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ORIGINAL PAPER

Wounds on *Rapanea melanophloeos* provide habitat for a large diversity of Ophiostomatales including four new species

Tendai Musvuugwa · Z. Wilhelm de Beer · Tuan A. Duong ·
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Abstract *Rapanea melanophloeos*, an important canopy tree in Afromontane forests, is commonly utilised for medicinal bark harvesting. Wounds created from these activities provide entrance for many fungi, including arthropod-associated members of the Ophiostomatales and Microascales (ophiostomatoid fungi). In this study we assessed the diversity of wound-associated Ophiostomatales on storm-damaged *R. melanophloeos* trees in the Afromontane forests of South Africa. Five species were identified based on micro-morphological and molecular phylogenetic analyses. These included *Ophiostoma* *stenoceras* and four newly described taxa *Sporothrix itsvo* sp. nov., *S. rapaneae* sp. nov., *S. uta* sp. nov. and *O. noisomeae* sp. nov. Four of these are members of the *S. schenckii–O. stenoceras* complex (*O. stenoceras*, *S. itsvo* sp. nov., *S. rapaneae* sp. nov., *S. uta* sp. nov.) while *O. noisomeae* groups basal in the Ophiostomatales alongside the *S. lignivora* complex and *Graphilbum*. In addition to other taxa known from this host, the present study shows that there is a rich, yet still poorly explored, diversity of Ophiostomatales associated with *R. melanophloeos* in Afromontane

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forests. More taxa are likely to be discovered with increased research effort. These must be assessed in terms of pathogenicity towards this ecologically and economically important tree.

Keywords Afromonatane forests · *Ophiostoma* · *Rapanea* · *Sporothrix*

Introduction

Ophiostomatoid fungi represent genera that are morphologically similar, although not phylogenetically closely related (De Beer et al. 2013a). The group includes genera such as *Ophiostoma* Syd., *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr., *Graphilbum* H.P. Upadhyay & W.B. Kendr., *Raffaelea* Arx & Hennebert, *Fragosphaeria* Shear and *Leptographium* Lagerb. & Melin in the Ophiostomatales, and *Ceratocystis* Ellis & Halst., *Knoxdaviesia* M.J. Wingf., P.S. van Wyk & Marasas, *Cornuvesica* Viljoen, M.J. Wingf. & Jacobs, *Custingophora* Stolk, Hennebert & Klopotek, *Sphaeronaemella* P. Karst. and *Graphium* Corda in the Microascales (De Beer et al. 2013b). A common morphological trait shared by these fungi is globose ascomata with usually elongated necks that give rise to masses of sticky spores at their tips, an entomochoric adaptation to allow spore dispersal via arthropods (Malloch and Blackwell 1993).

Members of the Ophiostomatales contain some of the best known tree pathogens. Some of these are responsible for very high rates of tree mortality, which has led to significant losses to forests (Brasier and Buck 2001; Harrington et al. 2008; Heybroek 1993; Wingfield et al. 1993). Well-documented examples include *Raffaelea quercivora* Kubono et Shin. Ito, responsible for oak die-back and mortality of Japanese oak trees (Kubono and Ito 2002) as well as *Ophiostoma ulmi* (Buismann) Nannf and *O. novo-ulmi* Brasier, responsible for the Dutch Elm disease pandemics in Europe and the United States of America (Brasier 2000, 2008; Pipe et al. 2000; Wingfield et al. 1993). A number of plant pathogenic *Leptographium* species have also been reported. Examples include *L. procerum* (W.B. Kendr) M.J. Wingf. associated with pine root disease (Jacobs and Wingfield 2001), *L. calophylli* (Wiehe) JF. Webber, K. Jacobs & MJ. Wingfield that causes vascular wilt disease of takamaka trees (Webber et al. 1999) and *L. wageneri*

(Goheen & F.W. Cobb) T.C. Harr. that causes black stain root disease on conifers (Harrington 1993). Some taxa are not pathogenic, or just weakly pathogenic, but may also cause significant problems in the forestry industry. For example, many species of *Ophiostoma* are responsible for sapstain of lumber and pulpwood, which degrades wood quality and results in substantial economic losses (Seifert 1993a, b).

Several Ophiostomatales infect their hosts via wounds (Wingfield et al. 1993). Such wounds are caused by various agents, including animals (e.g. arthropods), natural phenomena such as hail, frost or heavy rains as well as human practises such as bark stripping and silviculture (Nkuekam et al. 2008; Moller and Devay 1968; Vermeulen et al. 2012). *Ophiostoma minus*, for example, colonises wounds created through mechanical injury or partial debarking (Gibbs 1993). Fungi are transported to these wounds through agents such as arthropods (Harrington and Wingfield 1998; Hinds 1972; Moller and Devay 1968) or wind in the case of asexual states of *Sporothrix* Hektoen & C.F. Perkins with dry spores (Wingfield et al. 1993). Despite the importance of Ophiostomatales as tree pathogens, reports of these fungi in Africa, including South Africa, are very limited. A few studies focused on taxa associated with native tree hosts (De Beer et al. 2003; Nkuekam et al. 2008; Marais and Wingfield 1997, 2001; Roets et al. 2006, 2008), but most knowledge of the fungi in South Africa is based on species associated with non-native host tree species (De Beer et al. 2003; Nkuekam et al. 2012; Zhou et al. 2006).

Rapanea melanophloeos (L.) Mez is one of the more abundant and very important canopy trees found in these forests. It is a fast growing, evergreen forest pioneer, but can also be found as large trees in mature forests (VanWyk and VanWyk 1997). The bark of this plant is often harvested for medicinal purposes, creating wounds through which fungi can infect (Vermeulen et al. 2012). In South Africa, wound-associated ophiostomatoid fungi have been collected from this species, some of which have proven to be pathogenic (Nkuekam et al. 2008). For example, *Ceratocystis tsitsikammensis* Kamg. Nkuek. & Jol. Roux was isolated from wounds on *R. melanophloeos* in the Tsitsikamma forests and could kill seedlings of this species within a relatively short period (Nkuekam et al. 2008). Despite pathogens being critically important to ecosystem function (Castello et al.

1995), there has been little focus on them, especially on those associated with native trees in the Afromontane forests of South Africa (Taylor et al. 2001). The recently described virulent pathogen, *Immersiporthe knoxdaviesiana* S.F. Chen, M.J. Wingf. & Jol. Roux, which causes the death of *Rapanea* trees in the Harold Porter National Botanical garden (Chen et al. 2013), illustrates the importance of documenting these fungal taxa.

In addition to *C. tsitsikammensis*, only a few other wound infecting ophiostomatoid fungi have been isolated from *R. melanophloeos*. These include a *Graphilbum fragrans* (Mathiesen-Käärik) Z.W. de Beer, Seifert & M.J. Wingf.—like fungus and *O. quercus* (Georgévitch) Nannf. (Nkuekam et al. 2008). More recently, a few beetle associated taxa have also been identified from this host. *Raffaelea rapaneae* T. Musvuugwa, Z.W. de Beer, L.L. Dreyer & F. Roets was isolated from a Platypodinae beetle and *Sporothrix aemulophila* T. Musvuugwa, Z.W. de Beer, L.L. Dreyer & F. Roets was associated with *Xyleborinus aemulus* Wollaston (Scolytinae) on *R. melanophloeos* (Musvuugwa et al. 2015). This tree species may be under considerable threat from fungi, and there is thus an urgent need to study taxa associated with *R. melanophloeos* in their natural habitats. The objective of this study was therefore to assess the diversity of wound-associated Ophiostomatales on storm damaged *Rapanea* trees at various sites across its distribution range in South Africa.

Materials and methods

Sampling of plant material and fungal isolates

The Afromontane forests of South Africa are found from Table Mountain in the extreme southwest of the Cape Floristic Region, are most extensive in the Tsitsikamma area of the southern Cape coastal regions, and extend northwards along the coastal mountains into the KwaZulu-Natal Province (Lubke and Mackenzie 1996). They occur as small fragmented patches within river valleys, on mountains, foothills and on coastal platforms, and represent the outliers of the tropical African Afromontane forests (Turpie et al. 2003).

