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ORIGINAL PAPER

Ophiostomatalean fungi associated with wood boring beetles in South Africa including two new species

Wilma J. Nel · Michael J. Wingfield · Z. Wilhelm de Beer · Tuan A. Duong

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Abstract Ambrosia beetles are small wood inhabiting members of the Curculionidae that have evolved obligate symbioses with fungi. The fungal symbionts concentrate nutrients from within infested trees into a usable form for their beetle partners, which then utilize the fungi as their primary source of nutrition. Ambrosia beetle species associate with one or more primary symbiotic fungal species, but they also vector auxiliary symbionts, which may provide the beetle with developmental or ecological advantages. In this study we isolated and identified ophiostomatalean fungi associated with ambrosia beetles occurring in a native forest area in South Africa. Using a modified Bambara beetle trap, living ambrosia beetle specimens were collected and their fungal symbionts isolated. Four beetle species, three Scolytinae and one Bostrichidae, were collected. Five species of ophiostomatalean fungi were isolated from the beetles and were identified using both morphological characters and DNA sequence data. One of these species, *Raffaelea sulphurea,* was recorded from South Africa for the first time and two novel species were described as *Ceratocystiopsis lunata* sp. nov. and *Raffaelea promiscua* sp. nov.

Keywords Bostrichidae · *Ceratocystiopsis* · Ophiostomatales · *Raffaelea* · Xyleborini

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W. J. Nel (✉) · M. J. Wingfield · Z. W. de Beer · T. A. Duong
Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa
e-mail: janine.nel@fabi.up.ac.za; nel.wilma2@gmail.com

Introduction

Ambrosia beetles are small wood boring insects that reside in the true weevil family Curculionidae. Approximately 3500 species have been described in two sub-families (Platypodinae and Scolytinae), all of which have obligate associations with filamentous fungi (Six 2012; Jordal 2015). These fungi are primarily Ascomycota that are cultivated along the gallery walls and serve as the primary food source of the beetles and their growing broods (Batra 1966; Massoumi-Alamouti et al. 2009). While developing in the brood galleries, the beetles collect the spores of the fungal symbionts and store them within specially evolved structures known as mycangia (Klepzig and Six 2004). This not only maintains the association of the fungal symbionts within and between different generations of the beetles, but also provides the fungi with a consistent means of dispersal and introduction into a relatively competition-free environment in which they proliferate (Six 2012). In return, the fungi

concentrate available nutrients from the host into a form usable for their beetle partners (De Fine Licht and Biedermann 2012).

The symbiotic fungi of bark and ambrosia beetles represent a polyphyletic assemblage of filamentous fungal genera, which have evolved convergent morphological traits that favour insect dispersal (Cassar and Blackwell 1996; Zipfel et al. 2006). Most fungal symbionts of ambrosia beetles reside in the orders Ophiostomatales and Microascales, although some species of Hypocreales and Basidiomycota have also been discovered (Kolařík and Kirkendall 2010; Kasson et al. 2013, 2016; Machingambi et al. 2014; Lynn et al. 2020). In the Ophiostomatales, there are three genera regarded as primary ambrosia beetle symbionts. These include *Affroraffaelea* (Bateman et al. 2017), *Aureovirgo* (van der Linde et al. 2016), and *Raffaelea* sensu lato (Dreaden et al. 2014). Most of the remaining genera in this order associate with bark beetles although some species, are associates of other arthropods such as mites or they occur in non-insect niches such as soil (De Beer and Wingfield 2013). Additionally, the family includes species such as *Hawksworthiomyces lignivorus*, which was originally isolated from decaying telephone poles (De Meyer et al. 2008), as well as a small number of species in the *Sporothrix schenckii* clade that are opportunistic human and animal pathogens (López-Romero et al. 2011).

It has been argued for a relatively long time that obligate insect-fungus mutualisms, such as the ambrosia symbioses, represented a one-on-one relationship (Hubbard 1896; Talbot 1977; Cook and Rasplus 2003). This hypothesis appeared to hold true for ambrosia beetles as they were typically found associated with a single, dominant primary symbiont. However, Batra (1966, 1967) opposed this view and a few recent studies have shown that ambrosia beetles can also associate with multiple secondary (or auxiliary) fungal species (Kolařík and Kirkendall 2010; Carrillo et al. 2019). In many cases, these auxiliary associates reside in the same orders as the primary symbionts, and in some cases the primary symbiont of one beetle species may serve as an auxiliary species of another (Batra 1966). However, unlike the primary fungal symbionts, the roles of auxiliary species remain unclear although various hypotheses have been proposed. These include (1) serving as a nutritional source during brood development and succession (Freeman et al. 2016); (2) enabling a beetle to adapt to a new host or environment (Carillo et al. 2014) and (3); increasing beetle fitness by reducing host tree defences and allowing colonization by the primary symbiont (Saucedo et al. 2018).

In many Southern hemisphere countries, including South Africa, ambrosia beetles and their associated fungi are poorly known. This is attributed to the fact that most of these beetles are regarded as harmless, secondary pests infesting stressed or dying trees (Huclr et al. 2017). However, with increasing globalization and the introduction of invasive pests and their associated pathogenic fungi, interest regarding ambrosia beetles in their native ranges and their potential to become economically significant has increased (Liebhold et al. 1995; Ploetz et al. 2013; Hulcr et al. 2017). This elevated interest, as well as the importance of these insects and their fungal associates, prompted the present study to investigate the diversity ophiostomatalean fungi associated with some commonly encountered ambrosia beetles in a native forest area of South Africa.

Materials and methods

Collection of beetles and isolation of fungi

Beetle specimens were obtained from direct field sampling. Field collections were carried out at two locations in Tzaneen, Limpopo Province, South Africa (23° 42′ 29.491″ S 30° 5′ 57.638″ E and 23° 44′ 29.491″ S 30° 11′ 15.417″) using a modified Bambara beetle trap (Hulcr and McCoy 2015). The modified trap contained a wire mesh insert (gap size of 0.5 mm) between the entrance and collection zone, preventing the insects from making contact with the ethanol lure. Traps were set out in the late afternoon with 90% ethanol and left over-night, after which live beetles were collected early the following morning. Beetles were placed on the surfaces of 65 mm Petri dishes and allowed to walk over the agar. These Petri dishes contained malt extract agar (MEA: 2% malt extract and 2% Difco® agar, Biolab, Midrand, South Africa) amended with streptomycin (0.04%, Sigma-Aldrich, Missouri, United States) to control growth of bacteria and cycloheximide (0.03%, Sigma-Aldrich) that is selective for species in the Ophiostomatales. The beetles were removed from the plates after 24 h and

transferred into individual cryotubes containing 90% ethanol and stored at − 20 °C for species-level identification.

Petri dishes were inspected regularly for fungal growth and ophiostomatalean isolates were purified by transferring hyphal tips to new MEA plates. Multiple isolates with culture morphologies resembling those of the ophiostomatalean fungi were obtained and used for morphological and DNA sequence-based characterisation. Pure cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1) and representative isolates of novel taxa were also deposited in the culture collection (CBS) of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.

Identification of beetles

Beetle specimens were examined using an automated Zeiss Discovery V12 dissection microscope (Zeiss, Oberkocken, Germany). Based on overall morphology, beetles were first sorted into groups before they were identified to species level using the key of Rabaglia et al. (2006). The ventral, lateral and dorsal aspects of specimens were examined, and photographic images were captured using a Zeiss Axiocam IcC5 (Zeiss, Oberkocken, Germany). Focus-stacked photographs were produced for the dorsal aspects using Helicon Focus v. 5 (HeliconSoft, Kharkiv, Ukraine) with up to 30 different images.

One of the beetle specimens from which fungal isolates were obtained had obscure morphological characteristics and the specimen was subjected to PCR amplification and sequencing of the ribosomal large subunit (28S) gene region. DNA was extracted using the Macherey Nagel NucleoSpin Tissue Kit (Macherey–Nagel, Dueren, Germany) from the dissected head of the beetle. DNA extraction was performed following the manufacturer's protocols, except for the final elution volume that was reduced to 60 µl. PCR amplification of the partial ribosomal large subunit (28S) was done using the primers 3665 and 4068 (Belshaw and Quicke 1997; Cognato 2013) in 25 µL reaction volumes as described by Cognato (2013). PCR products were treated with ExoSAP-IT™ PCR Product Clean-up Reagent (ThermoFisher Scientific, Massachusetts, United States). Sequencing reactions were carried out in both the forward and reverse directions using the same primers used in PCR using the BigDye® Terminator v3.1 cycle sequencing kit (ThermoFisher Scientific) with an annealing temperature of 55 °C. Sequencing PCR products were precipitated using the sodium acetate/ethanol method and submitted to the DNA sanger sequencing facility based at the University of Pretoria for analyses on ABI PRISM®3500 Genetic Analyzer (Applied Biosystems, California, United States). The software package Sequence Scanner v. 1.0 (https://sequence-scanner-software.software.informer.com/) was used for quality assessment and editing of the obtained sequencing reads. The consensus sequence was used in a BLASTN search against NCBI GenBank nr/nt database (www.ncbi.nlm.nih.gov/Genbank) to confirm putative morphological identification. The newly obtained sequence has been deposited in NCBI GenBank with the accession number MT355516.

Identification of fungal isolates

The obtained fungal isolates were grouped based on culture morphology and DNA extraction was carried out using lyophilized mycelium of representative isolates following the method of De Beer et al. (2014). PCR amplification was carried out for the partial 28S ribosomal large subunit (LSU) using the primers LR0R and LR5 (Vilgalys and Hester 1990), the internal transcribed spacer region (ITS) using primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) and the partial Beta-tubulin (*β-tubulin*) gene using the primers T10 (O'Donnel and Cigelnik 1997) and Bt2B (Glass and Donaldson 1995). PCR reactions were carried out as described by De Beer et al. (2014) in 25 µL reaction volumes and the annealing temperature was set at 55 °C. PCR products were treated with ExoSAP-IT™ PCR Product Clean-up Reagent (ThermoFisher Scientific). Sequencing reactions, precipitation and quality assessment was carried out as described above. Reactions were performed in both the forward and reverse direction using the same primers used in PCR and an annealing temperature of 55 °C was used for all three gene regions. Newly obtained sequences have been deposited in NCBI GenBank with the accession numbers provided in Table 1.

