

New ophiostomatoid fungi from wounds on storm-damaged trees in Afromontane forests of the Cape Floristic Region

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Abstract

Ophiostomatoid fungi, a well-known, tree-associated group, includes some of the most important forest pathogens globally. Several ophiostomatoid species were reported already from *Rapanea melanophloeos* of the Afromontane forests from the Cape Floristic Region (CFR) of South Africa. The aim of this study was to investigate the diversity of ophiostomatoid fungi associated with wounds on other Afromontane forest tree species in the CFR. Storm-damaged trees were surveyed and fungi were isolated from bark and wood samples. Two undescribed ophiostomatoid species were identified based on micro-morphological characters and phylogenetic analyses. They are newly described here as *Graphilbum roseum* and *Sporothrix oleae*. A third taxon in the genus *Graphium* may also represent an undescribed species, but additional data is required to support this hypothesis. *Sporothrix oleae*, a species that groups within the *S. candida* species complex, was associated with *Olea capensis*. *Graphilbum roseum* was isolated from several host tree species including *Curtisia dentata*, *Halleria lucida* and *Pterocelastrus tricuspidatus*, while the *Graphium* species was isolated from *Ilex mitis*.

Key words: *Graphilbum*, *Graphium*, Ophiostomatales, *Sporothrix*, Microascales

Introduction

The Cape Floristic Region (CFR), an internationally recognised biodiversity hotspot at the southern tip of South Africa (Myers et al. 2000), is characterised by high levels of plant gamma-diversity and endemism (Goldblatt and Manning 2000). Even though taxa such as plants, birds and mammals have been well-documented in the CFR (Brooks et al. 2001; Kerley et al. 2003; Stattersfield et al. 1998), few studies have focussed on the diversity of less conspicuous groups such as fungi (Crous et al. 2006). Based on previous research, for example, Kamgan Nkuekam et al. (2008) and Musvuugwa et al. (2015, 2016a), a large proportion of these fungal taxa may be associated with trees in Afromontane forests, an important component of CFR vegetation (Goldblatt and Manning 2002; Mucina and Rutherford 2006).

Afromontane forests are evergreen and dominated by important canopy trees including Assegaihout (*Curtisia dentata* C.A. Sm.), various Yellowwoods (*Podocarpus* spp. and *Afrocarpus falcatus* (Thunb.) C.N. Page), Ironwood (*Olea capensis* L. ssp. *macrocarpa* (C.H. Wright) I. Verd.) and Cape Beech (*Rapanea melanophloeos* Mez) (Van Wyk and Van Wyk 1997). The largest continuous Afromontane forests are found in the Tsitsikamma area. Further westward they occur in small, fragmented patches on mountains, foothills, coastal platforms, river valleys and dunes along the coastal regions of the Western Cape Province, South Africa (Lubke and McKenzie 1996; Geldenhuys 2010). These forests are of ecological, economic and cultural importance. Tree species such as *Ocotea bullata* (Burch.) Baill, *Olinia ventosa* (L.) Cufod. and *Podocarpus* spp. are economically important for timber used in carpentry (Turpie et al. 2003). Others are used for medicinal purposes. The bark of *R. melanophloeos*, *C. dentata* and *O. bullata*, for example, is used by local people for the treatment of various ailments (Vermeulen et al. 2012).

The ophiostomatoid fungi represent one of the best-studied tree-associated fungal groups. This polyphyletic group (De Beer et al. 2013a) includes some of the best-known tree pathogenic fungi globally (Heath et al. 2009; Kamgan Nkuekam et al. 2012) as many of its members cause rot and wilting diseases and cankers that lead to death of infected trees (Roux and Wingfield 2013; Wingfield et al. 1993). The ophiostomatoid fungi are grouped into two orders, the Microascales (*Ceratocystis* Ellis & Halst., *Knoxdaviesia* M.J. Wingf., P.S. van Wyk & Marasas and *Graphium* Corda) and the Ophiostomatales [*Raffaelea* Arx & Hennebert, *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr., *Graphilbum* H.P. Upadhyay & W.B. Kendr., *Ophiostoma* Syd., *Sporothrix* Hektoen & C.F. Perkins and *Leptographium* Lagerb. & Melin (De Beer et al. 2013b,

2016)]. Some well-documented examples of pathogenic ophiostomatoid fungi include the *Ceratocystis fimbriata* species complex responsible for serious diseases of various economically important trees like mango, coffee, *Acacia* Mill. and *Eucalyptus* L'Hér. (Fourie et al. 2015; Oliveira et al. 2015), *C. platani* (Walter) Engelbr. & T.C. Harr. causing canker stain disease of plane trees (Tsopelas et al. 2017), *C. fagacearum* (Bretz) Hunt responsible for oak wilt disease (Juzwik et al. 2008), *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, the causal agents of Dutch elm disease in Europe and America (Brasier and Buck 2001; Pipe et al. 2000), *Raffaelela lauricola* T.C. Harr., Fraedrich & Aghayeva, responsible for the Laurel wilt disease in the southeastern USA (Harrington et al. 2008) and *R. quercivora* Kubono et Shin. Ito causing oak die-back and mortality of Japanese oak trees (Kubono and Ito 2002).