Wounds on *R. melanophloeos* were surveyed in the Harold Porter National Botanical Garden (S 34°20′54″ E 18°55′31″), Groenkop Forest Reserve (S 33°56′32″ E 22°32′50″) and Weza Forest (S 30°36′12″ E 29°39′59″) in the Afromontane forests of the Cape Floristic Region and KwaZulu-Natal Province. All wounds were presumably caused by weather damage during storms. Bark and wood samples were collected from wounds during late winter (August) of 2010–2012, by using a hammer and chisel or machete to cut off pieces measuring approximately 5 cm × 5 cm and not more than 3 cm deep. These samples were collected from fresh wounds characterized by oozing sap and that showed signs of fungal growth when examined using a field magnifying glass. In total, 45 bark and wood samples were collected, each from a different wound on a different tree. The samples were placed in separate sampling bags and transferred to the laboratory where they were examined for the presence of members of the Ophiostomatales using a Leica EZ4 microscope (Wetzlar, Germany). When fungi were present, masses of ascospores and/or conidia were collected from the apices of sporulating structures using a sterile needle. They were transferred to 2 % malt extract agar (MEA) emended with 0.05 g cyclohex-imide l^{-1} to select for members of the Ophiostomatales (Harrington 1981). When no fungi were present, collected material was placed in moisture chambers (resealable plastic bags with ddH$_2$O-moistened paper towels) at room temperature ($\sim$23 °C) in the dark for up to 4 weeks to stimulate fungal growth, after which fungal isolations were performed as outlined above. Isolates were stored in the dark at room temperature and examined daily for fungal growth. Isolates resembling anamorphic states of Ophiostomatales were purified by transferring single hyphal tips from the edges of actively growing fungal colonies to fresh MEA plates. All purified cultures were maintained on petri dishes containing MEA at 4 °C until further use. Representative cultures of all morpho-types collected in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa and in the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht in the Netherlands. Herbarium specimens of the anamorph and teleomorph states belonging to the new species were deposited in the National Fungus Collection (PREM), Pretoria, South Africa.

Morphological characterisation

Where available, ascocarps and ascospores of members of the Ophiostomatales were collected from plant material, mounted in clear lactophenol on microscope slides and studied using a Leica EZ4 microscope. 25 measurements of all morphologically and taxonomically useful structures were made of isolates suspected to represent new taxa. The maximum and minimum measurements for each taxonomically useful character were noted and means (±standard deviation) were calculated. Photographs of the structures were taken using a Leica digital camera mounted on the microscope.

Fungal identification

Based on colony and micro-morphological characteristics, all fungal cultures suspected to belong to the Ophiostomatales were grouped into morphotypes. Three or more pure isolates collected in this study, representing each morphotype, were randomly chosen for DNA sequence comparisons (Table 1). For DNA extraction, fungal mycelium was harvested from edges of 2 week old actively growing colonies of pure cultures on MEA using a sterile scalpel. DNA was extracted from the fungal mycelium using the PrepMan® Ultra sample preparation reagent following the manufacturer's instructions. To amplify the nuclear ribosomal internal transcribed spacer region (ITS1, ITS2), including the 5.8S gene region of the rDNA, the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) were used and where amplification was difficult, ITS1F was replaced with ITS1 (White et al. 1990). Preliminary placement of fungi using ITS was used as baseline to decide on additional markers to amplify for each fungal morpho-type (Table 1). For amplification of part of the beta-tubulin (βT) gene, the primers Bt2a and Bt2b (Glass and Donaldson 1995) were used, which were replaced in some cases with the primer pair Bt2b and T10 (O'Donnell and Cigelnik 1997). PCR amplification of the calmodulin gene region (CAL) was done using the primers CL2F and CL2R (Duong et al. 2012) and in cases where amplification was difficult, CL2R2 (Duong et al. 2012) was used instead of CL2R. For each marker, PCR reaction components, volumes and conditions followed those described by Musvuugwa et al. (2015).

All products were amplified using a Gene Amp^R, PCR System 2700 thermal cycler (Applied Biosystems, Foster City, U.S.A.). Amplified products were separated using agarose gel electrophoresis stained with GelRed (Biotium, Inc., California, USA) and visualized under ultraviolet light. All amplified PCR products were cleaned using EXO-SAP-IT (USB Corporation, Cleveland, Ohio, U.S.A.) following the manufacturer's instructions. Purified fragments were sequenced using the respective PCR primers and the Big Dye^TM Terminator v3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, CA, U.S.A.) and analysed on an ABI PRISM^TM 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, U.S.A.). PCR sequencing reaction volumes were the same as those used by Musvuugwa et al. (2015) and the same primers used for PCR amplifications were used to sequence both DNA strands. Resultant sequences were edited and used to construct consensus sequences using the CLC Genomics Workbench software package (CLCBio, Aarhus, Denmark).

BLAST algorithm (Altschul et al. 1990) searches were performed using ITS sequences to preliminary identify the fungal isolates and to compare and find similarities with sequences published on the GenBank sequence database (http://www.ncbi.nlm.nih.gov). Sequences of closely related taxa to isolates collected in this study (Table 1) were downloaded from the GenBank database and aligned with sequences generated in this study using MAFFT 6 (Katoh and Toh 2008) for each data set. Maximum likelihood (ML) and Bayesian inference (BI) analyses were conducted on all the datasets created. The online version of PhyML 3.0 (Guindon and Gascuel 2003, http://www.atgc-montpellier.fr/phyml) was used to perform ML analyses. To determine the best fit substitution models jModelTest 0.1.1 (Posada 2008) was used using Akaike information criteria (Akaike 1974). Statistical confidence support values for nodes were determined using 1000 replication bootstrap analyses. MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) was used to perform BI analyses employing a Markov chain Monte Carlo approach (MCMC). MrBayes's rjMCMC mixture model was used. Two Markov chains independent of each other were run simultaneously for 10 million generations starting from a random tree. A sample frequency of 2000 was implemented and the burn-in trees (first 2,500,000 generations) were discarded. The remaining trees were pooled into a 50 % majority rule consensus tree.

Table 1 Culture collection and GenBank accession numbers for strains of ophiostomatoid fungi isolated from *Rapanea melanophloeos* that were sequenced in this study

Fungal species	Collection numbers[1]	CBS accession number[2]	Type status	ITS	βT	CAL	OTU	% frequency of occurrence	Collection site
O. noisomeae	CMW 40329	CBS 141066	Ex-paratype	KU639634	KU639630	KU639611	4	26 %	Weza Forest
	CMW 40326	CBS 141065	Ex-holotype	KU639631	KU639628		4		
	CMW 40327	CBS 141067	Ex-paratype	KU639632		KU639612	4		
	CMW 40328		Ex-paratype	KU639633	KU639629	KU639610	4		
O. stenoceras	CMW 40372			KU595580	KU639619	KU639613	5	13 %	Weza Forest
	CMW 40373			KU595579	KU639620	KU639614	5		
	CMW 40374				KU639612	KU639615	5		
S. itsvo	CMW 40370	CBS 141063	Ex-holotype		KU639625		1	21 %	HPNBG[3]
	CMW 47169	CBS 141077	Ex-paratype	KU595582	KU639626		1		
	CMW 40371	CBS 141064	Ex-paratype		KU639628		1		
S. rapaneae	CMW 40367	CBS 141061	Ex-paratype		KU639622	KU639608	2	18 %	Groenkop Forest
	CMW 40368	CBS 141062	Ex-paratype	KU595581	KU639623		2		
	CMW 40369	CBS 141060	Ex-holotype	KU595583	KU639624	KU639609	2		
S. uta	CMW 40316	CBS 141069	Ex-paratype	KU595577	KU639616	KU639605	3	23 %	Weza Forest
	CMW 40317	CBS 141070	Ex-paratype	KU595578	KU639617	KU639606	3		
	CMW 40318	CBS 141068	Ex-holotype	KU595576	KU639618	KU639607	3		

[1] *CMW* Culture Collection of Michael Wingfield, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria

[2] *CBS* Culture Collection of the Centraalbureau voor Schimmelcultures, CBS, institute, Utrecht, The Netherlands

[3] *HPNBG* Harold Porter National Botanical Garden

Growth in culture

Optimal growth conditions of potential new species were determined by removing a disk of agar (10 mm diam) covered with mycelium from edges of actively growing, 1 week old cultures and placing these upside down in the centres of 90 mm fresh Petri dishes containing 20 mL MEA. Petri dishes were incubated in the dark at different temperatures ranging from 5 to 35 °C (at intervals of 5 °C) for a period of 10 days. Five replicates were used for each temperature interval. After 10 days, two colony diameters perpendicular to each other were measured for each colony, these measurements were averaged for each plate and then averaged for each species (±standard deviation).

