, CA, USA).

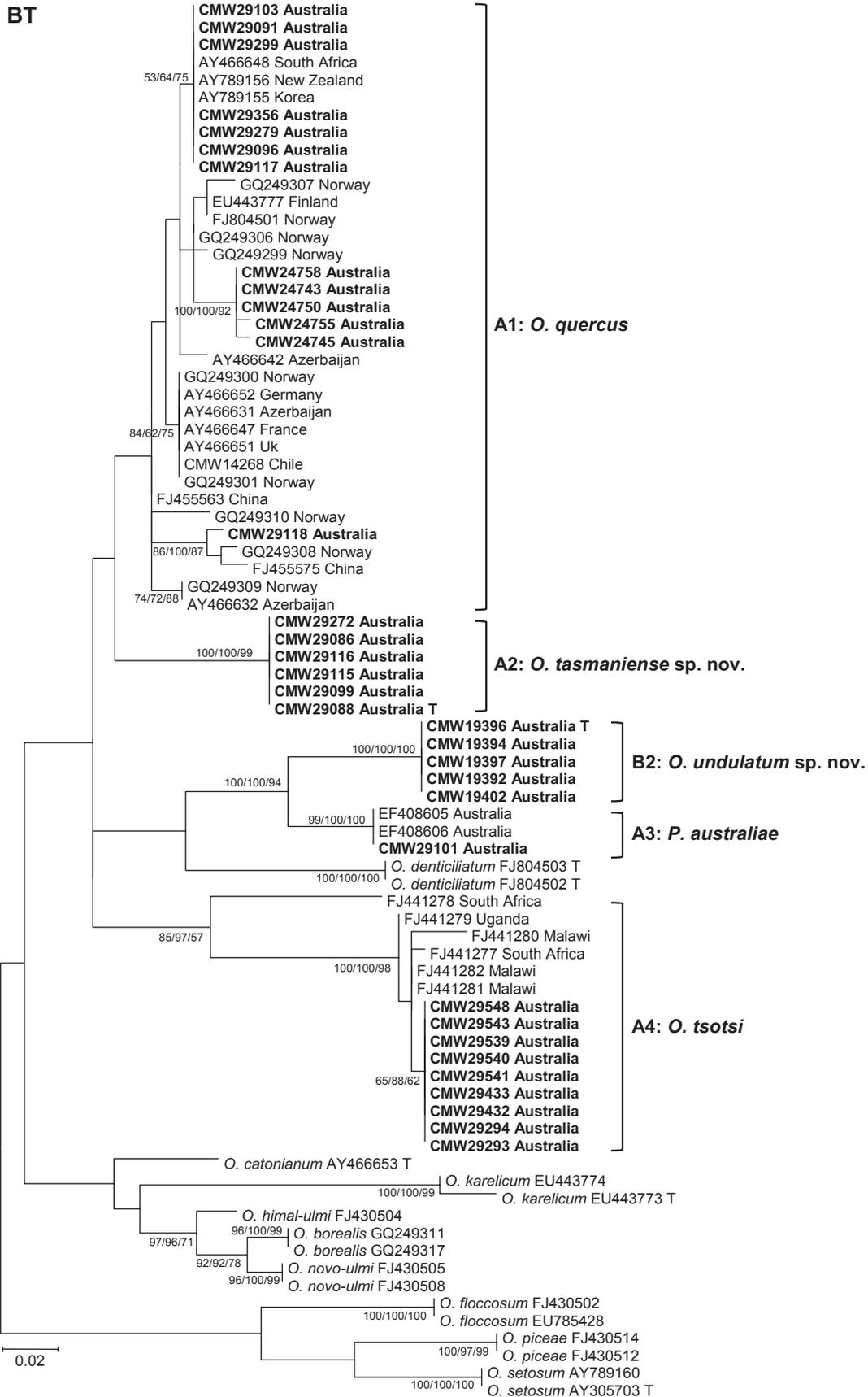
A preliminary identity for the isolates was obtained by performing a standard nucleotide BLAST against the GenBank database (<http://www.ncbi.nlm.nih.gov>). Datasets comprising sequences obtained in the present study, as well as reference sequences of related species obtained from GenBank, were compiled in MEGA 4.0.2 (Tamura *et al.* 2007). Sequences

were then aligned automatically by using the online version of MAFFT 6 (Kato *et al.* 2002). Data for the three gene regions sequenced were analysed separately from each other, and three types of analyses were carried out for each dataset. First, PAUP 4.0b10 (Sinauer Associates, Sunderland, MA, USA) was used to construct phylogenetic trees from the distance matrices by pairwise alignment of the sequences, using the maximum-parsimony (MP) method. Confidence levels of the phylogenies were estimated with 1000 bootstrap replicates. Additionally, Bayesian analyses were performed with MrBayes 3.1 (Ronquist and Heuelsenbeck 2003), based on Markov Chain Monte Carlo (MCMC) methods. Appropriate substitution models were determined using the Akaike information criterion (AIC) in MrModeltest 2.2 (<http://www.abc.se/~nylander/>). The models applied to the ITS, BT and TEF datasets were GTR+I+G, SYM+G and GTR+G, respectively. Four simultaneous MCMCs were run for 1 000 000 generations and trees were sampled every 100th generation. The burn-in procedure in MrBayes 3.1 was used to discard the first trees that formed before the point of convergence, and the posterior probabilities in the majority-rule consensus trees were calculated by MCMC sampling in MrBayes 3.1, using the best-fit model of evolution mentioned above. Last, maximum-likelihood (ML) analyses were conducted online by 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 = 0.6097; Pinvar = 0.6101), for the BT it was TVM+G (gamma shape parameter = 0.1904, Pinvar = 0) and GTR+G (gamma shape parameter = 1.0065, Pinvar = 0) for the TEF dataset.