Obtained consensus sequences were used in a BLASTN search against NCBI GenBank nr/nt database for putative identification to genus level. Based

Table 1 Isolates obtained in this study

Species	Isolate number	Beetle host	Locality	Genbank accession numbers		
				LSU	ITS	*β-tubulin*
Ceratocystiopsis lunata	CMW 55897[a]	*Xs. crassiusculus*	Tzaneen, Limpopo	MW028141	MW028169	MW066754
	CMW 55898	*Xs. crassiusculus*	Tzaneen, Limpopo	MW028140	MW028170	MW066755
Ophiostoma palustre	CMW 56170	Bostrichid beetle	Tzaneen, Limpopo	MW028135	MW028164	
	CMW 54253	Bostrichid beetle	Tzaneen, Limpopo	MW028137	MW028165	
	CMW 54254	Bostrichid beetle	Tzaneen, Limpopo	MW028139	MW028166	
	CMW 54255	Bostrichid beetle	Tzaneen, Limpopo	MW028138	MW028167	
	CMW 56171	Bostrichid beetle	Tzaneen, Limpopo	MW028136	MW028168	
Raffaelea arxii	CMW 55893	*Xbo. affinis*	Tzaneen, Limpopo	MW028147	MW028171	
	CMW 55894	*Xbo. affinis*	Tzaneen, Limpopo	MW028148		
	CMW 55895	*Xbo. affinis*	Tzaneen, Limpopo	MW028149	MW028172	
	CMW 55896	*Xbo. affinis*	Tzaneen, Limpopo	MW028150		
	WN19.12.18	*Xbo. affinis*	Tzaneen, Limpopo	MW028151		
Raffaelea promiscua	CMW 55899[a]	*Xbi.s saxesenii*	Tzaneen, Limpopo	MW028144	MW028176	MW066750
	CMW 55900	*Xbi. saxesenii*	Tzaneen, Limpopo	MW028145	MW028177	MW066751
	CMW 55901	*Xbi. saxesenii*	Tzaneen, Limpopo	MW028142		
	CMW 55902	*Xbi. saxesenii*	Tzaneen, Limpopo	MW028143		
	CMW 56172	*Xbi. saxesenii*	Tzaneen, Limpopo	MW028146	MW028178	MW066752
Raffaelea sulphurea	CMW 55779	*Xbi. saxesenii*	Tzaneen, Limpopo	MW028156		
	CMW 55780	*Xbi. saxesenii*	Tzaneen, Limpopo	MW028157	MW28174	
	CMW 55784	*Xbi. saxesenii*	Tzaneen, Limpopo	MW028158		
	CMW 55788	*Xbi. saxesenii*	Tzaneen, Limpopo	MW028159		
	CMW 55789	*Xbi. saxesenii*	Tzaneen, Limpopo	MW028160		
	CMW 55790	*Xbi. saxesenii*	Tzaneen, Limpopo	MW028161		
	CMW 55792	*Xbi. saxesenii*	Tzaneen, Limpopo	MW028152		
	CMW 55793	*Xbi. saxesenii*	Tzaneen, Limpopo	MW028153	MW028173	
	CMW 55794	*Xbi. saxesenii*	Tzaneen, Limpopo	MW028154		
	CMW 55795	*Xbi. saxesenii*	Tzaneen, Limpopo	MW028155		
	CMW 55781	*Xbi. saxesenii*	Tzaneen, Limpopo			
	CMW 55782	*Xbi. saxesenii*	Tzaneen, Limpopo			
	CMW 55783	*Xbi. saxesenii*	Tzaneen, Limpopo			
	CMW 55785	*Xbi. saxesenii*	Tzaneen, Limpopo			
	CMW 55786	*Xbi. saxesenii*	Tzaneen, Limpopo			
	CMW 55787	*Xbi. saxesenii*	Tzaneen, Limpopo			
	CMW 55791	*Xbi. saxesenii*	Tzaneen, Limpopo			
	CMW 55903	*Xbi. saxesenii*	Tzaneen, Limpopo			
	CMW 55796	*Xbi. saxesenii*	Tzaneen, Limpopo			
	CMW 55,797	*Xbi. saxesenii*	Tzaneen, Limpopo			
	CMW 55,798	*Xbi. saxesenii*	Tzaneen, Limpopo			

[a]Type strains

on these identifications, various datasets were prepared and analysed for each genus separately. Datasets were prepared using MEGA v. 7.0.26 (Kumar et al. 2016) and alignments were done using the online version of MAFFT v. 7 (Katoh and Standley 2013) with default settings. Due to high variability within the

three ITS datasets, alignment was subjected to Gblocks v. 0.91b (Castresana 2000) analysis with the less stringent options to remove ambiguous aligned positions before being used for phylogenetic analyses.

Maximum parsimony analyses were performed using MEGA v. 7.0.26. Phylogenies were generated using Subtree-Pruning-Regrafting algorithm starting with 10 random initial trees. Alignment gaps and missing data were included. Confidence levels for the nodes were tested using 1000 bootstrap replicates.

Maximum likelihood analyses were performed using the software raxmlGUI v. 2 (Silvestro and Michalak 2012; Elder et al. 2019) following the General Time Reversible + GAMMA (GTR + G) nucleotide substitution model. Ten random ML searches followed by 1000 bootstrap replicates were performed.

Bayesian inference analyses were performed using MrBayes v. 3.2.5 (Ronquist and Huelsenbeck 2003). Phylogenies were generated following the Markov Chain Monte Carlo (MCMC) method using the GTR + G model. Ten parallel runs, each with four independent MCMC chains were conducted. Trees were sampled every 1000 generations for 10 million generations. Trees sampled in the burn-in phase (25% of trees sampled) were discarded and posterior probabilities were calculated from the remaining trees. Multi-locus phylogenies were constructed for *Ceratocystiopsis* and *Raffaelea* using the same methods described above on the combined LSU, ITS and βT datasets.

Morphological observations

Microscopic structures—Fungal isolates were examined using a Zeiss AxioScop 2 compound microscope with an affixed Zeiss Axiocam 105 color camera and a Swift M3602-3DGL light microscope with a built-in 3-megapixel digital camera. Specimens of actively growing cultures were mounted in 80% lactic acid (ThermoFisher Scientific) and examined using bright field and differential interference contrast microscopy. Fifty to 100 measurements were made for all characteristic morphological structures using Zen Blue v. 2.6 (Zeiss). Measurements were taken for conidia, conidiophores, conidiogenous cells, and some additional characteristics when present. Values are presented as minimum—maximum.

Colony growth and morphology—A growth study was conducted to determine optimal growth conditions for two putative new species. Two isolates of each lineage were used to determine growth rate and culture morphology. Agar plugs of 5 mm in diameter were excised from the edge of an actively growing culture and placed, mycelium side down, onto the centre of a 65 mm 2% MEA plate. Plates were incubated in the dark at a temperature range from 15 to 30 °C at 5 °C intervals. Three replicates were carried out for each isolate at each temperature. Isolates were maintained for 10 days after which mean colony diameter was determined. Colony colours were determined using the colour charts of Rayner (1970).