Results

Fungal isolates and morphological characterisation

In total, 39 isolates morphologically resembling fungi in the Ophiostomatales were obtained from wounds on several *R. melanonophloeos* trees sampled at the different sites (Table 1). Based on colony characteristics and micro-morphology, the isolates collected in this study were grouped into five different morphotypes that all resembled species in the *Sporothrix schenckii–O. stenoceras* complex (De Beer and Wingfield 2013). Of the 39 isolates obtained, eight were from morphotype 1 collected from Harold Porter National Botanical Garden (Table 1). These were characterised by producing perithecia (produced on plant material only) with brownish ascomatal bases with hyphal ornamentation and produced whitish to cream-coloured colonies that were fluffy at the centre on MEA. Morphotype 2 consisted of seven isolates collected from the Groenkop Forest characterised by producing black ascomata (on plant material only), without hyphal ornamentation and white fluffy colonies on MEA. The third morphotype (morphotype 3) identified included nine isolates collected from Weza Forest that were characterised by producing black ascomata (produced on plant material only) and white colonies that grew slowly compared to other morphotypes. Morphotype 4 comprised of 10 isolates that were also collected from Weza Forest (Table 1).

They were characterised by producing black ascomata (on plant material only) and white colonies on MEA that turned blackish with age. Five isolates of morphotype 5 also from Weza Forest (Table 1) were characterised by producing black ascomata in culture after 3 weeks of incubation and colonies that either became dark brown or remained white with age. The frequency of occurrence per wound varied between 13 % for morphotype 5 and 26 % for morphotype 4 (Table 1).

Phylogenetic analyses

All fungal morphotypes were confirmed members of the Ophiostomatales based on initial placement using ITS data and represented discrete operational taxonomic units (OTU's). These were therefore further characterised using ITS, βT and CAL data. Alignment of data sets resulted in 84 taxa and 785 characters for ITS, 73 taxa and 280 characters for βT and 58 taxa and 720 characters for CAL. Statistical values obtained from the analyses of the different data sets are summarized in Table 2, along with the substitution models chosen. GenBank accession numbers for other taxa used in the phylogenetic analyses are presented on the respective trees for each data set (Figs. 1, 2, 3).

For OTU 1 collected from the Harold Porter National Botanical Garden, phylogenetic analyses were only performed using ITS and βT (Figs. 1, 2) and could not be performed for the CAL gene region due to amplification failure. Analysis of this particular OTU (hereafter referred to as: *Sporothrix itsvo* sp. nov.) using ITS data resulted in its isolates grouping together as a strongly supported distinct taxon sister to a clade comprising of *S. aemulophila* Musvuugwa, Z.W. de Beer, L.L. Dreyer & F. Roets, *O. candidum* Kamgan-Nkuek., Jol. Roux & Z. W. de Beer and the OTU from Groenkop forest collected in this study. Analyses using βT data resulted in it grouping as a strongly supported distinct taxon sister to *S. aemulophila*.

Analyses of ITS, βT and CAL data for OTU 2 collected from Groenkop forest (hereafter referred to as: *Sporothrix rapaneae* sp. nov.) resulted in trees with similar placement of its isolates than those of *S. itsvo*. The ITS data did not distinguish between *S. rapaneae*, *S. aemulophila* and *O. candidum*, which formed a single group (Fig. 1). Analyses of the βT data set showed that *S. rapaneae* grouped sister to *O.*

Table 2 Parameters used and statistical values obtained from maximum likelihood (ML) and Bayesian inference (BI) analyses of the three datasets

| | Dataset | ITS | βT | CAL |
	Figure	Figure 1	Figure 2	Figure 3
ML	Substitution model	GTR+I+G	K80+G	HKY+I+G
	Gamma shape	0.469	0.188	1.542
	Log-likelihood	−9084.95	−2040.08	−3891.25
	Relative rate parameters	A↔C 1.57550		
		A↔G 2.39500		
		A↔T 2.93976		
		C↔G 1.31852		
		C↔T 5.04703		
		G↔T 1.00000		
	Nucleotide frequencies	−f(A) = 0.16651	−f(A) = 0.25000	−f(A) = 0.24940
		−f(C) = 0.35674	−f(C) = 0.25000	−f(C) = 0.24091
		−f(G) = 0.28242	−f(G) = 0.25000	−f(G) = 0.29348
		−f(T) = 0.19433	−f(T) = 0.25000	−f(T) = 0.21622
BI	Likelihood	9221.97	−2240.28	−4023.97
	Average effective sample size of likelihood value	366	1368	1007
	GTR submodel probability	0.24	0.12	0.3
	Model string	[123454]	[123323]	[121321]
	Gamma shape	1.05	0.21	1.46
	Relative rate parameters	A↔C 0.10820	A↔C 0.16790	A↔C 0.08443
		A↔G 0.16567	A↔G 0.27235	A↔G 0.34859
		A↔T 0.21853	A↔T 0.09903	A↔T 0.08876
		C↔G 0.08272	C↔G 0.09205	C↔G 0.05901
		C↔T 0.34801	C↔T 0.28469	C↔T 0.33305
	Nucleotide frequencies	pi(A) = 0.16187	pi(A) = 0.31424	pi(A) = 0.24319
		pi(C) = 0.36121	pi(C) = 0.20805	pi(C) = 0.24811
		pi(G) = 0.28583	pi(G) = 0.28989	pi(G) = 0.29685
		pi(T) = 0.19109	pi(T) = 0.18783	pi(T) = 0.21185

candidum (Fig. 2), while analyses of the CAL dataset resulted in the isolates forming a strongly supported distinct taxon sister to *S. aemulophila* (Fig. 3).

Analyses of ITS data for OTU 3 collected from Weza Forest (hereafter referred to as: *Sporothrix uta* sp. nov.) resolved it as a distinct clade with strong support sister to *O. aurorae* X.D. Zhou & M.J. Wingf and also closely related to a larger clade comprising of the species *O. fusiforme* D.N. Aghayeva & M. J. Wingfield, *O. lunatum* D.N. Aghayeva & M. J. Wingfield and *O. abietinum* Marmolejo & Butin (Fig. 1). Analyses of βT and CAL data proposed a similar placement of *Sporothrix uta* (Figs. 2, 3), where it also grouped as a strongly supported distinct taxon

sister to a large clade containing species such as *O. fusiforme*, *O. lunatum* and *O. abietinum*.