Most research published on ophiostomatoid fungi in South Africa focussed on members associated with exotic plantation trees (De Beer et al. 2003; Kamgan Nkuekam et al. 2012, 2013; Zhou et al. 2006) and a few of these have proven to be pathogenic on their hosts. For example, *Ceratocystis albifundus* M.J. Wingf., De Beer & M.J. Morris, which is responsible for wattle wilt disease in *Acacia mearnsii* De Wild., has led to significant economic losses in South African plantations (Roux and Wingfield 2013). Several other *Ceratocystis* species with varying levels of pathogenicity have been reported on *Eucalyptus* (Kamgan Nkuekam et al. 2013). Other species of ophiostomatoid fungi were collected from tree wounds on native trees in Afromontane forests caused by weather or animal or human activities. Documented examples in the Microascales include *C. albifundus* and *C. savannae* Kamgan Nkuekam & Jol. Roux that are associated with several native hosts (Roux and Wingfield 2013), *C. tsitsikammensis* Kamgan & Jol. Roux from *Ocotea bullata* and *Rapanea melanophloeos* (Kamgan Nkuekam et al. 2008), and *Graphium adansoniae* Cruywagen, Z.W. de Beer & Jol. Roux from baobabs (Cruywagen et al. 2010). Taxa in the Ophiostomatales known from wounds on trees in this region include *Ophiostoma quercus* (Georgev.) Nannf. from *R. melanophloeos* (De Beer et al. 2003; Kamgan Nkuekam et al. 2008), *Sporothrix stenoceras* (Robak) Nannf., *S. itsvo* Musvuugwa, L.L. Dreyer & F. Roets, *S. noisomeae* Musvuugwa, L.L. Dreyer & F. Roets, *S. uta* Musvuugwa, L.L. Dreyer & F. Roets, and *S. rapanae* Musvuugwa, Z.W. de Beer, L.L. Dreyer & F. Roets from *R. melanophloeos* trees (Musvuugwa et al. 2016a), *Ophiostoma palustre* J.A. Osorio, Z.W. de Beer & Jol. Roux from native mangroves (Osorio et al. 2016), *O. thermarum* J.A. van der Linde, Z.W. de Beer & Jol. Roux and *Aureovirgo volantis* J.A. van der Linde, Z.W. de Beer & Jol. Roux on *Euphorbia ingens* (Van der Linde et al. 2016). In addition, at least one currently undescribed

ophiostomatoid species is known from tree wounds in CFR forests (Musvuugwa et al. 2016b). A few taxa are known from subcortical beetles infesting native tree species in the Afromontane forests. These include *Sporothrix aemulophila* Musvuugwa, Z.W. de Beer, L.L. Dreyer & F. Roets and *Raffaelea rapanae* Musvuugwa, Z.W. de Beer, L.L. Dreyer & F. Roets from *R. melanophloeos*, *S. pallida* (Tubaki) Matsush. and *R. vaginata* T. Musvuugwa, Z.W. de Beer, L.L. Dreyer & F. Roets from *O. capensis* ssp. *macrocarpa* (Musvuugwa et al. 2015).

Very little is known about ophiostomatoid fungi associating with forest trees other than *R. melanophloeos* (Musvuugwa et al. 2016a). During surveys of fungi associated with wounds on other forest trees (Musvuugwa 2014; Musvuugwa et al. 2016b), three possibly new and undescribed ophiostomatoid taxa were collected. The present study sets out to evaluate the identity of these taxa based on morphological and molecular phylogenetic comparisons.

Materials and methods

Sampling of plant material and fungal isolation

Sampling was conducted in various native forests of the CFR, including Groenkop Forest Reserve (S 33°56'32" E 22°32'50"), Gouldveld (S 33°54'44" E 23°0'10"), Gouna (S 33°57'3" E 23°2'10") and Assegaaibosch (S 33°58'23" E 18°56'11") between 2010 and 2012. Bark and wood samples were collected from wounds on various storm-damaged native trees following the methods described in Musvuugwa et al. (2016a, b). In total 56 bark and wood samples were collected from 56 tree individuals. Samples were typically associated with wounds. Samples were stored in separate sampling bags and transferred to the laboratory where they were examined for ophiostomatoid fungi using a Leica EZ4 microscope (Wetzlar, Germany).