### Pathogenicity tests

Pathogenicity tests were conducted in a quarantine greenhouse, using *Eucalyptus grandis* clone TAG5. This clone was selected because it has been shown to be susceptible to a range of fungal pathogens in previous studies. Two strains each (Table 1), for four of the fungal species identified in the present study, were used in the inoculation experiment. Ten trees, approximately 2-years old (~1 cm diameter), grown in plastic bags, were inoculated with each test strain and two other trees of the same age were inoculated with a sterile agar disc to serve as controls. Inoculations were carried out at an average temperature of 25°C and natural day/night lighting conditions of ~13 h daylight and 11 h darkness, using the same technique as described before by Kamgan Nkuekam *et al.* (2008b). Six weeks (42 days) after inoculation, the lengths of lesions on the bark surface and in the xylem of each tree were measured. Re-isolations were made from the lesions to meet the requirements of Koch's postulates. All data were processed using the statistical software package SAS® V8 (SAS Inc., Cary, NC, US) running under VM/CMS on the main-frame computer at the University of Pretoria, where lesion lengths

**Fig. 1.** Phylogenetic tree produced from a maximum likelihood analysis of the internal transcribed spacer region (ITS) sequence data, showing the placement of *Ophiostoma* and *Pesotum* isolates from eucalypt trees in Australia among known species of *Ophiostoma*. Isolates sequenced in the study are in bold type font. Maximum-parsimony (MP) bootstrap values, Bayesian posterior probabilities and maximum-likelihood (ML) bootstrap values, respectively, are indicated at each relevant node. T = ex-type isolates.



were analysed using GENMOD with a POISSON distribution and a link function of log.

#### Mating compatibility

To obtain sexual states for isolates collected in the present study that did not produce teleomorphs on wood or in culture (Table 1), single conidial isolates were paired in all possible combinations on water agar (20 g L<sup>-1</sup> agar, Biolab, Midrand, South Africa and 1000 mL sterile deionised water) supplemented with sterile *Eucalyptus* chips (Kamgan Nkuekam *et al.* 2008a; Grobbelaar *et al.* 2010). Petri dishes were incubated at 25°C for 1 month and monitored weekly for the presence of ascomata producing ascospores.

In a separate experiment, strains of *Pesotum australiae* (CMW6606, CMW6589, CMW6588, CMW6590) collected from wounds of *A. mearnsii* trees, and identified in a previous study (Kamgan Nkuekam *et al.* 2008a), were crossed in the same manner with a strain (CMW29101) collected from a wound on a *Eucalyptus* tree in the present study. These latter pairings were conducted in an attempt to stimulate production of ascomata for *P. australiae*, which have not previously been seen for this species.

## Results

#### Collection of samples

In total, 66 isolates resembling species in the Ophiostomatales were collected from 200 eucalypt trees sampled during the surveys in New South Wales, Queensland and Tasmania. Samples from which fungi were isolated included wounds on two eucalypt genera, *Eucalyptus* and *Corymbia*, including eight different species. These tree species were *E. nitens* Deane & Maiden, *E. globulus* Labill, *E. pilularis* Sm., *E. saligna* Sm., *E. agglomerata* Maiden, *E. dunnii* Maiden, *E. grandis* and *C. variegata* (F.Muell.) K.D.Hill & L.A.S.Johnson. Apart from some discoloration of the wood around the wounds, there were no other symptoms suggestive of a disease problem.

#### Culture characteristics and morphology

Isolates could be assigned to two morphological groups on the basis of colony morphology and the type of fruiting bodies produced on either MEA or OMA. Isolates collected in 2008 all shared similar culture morphology (Table 1, Group A), with no sexual states observed, and resembled species of *Pesotum*. Colonies of these isolates sporulated best on OMA. They produced only synnemata, with creamy to white spore drops. No ascomata were produced in culture. Isolates collected in Queensland, near Brisbane in 2005, could be grouped in a second morphological group (Table 1, Group B), which was characterised by slower growth than that of isolates in Group A. Ascomata with long necks, exuding creamy ascospore drops, were often produced in culture. These isolates produced white

mycelial mats with undulating colony surfaces and *Sporothrix* anamorphs in culture.

#### DNA-sequence comparisons

Representative isolates from different hosts and geographic locations were selected for DNA sequencing (Table 1). All of these isolates generated fragments of ~600 bp for the ITS, 400 bp for the BT and 800 bp for the TEF gene regions. BLAST searches in the GenBank database showed that all isolates belonged in the genus *Ophiostoma* and its related anamorph genus, *Pesotum*. Alignments of ITS sequence data for isolates from Australia with those of related *Ophiostoma* spp. from GenBank, resulted in a total of 589 characters including gaps, with 489 constant characters, 20 parsimony-uninformative characters and 80 parsimony-informative characters. The aligned BT dataset consisted of a total of 241 characters including gaps, with 146 constant characters, nine parsimony-uninformative characters and 86 parsimony-informative characters. The TEF dataset consisted of a total of 552 characters including gaps, with 92 constant characters, 21 parsimony-uninformative characters and 439 parsimony-informative characters. For reference species, TEF sequences were not available in GenBank and they were consequently generated in the present study (Table 1).

