

# New species of Ophiostomatales from Scolytinae and Platypodinae beetles in the Cape Floristic Region, including the discovery of the sexual state of *Raffaelea*

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**Abstract** *Olea capensis* and *Rapanea melanophloeos* are important canopy trees in South African Afromontane forests. Dying or recently dead individuals of these trees are often infested by Scolytinae and Platypodinae (Curculionidae) beetles. Fungi were isolated from the surfaces of beetles emerging from wood samples and their galleries. Based on micro-morphological and phylogenetic analyses, four fungal species in the Ophiostomatales were isolated. These

were *Sporothrix pallida* and three taxa here newly described as *Sporothrix aemulophila* sp. nov., *Raffaelea vaginata* sp. nov. and *Raffaelea rapanae* sp. nov. This study represents the first collection of *S. pallida*, a species known from many environmental samples from across the world, from Scolytinae beetles. *S. aemulophila* sp. nov. is an associate of the ambrosia beetle *Xyleborinus aemulus*. *R. rapanae* sp. nov. and *R. vaginata* sp. nov. were associated with a *Lanurgus* sp. and Platypodinae beetle, respectively, and represent the first *Raffaelea* spp. reported from the Cape Floristic Region. Of significance is that *R. vaginata* produced a sexual state analogous with those of

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*Ophiostoma seticolle* and *O. deltoideosporum* that also grouped in our analyses in *Raffaelea* s. str., to date considered an asexual genus. The morphology of the ossiform ascospores and anamorphs of the three species corresponded and the generic circumscription of *Raffaelea* is thus emended to accommodate sexual states. The two known species are provided with new combinations, namely *Raffaelea seticollis* (R.W. Davidson) Z.W. de Beer and T.A. Duong comb. nov. and *Raffaelea deltoideospora* (Olchow. and J. Reid) Z.W. de Beer and T.A. Duong comb. nov.

**Keywords** *Olea* · *Ophiostoma* · Platypodinae · *Raffaelea* · *Rapanea* · Scolytinae

## Introduction

*Olea capensis* L. ssp. *macrocarpa* (C. H. Wright) I. Verd. (Oleaceae) and *Rapanea melanophloeos* Mez. (Myrsinaceae) are important canopy trees in the evergreen Afromontane forests of Africa. In southern Africa both species are distributed from Zambia to Cape Town, South Africa (Van Wyk and Van Wyk 1997). Although both trees are renowned for their attractive wood utilised for making furniture, not much is known regarding their associated organisms. The recent description of a serious stem canker disease of *R. melanophloeos* from the Cape Floristic Region (CFR) in South Africa, caused by the fungus *Immersiporthe knoxdaviesiana* S.F. Chen, M.J. Wingf. and Jol. Roux (Chen et al. 2013), highlighted the importance of understanding such relationships and led to the research reported here.

Apart from pathogens, wood boring insects, such as bark and ambrosia beetles (Coleoptera: Curculionidae, Scolytinae and Platypodinae), may also represent a significant, but grossly understudied threat to native trees in these forests. These beetles form part of a diverse group that bore into a broad spectrum of trees, shrubs and other woody plants (Atkinson and Equihua 1986; Atkinson and Peck 1994). They are rated as some of the globally most important forest pests (Paine et al. 1997; Harrington 2005; Avtzis et al. 2012), including more than 6000 species in 225 genera (Knižek and Beaver 2004; Avtzis et al. 2012; Six 2012). They are usually found infesting conifers and hardwood trees (Avtzis et al. 2012). The Platypodinae includes about 1500 described species of ambrosia beetles (Wood and

Bright 1992; Beaver and Liu 2013), most of which are found in tropical and sub-tropical regions (Schedl 1972). There they are of economic importance, often penetrating felled timber along with their associated fungi, causing wood staining (Browne 1968). The ecology of very few Scolytinae and Platypodinae beetle species has been well-studied and these studies have mainly focussed on Northern Hemisphere systems (Batra 1967; Farrell et al. 2001). In Africa, their diversity and effects on tree hosts, especially on native trees, is virtually unknown (Jordal et al. 2001; Beaver 2005).

For part of their life cycles, scolytine bark beetles reside in galleries under bark, whereas the galleries of scolytine- and platypodinae ambrosia beetles are found within the wood of their hosts. As they penetrate these tissues they can introduce a diverse array of fungi that colonises the wood from within the gallery system (Batra 1966; Whitney 1982). Ambrosia beetles depend on their fungal symbionts for nutrition (Batra 1966), while bark beetles mainly feed on the phloem of trees, with nutritional supplementation of their diets by some of their fungal associates (Harrington 2005; Six 2003). In some cases the relationship between these two organism groups is therefore mutualistic. The fungi provide nutrients for use during reproduction and hormone synthesis, to help overcome tree defence mechanisms and to protect beetles against antagonistic fungi (Berryman 1972; Harrington 1993; Wingfield et al. 1995; Paine et al. 1997; Klepzig et al. 2001; Klepzig and Six 2004). In turn, the fungi are transported by the beetles from one host tree to the next under favourable conditions (Klepzig et al. 2001; Klepzig and Six 2004). In many cases the interaction is so strong that specialised spore-carrying structures (mycangia) have evolved in the beetles, but spores may also be transported on the exoskeletons and within the alimentary canal of some taxa (Batra 1967; Beaver 1989; Paine et al. 1997; Six 2003; Klepzig and Six 2004).

Fungi from the Ophiostomatales are some of the most common fungal associates of bark and ambrosia beetles (Barras and Perry 1972; Bridges and Moser 1983; Jacobs and Wingfield 2001; Klepzig et al. 2001; Six 2003; Klepzig and Six 2004; Harrington 2005; Alamouti et al. 2009). The order includes genera such as *Ceratocystiopsis* Upadhyay and Kendrick, *Graphilbum* H.P. Upadhyay and W.B. Kendr., *Leptographium* Lagerb. and Melin, *Ophiostoma* Syd. and *Raffaelea* Arx and Hennebert (Upadhyay 1981; Zipfel et al. 2006; De Beer and Wingfield 2013), some of which contain

important tree pathogens (Heybroek 1993; Brasier and Buck 2001; Zhou et al. 2001). Well-documented examples are those of *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier that are transported by the European elm bark beetle (*Scolytus multistriatus* Marsham) (Webber 1990) and the American elm bark beetle (*Hylurgopinus rufipes* Eichhoff). These two pathogens cause Dutch elm disease in Europe and the United States of America (Brasier 2000; Pipe et al. 2000). *Raffaelea lauricola* T.C. Harr., Fraedrich and Aghayeva, transported by the ambrosia beetle *Xyleborus glabratus* Eichhoff (Harrington et al. 2008), is responsible for Laurel wilt disease in the southeastern United States of America (Harrington et al. 2008; Ploetz et al. 2013), and in Japan, the ambrosia beetle *Platypus quercivorus* Murayama and its associate fungus *Raffaelea quercivora* Kubono et Shin. Ito were responsible for the catastrophic mass mortality and die-back of Japanese oak trees (Kubono and Ito 2002).

There are very few reports of members of the Ophiostomatales associated with subcortical beetles on native trees in southern Africa. Inadequate information on these insects and their fungal symbionts hinders our understanding of the death of some of these native tree species. The recent discovery of some ambrosia beetles and their associated fungi causing the decline of the native *Euphorbia ingens* E. Meyer: Boissier in South Africa (Roux et al. 2009; Van der Linde et al. 2012) highlighted the need for more research on these beetles and their effects on other native tree taxa in South Africa. During recent surveys of native trees in South Africa, many individuals of *R. melanophloeos* and *O. capensis* ssp. *macrocarpa* were found to be infested by bark and ambrosia beetles. These beetles were most often associated with dying or recently dead trees. Given the gap in our knowledge, and the importance of beetle-associated Ophiostomatales to tree health, this study aimed to document the fungi associated with the bark and ambrosia beetles from these two tree taxa, focussing specifically on Afromontane forests of the CFR of South Africa.