Results

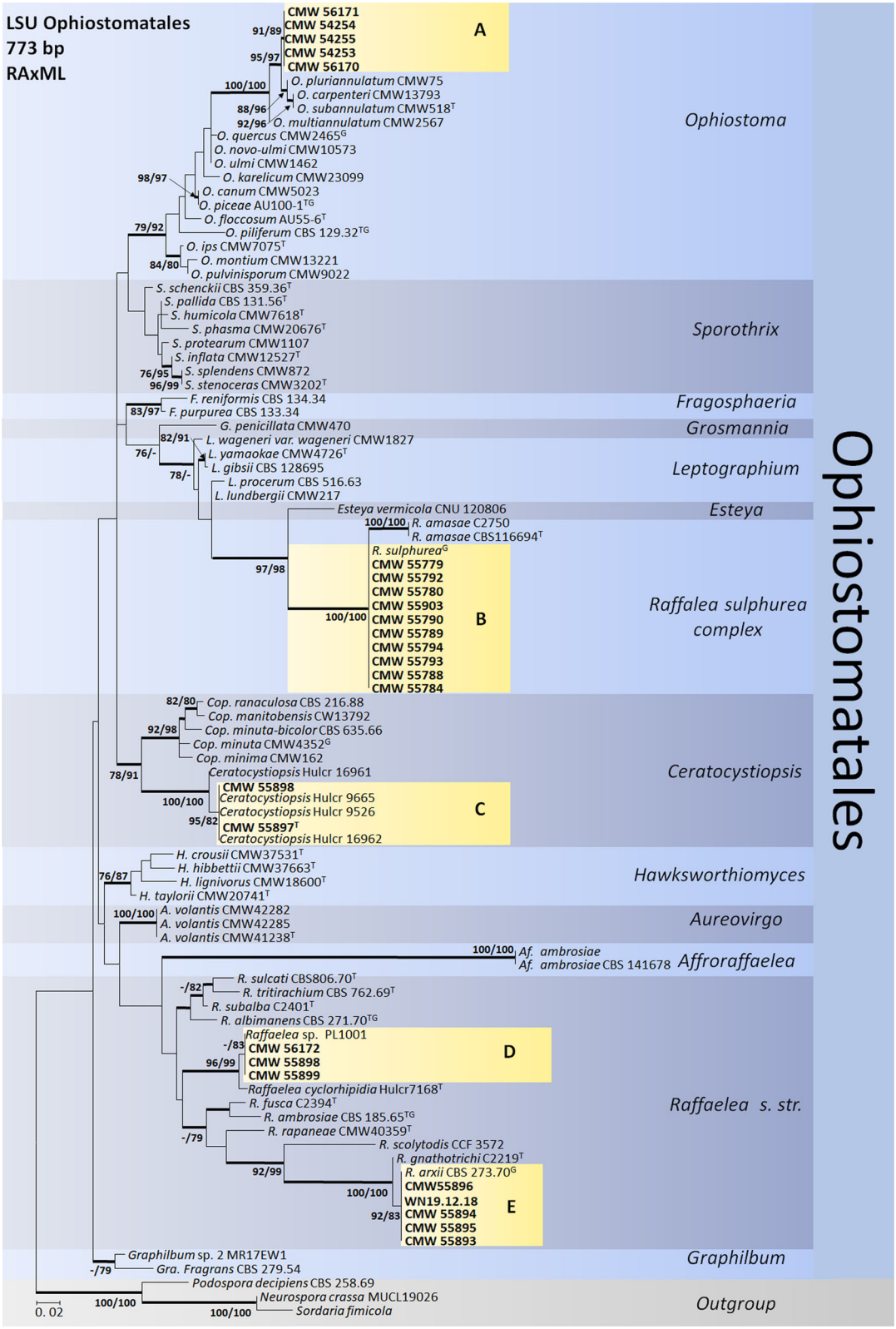
Identification of fungi

The collected beetle specimens yielded 38 isolates with morphologies resembling those of ophiostomatalean fungi. These included five distinct morphological groups and of these, 27 isolates (Table 1) were selected for DNA extraction, PCR amplification and sequencing of the LSU region.

BLAST searches using the LSU sequences revealed that all sequenced isolates were members of the Ophiostomatales. Based on the preliminary taxonomic groupings identified using BLAST, an LSU dataset (LSU-Ophiostomatales) was prepared and analysed to identify generic placement of the isolates. Phylogenetic analyses of the LSU-Ophiostomatales dataset (Fig. 1) including representative isolates of all genera of the Ophiostomatales, showed that the isolates considered in this study resided in in three different genera, *Ceratocystiopsis, Ophiostoma,* and *Raffaelea,* and represented five separate lineages (lineages A–E, Fig. 1).

Based on the genus and species complex assignments obtained from analyses of the LSU dataset (Fig. 1), seven genus level datasets were prepared and analysed (Tables 2, 3). The number of characters of each aligned dataset, numbers of parsimony informative characters, and additional information regarding phylogenetic analyses are presented in Table 3.

Ophiostoma—Based on the analyses of the LSU-Ophiostomatales dataset, five isolates grouped as a


LSU Ophiostomatales
773 bp
RAxML
CMW 56171
CMW 54254
CMW 54255
CMW 54253
CMW 56170
A
O. pluriannulatum CMW75
O. carpenteri CMW13793
O. subannulatum CMW518T
O. multiannulatum CMW2567
O. quercus CMW2465G
O. novo-ulmi CMW10573
O. ulmi CMW1462
O. karelicum CMW23099
O. canum CMW5023
O. piceae AU100-1TG
O. floccosum AU55-6T
O. piliferum CBS 129.32TG
O. ips CMW7075T
O. montium CMW13221
O. pulvinisporum CMW9022
Ophiostoma
S. schenckii CBS 359.36T
S. pallida CBS 131.56T
S. humicola CMW7618T
S. phasma CMW20676T
S. protearum CMW1107
S. inflata CMW12527T
S. splendens CMW872
S. stenoceras CMW3202T
Sporothrix
F. reniformis CBS 134.34
F. purpurea CBS 133.34
Fragosphaeria
G. penicillata CMW470
Grosmannia
L. wageneri var. wageneri CMW1827
L. yamaokae CMW4726T
L. gibsii CBS 128695
L. procerum CBS 516.63
L. lundbergii CMW217
Leptographium
Esteya vermicola CNU 120806
R. amasae C2750
R. amasae CBS116694T
Esteya
R. sulphureaG
CMW 55779
CMW 55792
CMW 55780
CMW 55903
CMW 55790
CMW 55789
CMW 55794
CMW 55793
CMW 55788
CMW 55784
B
Raffalea sulphurea complex
Cop. ranaculosa CBS 216.88
Cop. manitobensis CW13792
Cop. minuta-bicolor CBS 635.66
Cop. minuta CMW4352G
Cop. minima CMW162
Ceratocystiopsis Hulcr 16961
CMW 55898
Ceratocystiopsis Hulcr 9665
Ceratocystiopsis Hulcr 9526
CMW 55897T
Ceratocystiopsis Hulcr 16962
C
Ceratocystiopsis
H. crousii CMW37531T
H. hibbettii CMW37663T
H. lignivorus CMW18600T
H. taylorii CMW20741T
Hawksworthiomyces
A. volantis CMW42282
A. volantis CMW42285
A. volantis CMW41238T
Aureovirgo
Af. ambrosiae
Af. ambrosiae CBS 141678
Affroraffaelea
R. sulcati CBS806.70T
R. tritirachium CBS 762.69T
R. subalba C2401T
R. albimanens CBS 271.70TG
Raffaelea sp. PL1001
CMW 56172
CMW 55898
CMW 55899
D
Raffaelea cyclorhipidia Hulcr7168T
R. fusca C2394T
R. ambrosiae CBS 185.65TG
R. rapaneae CMW40359T
R. scolytodis CCF 3572
R. gnathotrichi C2219T
R. arxii CBS 273.70G
CMW55896
WN19.12.18
CMW 55894
CMW 55895
CMW 55893
E
Raffaelea s. str.
Graphilbum sp. 2 MR17EW1
Gra. Fragrans CBS 279.54
Graphilbum
Podospora decipiens CBS 258.69
Neurospora crassa MUCL19026
Sordaria fimicola
Outgroup
0. 02
Ophiostomatales

◀**Fig. 1** RAxML phylogram derived from analysis of LSU data including all major groups in the Ophiostomatales. Bold branches indicate posterior probabilities ≥ 95%. Bootstrap values ≥ 75% for maximum likelihood and maximum parsimony analyses are indicated at nodes as ML/MP. G = sequence extracted from available genome. T = Ex-Type isolate

single lineage (A) in the *O. pluriannulatum* complex. Phylogenetic analyses of the ITS-*Oph* dataset (Fig. 2), that included a subset of *Ophiostoma* species focussed on the *O. pluriannulatum* complex, identified this species as *O. palustre*.

Ceratocystiopsis—Two isolates resided in the genus *Ceratocystiopsis* based on the analyses of the LSU-Ophiostomatales dataset. These isolates formed a distinct clade with high statistical support together with sequences of three undescribed isolates available from GenBank (lineage C). Phylogenetic analyses of the genus level datasets (LSU, ITS, and *β-tubulin*) (Fig. 3a-c) all supported this grouping. No ITS and *β-tubulin* sequence data were available for these undescribed isolates, but based on their grouping and sequence conservation in the LSU analysis, they likely belong to the same species as the isolates recovered from the current study.

Raffaelea—Twenty isolates, representing three morphological groups, grouped in three separate lineages within *Raffaelea* sensu lato (Fig. 1). Ten isolates grouped in the *Raffaelea sulphurea* complex (lineage B) and were identified as *R. sulphurea* based on phylogenetic analyses of LSU and ITS sequence data (Figs. 1 and 4a and b). The remaining ten isolates grouped as two distinct lineages (D and E) within *Raffaelea* sensu stricto (Figs. 1, 4). Five isolates were identified as *R. arxii* based on phylogenetic analyses of LSU and ITS sequence data (Figs. 1, 4a and b). The five remaining isolates (lineage D) together with an additional isolate labelled as *Raffaelea* sp. PL1001 (Eskalen & McDonald 2011; Dreaden et al. 2014), for which LSU and *β-tubulin* sequences are available in GenBank, formed a distinct lineage separate from all other species in the genus, but closely related to *R. cyclorhipidia* (Fig. 4b and c). An additional isolate labelled as *Raffaelea* sp. Hulcr7507 (Simmons et al. 2016), for which ITS and *β-tubulin* sequences are available on GenBank, grouped peripheral to this clade. Based on phylogenetic placement and ITS and *β-tubulin* sequence conservation this isolate most likely belongs to the same species as *Raffaelea* sp. PL1001 and the five isolates recovered from the current study.

Identification of beetles

The fungal isolates obtained in this study originated from 16 beetle specimens. Based on their morphological characters, these beetles were identified as four different species (Fig. 5). Three of these species resided in the Xyleborini (Scolytinae), namely *Xyleborinus (Xbi.) saxesenii; Xylosandrus (Xs.) crassiusculus;* and *Xyleborus (Xbo.) affinis* (Fig. 5b-d). The fourth was a species of Bostrichidae (Fig. 5a). Isolates used in this study originated from thirteen *Xbi. saxesenii* specimens, and a single specimen each of *Xs. crassiusculus, Xbo. affinis* and the unknown Bostrichid. Due to some obscured morphological characters for the *Xbo. affinis* specimen, DNA sequencing of the ribosomal large subunit was carried out and results positively confirmed our identification.

Beetle-fungus associations

Thirty-eight isolates with ophiostomatalean-like morphologies were isolated from 16 living beetle specimens. After initial separation based on morphology, 27 representative isolates were selected for further DNA sequence-based characterisation. Five of these fungal isolates were identified as *O. palustre* (Figs. 1, 2—lineage A) and all five isolates originated from a single beetle specimen in the Bostrichidae. Twenty-one isolates recovered from *Xbi. saxesenii* specimens were putatively identified as *R. sulphurea* based on morphology and this was confirmed by DNA sequencing of ten representative isolates originating from different beetles (Figs. 1,4—lineage B). Another five isolates also obtained from *Xbi. saxesenii* specimens were identified as a new species of *Raffaelea* (Figs. 1, 4—lineage D). Five isolates identified as *R. arxii* (Figs. 1, 4—lineage E) were obtained from a single beetle specimen identified as *Xbo. affinis*. Two isolates were obtained from a single specimen of *Xs. crassiusculus* and were identified as a new species of *Ceratocystiopsis* (Figs. 1, 3—lineage C).