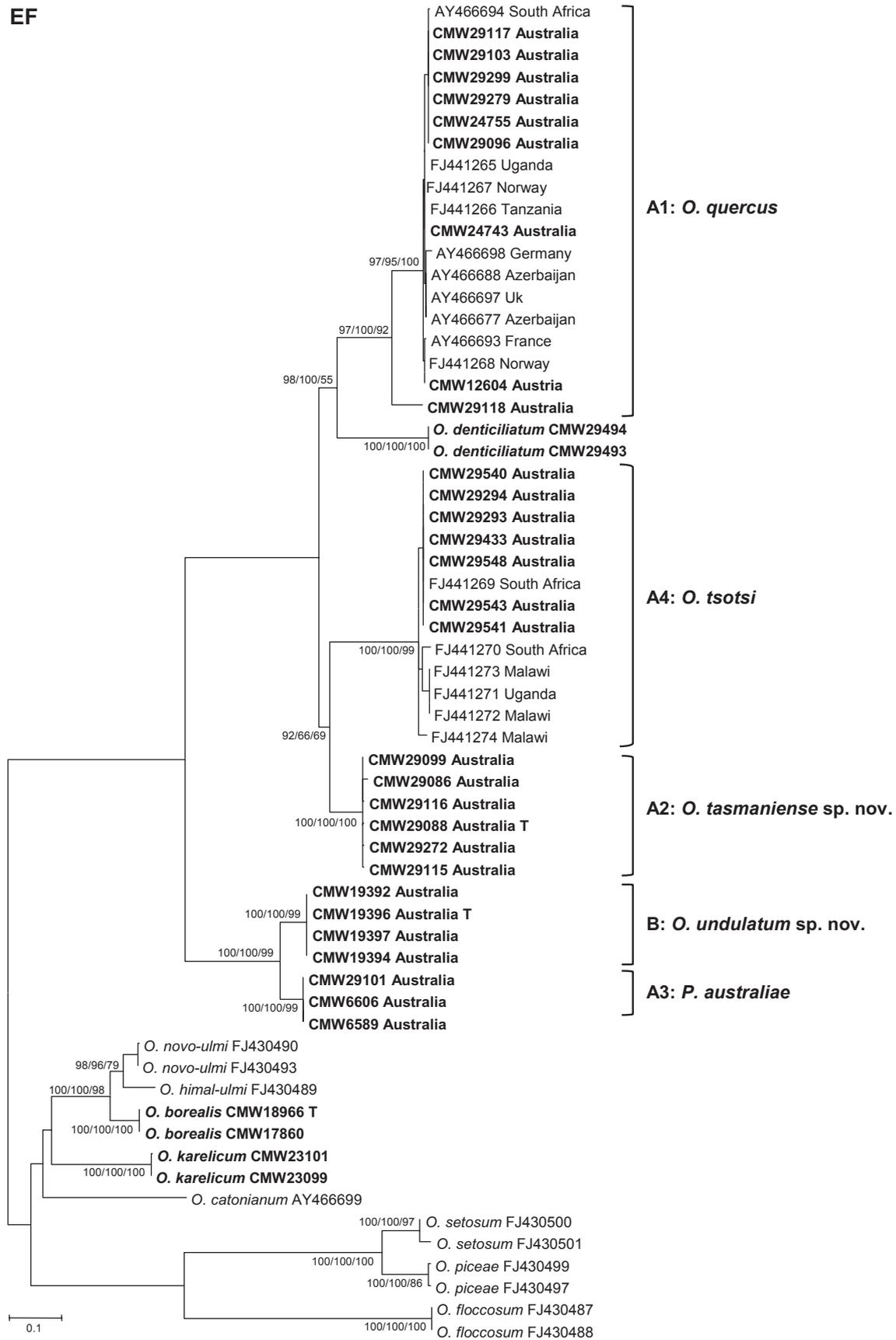
Phylogenetic analyses using MP generated 144 trees for the ITS, 273 trees for the BT, and 1117 trees for the TEF datasets. The consistency index (CI) values for the three datasets were 0.778, 0.634 and 0.679, respectively, whereas the retention index (RI) values were 0.918, 0.914 and 0.925, respectively. For each dataset, a 50% majority-rule tree was obtained from Bayesian analyses and a bootstrap tree was also obtained from a MP as well as from ML analyses. For each dataset, a ML tree is presented (Figs 1–3), indicating at the relevant nodes, MP bootstrap values, Bayesian posterior probabilities, and ML bootstrap values, respectively.

Isolates representing both morphological groups could not be resolved fully in the phylogenetic analyses using the ITS gene region (Fig. 1) and were scattered within the tree and intermingled with species such as *O. quercus*, *O. tsotsi* Grobbelaar, Z.W.de Beer & M.J.Wingf., *O. denticulatum* and *P. australiae*. However, within this complex, isolates representing Group B formed a distinct clade, which was most closely related to *O. quercus*, with 100% support in all three analyses.

In the BT tree (Fig. 2), Group A isolates separated into four lineages. The first set of isolates grouped with strains of *O. quercus* (A1). The second set of isolates formed a well supported clade (A2), distinct from, but most closely related to *O. quercus*. A single isolate grouped with the two *Pesotum australiae* isolates (A3), whereas the fourth set of isolates grouped in a well supported lineage with strains of *O. tsotsi* (A4). As was the case for the ITS region, isolates in Group B formed a well supported, distinct clade, although this time most closely related to *P. australiae*.

**Fig. 2.** Phylogenetic tree produced from a maximum likelihood analysis of the  $\beta$ -tubulin gene (BT) sequence data, showing the relationship of *Ophiostoma* and *Pesotum* isolates from eucalypt trees in Australia with known species of *Ophiostoma*. Isolates sequenced in the study are in bold type font. Maximum-parsimony (MP) bootstrap values, Bayesian posterior probabilities and maximum-likelihood (ML) bootstrap values, respectively, are indicated at each relevant node. T = ex-type isolates.