## Materials and methods

### Beetle sampling

Sampling was conducted in forests adjacent to the Harold Porter National Botanical Garden (S 34°20'54"

E 18° 55'31"), Gouna Forest (S 33°57'3" E 23°2'10") and Goudveld Forest (S 33°54'44" E 23°0'10") in the Western Cape Province of South Africa between 2010 and 2012. Bark and wood samples were collected from dying or recently dead *R. melanophloeos* and *O. capensis* ssp. *macrocarpa* trees that showed signs of subcortical beetle activity. The collected plant material was transferred to the laboratory and placed in insect emergence cages constructed from sealed cardboard boxes (ca. 49 × 49 × 32.6 cm) fitted with two transparent plastic bottles (5.7 cm diameter) on one side. Light penetrating through the plastic containers attracted emerging beetles, causing them to accumulate in the bottles, from where they could easily be collected. Emergence cages were maintained at room temperature and inspected every 2–3 days for a period of 50 days. Individual beetles were aseptically placed in sterile vials, grouped into morpho-species, their numbers recorded and stored at 4 °C until further use (but not longer than 5 days). Reference collections of all beetle taxa collected in this study were stored in 70 % ethanol. Reference material was sent for identification by expert taxonomists and is maintained in the Insect Collection of Stellenbosch University (USEC), Stellenbosch, South Africa.

### Fungal isolation from beetles and beetle galleries

Wood from emergence cages was examined for the presence of ascocarps of members of the Ophiostomatales. When present, ascospores were removed from the apices of ascomatal necks with a dissecting needle and transferred to 2 % Malt Extract Agar (MEA; Biolab, Midrand, South Africa) amended with 0.05 g/L cycloheximide (Harrington 1981). Depending on availability, 3 to 50 beetle individuals per beetle morpho-species were placed in eppendorf tubes containing 0.2 mL ddH<sub>2</sub>O and vigorously shaken for 1 min on a vortex mixer. A subset of individuals were also crushed and homogenized in 0.2 mL ddH<sub>2</sub>O. After washing, the arthropods were removed (when not crushed) and stored in 70 % ethanol for later identification. Water suspensions were spread on Petri dishes containing 2 % MEA, streptomycin sulphate (0.04 g/L) and cycloheximide (0.05 g/L). When present, a single colony of all the suspected Ophiostomatales morpho-types growing on these primary isolation plates was chosen at random and purified as representatives of the different fungal taxa. Once

purified, all 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. Type material has been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM) and the fungal collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

### Morphological characterisation

Where possible/available, fruiting structures (ascocarps with ascospores) of the Ophiostomatales taxa found in beetle galleries were collected and mounted in clear lactophenol on microscope slides. These were studied using a Leica EZ4 microscope (Leica Microsystems (Schweiz) AG, Taiwan). For isolates chosen as the types of new species, 25 measurements of all characteristic morphologically and taxonomically useful structures were made and the means ( $\pm$ standard deviation) were calculated. A Leica digital camera mounted on the microscope was used to take photographs.

### DNA extraction, amplification and sequencing

Three or more isolates from each beetle species and their galleries, representing each fungal morpho-type, were selected for DNA sequence comparisons (Table 1). Using a sterile scalpel, fungal mycelium was harvested from 2 week old, actively growing colonies on MEA. Following the manufacturer's instructions, genomic DNA was extracted using a Sigma-Aldrich™ plant extraction kit (USA). Preliminary placement of fungi into genera was done using nuclear ribosomal internal transcribed spacer region (ITS1, ITS2) and the 5.8S gene region of the rDNA (ITS), and according to this, amplification of other gene regions for specific clades were chosen based on previous studies (De Beer et al. 2013). Primers ITS1-f (Gardes and Bruns 1993) and ITS4 (White et al. 1990) were used to amplify ITS. In cases where amplification was difficult, ITS1-f was replaced with ITS1 (White et al. 1990). For DNA amplification, the reaction mixture for the Polymerase Chain Reaction (PCR) was 25  $\mu$ L, consisting of 11.3  $\mu$ L ddH<sub>2</sub>O, 2  $\mu$ L DNA,

2.5  $\mu$ L 10X PCR reaction buffer (with MgCl<sub>2</sub>), 2.5  $\mu$ L deoxyribonucleotide triphosphate mix (dNTP) (5 mM), 5  $\mu$ L GC rich solution (Roche Applied Science, Mannheim, Germany), 0.5  $\mu$ L of each primer (10 mM), 0.5  $\mu$ L extra MgCl<sub>2</sub> (25 mM) and 0.2  $\mu$ L FastStart Taq DNA Polymerase (Roche Applied Science, Mannheim, Germany).

For the relevant fungal morpho-types, primers T10 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) were used to amplify part of the Beta-tubulin (Bt) gene region and where amplification did not work, Bt2a (Glass and Donaldson 1995) replaced T10. CL2F and CL2R (Duong et al. 2012) were used for PCR for part of the Calmodulin (CAL) gene, and where amplification was difficult, either CL2R2 (Duong et al. 2012) was used in place of CL2R or a combination of the primers CL3F and CL3R was used. For the ribosomal LSU and SSU, primer pairs LR0R and LR5 (Vilgalys and Hester 1990), and NS1 and NS4 (White et al. 1990), were respectively used. The same PCR volumes used for ITS primers were used for DNA amplification of the other gene regions, except that no GC solution was added. PCR conditions comprised of an initial denaturation for 5 min at 95 °C, followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C and 60 s elongation at 72 °C. A final elongation step at 72 °C for 8 min was performed before termination of the PCR process. In some cases the annealing temperature was lowered to 50 °C to enable amplification. The same PCR protocol was used for all markers and sequencing of fragments followed protocols described in Roets et al. (2006).

Both DNA strands were sequenced using the same primers as those used for PCR amplifications. The CLC Genomics Workbench software package (CLC Bio, Cambridge, Massachusetts) was used to edit and combine sequences from both strands for each isolate to create consensus sequences. Sequences generated in this study (Table 1) were compared to sequences available from the GenBank sequence database (<http://www.ncbi.nlm.nih.gov>) and to those from the relevant clades identified in previous studies (De Beer et al. 2013). Alignment of sequences with those downloaded from GenBank was done online using MAFFT 6 (Katoh and Toh 2008) for each dataset. All datasets created were subjected to Bayesian inference (BI) and maximum likelihood (ML) analyses. ML analyses were performed with the online version of PhyML 3.0 (Guindon and Gascuel 2003, [!\[\]\(aa53ad6fea213b8b2226d3077e30533a\_img.jpg\) Springer](http://www.atgc-</a></p>
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**Table 1** Culture collection details and GenBank accession numbers for strains of ophiostomatoid fungi isolated from bark beetles in this study