Table 2 Isolates used in phylogenetic analyses and their sequence accession numbers

Species	Collection number	Genbank accession numbers		
		LSU	ITS	*β-tubulin*
Ceratocystiopsis brevicomis	CBS 137839	MW028162	MW028175	MW066757
C. brevicomis	UM1452	EU913683	EU913722	EU913761
C. collifera	CBS 126.89	EU913681	EU913721	–
C. concentrica	WIN(M) 71–07	AF135571	–	–
C. longisporum	UM48	EU913684	EU913723	–
C. manitobensis	UM237	EU913674	EU913714	EU913753
C. manitobensis	CBS 118838	DQ294358	–	–
C. minuta	CMW 4352	MW026163	MW028179	MW066756
C. minima	CBS 182.86	DQ294361	–	DQ296081
C. minima	UM1462	EU913663	EU913704	–
C. minuta-bicolor	CBS 635.66	MH870571	MH858901	EU977482
C. minuta-bicolor	CBS 393.77	DQ294359	–	DQ296079
C. pallidobrunnea	UM51	EU913682	–	–
C. ranaculosa	CBS 216.88	EU913673	EU913713	EU913752
C. ranaculosa	CBS 119683	DQ294357	–	DQ296077
C. rollhanseniana	CBS 118669	DQ294362	–	DQ296082
C. rollhanseniana	UM113	EU913678	EU913718	EU913757
Ceratocystiopsis sp.	Hulcr16961	LC363548	–	–
Ceratocystiopsis sp.	Hulcr9665	LC363540	–	–
Ceratocystiopsis sp.	Hulcr16962	LC363547	–	–
Ceratocystiopsis sp.	Hulcr9526	LC363538	–	–
Ophiostoma canum	CMW5023	DQ294372	–	–
O. carpenteri	CMW13793	DQ294363	–	–
O. flocossum	AU55-6[a]	AF234836	AF198231	–
O. himal-ulmi	C1183	–	AF198233	
O. ips	CMW7075[a]	DQ294381	AY546704	–
O. karelicum	CMW23099	EU443756	–	–
O. longiconidiatum	CMW17574[a]	–	EF408558	
O. montium	CMW13221	DQ294379	AY546711	–
O. multiannulatum	CMW2567[a]	DQ294366	FJ959049	–
O. novae-zelandiae	CIEFAP423	–	KT362249	
O. novo-ulmi	CMW10573	DQ294375	FJ430478	–
O. novo-ulmi s. americana	C510	–	AF198236	
O. palustre	CMW44403	–	KU865593	
O. piceae	AU100-1	AF234837	AF081129	LC090730
O. piliferum	CBS 129.32	DQ294377	–	AF221628
O. pluriannulatum	CMW75	DQ294365	AY934517	–
O. pulvinisporum	CMW9022[a]	DQ294380	AY546714	–
O. quercus	CMW2465	DQ294376	AY466626	AY466647
O. sparsiannulatum	CMW17231[a]	–	FJ906817	
O. subannulatum	CMW518[a]	DQ294364	AY934522	–
O. ulmi	CMW1462	DQ294374	AF198232	
Raffaelea aguacate	Raff. sp. 272	MT629748	MT633065	–

Table 2 continued

Species	Collection number	Genbank accession numbers		
		LSU	ITS	*β-tubulin*
R. albimanens	CBS 271.70[a]	MT629749	MT633066	MT644111
R. amasae	C2750[a]	MF399174	–	–
R. amasae	CBS116694[a]	EU984295	–	EU977470
R. ambrosiae	CBS 185.65[a]	MT629751	MT633067	MT644094
R. arxii	CBS 273.70	MT629754	MH859604	MW066753
R. brunnea	CBS 378.68[a]	EU984284	–	EU977460
R. campbellii	CMW44800[a]	KR018414	–	KX267112
R. canadensis	CBS 168.66[a]	EU984299	GQ225699	EU977473
R. crossotarsa	Hulcr7182[a]	KX267103	KX267135	KX267114
R. cyclorhipidia	Hulcr7168[a]	KX267104	KX267136	KX267115
R. ellipticospora	C2395	EU177446	–	KJ909298
R. fusca	C2394[a]	EU177449	–	KJ909301
R. gnathotrichi	C2219[a]	EU177460	–	–
R. lauricola	Raff. sp. 570	MT629759	MT633071	MT644093
R. montetyi	CBS 451.94[a]	EU984301	–	EU977475
R. quercivora	MAFF410918	MAFF410918	GQ225697	GQ225691
R. quercus-mongolicae	KACC44405	MT629763	MT633074	MT644091
R. rapaneae	CMW40359[a]	KT182935	KT192601	–
R. santoroi	CBS 399.67[a]	MH870707	MH859006	EU977476
R. scolytodis	CCF 3572	AM267270	–	–
R. subalba	C2401[a]	EU177443	–	KJ909305
R. subfusca	Hulcr4717	–	KX267137	KX267122
R. sulcati	CBS 806.70[a]	MH871752	–	EU977477
R. sulphurea	CBS 380.68	MT629768	MT633077	MT644092
R. tritiracium	CBS 762.69[a]	MH871169	MH859401	EU977478
R. xyleborina	Hulcr6099	KX267110	–	–
Raffaelea sp.	PL1001	KJ909293	–	KJ909295
Raffaelea sp.	Hulcr7507	–	KX267141	KX267128
Fragosphaeria purpurea	CBS 133.34	AF096191	–	–
F. reniformis	CBS 134.34	AB189155	–	–
Grosmannia penicillata	CMW470	DQ294385	–	–
Leptographium gibsii	CBS 128695	MH876512	–	–
L. lundbergii	CMW217	DQ294388	–	–
L. procerum	CBS 516.63	MH869960	–	–
L. yamaokae	CMW4726[a]	JN135315	–	–
L. wageneri var. wageneri	CMW1827	DQ294397	–	–
Esteya vermicola	CNU 120806	EU627684	–	–
Sporothrix humicola	CMW7618[a]	EF139114	–	–
S. inflata	CMW12527[a]	DQ294351	–	–
S. pallida	CBS 131.56[a]	EF139121	–	–
S. phasma	CMW20676[a]	DQ316151	–	–
S. protearum	CMW1107	DQ316145	–	–
S. splendens	CMW872	AF221013	–	–

Table 2 continued

Species	Collection number	Genbank accession numbers		
		LSU	ITS	*β-tubulin*
S. schenckii	CBS 359.36[a]	KX590890	–	–
S. stenoceras	CMW3202[a]	DQ294350	–	–
Hawksworthiomyces crousii	CMW37531[a]	KX396548	–	–
H. hibbettii	CMW37663[a]	KX396547	–	–
H. lignivorus	CMW18600[a]	EF139119	–	–
H. taylorii	CMW20741[a]	KX396546	–	–
Aureovirgo volantis	CMW42282	KR051133	–	–
Au. volantis	CMW42285	KR051134	–	–
Au. volantis	CMW41238[a]	KR051131	–	–
Affroraffaelea ambrosiae	n/a	KX620930	–	–
Af. ambrosiae	CBS 141678	NG057115	–	–
Graphilbum fragrans	CBS 279.54	MH868872	–	–
Graphilbum sp.2	MR17EW1	AY672929	–	–
Podospora decipiens	CBS 258.69	AY780073	–	–
Sordaria fimicola	n/a	AY545728	–	–
Neurospora crassa	MUCL19026	AF286411	–	–

[a]Type

Table 3 Number of characters strains and substitutional models used in phylogenetic analyses

	Dataset	LSU ophiostomatales	ITS *Oph*	LSU *Cer*	ITS *Cer*	βT *Cer*	LSU *Raf*	ITS *Raf*	βT *Raf*
Number of taxa		95	27	26	15	16	48	26	29
Number of characters	Total	496	567	690	565	283	530	403	473
	VPUC	13	34	26	63	11	29	24	56
	Constant	326	338	592	281	189	382	184	222
	PIC	157	195	72	221	83	119	195	195
MP	Tree length	649	408	159	633	178	341	770	1073
	CI	0.404	0.782	0.711	0.727	0.708	0.540	0.508	0.467
	RI	0.847	0.910	0.877	0.747	0.790	0.870	0.687	0.548

VPUC variable parsimony uninformative characters; *PIC* parsimony informative characters; *MP* maximum parsimony; *CI* consistency index; *RI* retention index

Taxonomy

Ceratocystiopsis lunata W.J. Nel sp. nov. Figure 6.

MycoBank MB838616 *Etymology*: Name reflects the crescent shaped conidia.