EF



Analyses of the TEF dataset gave results similar to those obtained with the BT dataset (Fig. 3). These data confirmed with good support the distinction of especially Clades A2 and B2 from, respectively, *O. tsotsi* and *P. australiae*. Furthermore, TEF data of *O. denticiliatum*, *P. australiae*, *O. karelicum* and *O. borealis* isolates separated all four of these species into lineages that are distinct from other species in the complex.

#### Mating compatibility

The *Pesotum* isolates (Table 1, Goup A2) that did not produce ascospores in culture and that were paired in all possible combinations, produced sexual fruiting bodies typical of the genus *Ophiostoma* on *Eucalyptus* chips. The crosses between the reference *P. australiae* isolates (Table 1) from a previous study, and the single isolate (CMW29101) identified as *P. australiae* on the basis of DNA sequences from the present study, did not produce ascospores.

#### Taxonomy

On the basis of morphological examinations and multigene sequence phylogenies, two *Ophiostoma* spp. from Australia emerged as distinct and previously undescribed species. The following descriptions are provided for them.

##### *Ophiostoma undulatum* Kamgan-Nkuek, M.J.Wingf. & Jol.Roux, sp. nov. (Fig. 4)

*Etymology*: The name refers to the undulating mycelium of colonies on growth media.

Coloniae albae vel bubalinae in MEA in 10 diebus in 25°C ad 16 mm crescentes. Mycelium tegetes crassas undulantes circulares concentricas in superficie agaris formans. Bases ascum nigras, globosas sine decoribus. Colla ascum nigra sine hyphis ostiolaribus. Ascospores reniformes non-septatae, hyalinae (5–)5.5–6(–6.5) × (1.5–)1.5–2(–2) μm. Anamorph *Sporothrix* conidiophoris hyalinis apicem versus ramosis. Conidia non-septata, hyalina oblonga vel cylindrica (5–)5.5–7(–8) × (2–)2.5–3(–3.5) μm.

*Colonies* white to Buff (19''f) on MEA. Reverse Hazel (17''b). Colony diameters reaching 16 mm in 10 days on MEA at 25°C. Optimal growth at 25°C. No growth at 5°C and at 35°C. Mycelia forming thick, undulating, circular and concentric mats on agar surface. *Ascospores* produced by only a few strains, scattered over the colony surface and embedded within mycelium, producing white, creamy spore drops at the neck apices. *Ascospore* necks black (68.5–)181.5–348(–378) μm long. *Ascospore* bases black, globose (34.5–)66.5–135(–193.5) μm long and (35–)69–140.5(–206) μm wide, without ornamentations. Neck bases smooth. *Ostiolar hyphae* absent. *Asci* rarely seen, evanescent, deliquescing early in the development. *Ascospores* reniform, aseptate, hyaline (5–)5.5–6(–6.5) × (1.5–)1.5–2(–2) μm.

*Anamorph*: *Sporothrix*, conidiophores, hyaline, branched towards the apex, (9.5–)16–27(–34) × (1.5–)2–2.5(–3) μm.

Conidia, aseptate, hyaline, oblong to cylindrical (5–)5.5–7(–8) × (2–)2.5–3(–3.5) μm.

*Specimens examined*: Australia, state of Queensland, isolated from wet *E. grandis* stems infested by *Phoracantha* beetles, August 2005, Michael J. Wingfield, holotype PREM60443, living culture CMW19396 = CBS127183.

*Additional specimens examined*: Australia, state of Queensland, isolated from *E. grandis* stems infested by *Phoracantha* beetles, August 2005, Michael J. Wingfield, paratype, living culture CMW19394 = PREM60438 = CBS127182, CMW19397 = PREM60437 = CBS127184.

##### *Ophiostoma tasmaniense* Kamgan-Nkuek, Jol.Roux & Z.W.de Beer, sp. nov. (Fig. 5)

*Etymology*: The name refers to the state of Tasmania, Australia, where the fungus was first collected.