Fungal species	Isolate numbers <sup>a</sup>	LSU	ITS	βT	CAL	Host plant	Arthropod sp.	Collection site
<i>Raffaelea rapaneae</i>	CMW 40358	KT182934	KT192597			<i>Rapanea melanophloeos</i>	Platypodinae sp.	Goudveld Forest
	CMW 40359	KT182935	KT192601			<i>R. melanophloeos</i>	Platypodinae sp.	Goudveld Forest
	CMW 40357	KT182930	KT192596			<i>R. melanophloeos</i>	Platypodinae sp.	Goudveld Forest
<i>R. vaginata</i>	CMW 40363	KT182931	KT192598			<i>Olea capensis</i>	<i>Lanurgus</i> sp. 1	Goudveld Forest
	CMW 40364		KT192599			<i>O. capensis</i>	<i>Lanurgus</i> sp. 1	Goudveld Forest
	CMW 40365	KT182932	KT192602			<i>O. capensis</i>	<i>Lanurgus</i> sp. 1	Goudveld Forest
	CMW 40366	KT182933	KT192600			<i>O. capensis</i>	<i>Lanurgus</i> sp. 1	Goudveld Forest
<i>Sporothrix aemulophila</i>	CMW 40381		KT192603	KT192607		<i>R. melanophloeos</i>	<i>X. aemulus</i>	Harold Porter National Botanical Garden
	CMW 40382			KT192608	KT192613	<i>R. melanophloeos</i>	<i>X. aemulus</i>	Harold Porter National Botanical Garden
	CMW 40383			KT192609		<i>R. melanophloeos</i>	<i>X. aemulus</i>	Harold Porter National Botanical Garden
<i>S. pallida</i>	CMW 40330		KT192606	KT192610		<i>O. capensis</i>	<i>Ctonoxylon</i> sp. 1	Gouldveld Forest
	CMW 40331		KT192605	KT192611		<i>O. capensis</i>	<i>Lanurgus</i> sp. 1	Groenkop Forest
	CMW 40332		KT192604	KT192612	KT192614	<i>O. capensis</i>	<i>Lanurgus</i> sp. 1	Goudveld Forest

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

[montpellier.fr/phyml](http://montpellier.fr/phyml)). jModelTest 0.1.1 (Posada 2008) was used to determine the best fit substitution models using Akaike information criteria (Akaike 1974) and confidence support values for nodes were estimated using 1000 replication bootstrap analyses. Bayesian inference analyses were performed based on a Markov chain Monte Carlo approach (MCMC) using MrBayes v. 3.2 (Ronquist et al. 2012). MrBayes’s rjMCMC mixture model was used. Starting from a random tree, two Markov chains independent of each

other were run simultaneously for 10 million generations. At every 2000th generation, trees were sampled and burn-in trees (first 2,500,000 generations) were discarded. The remaining trees were pooled into a 50 % majority rule consensus tree.

#### Growth in culture

The optimal growth conditions of suspected new species were determined using designated type

cultures for taxa identified as undescribed. A disk of agar (10 mm diam) that was covered with mycelium was removed from the edges of actively growing 1 week old cultures. They were placed mycelial side downward facing in the centres of 90 mm Petri dishes containing 20 mL MEA and incubated for 10 days in the dark at different temperatures ranging from 5 °C to 35 °C at intervals of 5 °C. For each temperature interval, five replicates were used. Two colony diameters perpendicular to each other were taken after incubation and averaged for each isolate and means and standard deviations for each fungal species were calculated.

## Results

### Beetles

Three Scolytinae and one Platypodinae species were collected from *R. melanophloeos* and *O. capensis* ssp. *macrocarpa* trees. All were associated with members of the Ophiostomatales. *Lanurgus* sp. 1 Eggers and *Ctonoxylon* sp. 1 Hagedorn (Scolytinae) were both collected from *O. capensis* ssp. *macrocarpa*, while *Xyleborinus aemulus* Wollaston (Scolytinae) and the Platypodinae species were collected only from *R. melanophloeos*. About 2000 individuals of *Lanurgus* sp. 1, 300 individuals of *Ctonoxylon* sp. 1, six individuals of *X. aemulus* and only three individuals of the Platypodinae species were collected.

### Fungal isolates and morphological characterisation

A total of 38 fungal isolates that morphologically resembled members of the Ophiostomatales were obtained from the four beetle species. Of these, 15 were isolated from *Lanurgus* sp. 1, 10 from *Ctonoxylon* sp. 1, eight from *X. aemulus* and five from the Platypodinae species. Based on micro-morphology and colony characteristics, these isolates were grouped into four different operational taxonomic units (OTUs). The first OTU came from *Lanurgus* sp. 1 from *O. capensis* ssp. *macrocarpa* and had a reduced hyalorhinocladia-like anamorph and ascospores with sheathed, ossiform ascospores (Type E) (De Beer and Wingfield 2013). The Platypodinae beetle from *R. melanophloeos* was associated with the second OTU

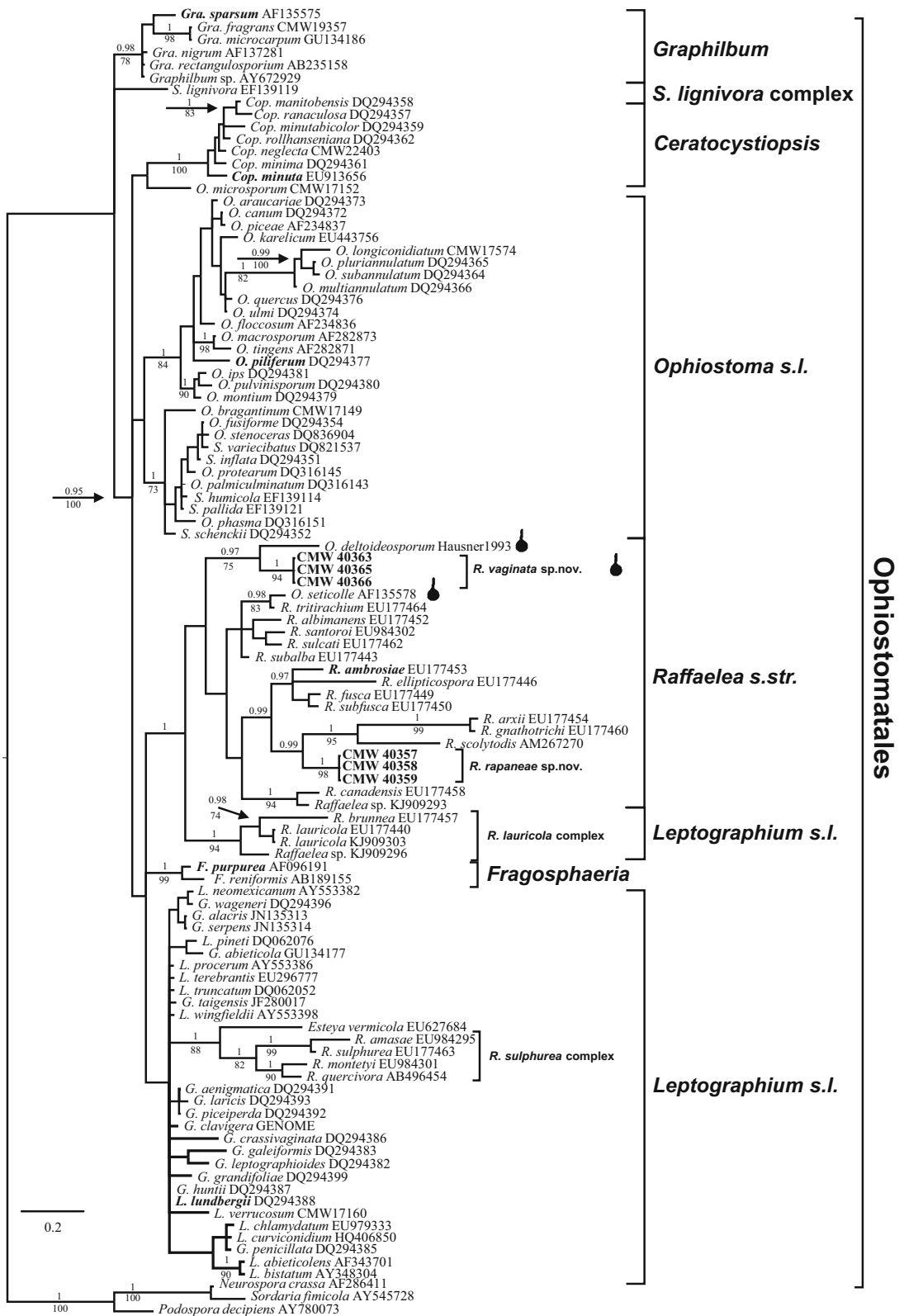
**Fig. 1** Bayesian Inference consensus tree (LSU data) of taxa related to species isolated in the present study. Values above nodes indicate posterior probabilities (>0.95) obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates, >70) obtained from Maximum Likelihood analysis. Isolates in *bold* were collected in this study and/or present type species for the various genera. Other isolates (GenBank accession numbers and isolate numbers shown (when available)) were selected for comparative purposes. ♣ = tele-morph known