Operational taxonomic unit 4 from Weza Forest (hereafter referred to as: *Ophiostoma noisomeae* sp. nov.) grouped strongly as distinct taxon in analyses of all three data sets. Analyses of ITS data grouped it as sister to the genus *Graphilbum* (Fig. 1), while analyses of βT data grouped it as sister to the *Sporothrix lignivora* complex (De Beer and Wingfield 2013) (Fig. 2). Analysis of CAL data strongly supported it as a distinct clade, but unfortunately CAL data for the above-mentioned closely related taxa was not available (Fig. 3). Analyses of ITS, βT and CAL data showed that the OTU 5 grouped with isolates of
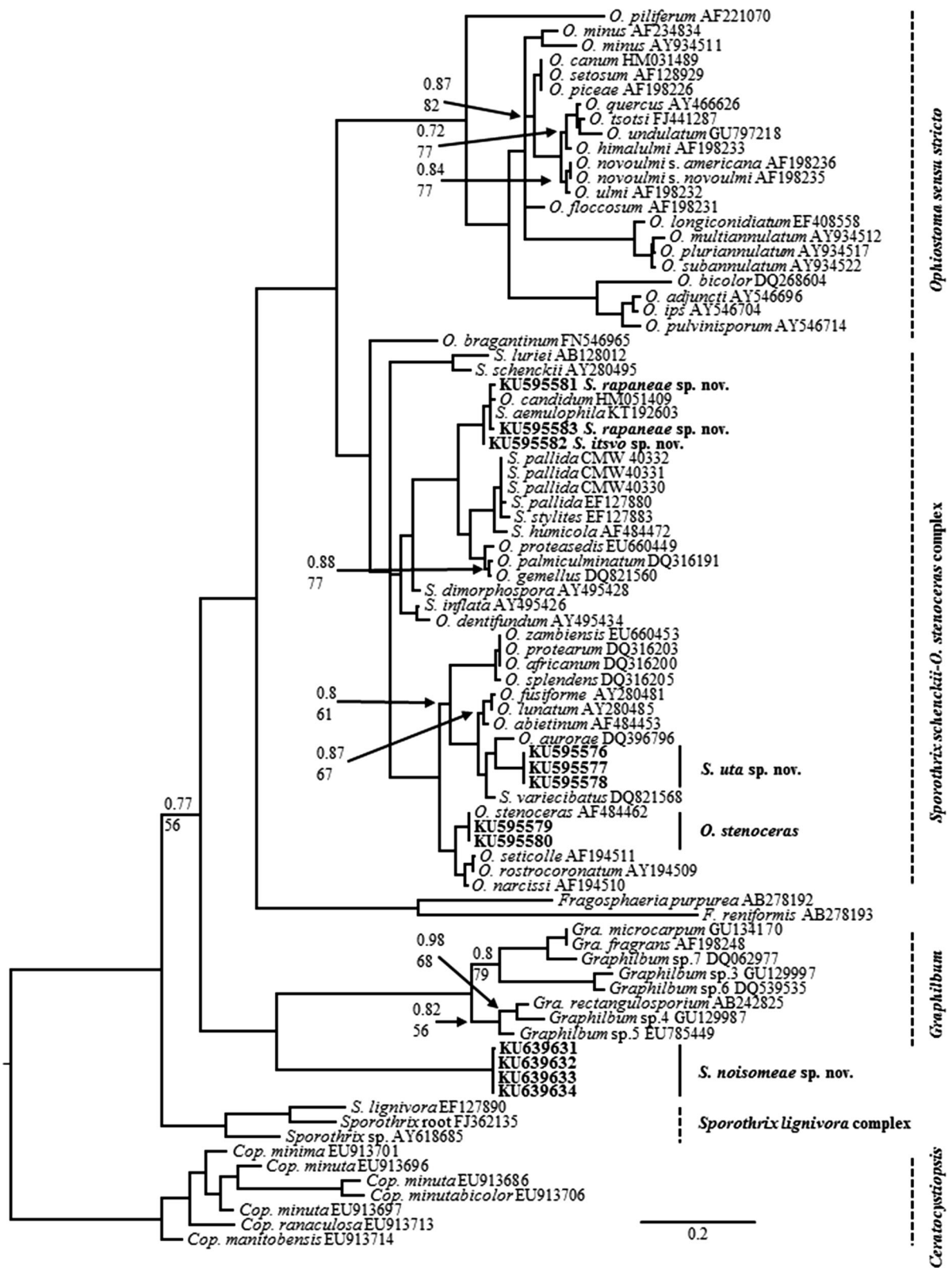
◄ **Fig. 1** Bayesian Inference consensus tree (ITS data) of taxa related to species isolated in the present study (bold type). Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood analysis. Other isolates [GenBank accession numbers and isolate numbers shown (when available)] were selected for comparative purposes. Dashed lines demarcate different genera or species complexes

Ophiostoma stenoceras (Robak) Nannf. with strong support (Fig. 1, 2, 3).

Taxonomy

Based on phylogenetic analyses and micro-morphological characteristics four of the five collected taxa (all except *Ophiostoma stenoceras*) were recognised as distinct, undescribed species. The three taxa belonging to the *Sporothrix schenckii–Ophiostoma stenoceras* complex are described below as species of *Sporothrix*, as this complex will soon be redefined to represent the genus *Sporothrix* (Z.W. de Beer, personal communication), which is distinct from the genus *Ophiostoma s.str.* (De Beer and Wingfield 2013). The fourth species that groups alongside, but not within *Graphilbum* and the *S. lignivora* complex, cannot be placed with confidence in any of these genera based on the ITS data. Until all genera in the Ophiostomatales are redefined based on more conserved gene regions, we follow the interim recommendations by De Beer and Wingfield (2013) and describe the fourth species in *Ophiostoma s.l.*:

Sporothrix itsvo Musvuugwa, LL. Dreyer & F. Roets sp. nov. Fig. 4.

Mycobank: MB816106

Etymology: The epithet itsvo (itsvo = kidney in the African native Shona language) refers to the kidney-shaped ascospores produced by this species.

Ascomata superficial or embedded on the host substrate, bases globose, dark brown, ornamental hyphae present, 95–178 (134 ± 28) μm diam; necks black, 384–777 (530 ± 121) μm long, 15–30 (24 ± 5) μm wide at the base, 5–12 (8 ± 2) μm wide at the apex, ostiolar hyphae hyaline, aseptate, glossy 13–17 (15 ± 1.2) μm long (Fig. 4a, c, d). *Asci* evanescent. *Ascospores* kidney-shaped, aseptate, hyaline, sheaths absent, 1.3–2.4 (1.84 ± 0.46) × 0.3–0.5 (0.37 ± 0.12)

μm (Fig. 4b), accumulating in a transparent gelatinous droplet at the apex of the neck, becoming dry when old. *Colonies* whitish to cream-coloured, on MEA fluffy in the centre only. Odourless, circular with entire edge (Fig. 4e). Colony diameter reaching 35.7 (±1.3) mm after 10 days on MEA at an optimal growth temperature of 25 °C. No growth below 5 °C or above 35 °C. *Conidiophores* mononematous, cylindrical, hyaline, 5.1–7.9 (6.18 ± 1.24) × 0.5–1.1 (0.83 ± 0.28) μm (Fig. 4f). *Conidiogenous* cells arising directly from tips of conidiophores, hyaline, forming denticles 0.5–1.3 (0.9 ± 0.3) μm long (Fig. 4g). *Conidia* hyaline, aseptate, obovate, 3.4–4.4 (3.9 ± 0.42) × 0.6–0.9 (0.75 ± 0.13) μm (Fig. 4h).

Substrate: Isolated from damaged wood and inner bark of *Rapanea melanophloeos*

*Distribution*: South Africa, Western Cape Province

Specimens examined: South Africa, Western Cape Province, Harold Porter National Botanical Garden, Betty's Bay. Isolated from *Rapanea melanophloeos*, August 2010, T. Musvuugwa, **holotype** PREM 61325, culture ex-holotype CMW 40370 = CBS 141063, **paratype** PREM 61327, culture ex-paratype CMW 47169 = CBS 141077, **paratype** PREM 61326, culture ex-paratype CMW 40371 = CBS 141064.

Sporothrix rapaneae Musvuugwa, LL. Dreyer & F. Roets sp. nov. Fig. 5.

Mycobank: MB816107

Etymology: The epithet *rapaneae* refers to the genus name of the host plant (*Rapanea melanophloeos*) from which this species was collected.