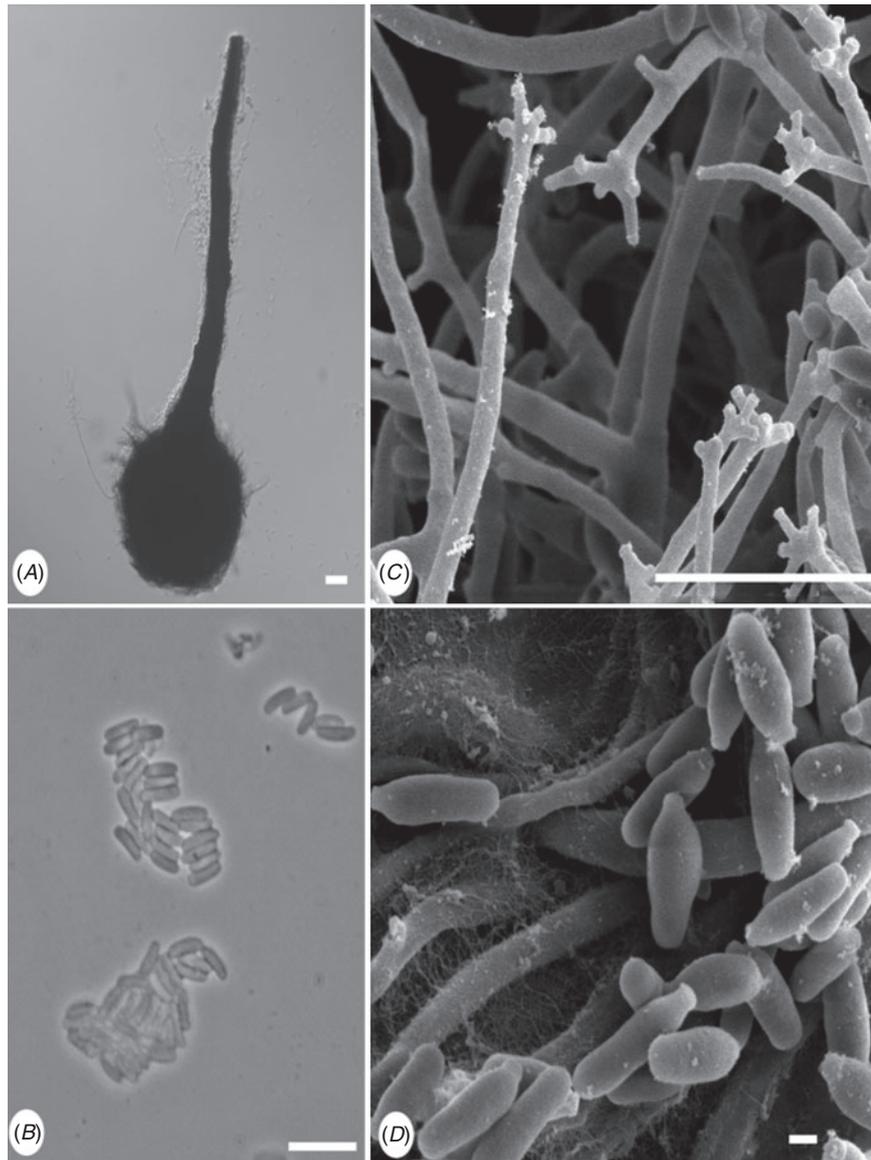
Coloniae in OMA fumeae, in MEA bubalinae in 10 diebus in 25°C ad 24 mm crescentes. Bases ascum nigras, globosas cum pilis ostiolaribus. Colla ascum nigra cum hyphis ostiolaribus divergentibus. Ascospores allantoideae non-septatae (4.5–)5–5.5(–6) μm longae (1–)1–1.5(–2) μm latae. Anamorph biformus. Gradus *Pesotum* conidiophoris erectis atrobunneis apicem versus pallescentibus. Cellulae conidiogenae hyalinae acerosae. Conidia non-septata, hyalina (3.5–)4–4.5(–5) μm × (1–)1–1.5(–2) μm. Gradi *Sporothrix* conidiophorae hyalinae cylindricae cum denticulis prominentibus. Conidia saepe septata, hyalina oblonga vel cylindrica interdum distincte pediformia (8.5–)11–19.5(–30.5) × (2–)2.5–4(–5) μm.

*Colonies* Smoke grey (21''d) on OMA, with conidiophores forming light-coloured slimy heads, arranged in mostly circular rings or scattered over the colonies. On MEA colonies Buff (19''d), with conidiophores forming cream-coloured slimy heads scattered over the colonies, mostly surrounded by a mat of mycelia, reverse colonies Buff (19''d), becoming Honey (19''b) towards the middle of the plates. Colony diameters reaching 24 mm in 10 days on MEA at 25°C. Optimal growth temperature 25°C, no growth at 5°C or at 35°C.

*Teleomorph* state produced on *Eucalyptus* chips after random crossing between strains after about 1 month. *Ascospore* bases black, globose (66.5–)95.5–144(–171.5) μm long and (71–)98.5–159(–197.5) μm wide, with hyphal hairs. *Ascospore* necks black (101–)499.5–1105.5(–1274) μm long, neck bases smooth (29–)34.5–46(–53) μm wide, middle of necks (17.5–)19.5–25.5(–29.5) μm wide, tips of necks (10.5–)11.5–17.5(–22) μm wide. *Ostiolar hyphae* present, divergent (11.5–)15.5–20.5(–21.5) μm long. *Asci* evanescent. *Ascospores* allantoid in side view, aseptate (4.5–)5–5.5(–6) μm long and (1–)1–1.5(–2) μm wide. *Ascospores* accumulating in round, creamy spore drops.

*Anamorph*: *Pesotum*, conidiophores synnematal, erect, dark brown at the bases, becoming lighter towards the apex, (472–)553.5–708.5(–774.5) μm long, (46.5–)67.5–110.5(–170) μm wide in the middle, (46–)54–90(–129.5) μm wide at the base. *Conidiophore heads* (160–)198–328(–452) μm across the widest

**Fig. 3.** Phylogenetic tree produced from a maximum likelihood analysis of the transcription elongation factor-1α gene (TEF) sequence data, showing the relationship between *Ophiostoma* and *Pesotum* isolates from eucalypt trees in Australia with known species of *Ophiostoma*. Isolates sequenced in the study are in bold type font. Maximum-parsimony (MP) bootstrap values, Bayesian posterior probabilities and maximum-likelihood (ML) bootstrap values, respectively, are indicated at each relevant node. T = ex-type isolates.



**Fig. 4.** Morphological characteristics of *Ophiostoma undulatum* sp. nov. (A) Globose ascogonial base and a relatively short neck (scale bar = 20  $\mu\text{m}$ ), (B) reniform ascospores (scale bar = 10  $\mu\text{m}$ ), (C) conidiogenous cells with denticles (scale bar = 10  $\mu\text{m}$ ) and (D) conidia, oblong to cylindrical, acerose (scale bar = 1  $\mu\text{m}$ ).

part, light brown, becoming hyaline towards the apex. *Conidiogenous cells*, hyaline, acerose (11.5–)14.5–28.5 (–41.5)  $\mu\text{m}$  long, (1–)1–1.5(–2)  $\mu\text{m}$  wide, tapering towards the apex. *Conidia* produced through holoblastic, annellidic development. Conidia aseptate, hyaline, oblong to obovoid, accumulating in slimy heads on the apices of the synnemata, (3.5–)4–4.5(–5)  $\mu\text{m}$   $\times$  (1–)1–1.5(–2)  $\mu\text{m}$ .