with only an asexual state with inflated conidia resembling those produced by species in the genus *Raffaelea*. *Xyleborinus aemulus*, collected from *R. melanophloeos*, was associated with a third OTU with a sporothrix-like asexual state and ascospores (Type A) resembling those of species in the *S. schenckii*-*O. stenoceras*- complex (De Beer and Wingfield 2013). The fourth OTU also had an asexual state that resembled those of *Sporothrix* spp. and was associated with both *Lanurgus* sp. 1 and *Ctonoxylon* sp. 1 collected from *O. capensis* ssp. *macrocarpa*.

### Phylogenetic analyses

ITS data confirmed the ordinal placement of all four of the collected fungal OTUs in the Ophiostomatales. The phylogenetic placement of the OTUs with the hyalorhinocladia- and raffaelea-like asexual states was determined using LSU and ITS data (ITS dat set I). The aligned LSU data set included 106 taxa and 750 characters, while the ITS data set I included 53 taxa and 701 characters. Statistical values obtained from analyses of the different data sets and the substitution models chosen are presented in Supplementary resource 1. Both the LSU (Fig. 1) and ITS (Fig. 2) data showed that these two taxa grouped in *Raffaelea* s. str., but distinct from all the known species in this group. Hereafter these two taxa are referred to as *R. vaginata* sp. nov. and *R. rapanaeae* sp. nov.

ITS data of the remaining two OTUs with sporothrix-like anamorphs suggested that these two taxa belonged to the *S. schenckii*-*O. stenoceras* complex in *Ophiostoma* s.l. as defined by De Beer and Wingfield (2013). Since ITS data between the different genera in the Ophiostomatales vary too much to reliably align in a single data set (De Beer and Wingfield 2013), the ITS data of these two OTUs were not included in the same data set as the *Raffaelea* spp. They were instead included in a second ITS data set



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(ITS data set II) that had reference sequences of species in the *S. schenckii*-*O. stenoceras* complex (data not shown). The phylogenetic placement of these two OTUs were further investigated using  $\beta$ T and CAL sequences. The aligned ITS data set II consisted of 54 taxa and 723 characters, the  $\beta$ T data set consisted of 45 taxa and 276 characters, while the CAL data set contained 38 taxa and 684 characters. Analyses of ITS (data set II, tree not shown),  $\beta$ T (Fig. 3) and CAL (Supplementary resource 2) data showed that the sporothrix-like OTU associated with *Lanurgus* sp. 1 and *Ctonoxylon* sp. 1 on *O. capensis* ssp. *macrocarpa* grouped in a clade with strong support that included the ex-type isolates of *Sporothrix pallida* (Tubaki) Matsushima and its synonyms *S. albicans* S.B. Saksena and *S. nivea* Kreisel and F. Schauer (De Meyer et al. 2008). The other sporothrix-like OTU from *Xyleborinus aemulus* on *R. melanophloeos* (hereafter referred to as: *Sporothrix aemulophila* sp. nov.) grouped strongly as distinct, but sister to *Ophiostoma candidum* Kamgan-Nkuek., Jol. Roux and Z. W. de Beer, using the same three markers (Fig. 3 and Supplementary resource 2). All data matrices and Bayesian 50 % majority-rule consensus trees are available from Treebase (<http://treebase.org/treebase-web/search/study/summary.html?id=17791>).

### Growth in culture

After 10 days of growth in the dark, *R. vaginata* sp. nov. grew optimally at 30 °C, with a mean colony diameter of 23.8 mm ( $\pm 1.3$ ). *R. rapanae* sp. nov. had a colony diameter of 21 mm ( $\pm 0.95$ ) when grown at its optimal temperature of 20 °C and *S. aemulophila* sp. nov. had a mean colony diameter of 24.2 mm ( $\pm 1.4$ ) at an optimal temperature of 30 °C.

### Taxonomy

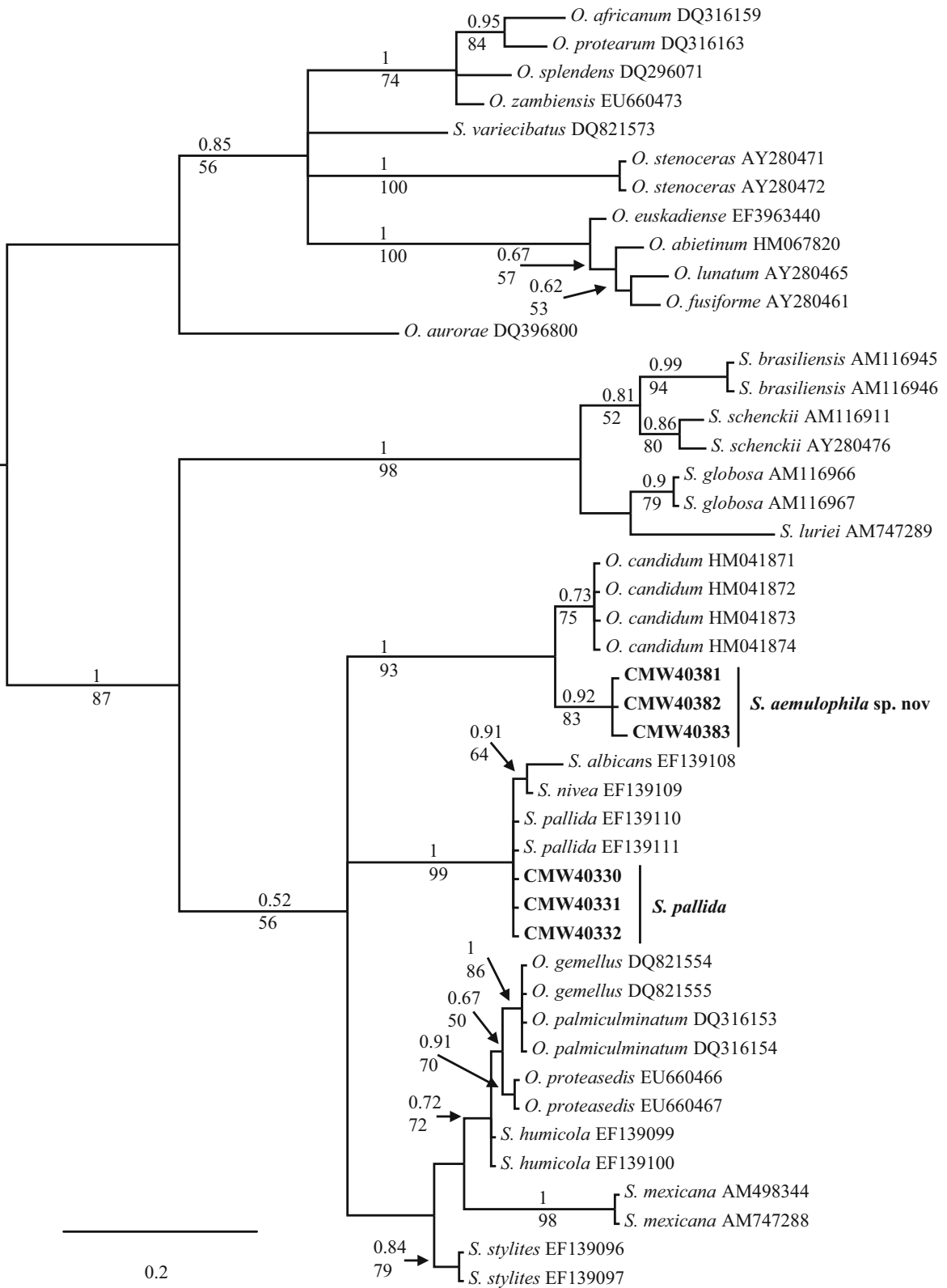
Based on micro-morphological and phylogenetic analyses three of the four collected taxa are recognised as distinct and undescribed taxa. Two of the novel species grouped in the genus *Raffaelea* and are described here. One of these presented a sexual state in culture and thus the generic description of *Raffaelea* is emended below, as the genus to date only accommodated species known from the asexual states. Furthermore, our analyses confirmed the placement of two *Ophiostoma* spp. with sexual states in the genus