Ascomata superficial on the host substrate, bases globose, black, with no ornamental hyphae, 125–165 (147 ± 11) μm diam; necks black, 473–905 (670 ± 129) μm long, 11–30 (24 ± 5) μm wide at the base, 3–6 (4 ± 1) μm wide at the apex, ostiolar hyphae present, hyaline, aseptate, pale brown to glossy brown, 22–35 μm (26 ± 3) long (Fig. 5a, c). *Asci* evanescent. *Ascospores* allantoid, slightly curved, aseptate, hyaline, 2.8–4.7 (3.75 ± 0.98) × 0.3–0.6 (0.45 ± 0.13) μm (Fig. 5b), accumulating in a sticky gelatinous droplet at the tip of the neck, becoming white to cream colored when dry. *Colonies* white, fluffy, on MEA, circular, edges entire (Fig. 5d). Colony diameter reaching 34.9 (±0.7) mm after 10 days on MEA at an optimal growth temperature of 25 °C. No growth below 5 °C or above 30 °C. *Conidiophores* hyaline, cylindrical, 4.1–7.9 (5.95 ± 1.59) × 0.7–1.2 (0.98 ± 0.22) μm (Fig. 5e). *Conidiogenous* cells forming directly from tips of
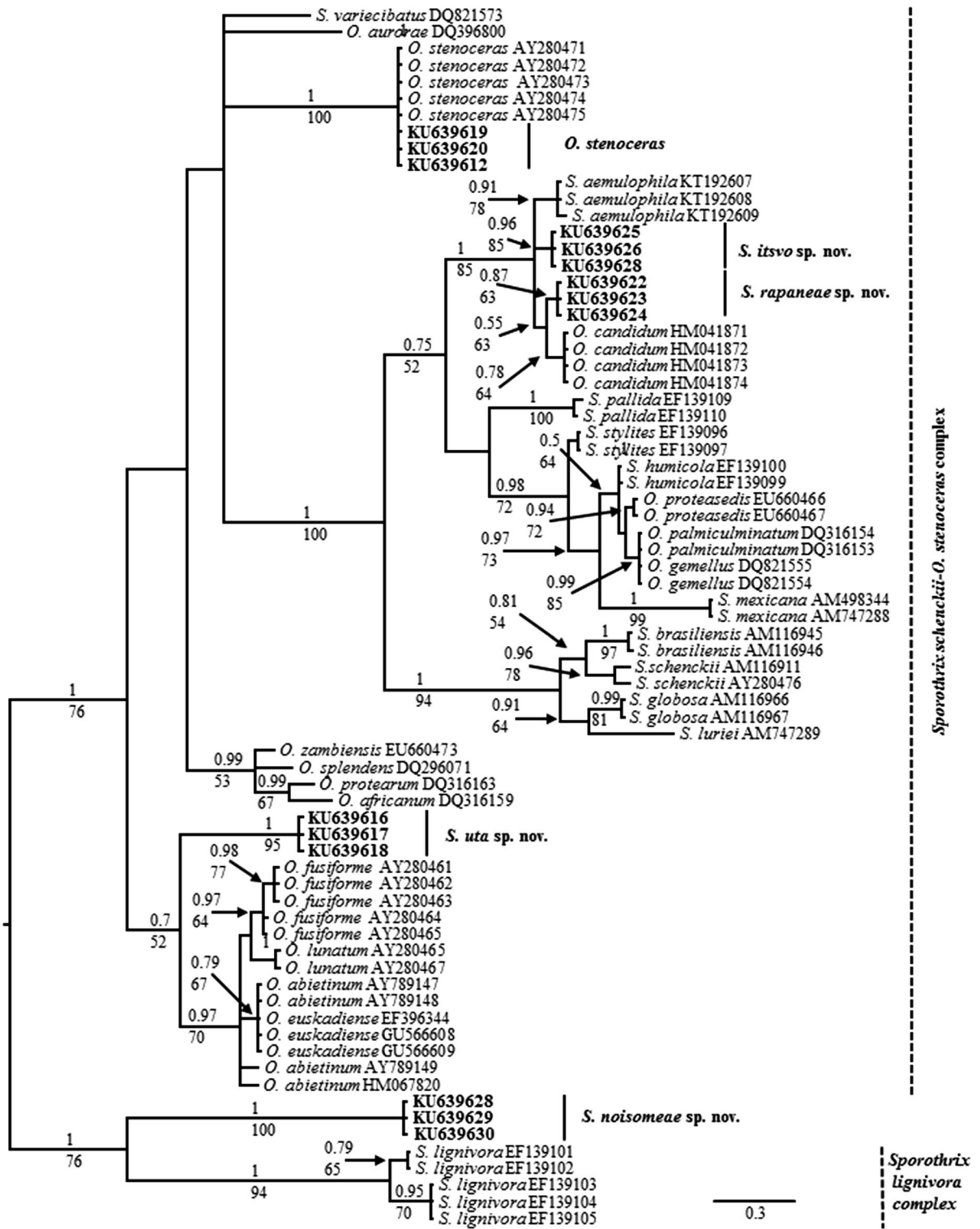
◄ **Fig. 2** Bayesian Inference consensus tree (βt data) of taxa related to species isolated in the present study (*bold* type). Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood analysis. Other isolates [GenBank accession numbers and isolate numbers shown (when available)] were selected for comparative purposes. Dashed lines demarcate different species complexes

conidiophores, hyaline, with prominent, denticles 0.4–1.3 (0.86 ± 0.2) μm long (Fig. 5e). *Conidia* hyaline, aseptate, oblong, 3.9–5.5 (4.7 ± 0.67) × 0.5–1.1 (0.85 ± 0.26) μm (Fig. 5f).

Substrate: Isolated from inner bark of *Rapanea melanophloeos*

Distribution: South Africa, Western Cape Province

Specimens examined: South Africa, Western Cape Province, Groenkop. Isolated from *Rapanea melanophloeos*, October 2011, T. Musvuugwa, **holotype** PREM 61322, culture ex-holotype CMW 40369 = CBS 141060, **paratype** PREM 61323, culture ex-paratype CMW 40367 = CBS 141061, **paratype** PREM 61324, culture ex-paratype CMW 40368 = CBS 141062.

Sporothrix uta Musvuugwa, LL. Dreyer & F. Roets sp. nov. Fig. 6.

Mycobank: MB816108

Etymology: The epithet uta (uta = crescent/bow shaped in the African native Shona language) refers to the crescent or bow-shaped ascospores produced by this species.

Ascomata embedded or superficial on the host substrate, bases globose, brownish to black, with no hyphal ornamentation, 87–101 (94 ± 5) μm diam; necks dark brown, 1212–1501 (1319 ± 103) μm long, 19–30 (23 ± 4) μm wide at the base, 6–9 (7 ± 1) μm wide at the tip, ostiolar hyphae hyaline, aseptate, pale brown, 12–17 (15 ± 2) long (Fig. 6a, c). *Asci* evanescent. *Ascospores* crescent-shaped, hyaline, aseptate, 1.9–2.6 (2.28 ± 0.29) × 0.5–0.8 (0.65 ± 0.13) μm (Fig. 6b), accumulating transparent sticky masses at the apex of the neck, becoming white with age. *Colonies* white, fluffy appearance on MEA. Odourless, circular with entire edges (Fig. 6d). Colony diameter reaching 38 (±1.2) mm after 10 days on MEA at optimal temperature of 25 °C. No growth below 5 °C or above 35 °C. *Conidiophores* hyaline, cylindrical, 3.9–8 (5.7 ± 2.1) × 0.9–1.4 (1.2 ± 0.22) μm (Fig. 6e, f). *Conidiogenous* cells formed from tips

of conidiophores, hyaline, with 0.9–2.8 (1.6 ± 0.6) μm long denticles (Fig. 6e, f). *Conidia* hyaline, aseptate, oblong, 4.2–4.8 (4.5 ± 0.26) × 0.7–1.2 (0.95 ± 0.24) μm (Fig. 6f).

Substrate: Isolated from wood and inner bark of *Rapanea melanophloeos*

Distribution: South Africa, KwaZulu-Natal Province

Specimens examined: South Africa, KwaZulu-Natal Province, Weza Forest Reserve. Isolated from *Rapanea melanophloeos*, September 2010, T. Musvuugwa, **holotype** PREM 61331, culture ex-holotype CMW 40318 = CBS 141068, **paratype** PREM 61332, culture ex-paratype CMW 40316 = CBS 141069, **paratype** PREM 61333, culture ex-paratype CMW 40317 = CBS 141070.

Ophiostoma noisomeae Musvuugwa, LL. Dreyer & F. Roets sp. nov. Fig. 7

Mycobank: MB816109

Etymology: The epithet *noisomeae* (*noisome* = foal smell) refers to the foal smell produced by colonies of this species.

Ascomata superficial on host substrate, bases globose, black, with no ornamental hyphae, 104–146 (126 ± 15) μm diam; necks black, 658–1270 (1034 ± 265) μm long, 12–31 (23 ± 5) μm wide at the base, 4–9 (7 ± 1) μm wide at the apex, ostiolar hyphae hyaline, aseptate, glossy 24–33 (28 ± 3) μm long (Fig. 7a, b). *Asci* evanescent. *Ascospores* allantoid, aseptate, hyaline, 2.0–2.6 (2.34 ± 0.26) × 0.5–0.8 (0.65 ± 0.13) μm, accumulating a gelatinous droplet at the tip of the neck, becoming whitish with age (Fig. 7c). *Colonies* white, turning blackish with age on MEA. Foal smelling when older, circular, edges entire (Fig. 7d). Optimal growth at 25 °C with a colony diameter of 28.7 (±1.1) mm after 10 days on MEA. No growth below 5 °C or above 30 °C. *Conidiophores* 18.3–21.4 (19.68 ± 1.35) × 0.9–1.8 (1.35 ± 0.39) μm, hyaline, cylindrical (Fig. 7d, e). *Conidiogenous* cells arising from apex of conidiophores, hyaline (Fig. 7d). *Conidia* aseptate, hyaline, thin-walled, smooth, spherical, form in masses, becoming brownish with age, 2.2–3.8 (3.08 ± 0.72) × 2.2–3.4 (2.78 ± 0.53) μm (Fig. 7f).