Description: Conidiophores mononematous, macronematous, arising from vegetative hyphae, simple, upright, straight, curved or undulate, 5–97 µm long (avg. 26.1 ± 15.8 µm). Conidiogenous cells integrated, hyaline, blastic, sometimes denticulate, 3–42 × 1.5–3.1 µm (avg. 13.7 ± 5.5 × 2.1 ± 0.3 µm). Conidia hyaline, aseptate, two types falcate to crescent shaped, no sheath 4.9–9.5 × 1.3–2.8 µm (avg. 6.6 ± 0.8 × 1.9 ± 0.3 µm) and oblong with the upper part swollen, apex round, tapering toward base,

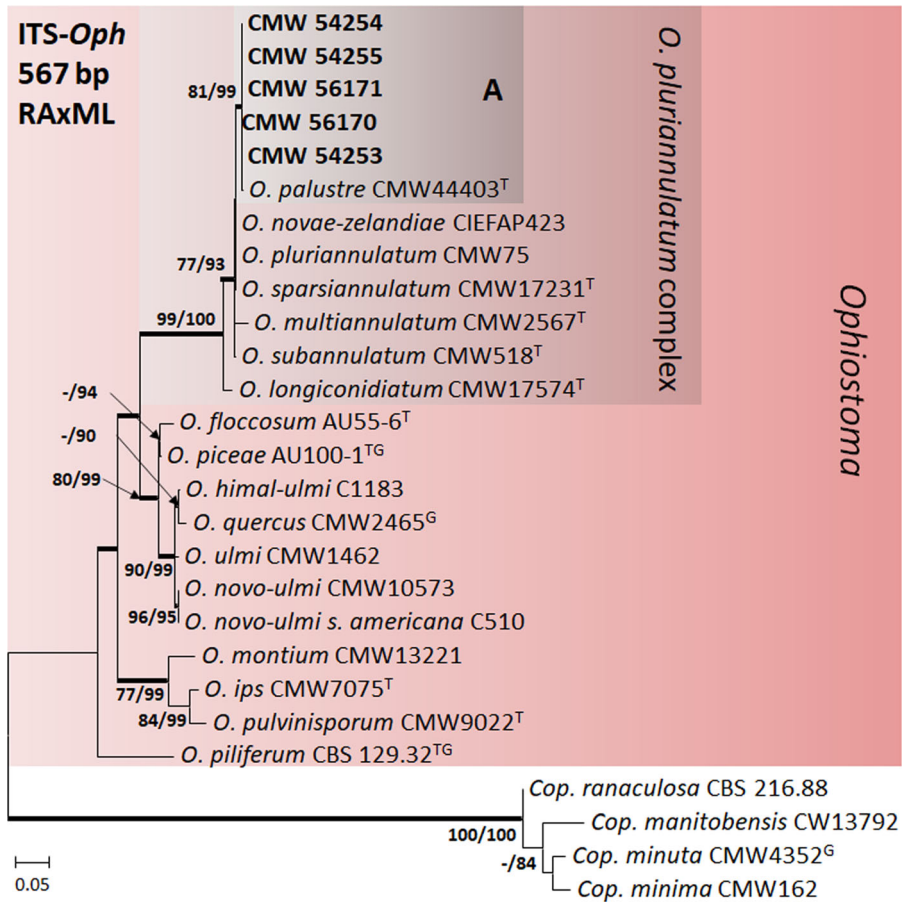


Fig. 2 RAxML phylogram derived from analysis of ITS-*Ophiostoma* data including taxa from the genus *Ophiostoma* specifically focussing on the *O. pluriannulatum* complex. Bold branches indicate posterior probabilities ≥ 95%. Bootstrap values ≥ 75% for maximum likelihood and maximum parsimony analyses are indicated at nodes as ML/MP. G = sequence extracted from available genome. T = Ex-Type isolate

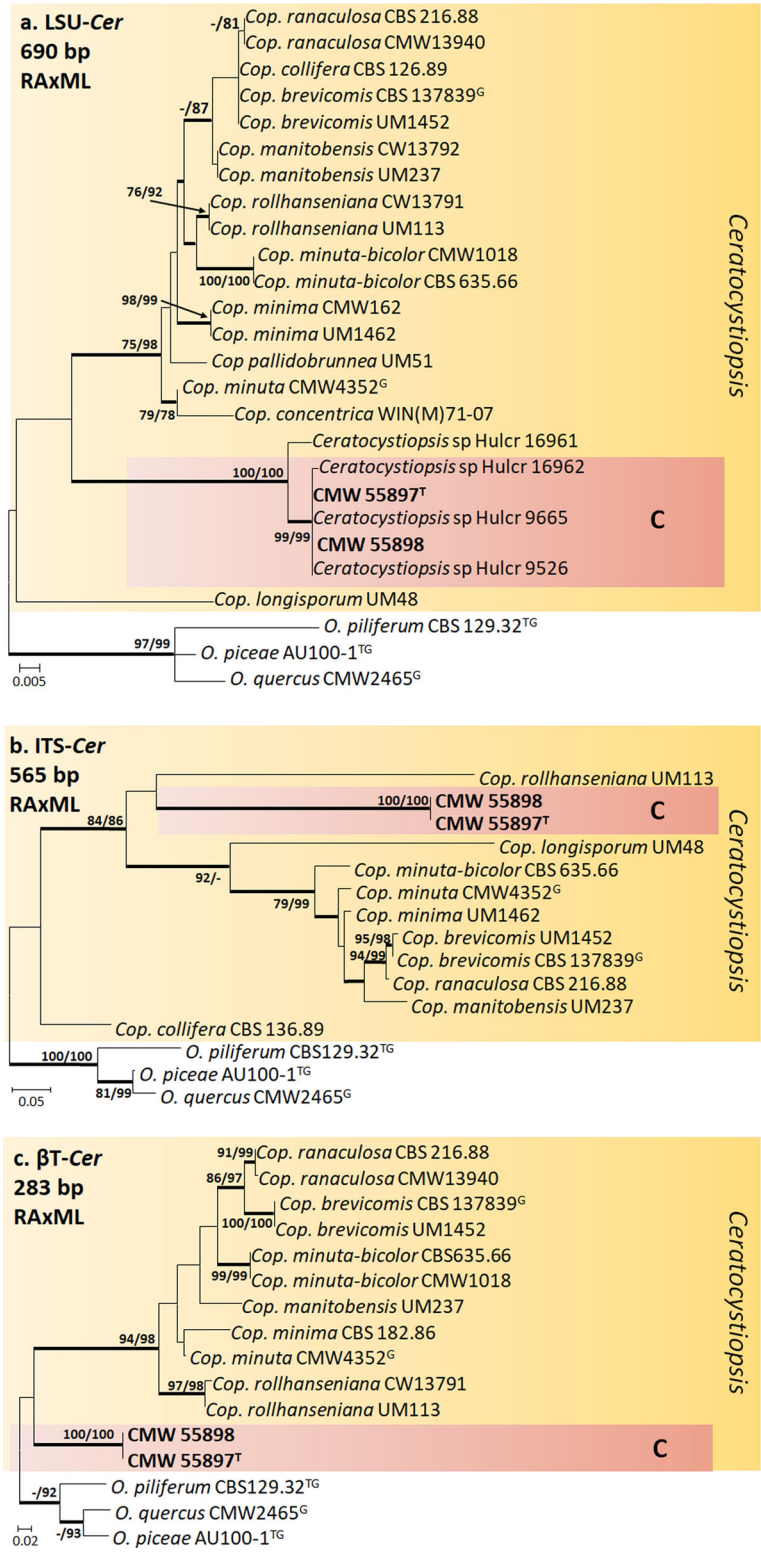
base truncated, 2–6 × 1.2–3.1 µm (avg. 4.5 ± 0.6 × 2.1 ± 0.4 µm), yeast-like budding observed in fresh culture.

Specimens examined: South Africa, Limpopo, Tzaneen, isolated from a living *Xs. crassciusculus* beetle, W. J. Nel. 21 September 2019, holotype (PREM 63099, living culture ex-holotype CMW 55897 = CBS 147171).

Additional specimens: South Africa, Limpopo, Tzaneen, isolated from a living *Xs. crassciusculus* beetle, W. J. Nel. 21 September 2019, paratype (PREM 63100, living culture CMW 55898 = CBS 147172).

Cultures: Moderate growth rate on 2% MEA in dark. Grows best at 30 °C reaching an average of 48.8 mm (± 0.9 mm) in 10 d. Colony growth circular with smooth margins, both abundant aerial and submerged mycelia present, flat, whitish to creamy in colour.

Notes: *Ceratocystiopsis lunata* can be distinguished from other species of *Ceratocystiopsis* based on its conidial morphology. *Ceratocystiopsis lunata* produces both falcate and oblong conidia whereas other species of *Ceratocystiopsis* typically only produce oblong conidia (De Beer and Wingfield 2013).


a. LSU-Cer
690 bp
RAxML
-/81
Cop. ranaculosa CBS 216.88
Cop. ranaculosa CMW13940
Cop. collifera CBS 126.89
Cop. brevicomis CBS 137839G
-/87
Cop. brevicomis UM1452
Cop. manitobensis CW13792
Cop. manitobensis UM237
76/92
Cop. rollhanseniana CW13791
Cop. rollhanseniana UM113
Cop. minuta-bicolor CMW1018
100/100
Cop. minuta-bicolor CBS 635.66
98/99
Cop. minima CMW162
Cop. minima UM1462
75/98
Cop pallidobrunnea UM51
Cop. minuta CMW4352G
79/78
Cop. concentrica WIN(M)71-07
Ceratocystiopsis sp Hulcr 16961
100/100
Ceratocystiopsis sp Hulcr 16962
CMW 55897T
Ceratocystiopsis sp Hulcr 9665
99/99
CMW 55898
Ceratocystiopsis sp Hulcr 9526
C
Cop. longisporum UM48
O. piliferum CBS 129.32TG
97/99
O. piceae AU100-1TG
O. quercus CMW2465G
0.005
Ceratocystiopsis
b. ITS-Cer
565 bp
RAxML
Cop. rollhanseniana UM113
100/100
CMW 55898
CMW 55897T
C
84/86
Cop. longisporum UM48
92/-
Cop. minuta-bicolor CBS 635.66
79/99
Cop. minuta CMW4352G
Cop. minima UM1462
95/98
Cop. brevicomis UM1452
94/99
Cop. brevicomis CBS 137839G
Cop. ranaculosa CBS 216.88
Cop. manitobensis UM237
Cop. collifera CBS 136.89
100/100
O. piliferum CBS129.32TG
O. piceae AU100-1TG
81/99
O. quercus CMW2465G
0.05
Ceratocystiopsis
c. βT-Cer
283 bp
RAxML
91/99
Cop. ranaculosa CBS 216.88
86/97
Cop. ranaculosa CMW13940
Cop. brevicomis CBS 137839G
100/100
Cop. brevicomis UM1452
Cop. minuta-bicolor CBS635.66
99/99
Cop. minuta-bicolor CMW1018
Cop. manitobensis UM237
Cop. minima CBS 182.86
94/98
Cop. minuta CMW4352G
97/98
Cop. rollhanseniana CW13791
Cop. rollhanseniana UM113
100/100
CMW 55898
CMW 55897T
C
-/92
O. piliferum CBS129.32TG
O. quercus CMW2465G
0.02
-/93
O. piceae AU100-1TG
Ceratocystiopsis

◀**Fig. 3** RAxML phylogenies derived from analyses of various *Ceratocystiopsis* datasets including all major taxa described in the genus. **a** Phylogram derived from the LSU analyses; **b** Phylogram derived from the ITS analyses; **c** Phylogram derived from the *β-tubulin* analyses. Bold branches indicate posterior probabilities ≥ 95%. Bootstrap values ≥ 75% for maximum likelihood and maximum parsimony analyses are indicated at nodes as ML/MP. G = sequence extracted from available genome. T = Ex-Type isolate

Raffaelea promiscua W.J. Nel sp. nov. Figure 7.