*Raffaelea*, and new combinations are provided for these two taxa. The other two taxa isolated in the present study belonged to the *S. schenckii*-*O. stenoceras* complex. The one was identified as *S. pallida* and the other as a new species, described below.

***Raffaelea*** Arx and Hennebert, Mycopathol. Mycol. Appl. 25: 310.1965. emend. Z.W. de Beer and T.A. Duong. [type species *R. ambrosiae* Arx and Hennebert, Mycopathol. Mycol. Appl. 25: 310.1965].

*Ascocarps* light brown to black, bases globose to subglobose; necks dark brown to black, slightly bulbous to cylindrical to tapered, straight or slightly curved; ostiolar hyphae present or absent. *Asci* evanescent. *Ascospores* hyaline, aseptate, cylindrical to oblong, surrounded with a hyaline, gelatinous, ossiform to rectangular sheath. *Conidiophores* single to aggregated in sporodochia, hyaline, unbranched or sparingly branched, one-celled to septate, producing conidia holoblastically. *Conidiogenous cells* proliferating percurrently or sympodially, leaving denticles, inconspicuous scars, or annellations. *Conidia* hyaline, elliptical to ovoid to globose, sometimes T- or Y-shaped, succession schizolytic, producing yeast-like growth through budding. Tolerating cycloheximide in culture. Phylogenetically classified in the Ophiostomatales. Associated with ambrosia beetles.

*Notes* Harrington et al. (2010) emended the generic description of *Raffaelea* to accommodate asexual ambrosial species in the Ophiostomatales. De Beer and Wingfield (2013) and Dreaden et al. (2014) showed that *Raffaelea* is not monophyletic as was suggested by Harrington et al. (2010). De Beer and Wingfield (2013) also showed that two *Ophiostoma* species with similar sexual states, *O. seticolle* (R.W. Davidson) de Hoog and Scheffer, and *O. deltoideosporum* (Olchow. and J. Reid) Hausner, J. Reid and Klassen, grouped among *Raffaelea* s. str. isolates, but were hesitant to include these species in *Raffaelea*. The discovery in the present study of yet another species that produces sexual structures with ascospores similar to those of *O. seticolle* and *O. deltoideosporum*, confirmed that the genus *Raffaelea* does not only consist of asexual species as was previously considered (Cassar and Blackwell 1996; Alamouti et al. 2009; Harrington et al. 2010). Based on the one fungus one name principles recently adopted into the International Code of Nomenclature for Algae, Fungi and Plants (Hawksworth 2011; Hawksworth et al. 2011), we here emend the generic



◀ **Fig. 3** Bayesian Inference consensus tree ( $\beta$ t data) of members of the *S. schenckii*-*O. stenoceras* complex (De Beer et al. 2013). 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

diagnosis of *Raffaelea*. We largely followed the emended description for *Raffaelea* by Harrington et al. (2010) for the asexual forms produced in the genus, and based the diagnosis of the sexual states on the protologues of three species producing sexual states. We furthermore provide new combinations for *O. seticolle* and *O. deltoideosporum* in *Raffaelea*.

***Raffaelea seticollis*** (R.W. Davidson) Z.W. de Beer and T.A. Duong comb. nov., MycoBank: MB813625.

≡ *Ceratocystis seticollis* R.W. Davidson, Mycopath. Mycol. Appl. 28: 282. 1966. (basionym).

≡ *Ophiostoma seticolle* (R.W. Davidson) de Hoog and Scheffer, Mycologia 76: 297. 1984.

**Descriptions:** Davidison (1966, pp. 282–285, Figs. 4–5); Upadhyay (1981, p. 83, Figs. 286–289).

**Notes:** This species was originally isolated from ambrosia galleries in a hemlock (*Tsuga canadensis*) stump in New York State (Davidison 1966) and, to our knowledge, never reported again. The LSU sequence produced for the ex-type isolate of this species by Hausner and Reid (2003), placed this species peripheral to other *Ophiostoma* spp. but no *Raffaelea* spp. were included in their analyses. In the more comprehensive data set analysed by De Beer and Wingfield (2013), the species grouped in *Raffaelea s. str.* However, they treated the species as of uncertain generic status (De Beer and Wingfield 2013; De Beer et al. 2013) because an ITS sequence produced by Jacobs et al. (2003) grouped in the *S. schenckii*-*O. stenoceras* complex. The latter sequence was clearly erroneous as the morphology of the sexual and asexual states, as well as its ambrosial habit, supports its placement in *Raffaelea s. str.* as confirmed by the LSU sequence of Hausner and Reid (2003). Attempts to revive the ex-holotype isolate of *C. seticolle* for more DNA sequences were not successful, so the only reliable and usable sequence for this species is the one produced by Hausner and Reid (2003).

***Raffaelea deltoideospora*** (Olchow. and J. Reid) Z.W. de Beer and T.A. Duong comb. nov., MycoBank: MB813626.

≡ *Ceratocystis deltoideospora* Olchow. and J. Reid, Can. J. Bot. 52: 1691. 1974. (basionym).

≡ *Ophiostoma deltoideosporum* (Olchow. and J. Reid) Hausner, J. Reid and Klassen, Can. J. Bot. 71: 1264. 1993.