Substrate: Isolated from wood and inner bark of *Rapanea melanophloeos*

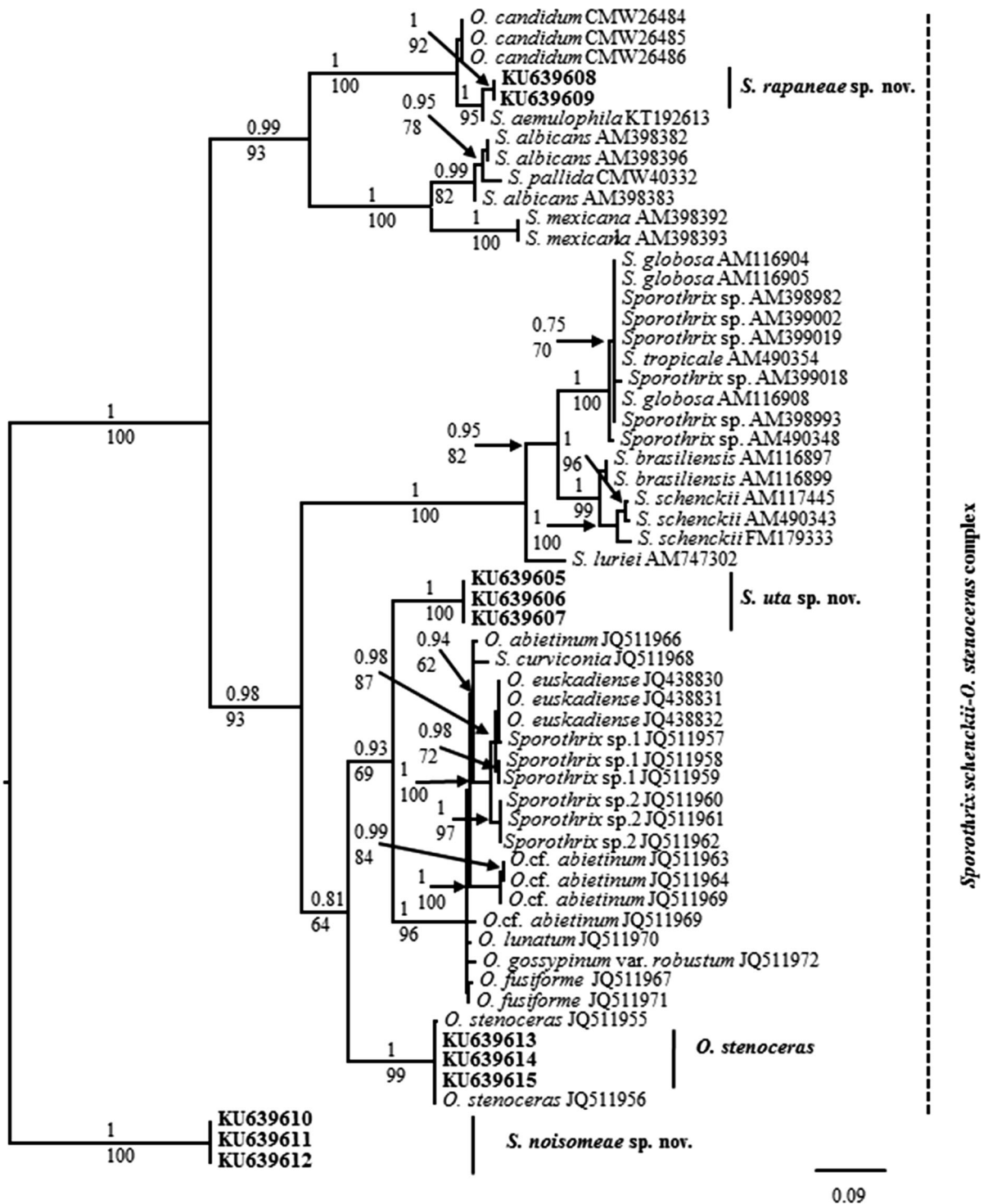*Distribution*: South Africa, KwaZulu-Natal Province

Fig. 3 Bayesian Inference consensus tree (CAL data) of taxa related to species isolated in the present study. Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood analysis. Isolates in bold were collected in this study. Other isolates [GenBank accession numbers and isolate numbers shown (when available)] were selected for comparative purposes
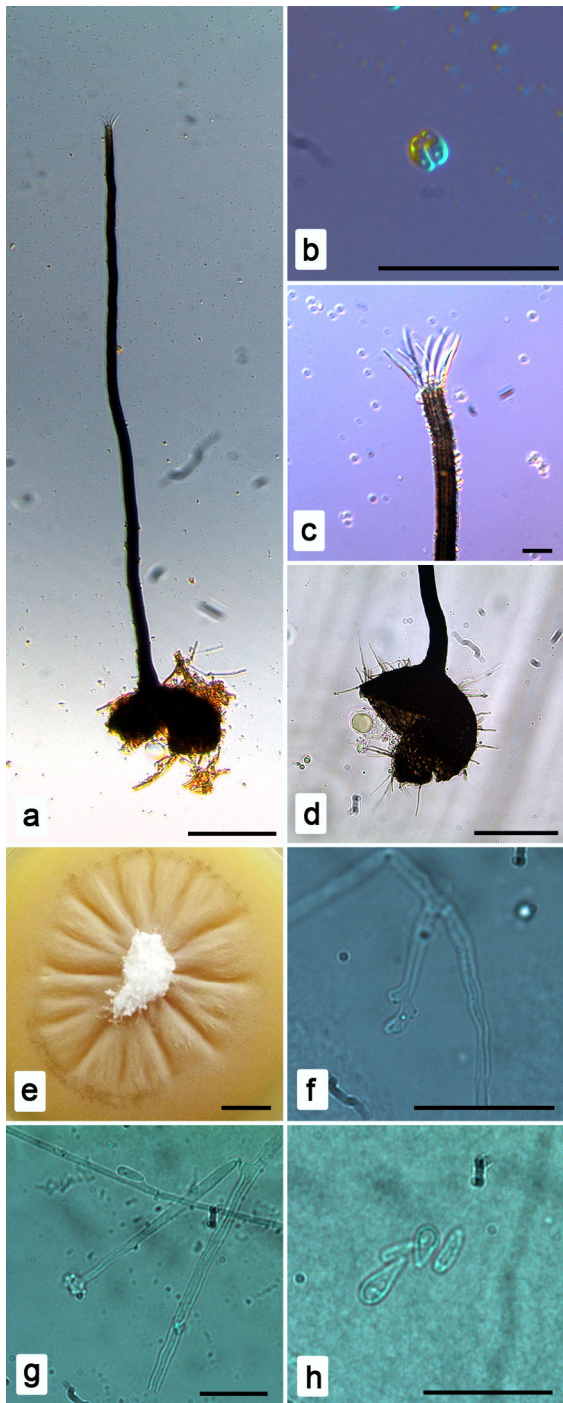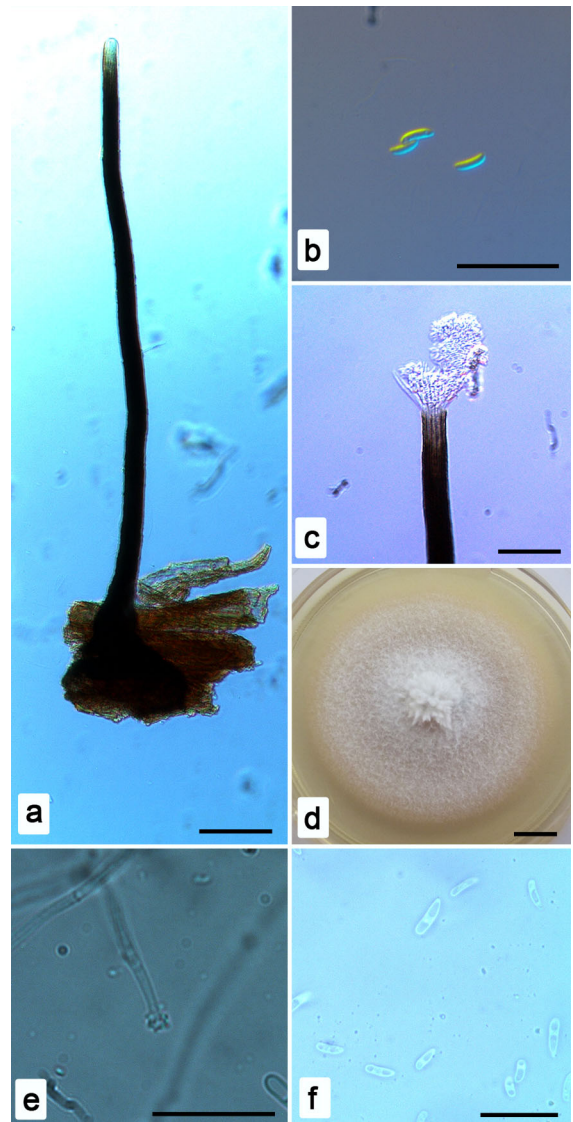
Fig. 4 Micrographs of *Sporothrix itsvo*. **a** Perithecium removed from the bark of *Rapanea melanophloeos* (*scale bar* 100 μm); **b** ascospores (*scale bar* 10 μm); **c** tip of perithecial neck (*scale bar* 10 μm); **d** globose base showing ornamental hyphae (*scale bar* 100 μm); **e** two-week-old colony on MEA at 25 °C (*scale bar* 10 mm); **f** conidiophores (*scale bar* 10 μm); **g** Conidiogenous cells showing denticles (*scale bar* 10 μm); **h** conidia (*scale bar* 10 μm)