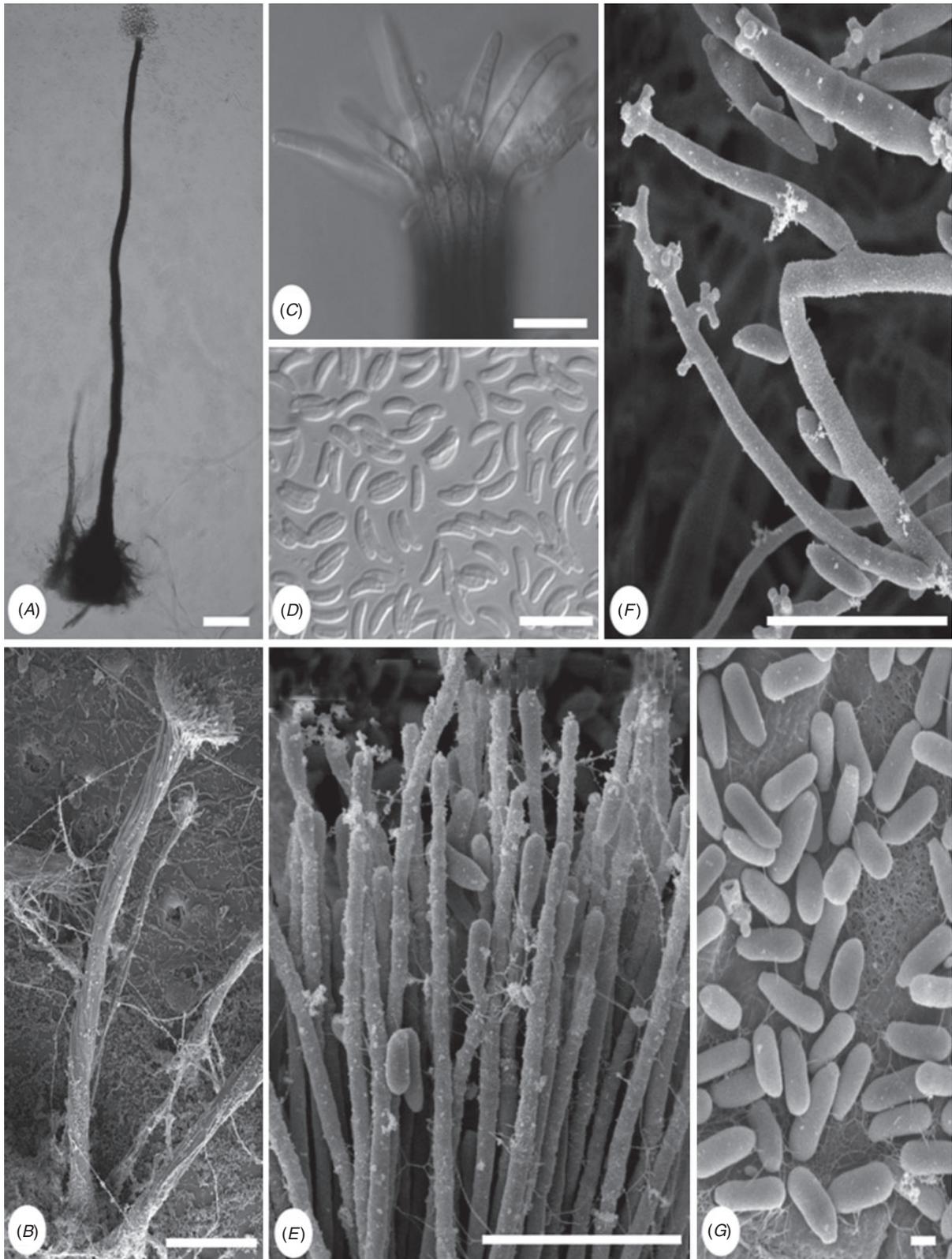
*Anamorph*: *Sporothrix*, conidiophores, hyaline, cylindrical and branched, tapering towards the apex, (10.5–)27–61.5 (–108.5)  $\times$  (1.5–)2–2.5(–3)  $\mu\text{m}$ , prominent denticles present. *Conidia*, often septate, hyaline, oblong to cylindrical (8.5–)11–19.5(–30.5)  $\times$  (2–)2.5–4(–5)  $\mu\text{m}$  in size.

*Specimens examined*: Australia, state of Tasmania, Tarraleah State forest, isolated from cut stumps of *Eucalyptus nitens*, August 2008, G. Kamgan Nkuekam, holotype PREM60439, living culture CMW29088 = CBS127212.

*Additional specimens examined*: Australia, state of Tasmania, Burnie, isolated from *E. globulus* stumps. August 2008, G. Kamgan Nkuekam, paratype, living cultures CMW29115 = PREM60440 = CBS127213, CMW29116 = PREM60444 = CBS127214.

#### *Distribution and host range of Ophiostoma spp.*

Five *Ophiostoma* species were identified from areas sampled in three different states of Australia. In NSW, two *Ophiostoma* spp. were identified. These comprised *O. quercus*, collected from four eucalypt tree species (*E. grandis*, *E. dunnii*, *E. agglomerata* and *C. variegata*) and *O. tsotsi* that was found on two *Eucalyptus* spp. (*E. pilularis* and *E. grandis*). In Tasmania, three *Ophiostoma* spp. were collected, including *O. quercus* from two *Eucalyptus* spp. (*E. nitens* and *E. globulus*), *O. tasmaniense* collected from three *Eucalyptus* spp. (*E. globulus*, *E. nitens* and



**Fig. 5.** Morphological characteristics of *Ophiostoma tasmaniense* sp. nov. (A) Globose ascomatal base (scale bar = 100 μm), (B) synnematal anamorph (scale bar = 100 μm), (C) divergent ostiolar hyphae (scale bar = 10 μm), (D) allantoid ascospores (scale bar = 10 μm), (E) conidiogenous cell with annelidic proliferation of conidia (scale bar = 10 μm), (F) *Sporothrix* conidiogenous cells with denticles and mycelial conidia (scale bar = 10 μm) and (G) oblong to cylindrical conidia (scale bar = 1 μm).