**Descriptions** Olchowecki and Reid (1974, pp. 1691–1692, Figs. 132–141); Upadhyay (1981, p. 77–78, Figs. 253–256).

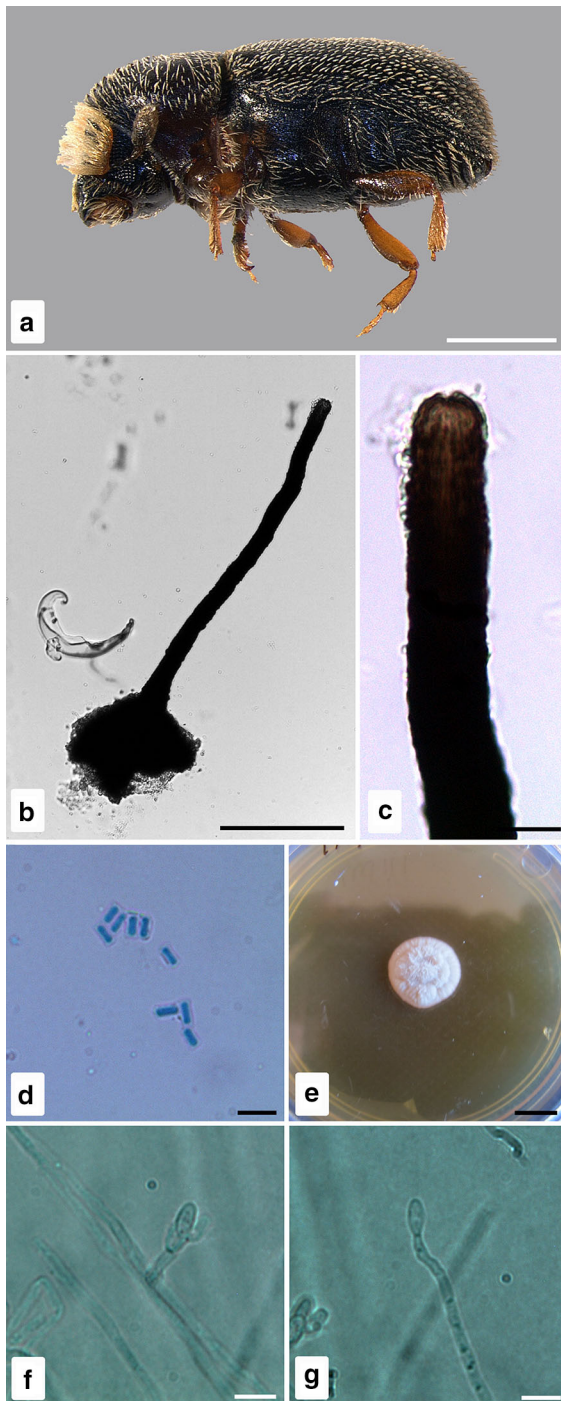
**Notes** This species was originally isolated from *Pinus banksiana*, *P. resinosa* and *P. strobus* wood in Manitoba and Ontario, Canada (Olchowecki and Reid 1974), and subsequently from cerambycid pupal chambers in *P. resinosa* and *P. banksiana* in Wisconsin, USA (Wingfield 1987). However, the latter identification was based on morphology only. The LSU sequence of this species grouped peripheral to *Ophiostoma* s.l. and *Leptographium* s.l. in the analyses of Hausner et al. (1993) and Hafez et al. (2013), but no *Raffaelea* spp. were included in those studies. The analyses of De Beer and Wingfield (2013) and our results (Fig. 1) showed that this species groups in *Raffaelea s. str.* based on LSU data. Similarly, based on the study of De Beer and Wingfield (2013) and on our results (Fig. 2), the ITS sequence (Mullineux and Hausner 2009) grouped in a lineage with *R. canadensis*, the only species of *Raffaelea s. str.* for which ITS sequence data is currently available. The hyalorhinocladia-like conidiogenesis and ossiform ascospores resemble those of both *R. seticollis* and *R. vaginata*, and validate its treatment in *Raffaelea s. str.*

***Raffaelea vaginata*** T. Musvuugwa, Z.W. de Beer, L.L. Dreyer and F. Roets sp. nov. Figure 4b–g.

MycoBank: MB812910.

**Etymology:** The epithet *vaginata* is derived the Latin vagina, meaning a sheath or covering, and refers to the sheathed ascospores produced by this species.

**Ascomata** superficial on the host substrate, bases globose, black, with no hyphal ornamentation, (85–) 92–142 (–149)  $\mu$ m diam, necks black, (308–) 311–692 (–698) long, (19–) 24–40 (–45)  $\mu$ m wide at base, (4–) 10–18 (–23)  $\mu$ m wide at the apex, osteolar hyphae absent (Fig. 4b, c). **Asci** evanescent. **Ascospores** cylindrical, aseptate, hyaline, sheaths present, (1.5–) 2.3–3.4 (–3.8)  $\times$  (0.3–) 0.8–1.5 (–1.9)  $\mu$ m (Fig. 4d), accumulating in a gelatinous droplet at the tip of the neck, becoming white to cream colored when dry. **Colonies** white to cream coloured on MEA. Odourless, circular with entire edge and rough surface (Fig. 4e). Colony diameter reaching 23.8 mm ( $\pm$ 1.3) after



**Fig. 4** **a** Beetle associate (*Lanurgus* sp. 1) of *Raffaelea vaginata* sp. nov. (scale bar 1 mm). Micrographs of *R. vaginata* sp. nov.: **b** Perithecium removed from the gallery of *Lanurgus* sp. 1 in wood of *Olea capensis* ssp. *macrocarpa* (scale bar 100 µm); **c** Tip of perithecial neck (scale bar 10 µm); **d** Ascospores (scale bar 5 µm); **e** Two-week-old colony on MEA (scale bar 1 cm); **f–g** Conidiophores with conidia (scale bars 5 µm)

apex of conidiophores, hyaline, (Fig. 4f), *Conidia* produced sympodially, aseptate, hyaline, thick walled, obovate, (3–) 3.5–4.9 (–5.4) µm long, (0.4–) 0.7–1.1 (–1.8) µm wide (Fig. 4f–g). *Conidia* produced singly, directly from hyphae (Fig. 4f–g).

**Substrate:** Isolated from *Lanurgus* sp. 1 (Scolytinae) (Fig. 4a) and its host plant *Olea capensis* ssp. *macrocarpa*.

**Distribution:** South Africa, Western Cape Province

**Specimens examined:** South Africa, Western Cape Province, Gouldveld and Gourn forest. Isolated from *Lanurgus* sp. associated with *Olea capensis*, October 2011, T. Musvuugwa, holotype PREM 61274, culture ex-holotype CMW 40365 = CBS 140086, paratype PREM 61273, culture ex-paratype CMW 40363, paratype culture ex-paratype CMW 40366.

***Raffaelea rapaneae*** T. Musvuugwa, Z.W. de Beer, L.L. Dreyer and F. Roets sp. nov. Figure 5b–e.

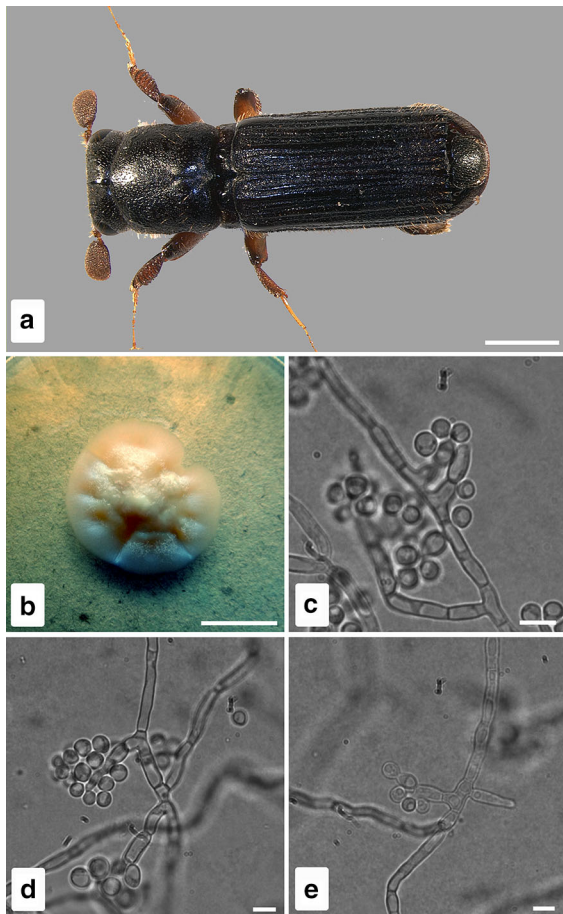
Mycobank: MB812911.