Fig. 5 Micrographs of *Sporothrix rapaneae*. **a** Perithecium removed from bark of *Rapanea melanophloeos* (*scale bar* 100 μm); **b** ascospores (*scale bar* 10 μm); **c** tip of perithecial neck (*scale bar* 10 μm); **d** two-week-old colony on MEA (*scale bar* 10 mm); **e** conidiogenous cells showing prominent denticles (*scale bar* 10 μm); **f** conidia (*scale bar* 10 μm)

Specimens examined: South Africa, KwaZulu-Natal Province, Weza Forest Reserve. Isolated from *Rapanea melanophloeos*, September 2010, T. Musvuugwa, **holotype** PREM 61328, culture ex-holotype CMW 40326 = CBS 141065, **paratype** PREM 61329, culture ex-paratype CMW 40329 = CBS 141066, **paratype** PREM 61330, culture ex-paratype CMW 40327 = CBS 141067.
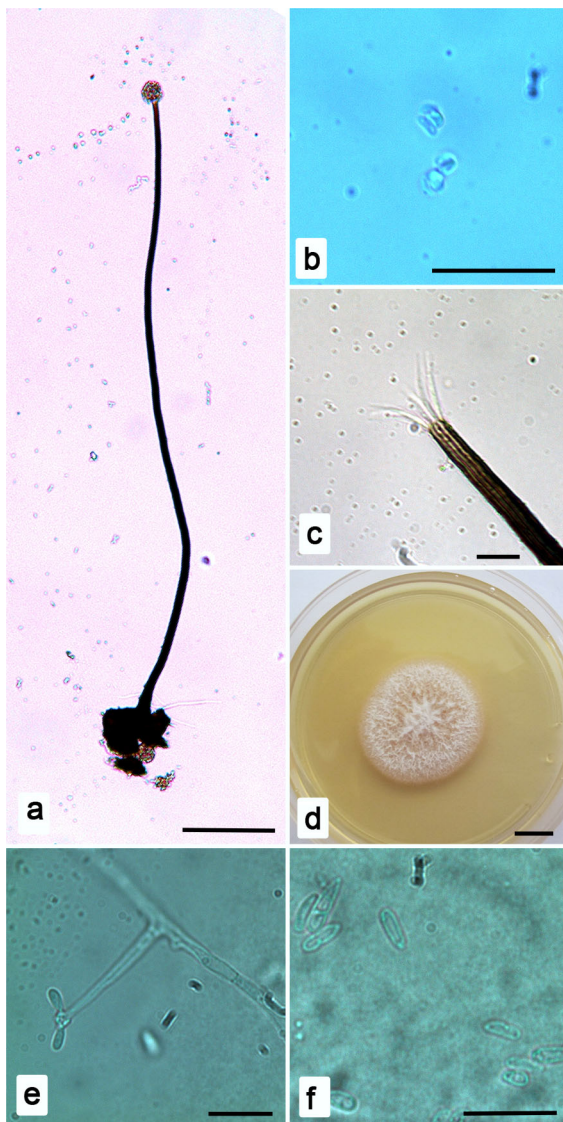
Fig. 6 Micrographs of *Sporothrix uta*. **a** Perithecium removed from bark of *Rapanea melanophloeos* (*scale bar* 100 μm); **b** ascospores (*scale bar* 10 μm); **c** tip of perithecial neck (*scale bar* 10 μm); **d** two-week-old colony on MEA (*scale bar* 10 mm); **e** conidiogenous cells showing prominent denticles (*scale bar* 10 μm); **f** conidia (*scale bar* 10 μm)

Discussion

This study reports five members of the Ophiostomatales associated with wounds on *R. melanophloeos* in Afromontane forests of South Africa. Four of these are newly described here (*Sporothrix itsvo*, *S. rapaneae*, *S. uta* and *Ophiostoma noisomeae*). This demonstrates that there is a rich and yet still poorly studied diversity
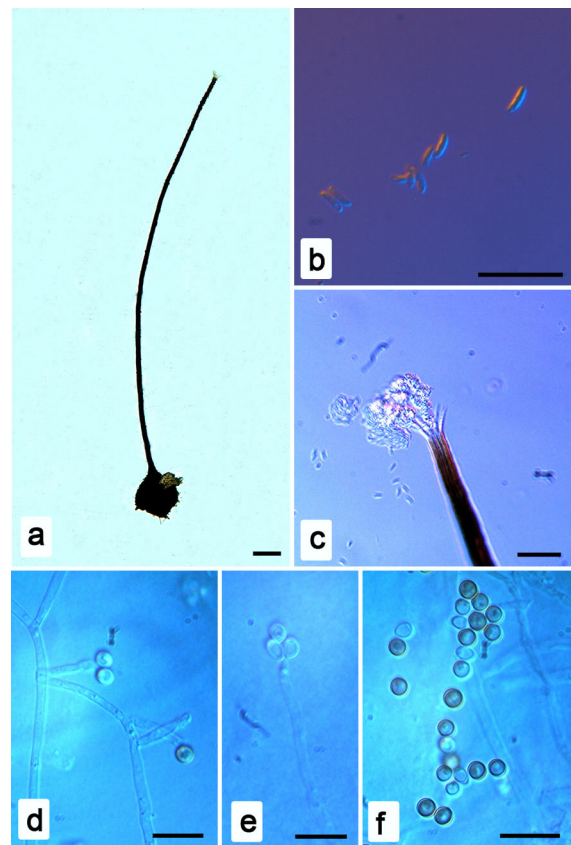

Fig. 7 Micrographs of *Ophiostoma noisomeae*. **a** Perithecium removed from bark of *Rapanea melanophloeos* (*scale bar* 100 μm); **b** ascospores (*scale bar* 10 μm); **c** tip of perithecial neck (*scale bar* 10 μm); **d**, **e** conidiophores (*scale bar* 10 μm); **f** conidia (*scale bar* 10 μm)

of Ophiostomatales associated with *R. melanophloeos* in Afromontane forests. The five reported species are in addition to several other species that have previously been isolated from wounds (Nkuekam et al. 2008) and subcortical beetles (Musvuugwa et al. 2015) from the same host. This brings the total number of species in this order now known from this host to nine and includes *Ophiostoma quercus*, *Graphilbum fragrans*, *Raffaelea rapaneae* T. Musvuugwa, Z.W. de Beer, L.L. Dreyer and F. Roets, and *Sporothrix aemulophila* T. Musvuugwa, Z.W. de Beer, L.L. Dreyer and F. Roets (Nkuekam et al. 2008; Musvuugwa et al. 2015). Given the relatively high number Ophiostomatales collected from *R. melanophloeos* from only a few studies in these forests, there is reason to believe that numerous additional species still await discovery with the inclusion of additional sites in future studies.