MycoBank MB838615 Etymology: Name refers to the promiscuous (*Promiscuum* L.) nature of the species that is associated with different ambrosia beetles.

Description: Conidiophores mononematous, macronematous, arising from vegetative hyphae, mostly simple, occasionally branched, upright, straight, curved or undulate, tapering towards apex, 14–195 µm long, reduced to conidiogenous cell. Conidiogenous cells integrated, hyaline to lightly pigmented, cylindrical, or peg-like, tapering towards apex, blastic, 5–56 × 1–3.9 µm (avg. 21.9 × 2.4 µm). Conidia hyaline, aseptate, majority oblong with the upper part swollen, apex round, tapering toward base, base truncated, 2–9 × 1–4 µm (avg. 5.1 × 2.5 µm), yeast-like budding observed in fresh culture.

Specimens examined: South Africa, Limpopo, Tzaneen, isolated from a living *Xbi. saxesenii* beetle, W. J. Nel. 21 September 2019, holotype (PREM 63101, living culture CMW 55899 = CBS 147173).

Additional specimens: South Africa, Limpopo, Tzaneen, isolated from a living *Xbi. saxesenii* beetle, W. J. Nel. 21 September 2019, paratype (PREM 63102, living culture CMW 55900 = CBS 147174). South Africa, Limpopo, Tzaneen, isolated from a living *Xbi. saxesenii* beetle, W. J. Nel. 21 September 2019 (living culture CMW 55901 = CBS 147175). South Africa, Limpopo, Tzaneen, isolated from a living *Xbi. saxesenii* beetle, W. J. Nel. 21 September 2019 (living culture CMW 55902 = CBS 147176).

Cultures: Slow growing on 2% MEA in dark. Grows best at 25 °C reaching and average of 34 mm (± 1.9 mm) in 10 d. Colonies circular with smooth margins, mycelia mostly submerged, aerial hyphae present, flat, initially whitish turning brownish olive to dark greyish brown with age starting at the centre of the colony. Yeast-like growth often present at inoculation site from initial growth of colony.

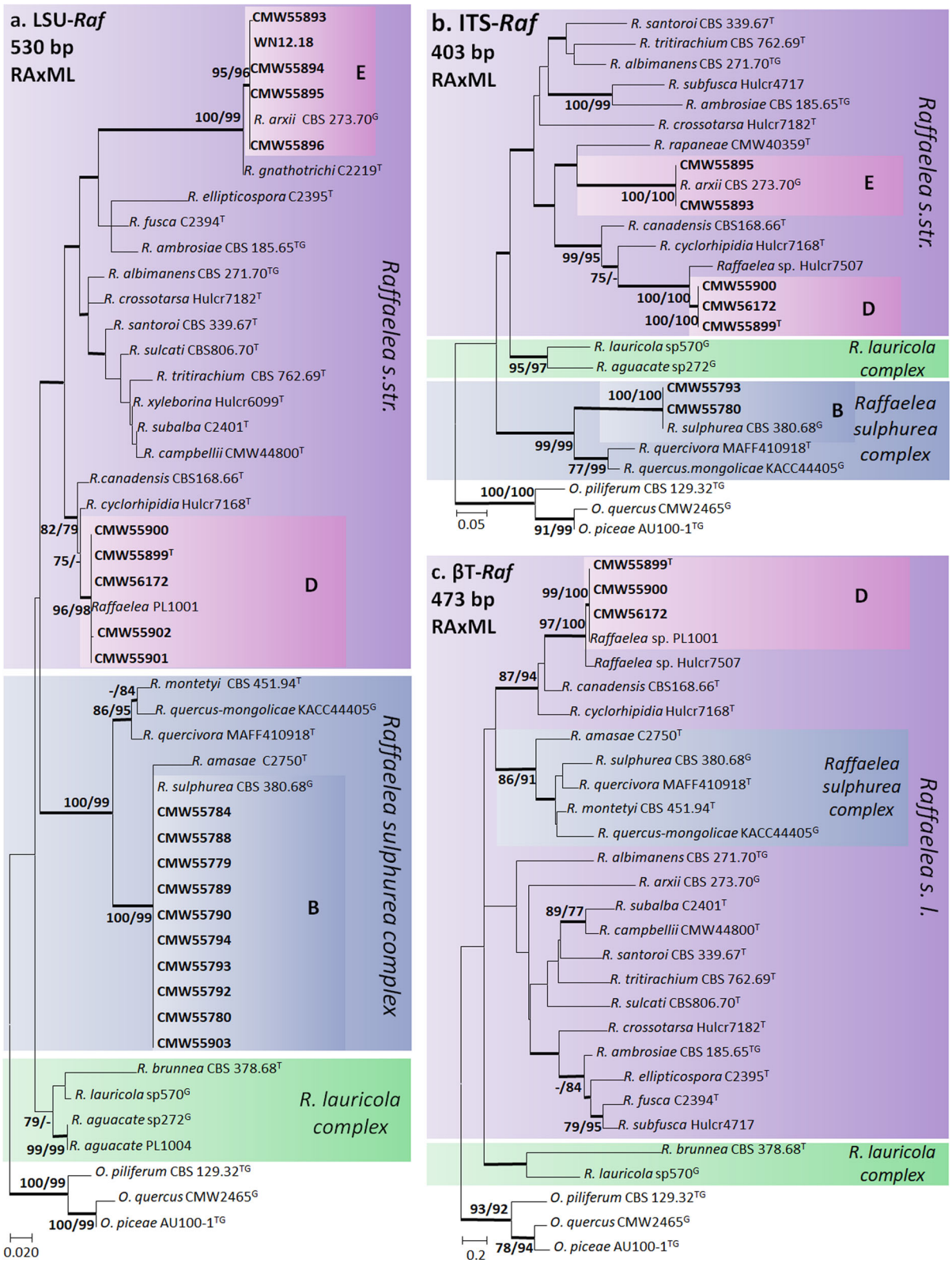
Notes: *Raffaelea promiscua* can be distinguished from its sister taxon *R. cyclorhipidia* (Simmons et al. 2016) by its smaller conidia and the fact that these are predominantly oblong with enlarged apices, and are 5.1 × 2.5 µm on average, whereas those of *R. cyclorhipidia* are elliptical to elongate and 7.3 × 3.5 µm on average. *Raffaelea promiscua* colonies are smooth with aerial hyphae whereas *R. cyclorhipidia* has a tough and wrinkled appearance.

Discussion

A total of 38 ophiostomatalean fungal isolates were obtained from 16 adult beetles representing *Xs. crassiusculus, Xbi. saxesenii, Xbo. affinis*, and an unidentified species of Bostrichidae. The fungi were identified as five distinct species in the Ophiostomatales, one of which was recorded from South Africa for the first time and two represented novel species described here as *C. lunata* and *R. promiscua*.

An unusual association of *O. palustre* with a species of Bostrichidae emerged from this study. Although some small species of Bostrichidae can easily be confused with species of Scolytinae (Ivie 2002), these beetles are not known to associate with fungi, preferentially infesting wood with a low moisture content (Creffield 1996; Ivie 2002). However, some species of *Ophiostoma,* including many species in the *O. pluriannulatum* complex such as *O. palustre* (Osorio et al. 2016), are associated with wounds on trees. It is consequently possible that the bostricid beetle accidentally picked up spores of *O. palustre* colonizing the wounded tissue induced by its gallery. Many ophiostomatalean fungi also associate with phoretic mites vectored between hosts by various beetles (Hofstetter et al. 2013) and it is also possible that the fungus originated from a mite carried by the beetle.