Where ophiostomatoid-like fungi were present, a sterile needle was used to collect and transfer spores from the tips of sporulating structures to 2% malt extract agar (MEA, Biolab, Midrand, South Africa). Cultures producing conidia in sticky droplets on upright stipes and/or ascospores in sticky droplets on ascomata with elongated necks were investigated. Brown or black discoloration of wood was sampled by removing small pieces of wood tissues with a sterile scalpel from the leading edge of the stain and placing on MEA. Primary isolation plates were incubated in the dark at room temperature and examined daily for fungal growth. Colonies that produced structures that resembled anamorphic characteristics of ophiostomatoid fungi were purified by a single hyphal tip from edges of actively growing colonies to fresh MEA plates. Purified

cultures were maintained on Petri dishes at 4°C for further use. Representatives of pure cultures of morphotypes collected in this study were preserved in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa and in the culture collection (CBS) of the Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands. Herbarium type specimens of the new species were deposited in the National Collection of Fungi (PREM), Roodeplaat, South Africa.

Morphological characterisation

Where available, perithecia and ascospores of ophiostomatoid fungi were collected from plant material and mounted in 85% lactic acid for observation. Perithecia, conidia and conidiophores that formed in culture were treated similarly and all structures were studied using Leica EZ4, Nikon SMZ18 or Nikon Eclipse Ni microscopes. Photographs of microscopic structures were taken with a Nikon digital camera (DS-Ri2, Nikon, Tokyo, Japan). Twenty-five measurements of all morphologically and taxonomically informative structures were made for representative isolates including those chosen as ex-type strains of the undescribed taxa. The maximum and minimum measurements for each taxonomically informative structure was noted. Rayner's colour chart (1970) was used for the description of colony characteristics.

DNA extraction, amplification and sequencing

Fungal cultures resembling ophiostomatoid fungi were grouped into morphotypes based on micro-morphological and colony characteristics. At least three isolates representing each morphotype were randomly chosen for DNA sequencing (Table 1). A sterile scalpel was used to harvest fungal mycelium from the edges of actively growing pure cultures on MEA. Following the manufacturer's instructions, a Sigma-Aldrich™ plant extraction kit (USA) was used for the extraction of genomic DNA. To amplify the internal transcribed spacer (ITS1, ITS2) and 5.8S gene regions, the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) were used. Preliminary phylogenetic placements were based on ITS sequence data and additional gene regions required for more accurate identifications were selected according to these preliminary analyses and following De Beer et al. (2013a, b).

A fragment of the Beta-tubulin (β T) gene was amplified with primers Bt2a and Bt2b (Glass and Donaldson 1995) for selected isolates (Table 1). Part of the transcription elongation factor-1 α gene was amplified using primers EF1-F and EF2-R (Jacobs et al. 2004). Choice of particular markers used for phylogenetic analyses was based on

availability of sequences for comparisons of the different taxa in GenBank and on the most informative markers for those particular taxa based on previous studies (De Beer et al. 2016; Lynch et al. 2016; Reid and Hausner 2015). PCR conditions were as described by Musvuugwa et al. (2015). PCR reactions were conducted using a Gene Amp^R PCR system 2700 thermal cycler (Applied Biosystems, Foster City, U.S.A.).

Table 1. Culture collection and GenBank accession numbers for strains of ophiostomatoid fungi isolated from different native CFR trees collected in this study

Species	CMW	CBS	ITS	βT	EF	Host tree	Site
<i>Graphilbum roseum</i> sp. nov.	40349	141074	KY05075	n.a	n.a	<i>Curtisia dentata</i>	Gouna Forest
			1				
	40350	141075	KY05075	n.a	n.a	<i>C. dentata</i>	Gouna Forest
			3				
<i>Graphium</i> sp.	40351	141076	KY05075	n.a	n.a	<i>Pterocelastrus tricuspidatus</i>	Groenkop Forest
			2				
	40353	n.a	KY05075	n.a	n.a	<i>Halleria lucida</i>	Gouna Forest
		4					
<i>Graphium</i> sp.	40354	141072	KY05075	n.a	n.a	<i>Ilex mitis</i>	Assegaaibosch
			7				
	40355	141071	KY05075	n.a	KY321426	<i>I. mitis</i>	Assegaaibosch
		5					
<i>Sporothrix oleae</i> sp. nov.	40356	141073	KY05075	n.a	n.a	<i>I. mitis</i>	Assegaaibosch
			6				
	40360	142026	KY05075	KY094078	n.a	<i>Olea capensis</i> ssp. <i>macrocarpa</i>	Gouldveld Forest
		8					
<i>Sporothrix oleae</i> sp. nov.	40361	142027	MN29885	KY094079	n.a	<i>O. capensis</i> ssp. <i>macrocarpa</i>	Goudveld Forest
			0				
<i>Sporothrix oleae</i> sp. nov.	40362	142082	MN29885	KY094080	n.a	<i>O. capensis</i> ssp. <i>macrocarpa</i>	Gouldveld Forest
			1				