Four of the species collected in this study belong to the *S. schenckii–O. stenoceras* complex (De Beer et al. 2013b), including three of the newly described species. This adds to an already high diversity of species in this complex from southern Africa (De Meyer et al. 2008; Nkuekam et al. 2012; Musvuugwa et al. 2015; Roets et al. 2007, 2009; Zhou et al. 2006). This complex includes species collected from many different substrates, including mite-associated species from *Protea* infructescences (Roets et al. 2007, 2009), human pathogens (Marimon et al. 2007) and subcortical beetle-associated species (Musvuugwa et al. 2015). Many taxa are also known from "environmental" samples such as soil, air and decaying wood (De Beer et al. 2013b). The ecology of species in this complex thus varies greatly and it would be interesting to determine the ecological role of the taxa associated with wounds on *Rapanea*.

Sporothrix itsvo was collected from *Rapanea* hosts in indigenous forests surrounding the Harold Porter National Botanical Garden. The species is closely related to *S. aemulophila*, a recently described species from the same *R. melanophloeos* host (Musvuugwa et al. 2015). *Sporothrix aemulophila* is an associate of the ambrosia beetle *Xyleborinus aemulus* Wollaston (Scolytinae) that infest *R. melanophloeos* trees at the same site (Harold Porter National Botanical Garden) from which *S. itsvo* was collected (Musvuugwa et al. 2015). Besides being isolated from the same host tree and from the same area, these two taxa are also similar in characters such as the presence of ostiolar hyphae and similar colony growth forms on MEA (Musvuugwa et al. 2015). The two species differ in other traits such as the presence of ornamental hyphae on perithecial bases in *S. itsvo*, which are absent in *S. aemulophila* and the kidney-shaped ascospores in *S. itsvo*, while *S. aemulophila* has allantoid-shaped spores (Musvuugwa et al. 2015).

Sporothrix rapaneae was collected from Groenkop forest and analyses of βT and CAL data resolved it as sister to *O. candidum* and *S. aemulophila*, respectively. The relationship with *S. aemulophila* was, however, more strongly supported than that with *O. candidum*. Similarities shared with *S. aemulophila* include the presence of ostiolar hyphae and the production of allantoid-shaped ascospores. *Sporothrix rapaneae* differs from *S. aemulophila* in its optimal growth temperatures (30 °C for *S. aemulophila* and 25 °C *S. rapaneae*) and average sizes of

morphological structures (Musvuugwa et al. 2015). *Sporothrix rapaneae* and *S. aemulophila* differ from *O. candidum* in basal ornamentation of perithecia, which is present in *O. candidum*, but absent in *S. rapaneae*, the production of ascomata on MEA by *O. candidum*, but not by *S. rapaneae* as well as in average sizes of morphological structures (Nkuekam et al. 2012). They are similar in having the same optimal growth temperatures and the presence of ostiolar hyphae (Nkuekam et al. 2012). Phylogenetic analyses of the three gene regions suggest that *Sporothrix uta* is closely related to several species (e.g. *O. fusiforme*, *O. lunatum* and *O. abietinum*), although ITS data showed it to be sister to *O. aurorae*. *Ophiostoma aurorae* was isolated from root-feeding *Hylastes angustatus* Herbst that infest *Pinus patula* Schiede & Deppe in South Africa (Zhou et al. 2006). It is characterised by bases ornamented with light grey hyphae and allantoid ascospores (Zhou et al. 2006). These characters differentiate it from *S. uta*. Several species closely related to *S. uta* are wood-inhabiting (De Beer et al. 2013b). *Ophiostoma fusiforme*, for example, was isolated from *Populus nigra* L. and *Quercus petraea* (Matt.) Liebl. in Azerbaijan and Austria, respectively. *Ophiostoma lunatum* is known from Austria, where it was isolated from *Carpinus betulus* L. and *Larix decidua* Mill., while *O. abietinum* was isolated from pine wood (Aghayeva et al. 2004). Although they are all wood inhabiting species with *Sporothrix*-like anamorphs and share other similarities such as the presence of ostiolar hyphae, they differ in several traits such as their basal hyphal ornamentation (present in *O. fusiforme* and *O. lunatum*), the shape of their ascospores and the average measurements of morphological structures (Aghayeva et al. 2004).

The isolates of *Ophiostoma stenoceras* collected in this study were from Weza Forest. It is a well-known sapwood colonising fungus that was first described in Norway from ground wood pulp (Robak 1932), but are now known from several hardwood and coniferous hosts, mostly in the Northern Hemisphere (Griffin 1968). This wide host range is reflected in the existence of several synonyms for this species, including *O. albidum* Math.-Käärik, *Ceratocystis gossypina var. robusta* T.E. Hinds & R.W. Davidson, *O. ponderosae* (T.E. Hinds & R.W. Davidson) Hausner, J. Reid & Klassen, and *Ceratocystis eucastaneae* R.W. Davidson (De Beer et al. 2003b). *Ophiostoma stenoceras* has previously also been collected in the

Southern Hemisphere, although only from non-native hosts such as *Pinus* spp. in New Zealand (Schirp et al. 1999) and *Eucalyptus* spp. in South Africa (De Beer et al. 2003). Although this species causes gray staining of pine wood (Käärik 1960), it is not considered economically important (Griffin 1968).

Ophiostoma noisomeae, the other newly described species from Weza Forest, groups between *Graphilbum* and the *Sporothrix lignivora* complex based on ITS data. The βT intron composition of *O. noisomeae* is (3/4/5), while that of the *S. lignivora* complex is –/4/5 and that of the *S. schenckii–O. stenoceras* complex is 3/–/5 or –/–/5 (De Beer et al. (2013b). However, in the βT data set used for our analyses, only data for exons 4, 5, and 6, interspersed with introns 4 and 5, were included. This explains why *O. noisomeae* grouped closer to the *S. lignivora* complex (Fig. 2). Based on CAL data, *O. noisomeae* formed a distinct clade, but its affinities are unclear as CAL data of *S. lignivora* and *Graphilbum* species are still lacking. Our analyses support results of De Beer et al. (2013b) and Linnakoski et al. (2010) that the *S. lignivora* complex and *O. noisomeae* form distinct lineages with significant distance from other genera and complexes in the Ophiostomatales. De Beer et al. (2013b) suggested that, due to the distance at which the *S. lignivora* complex groups from other complexes and genera in the Ophiostomatales, the group may represent a separate genus. There is, however, uncertainty about the phylogenetic placement of this group as it differs depending on the specific marker used (De Beer et al. 2013b). Sequences for more conservative gene regions will be required to determine the exact generic placements of the *S. lignivora* complex and *O. noisomeae*.

The frequency of occurrence on wounds was highest for *O. noisomeae* and lowest for *O. stenoceras*. Although not tested here this may be very relevant if it is shown that species with high frequency of occurrence is also pathogenic to *R. melanophloeos* and could become problematic with increased creation of wounds for bark harvesting. This high frequency also point towards mechanisms that allow these species to easily disperse and infect wounds. Future studies should focus on determining the dispersal mechanisms of the different Ophiostomatales species between wounds to verify whether differences in their frequency of occurrence may be related to the abundance/dispersal abilities of the main vectors involved and/or

whether different fungal taxa may prefer different aged wounds (i.e. succession studies).

In this study we collected five members of the Ophiostomatales, including four new species, associated with wounds on *R. melanophloeos*. Four of the species belong to the *S. schenckii–O. stenoceras* complex, while the fifth species groups basal in the Ophiostomatales alongside the *S. lignivora* complex and *Graphilbum*. Based on these results and those from previous studies, there is evidence of a relatively high diversity of Ophiostomatales associated with this host tree in the Afromontane forests of South Africa. It is recommended that future studies investigate the total number of taxa associated with this important tree, and that the pathogenicity of all the taxa towards this host is determined. If many prove to be virulent pathogens, conservation managers may need to restrict bark harvesting of this species.

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