Xyleborinus saxesenii is an ambrosia beetle with a cosmopolitan distribution and is among the most


a. LSU-Raf
530 bp
RAxML
CMW55893
WN12.18
95/96
CMW55894
CMW55895
E
100/99
R. arxii CBS 273.70G
CMW55896
R. gnathotrichi C2219T
R. ellipticospora C2395T
R. fusca C2394T
R. ambrosiae CBS 185.65TG
R. albimanens CBS 271.70TG
R. crossotarsa Hulcr7182T
R. santoroi CBS 339.67T
R. sulcati CBS806.70T
R. tritirachium CBS 762.69T
R. xyleborina Hulcr6099T
R. subalba C2401T
R. campbellii CMW44800T
Raffaelea s.str.
R.canadensis CBS168.66T
R. cyclorhipidia Hulcr7168T
82/79
CMW55900
75/-
CMW55899T
CMW56172
D
96/98
Raffaelea PL1001
CMW55902
CMW55901
-/84
R. montetyi CBS 451.94T
86/95
R. quercus-mongolicae KACC44405G
R. quercivora MAFF410918T
R. amasae C2750T
R. sulphurea CBS 380.68G
100/99
CMW55784
CMW55788
CMW55779
CMW55789
100/99
CMW55790
B
CMW55794
CMW55793
CMW55792
CMW55780
CMW55903
Raffaelea sulphurea complex
R. brunnea CBS 378.68T
R. lauricola sp570G
79/-
R. aguacate sp272G
99/99
R. aguacate PL1004
R. lauricola complex
100/99
O. piliferum CBS 129.32TG
O. quercus CMW2465G
100/99
O. piceae AU100-1TG
0.020
b. ITS-Raf
403 bp
RAxML
R. santoroi CBS 339.67T
R. tritirachium CBS 762.69T
R. albimanens CBS 271.70TG
R. subfusca Hulcr4717
100/99
R. ambrosiae CBS 185.65TG
R. crossotarsa Hulcr7182T
R. rapaneae CMW40359T
CMW55895
R. arxii CBS 273.70G
E
100/100
CMW55893
R. canadensis CBS168.66T
99/95
R. cyclorhipidia Hulcr7168T
75/-
Raffaelea sp. Hulcr7507
100/100
CMW55900
CMW56172
D
100/100
CMW55899T
Raffaelea s.str.
95/97
R. lauricola sp570G
R. aguacate sp272G
R. lauricola complex
100/100
CMW55793
CMW55780
B
R. sulphurea CBS 380.68G
Raffaelea sulphurea complex
99/99
R. quercivora MAFF410918T
77/99
R. quercus.mongolicae KACC44405G
100/100
O. piliferum CBS 129.32TG
O. quercus CMW2465G
91/99
O. piceae AU100-1TG
0.05
c. βT-Raf
473 bp
RAxML
CMW55899T
99/100
CMW55900
D
CMW56172
97/100
Raffaelea sp. PL1001
Raffaelea sp. Hulcr7507
87/94
R. canadensis CBS168.66T
R. cyclorhipidia Hulcr7168T
R. amasae C2750T
R. sulphurea CBS 380.68G
86/91
R. quercivora MAFF410918T
Raffaelea sulphurea complex
R. montetyi CBS 451.94T
R. quercus-mongolicae KACC44405G
R. albimanens CBS 271.70TG
R. arxii CBS 273.70G
89/77
R. subalba C2401T
R. campbellii CMW44800T
R. santoroi CBS 339.67T
R. tritirachium CBS 762.69T
R. sulcati CBS806.70T
R. crossotarsa Hulcr7182T
R. ambrosiae CBS 185.65TG
-/84
R. ellipticospora C2395T
R. fusca C2394T
79/95
R. subfusca Hulcr4717
Raffaelea s. l.
R. brunnea CBS 378.68T
R. lauricola sp570G
R. lauricola complex
93/92
O. piliferum CBS 129.32TG
O. quercus CMW2465G
78/94
O. piceae AU100-1TG
0.2

◀**Fig. 4** RAxML phylogenies derived from analyses of various *Raffaelea* datasets including all major taxa described in the genus. **a** Phylogram derived from the LSU analyses; **b** Phylogram derived from the ITS analyses; **c** Phylogram derived from the *β-tubulin* analyses. Bold branches indicate posterior probabilities ≥ 95%. Bootstrap values ≥ 75% for maximum likelihood and maximum parsimony analyses are indicated at nodes as ML/MP. G = sequence extracted from available genome. T = Ex-Type isolate

common ambrosia beetles found globally. This species was originally described from Germany (Ratzeburg 1837) and has been detected on every continent except Antarctica (Wood and Bright 1992). Considerable research has been conducted on the fungal associates of *Xbi. saxesenii*, with its primary fungal symbiont *Raffaelea sulphurea* first described by Batra (1967). Subsequently, a second dominant mycangial symbiont of this insect, *Fusicolla acetilerea*, was detected by Biedermann et al. (2013). *Xbi. saxesenii* is also associated with a number of less dominant fungi including species of *Paecilomyces, Cladosporium, Ramularia,* and *Aureobasidium* (Biedermann et al. 2013; Malacrinò et al. 2017).

Xyleborinus saxesenii was first reported in South Africa by Schedl (Schedl 1975) where it was collected in the Western Cape and Kwazulu-Natal provinces, but its fungal associates were not considered. In the present study, 26 fungal isolates were obtained from specimens of *Xbi. saxesenii*. Of these, 21 were identified as *R. sulphurea* and this represents the first report of the fungus from South Africa. The remaining five fungal isolates grouped together with an undescribed *Raffaelea* sp. isolated from an unknown ambrosia beetle recorded as PL1001 by Eskalen and McDonald (2011). Subsequent to its first report, this undescribed fungus has been identified in several studies associated with various Xyleborini including *Xbo. bispinatus, Xbo. volvulus* and *Xbi. saxesenii* (Cruz et al. 2018, 2019; Saucedo-Carabez et al. 2018). Based on their distinct morphology and phylogenetic grouping separating them from their closest relative, *R. cylcorhipidia,* the isolates from the present study, including *Raffaelea* sp. PL1001, were described as *R. promiscua.*

Xylosandrus crassiusculus is a commonly encountered, cosmopolitain ambrosia beetle. Yet relatively little is known regarding the fungi associated with this

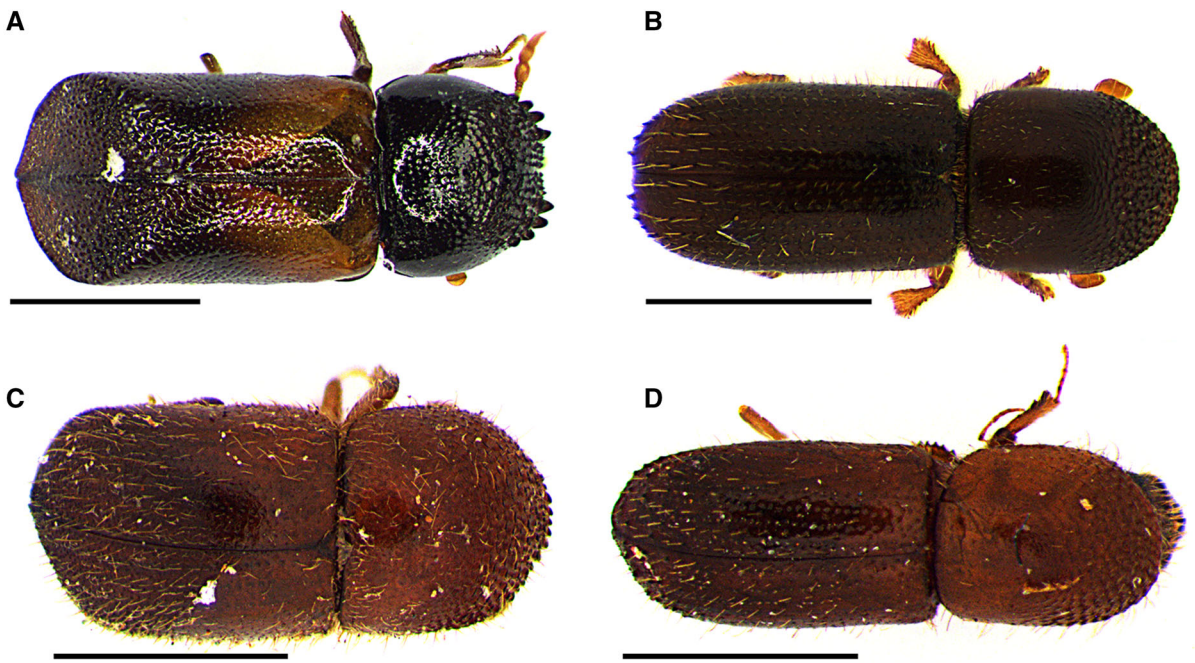


Fig. 5 Beetle species from which fungal isolates were obtained; **a** A unknown species of Bostrichidae beetle; **b** *Xyleborinus saxesenii*; **c** *Xylosandrus crassiusculus*; **d** *Xyleborus affinis*. Scale = 1 mm