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

**Ascomata** not observed. **Colonies** cream turning blackish on MEA, odourless, surface tough and leathery, which wrinkles and forms cracks under the wrinkled area (Fig. 5b). Colony diameter reaching 21 mm after 10 days on MEA at 20 °C. Optimal growth at 20 °C. No growth below 10 °C or above 25 °C, cream in the center and black along the edges, somewhat circular with rough surface and edge. **Conidiophores** hyaline, tapering towards the tips, with segmented appearance, (9.9–) 10.6–15.5 (–17.5) µm long, (1–) 1.5–1.7 (–2.1) µm wide (Fig. 5c–e), **Conidiogenous** cells phialidic, arising from tips of conidiophores, hyaline (Fig. 5d–e), **Conidia** aseptate, hyaline, turning brown when mature, thick walled, spherical, form in masses, (1.6–) 2.3–3.5 (–4.3) µm long, (1.7–) 2.4–3.4 (–3.9) µm wide (Fig. 5c–e).

**Substrate:** Isolated from a Platypodinae beetle (Fig. 5a) collected from the wood of *Rapanea melanophloeos*.

10 days of growth on MEA at optimal growth temperature of 30 °C in the dark. No growth below 10 °C or above 35 °C. **Conidiophores** hyaline, (6.6–) 7.1–8.9 (–9.2) µm long, (0.3–) 0.7–1.1 (–1.5) µm wide (Fig. 4f), **Conidiogenous** cells forming directly from



**Fig. 5** **a** Beetle associate (Platypodinae sp. 1) of *Raffaelea rapanae* sp. nov. (scale bar 1 mm). Micrographs of *R. rapanae* sp. nov.; **b** Two-week-old colony on MEA (scale bar 1 cm); **c–e** Conidiophores with conidia (scale bars 5 µm)

**Distribution:** South Africa, Western Cape Province  
**Specimens examined:** South Africa, Western Cape Province, Gourna forest. Isolated from Platypodinae sp. associated with *Rapanea melanophloeos*, February 2012, T. Musvuugwa, holotype PREM 61271, culture ex-holotype CMW 40357 = CBS 140084, paratype PREM 61272, culture ex-paratype CMW 40359 = CBS 140085, paratype culture ex-paratype CMW 40357.

***Sporothrix aemulophila*** T. Musvuugwa, Z.W. de Beer, L.L. Dreyer and F. Roets sp. nov. Figure 6b–g. Mycobank: MB812912.

**Etymology:** The epithet *aemulophila* refers to the apparent close association between this species and the beetle *Xyleborinus aemulus*.

**Ascomata** embedded in and superficial on the host substrate, bases black, globose, (72–) 81–162 (–170)

µm diam, hyphal ornamentation absent. **Ascomatal** necks black, (270–) 278–743 (–750) µm long, (18–) 22–45 (–51) µm wide at base, (4–) 7–11 (–13) µm wide at the tip (Fig. 6b), osteolar hyphae present, slightly curved, hyaline, (9–) 13–20 (–24) µm long (Fig. 6c). **Asci** evanescent. **Ascospores** allantoid, aseptate, hyaline, sheaths absent, (2–) 2.9–4.5 (–5.5) × (0.2–) 0.9–1.3 (–1.8) µm (Fig. 6d), accumulating in a sticky droplet at the tip of the neck, becoming whitish when dry. **Colonies** of sporothrix-like asexual state whitish in colour on MEA, odourless, circular with entire edge and fluffy (Fig. 6e). Colony diameter reaching 24.2 mm (±1.4) after 10 days of growth on MEA at an optimum growth temperature of 30 °C in the dark. No growth below 5 °C or above 35 °C. **Conidiophores** hyaline, tapering towards the tip, (3.8–) 4.5–8.2 (–8.7) µm long, (0.3–) 0.8–1.2 (–1.8) µm wide (Fig. 6f), **Conidiogenous** cells forming directly from tips of conidiophores, hyaline, prominent denticles present (0.3–) 0.6–1.4 (–1.7) µm long (Fig. 6f). **Conidia** produced sympodially, hyaline, aseptate, oblong, holoblastic, (3.2–) 3.8–7.2 (–7.6) µm long, (0.4–) 0.9–1.5 (–1.9) µm wide (Fig. 6g).

**Substrate:** Isolated from *Xyleborinus aemulus* (Fig. 6a) and its galleries on *Rapanea melanophloeos*

**Distribution:** South Africa, Western Cape Province

**Specimens examined:** South Africa, Western Cape Province, Harold Porter National Botanical Garden. Isolated from *Xyleborinus aemulus* associated with *Rapanea melanophloeos* April, 2010, T. Musvuugwa, holotype PREM 61275, culture ex-holotype CMW 40381 = CBS 140087, paratype PREM 61276, culture ex-paratype CMW 40382 = CBS 140088, paratype culture ex-paratype CMW 40383.

## Discussion

This represents the first study on the Ophiostomatales associated with sub-cortical beetles from trees native to the Afromontane forests of the CFR, South Africa. Previous studies focussed on either those taxa associated with wounds on native trees (e.g. Kamgan Nkuekam et al. 2008) or on the beetle associates of exotic tree taxa (e.g. Zhou et al. 2001). Three species from two genera were newly described here from only four beetle species occurring on two tree species. Considering the large diversity of native plants, bark beetles and ambrosia beetles associated with these

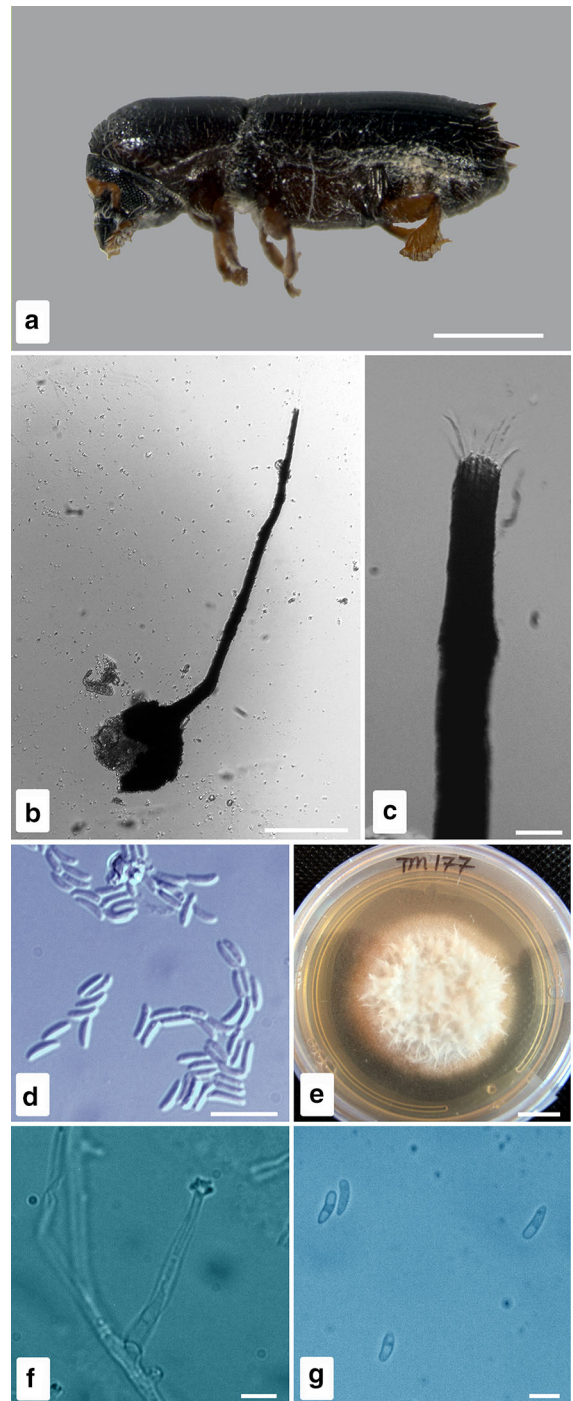
**Fig. 6** **a** Beetle associate (*Xyleborinus aemulus*) of *Sporothrix aemulophila* sp. nov. (scale bar 1 mm). Micrographs of *S. aemulophila* sp. nov.; **b** Perithecium removed from bark of *Rapanea melanophloeos* colonised by *Xyleborinus aemulus* (scale bar 100  $\mu$ m); **c** Tip of perithecial neck showing ostiolar hyphae (scale bar 10  $\mu$ m); **d** Ascospores (scale bar 10  $\mu$ m); **e** Two-week-old colony on MEA (scale bar 1 cm); **f** Conidiophore with conidiogenous cells showing denticles (scale bar 2  $\mu$ m); **g** Conidia (scale bar 5  $\mu$ m)

forests, it is reasonable to assume that many taxa still await discovery. This study therefore forms a platform for future studies on the diversity, evolution and ecology of these important fungi. In addition, this study placed three species with sexual states in *Raffaelea*, which had previously contained only asexual taxa.