*E. saligna*), and *P. australiae* collected from *E. nitens*. In Queensland, only *O. undulatum* was found on *E. grandis* trees.

### Pathogenicity tests

Six weeks after inoculation, *Eucalyptus* trees were assessed for lesion development seen on the bark or at the cambial surface. All four fungal species used for inoculation, *O. quercus*, *O. tsotsi*, *O. tasmaniense* sp. nov. and *O. undulatum* sp. nov., produced very small lesions on both the bark and the xylem of inoculated *E. grandis* trees. Because no lesions could be measured for many inoculations, lesion lengths were analysed using GENMOD with a POISSON distribution and a link function of log. Significant ( $P < 0.0001$ ) differences in lesion lengths on either the bark or the xylem were, however, not found between the four fungal species and their replicates when compared with each other (Figs 6, 7). At the time when lesion lengths were recorded, trees were healthy with no signs of disease. All species other than *O. undulatum* were successfully isolated from the lesions. Control inoculations were covered with callus when the experiment was terminated and none of the test fungi was isolated from them.

### Discussion

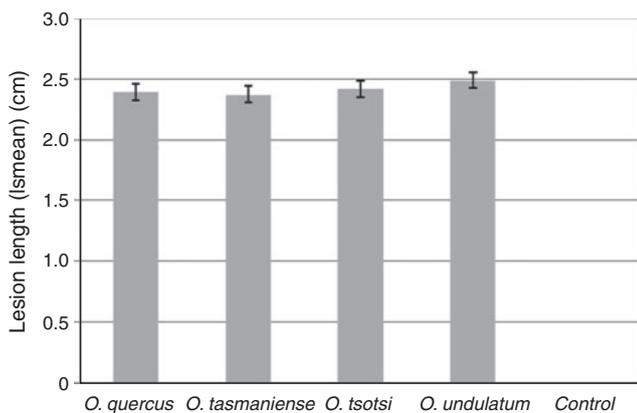
In the present study, five *Ophiostoma* spp. were identified from a survey of wounds on eucalypt trees in Australia. Two of these fungi were previously undescribed species for which the names *O. undulatum* and *O. tasmaniense* have been provided. *O. tsotsi* and *O. quercus* are reported for the first time from *Eucalyptus* trees in Australia, and the host and geographic ranges of *P. australiae* are expanded to include *Eucalyptus nitens* in Tasmania. The four species (*O. quercus*, *O. tsotsi*, *O. tasmaniense* sp. nov. and *O. undulatum* sp. nov.) inoculated onto *E. grandis* trees caused very small lesions, suggesting that they are not pathogens.

All five species identified in the study fall within a group of fungi commonly known as the *O. piceae* complex. This complex was first recognised to include nine morphologically similar

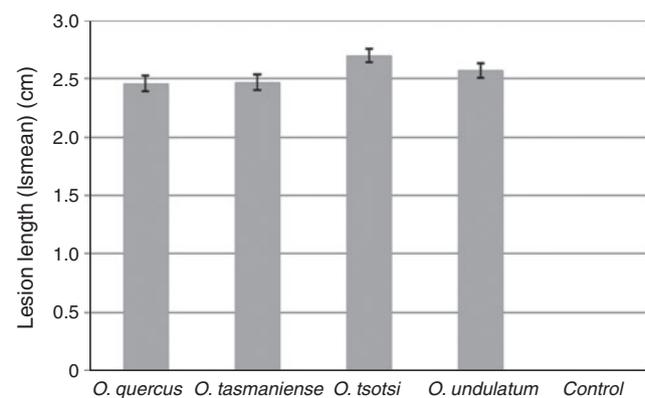
species (Harrington et al. 2001), and has been the subject of considerable taxonomic confusion (Przybyl and de Hoog 1989; Harrington et al. 2001). *O. tasmaniense*, one of the new taxa described in the study, along with *O. tsotsi*, *O. quercus* and *P. australiae*, could not be differentiated in phylogenetic analyses by using only the ITS gene regions. This is not surprising as ITS gene regions have previously been shown to be insufficient to differentiate closely related members of the *O. piceae* complex (Chung et al. 2006; Kamgan Nkuekam et al. 2008a, 2010); thus, sequences for the BT gene region (Chung et al. 2006; Kamgan Nkuekam et al. 2008a, 2010) and, more recently the TEF gene region (Grobbelaar et al. 2009a) have been useful in distinguishing these species. On the basis of BT and TEF sequence data, *O. tasmaniense* described in the present study is most closely related to *O. quercus*. This expands the host and geographic ranges of members of the *O. piceae* complex in the southern hemisphere.

It was not surprising to find *P. australiae* infecting wounds of *Eucalyptus* trees in Australia. This fungus was recently described from wounds on native *Acacia mearnsii* trees near Cann River, Victoria, Australia (Kamgan Nkuekam et al. 2008a). Its teleomorph has not been seen and it could not be induced in the present study by crossing it with different strains isolated from *A. mearnsii* and *Eucalyptus* trees. Isolation of this fungus from Tasmania, and from *Eucalyptus* spp., expands its host and geographic range. It provides further evidence that it is native to the continent, because surveys for ophiostomatoid fungi on both *A. mearnsii* and *Eucalyptus* spp., for example in Africa, have not yielded this fungus.