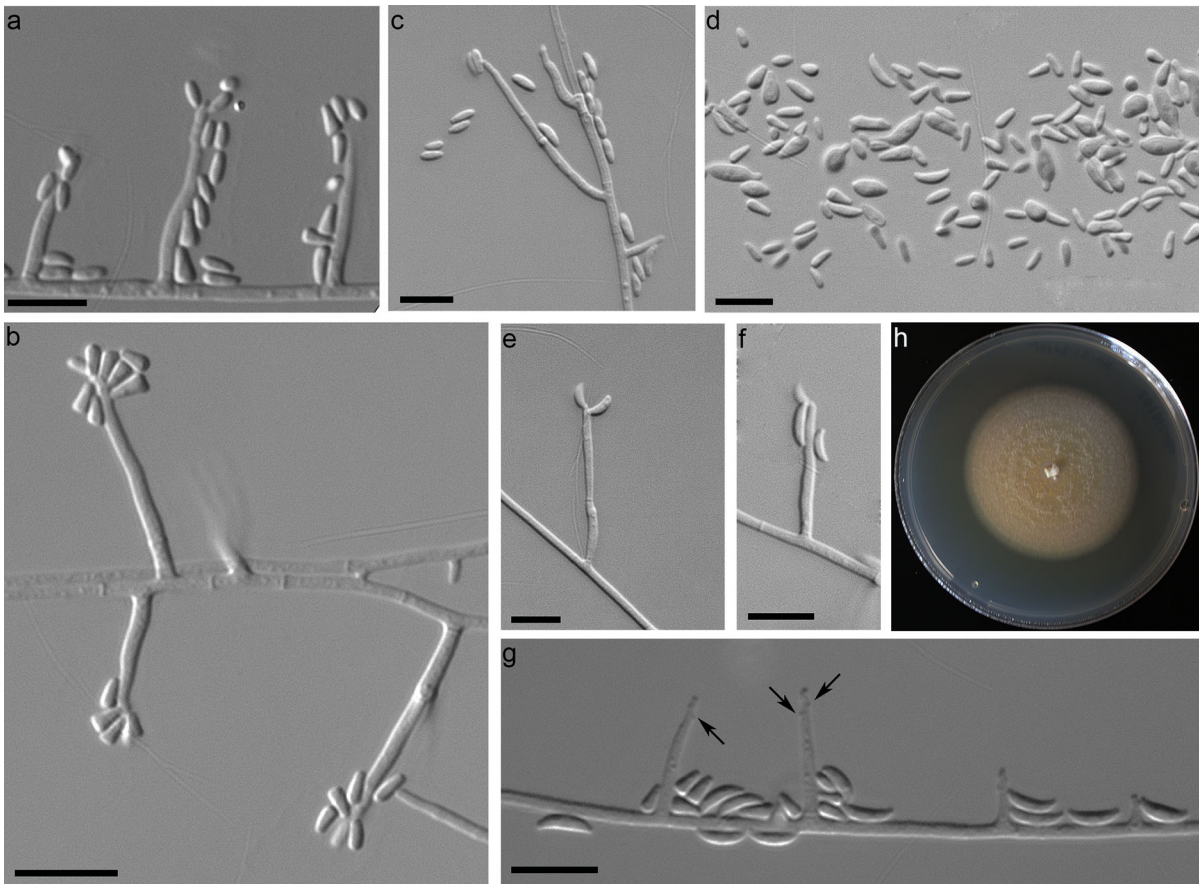


Fig. 6 Morphological characteristics of asexual structures of *Ceratocystiopsis lunata* sp. nov. **a–c** Conidiogenous cells giving rise to oblong conidia; **d** Conidia; **e–g** Conidiogenous cells giving rise to falcate conidia; **g** arrows Presence of denticles; **h** Pure culture gown on MEA in the dark for 12 days. Scale = 10 µm

beetle. Its primary fungal symbiont, *Ambrosiella roeperi*, was described relatively recently (Harrington et al. 2014), which is surprising given that the beetle was described as long ago as 1866. Recent pyrosequencing of the mycangial community of *Xs. crassiusculus* revealed that this niche is dominated by *Ambrosiella*, but other species including *Ceratocystis, Fusarium, Cladosporium* and various yeasts were also shown to be present (Kostovic et al. 2015).

In this study, two isolates of *Ceratocystiopsis* were obtained from a living *Xs. crassiusculus* specimen. Species of *Ceratocystiopsis* are common symbionts of scolytine bark beetles, which are close relatives of the ambrosia beetles. Previous studies investigating the fungal symbionts of ambrosia beetles in the Platypodinae showed that some species of *Ceratocystiopsis* have a promiscuous relationship with these insects (Inácio et al. 2012; Li et al. 2018). Li et al. (2018) concluded that their *Ceratocystiopsis* sp. 2 obtained from *Euplatypus compositus*, *E. parelellus* and *Oxoplatypus quadridentatus*, was the same species as *Ophiostoma* sp. X obtained from *Platypus cylindrus* in a study by Inácio et al. (2012). Phylogenetic analysis of the LSU region of the *Ceratocytiopsis* isolates obtained from *Xs. crassiusculus*in the present study, grouped these isolates along with those obtained by Li et al. (2018) and they were described here as *C. lunata*. This is the first time that *C. lunata* has been obtained from an ambrosia beetle in the Scolytinae. Our results, therefore, suggest that it could be an auxiliary symbiont of multiple species of ambrosia beetles.

Xyleborus affinis is a pan topical species of ambrosia beetle, native to tropical America (Rabgalia et al. 2006). This species has been introduced into Europe, Asia, Australia and Africa, where its presence was

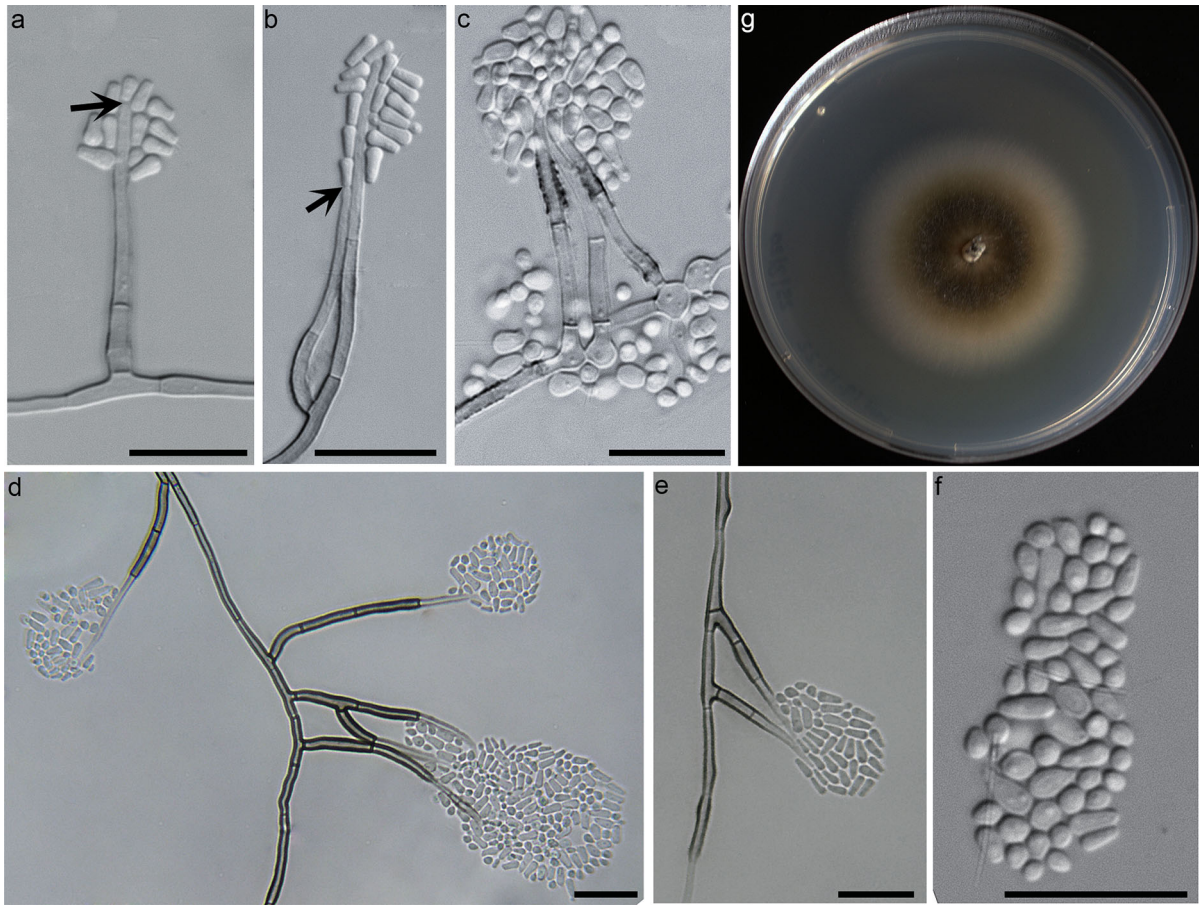


Fig. 7 Morphological characteristics of asexual structures of *Raffaelea promiscua* sp. nov. **a–e** Conidiogenous cells giving rise to conidia; **a** and **b** arrows indicate conidiogenesis taking place; **f** Conidia; **g** Pure culture gown on MEA in the dark for 12 days. Scale: **a–c**, **f** = 10 µm; **d–e** = 20 µm

later recorded in South Africa in the early 1980's (Schedl 1982; Rabgalia et al. 2006). Although the beetle was described more than a decade ago, its primary fungal symbiont is unknown. However, community pyrosequencing of the mycangia from 38 *Xbo. affinis* beetles showed that their fungal communities are highly diverse, including species from the Ophiostomatales, Microascales and a large variety of yeasts (Kostovic et al. 2015).

In this study, five isolates of *Raffaelea arxii* were obtained from a living specimen of *Xbo. affinis. Raffaelea arxii* was first described from South Africa as the primary fungal symbiont of *Xbo. torquatus* (= *Xbo. volvulus*) by Scott and Du Toit (1970). Aside from being the primary fungal symbiont of *Xbo. torquatus*, previous studies have found this species to be vectored by numerous other *Xyleborus* spp. including *Xbo. affinis* (Campbell et al. 2016; Saucedo-Carabez et al. 2018). Our findings provide additional support for *R. arxii* being the primary symbiont of both *Xbo. volvulus* and *Xbo. affinis,* as has previously been hypothesized (Saucedo et al. 2016). However, because *R. arxii* was obtained only from the body surfaces of the beetle specimen and not the mycangia, its symbiotic relationship with *Xbo. affinis* could not be deduced from this study.

Conclusions

Ambrosia beetles associate with various fungal symbionts that act as their source of nutrition, aid in their development and play an important part in their adaptive success. In this study, five species of

ophiostomatalean fungi were obtained from four species of wood-boring beetle, three species of scolytine ambrosia beetle and one species of bostrichid beetle. Based on morphological characters and DNA sequence data, two new species of ophiostomatalean fungi were described and one is reported from the country for the first time. This study, like many others investigating the ophiostomatalean fungi in South Africa has led to the discovery and description of new species, suggesting that there are more novel species to be discovered. However, with very few studies focussed on investigating the diversity of ophiostomatalean fungi associated with ambrosia beetles in South Africa, this appears to be a niche that warrants further investigation.

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Data availability All sequence data produced in the study have been made publicly available. All cultures have been deposited in accessible culture collections. Data sets generated and analysed in this study are available from the corresponding author upon request.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethics approval Approval obtained for study from institution. Registration number: NAS282/2019.

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