The two *Raffaelea* species collected in this study group with members of the *Raffaelea* s. str. clade (De Beer and Wingfield 2013). Although the two species group in different clades within *Raffaelea* s. str., they are closely related. Most of the species in *Raffaelea* s. str. are associated with ambrosia beetles (Harrington et al. 2010; De Beer and Wingfield 2013). This was also found to be the case with the two *Raffaelea* species collected here. Morphologically they generally exhibit hyalorhinocladia-like asexual states and reduced conidiogenous structures, often producing pigmented conidia (De Beer et al. 2013). Only a few other *Raffaelea* species have been reported from South Africa. These include *R. albimanens* D.B. Scott and J.W. du Toit, which was associated with the ambrosia beetle *P. externedentatus* Fairm. from *Ficus sycamorus* L. in the Dukuduku Forest in the KwaZulu-Natal Province, and *R. arxii* D.B. Scott and J.W. du Toit, associated with *Xyleborus torquatus* Eichh. and collected from *Schefflera* (= *Cussonia*) *umbellifera* (Sond.) Baill. (Scott and Du Toit 1970).

*R. vaginata* was associated with the ambrosia beetle *Lanurgus* sp. 1 from *Olea capensis* ssp. *macrocarpa* and represents the first report of any Ophiostomatales from both the beetle and host tree. Although part of *Raffaelea* s. str., it is phylogenetically and morphologically very distinct from the other species in the genus. Although most of the species in the *Raffaelea* s. str. clade are associated with ambrosia beetles, *R. vaginata* is the first species in the group that is associated with a beetle in the genus *Lanurgus*.

*R. rapanaeae* was isolated from a Platypodinae beetle associated with *R. melanophloeos*.



Phylogenetically it clearly belongs in the genus, but is also very distinct from all the other known species. Similar to the beetle associate of *R. rapanaeae*, the associates of many other *Raffaelea* species belong to the subfamily Platypodinae. *Raffaelea ambrosiae* Arx

and Hennebert, for example, is associated with *Platypus cylindricus* Fab. and *P. compositus* Say (Baker 1963, Batra 1967), *R. santoroi* Guerrero is associated with *P. sulcatus* Chap. (Guerrero 1966), and the South African *R. albimanens* is associated with *P. externedentatus* (Scott and du Toit 1970).

Both the *Sporothrix* species collected in this study belong to the *S. schenckii*-*O. stenoceras* complex, which forms part of a larger contingent, *Ophiostoma* sensu lato (De Beer and Wingfield 2013). Besides the human pathogens, such as *S. schenckii* and *S. luriei*, the *S. schenckii*-*O. stenoceras* complex mostly contains species that are associated with environmental samples such as soil (De Meyer et al. 2008). However, some species in this complex are arthropod-associated. Examples include nine species collected from *Protea* infructescences in Southern Africa, two of which have been confirmed to be specifically associated with mites (Roets et al. 2007, 2009). Other species that have been isolated infrequently from bark beetles infesting *Pinus* spp. include *O. aurorae* X.D. Zhou and M.J. Wingf. (Zhou et al. 2006), *O. cantabriense* P. Romón, Z.W. De Beer and M.J. Wingf. (Romón et al. 2014a), *O. euskadiense* P. Romón, Z.W. De Beer and M.J. Wingf. (Romón et al. 2014b), *O. abietinum* Marm. and Butin and *O. abietinum*-like isolates (Zhou et al. 2004, 2006; Linnakoski et al., 2010; Taerum et al. 2013). Given the number of South African species in this complex, it has been suggested that southern Africa may be the centre of diversity for this group of fungi (Kamgan Nkuekam et al. 2012).

Our isolates of *S. pallida* were collected from both *Lanurgus* sp. 1 and *Ctonoxylon* sp. 1. *Sporothrix pallida* is known from many environmental habitats (e.g. water sediments, soil and the sporophore of a slime-mould) and from various European countries (e.g. Germany, Spain, Italy, Netherlands, and England) as well as Japan (De Meyer et al. 2008). This fungus was also recently isolated from a human patient as the causative agent of a corneal ulcer (Morrison et al. 2013). To the best of our knowledge our findings represent the first example of this fungus in association with Scolytinae beetles. It is also the first example of a member of the Ophiostomatales associated with beetles belonging to the genera *Lanurgus* and *Ctonoxylon*.

Similar to *S. pallida*, the newly described *S. aemulophila* was associated with a Scolytinae beetle,

*Xyleborinus aemulus*. It is closely related to *O. candidum* that is associated with a cerambycid beetle on exotic *Eucalyptus* spp. trees in South African plantations (Kamgan Nkuekam et al. 2012). *Sporothrix aemulophila* and *O. candidum* differ in many characteristics, including optimal growth temperature (30 °C for *S. aemulophila* and 25 °C for *O. candidum*), ornamentation at the base of ascoma (present in *O. candidum* and absent in *S. aemulophila*) and dimensions of morphological characters (Kamgan Nkuekam et al. 2012). Despite quite extensive surveys from wounds on trees in the Afromontane forests where *S. aemulophila* occurs, including those on the same *R. melanophloeos* host, this species has never been found previously (Kamgan Nkuekam et al. 2008). Whether this fungus is involved in beetle nutrition is unknown, but the beetle has an ambrosial-type of tunnelling system, which is usually seen in beetle taxa that depend on fungi for survival (De Fine Licht and Biedermann 2012; Jordal and Cognato 2012). *Xyleborinus saxesenii*, an ambrosia beetle in the same genus as *X. aemulus*, is mutualistically associated with fungi in the Ophiostomatales (Biedermann 2012). This suggests that the association between *X. aemulus* and *S. aemulophila* may be mutualistic, but this needs to be confirmed in future studies.

The beetle species collected in this study were mostly associated with recently dead trees, although they were collected from weakened living trees in a few cases. These beetles can therefore be classified as secondary beetles, known to attack stressed, weakened and recently dead trees (Paine et al. 1997; Six and Wingfield 2011; Avtzis et al. 2012). Although secondary beetles have been responsible for the destruction of large populations of stressed trees (for example Breshears et al. 2005), we have seen no evidence of this at the sites included in this study.

Four members of the order Ophiostomatales were collected in this study from four subcortical beetles infesting *R. melanophloeos* and *O. capensis* ssp. *macrocarpa*. This represents the first report of subcortical beetles associated with the Ophiostomatales in the CFR. Phylogenetic analyses confirmed that one new species belongs to the *S. schenckii*-*O. stenoceras* complex, while two other new species belong to *Raffaelea* s. str. Given that three out of the four fungal species collected are new species, it is highly likely that there are more Ophiostomatales beetle-associates

awaiting discovery in the CFR. Future research should include other native tree taxa and sub-cortical beetles. Although this study was an important first step towards understanding relationships between subcortical beetles and their fungal symbionts on native trees in the CFR, the exact nature of these relationships are still unclear and needs to be a focus in future studies.

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#### Compliance with ethical standards

**Conflict of interest** None.

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