*Ophiostoma tsotsi*, another species collected in the present study, was for many years confused with *O. quercus*. Recent studies have, however, considered cryptic species in the *O. quercus* complex, using polymorphic sequence repeats (Grobbelaar et al. 2009b) and multigene DNA-sequence phylogenies (Grobbelaar et al. 2009a) of isolates morphologically resembling *O. quercus*, originating from various hardwood trees in Africa. These data revealed a unique clade among these isolates, resulting in the description



**Fig. 6.** Histogram showing results of inoculation trial (bark lesion) with *Ophiostoma quercus* (Lsmean = 2.3937), *O. tasmaniense* (Lsmean = 2.3749), *O. tsotsi* (Lsmean = 2.4216), *O. undulatum* (Lsmean = 2.4920) on *Eucalyptus grandis* trees. Lsmean was determined for two isolates used per species, as indicated in Table 1 ( $P < 0.0001$ , confidence limit = 95%).



**Fig. 7.** Histogram showing results of inoculation trial (xylem lesion) with *Ophiostoma quercus* (Lsmean = 2.4582), *O. tasmaniense* (Lsmean = 2.4676), *O. tsotsi* (Lsmean = 2.6975) and *O. undulatum* (Lsmean = 2.5726) on *Eucalyptus grandis* trees. Lsmean was determined for two isolates used per species, as indicated in Table 1 ( $P < 0.0001$ , confidence limit = 95%).

of *O. tsotsi* (Grobbelaar *et al.* 2010). *O. tsotsi* is known only from hardwood tree species, including *Eucalyptus* and *Acacia mearnsii* in southern Africa and China (Grobbelaar *et al.* 2010, 2011). Its discovery on *Eucalyptus* trees in Australia expands its geographic range. The discovery also supports the view (Grobbelaar *et al.* 2010, 2011) that its distribution is probably more extensive than is currently known. The origin of the species remains unknown and more extensive sampling and population-genetic tools will be necessary to determine this.

*Ophiostoma quercus* has previously been reported from *P. radiata* in Australia (Harrington *et al.* 2001) and is known to occur on *Eucalyptus* spp. in Africa (De Beer *et al.* 2003). However, the present study is the first to report the fungus on eucalypt species in Australia. *O. quercus* has a cosmopolitan distribution and a wide host range including hardwood and softwood trees and has been reported from numerous countries worldwide. The taxonomy of this fungus is, however, not fully resolved. It is suspected that there are cryptic species within what is now referred to as the *O. quercus* complex, based on the wide host and geographic ranges of isolates in this group. This is supported by the occurrence of subclades within the larger *O. quercus* clade in phylogenetic trees by using part of the ITS, BT (Kamgan Nkuekam *et al.* 2008a; Grobbelaar *et al.* 2009a; Linnakoski *et al.* 2009; Kamgan Nkuekam *et al.* 2010) and histone gene regions (Grobbelaar *et al.* 2009a). Isolates from Australia grouped in several different subclades of what is currently treated as the *O. quercus* complex (Kamgan Nkuekam *et al.* 2008a, 2010; Grobbelaar *et al.* 2009a; Linnakoski *et al.* 2009) that clearly deserves further taxonomic study.

*Ophiostoma tasmaniense* is morphologically most similar to *O. quercus*. The two fungi produce only a *Pesotum* anamorph and a mycelial synanamorph (*Sporothrix*) in culture. The teleomorph state of *O. tasmaniense* was generated in the present study by crossing different strains of the fungus in different combinations on agar plates supplemented with *Eucalyptus* chips, as was the case for *O. quercus* (Morelet 1992; De Beer *et al.* 2003). It is clearly heterothallic and mating tester strains for it were produced in the present study. On the basis of morphology, *O. tasmaniense* differs from *O. quercus* in having substantially shorter ascumatal necks, and the synnematal conidia of *O. tasmaniense* emerged from conidiogenous cells that are much more clearly annellidic than those of *O. quercus*, which have a deceptive appearance of developing sympodially (Wingfield *et al.* 1991).

The newly described *O. undulatum* is phylogenetically most closely related to *P. australiae*. Unlike other members of the *O. piceae* complex, *O. undulatum* does not have a *Pesotum* anamorph, which is one of the morphological characters that was used to define the *O. piceae* complex (Harrington *et al.* 2001). *O. undulatum*, although phylogenetically most closely related to *P. australiae*, does not share any morphological similarities with it. The new species produces thick and undulating mycelial mats, with ascumata embedded below the mycelium on both MEA and OMA. Furthermore, the anamorph state is a *Sporothrix*, in contrast to *P. australiae*, which has only a *Pesotum* anamorph (Kamgan Nkuekam *et al.* 2008a). Small differences were found in the growth rates of *O. undulatum* and *P. australiae* at optimum temperatures. Colonies of *O. undulatum* reached 16 mm in 10 days at 25°C, whereas in previous studies those of

*P. australiae* reached 14 mm at the same temperature (Kamgan Nkuekam *et al.* 2008a). However, *O. undulatum* could grow at 30°C, with colonies reaching 11 mm in 10 days, whereas *P. australiae* does not grow at 30°C or higher temperatures (Kamgan Nkuekam *et al.* 2008a).

The present study represents the most comprehensive consideration of fungi in the Ophiostomatales occurring on *Eucalyptus* and *Corymbia* trees in Australia. The results show that *Ophiostoma* spp. are more diverse on wounds on *Eucalyptus* trees in Australia than previously understood. Of the fungi collected in the present study, *O. quercus* and *O. tsotsi* have been reported from trees in countries other than Australia. This suggests intercontinental movement of these fungi, most likely through infected wood. The present study should provide a foundation for future studies considering trends in biodiversity, origin and pathogenicity of *Ophiostoma* in Australia and other countries.

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