All PCR products were amplified, separated using agarose gel electrophoresis stained with GelRed (Biotium Inc., California, U.S.A.) and visualized under ultraviolet light. Following the manufacturer's instructions, all amplified PCR products were cleaned using the EXOSAP-IT kit (USB Corporation, Cleveland, Ohio, U.S.A.). Purified fragments were sequenced using the respective PCR primers and a Big DyeTM Terminator v3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, CA, U.S.A.) and analysed on an ABI PRISMTM 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, U.S.A.). Sequencing conditions followed those used by

Musvuugwa et al. (2015) and both DNA strands were sequenced using the same primers used for PCR amplification. Consensus sequences were constructed from the resultant sequences using the CLC Genomics Workbench software package (CLCBio, Aarhus, Denmark). Sequences generated in this study were deposited in NCBI GenBank (<http://www.ncbi.nlm.nih.gov>) (Table 1).

Phylogenetic analyses

Using ITS sequences, BLAST algorithm (Altschul et al. 1990) searches were conducted on the GenBank sequence database in order to preliminarily identify the fungal isolates. Sequences of taxa that were closely related to isolates collected in this study (Table 1) were downloaded from GenBank and aligned with sequences generated in this study for each data set using MAFFT 6 (Kato and Toh 2008). Datasets were analysed using Bayesian inference (BI) with MrBayes 3.2 (Ronquist et al. 2012) and maximum likelihood (ML) methods [RAxML 8.2.10 (Stamatakis 2014) on CIPRES Science Gateway (Miller et al. 2010; <https://www.phylo.org/portal2>)]. A mixed model was used in MrBayes analyses to average models in the GTR family, and including a Γ correction. Two independent Markov chains were run simultaneously for 10 million generations starting from a random tree. A sample frequency of 2000 was implemented, and the first 2.5 million generations discarded as burnin. The remaining trees were pooled into a 50% majority rule consensus tree. Default settings and the default model (GTR+ Γ) were used for all RAxML analyses, and confidence support values for nodes were estimated using 1000 bootstrap replicates.

Growth in culture

The temperature for optimal growth of selected cultures of putative new taxa was determined by transferring mycelium-covered disks of agar (5 mm diam) from edges of actively growing 1 week old cultures to the centres of 90 mm fresh Petri dishes containing 20 mL 2% MEA. These plates were incubated in the dark at a range of different temperatures (from 5°C to 35°C at intervals of 5°C) for 10 days. The experiment was replicated five times, each replicate using a different strain. After the 10-day incubation period, colony diameters at the optimal growth temperature were determined by calculating the average of two perpendicular measurements per colony and then calculating the mean per taxon.

Results

Fungal isolates and Phylogenetic analyses

In total, 21 ophiostomatoid fungi were isolated and grouped on the basis of micro-morphological and colony characteristics into three different morphotypes; seven isolates resembling species in the genus *Sporothrix* collected from *Olea capensis* ssp. *macrocarpa* trees in Gouldveld, eight *Graphilbum* isolates collected from *Curtisia dentata*, *Halleria lucida* L., *Pterocelastrus tricuspidatus*, *Trichocladus crinitus* (Thunb.) Pers. and *O. capensis* ssp. *macrocarpa* from the Groenkop and Gouna forests; and six *Graphium* isolates from *Ilex mitis* (L.) Radlk. in Assegaaibosch (Table 1). Generic placement of these isolates were confirmed by ITS data.

The phylogenetic placement of the *Graphilbum* was assessed using only ITS data because no other loci have yet been provided for *Graphilbum* species (Reid and Hausner 2015) (Fig. 1). Placement of the *Sporothrix* taxon was first investigated using ITS data (Appendix 1), but also β T data as it is not possible to distinguish between closely related members in this clade based only on ITS data (De Beer et al. 2016) (Fig. 2). Placement of the *Graphium* taxon was assessed using ITS (Appendix 2) and EF (Fig. 3) data following Lynch et al. (2016). GenBank accession numbers for all other taxa used in analyses are presented on the respective phylogenetic trees (Figs. 1–3) and in Table 2.

Table 2. Culture collection and GenBank accession numbers for strains of ophiostomatoid used for phylogenetic analyses

Species	Isolate/Strain number	ITS	β t	EF	Substrate	Country of origin	References
<i>Graphilbum</i> sp.	CXY1939	MH55903	n.a	n.a	<i>Tomicus yunnanensis</i>	China	HuiMin et al. 2019
<i>Graphilbum</i> sp.	KFL342NDB16AO	MH740929	n.a	n.a	Hardwood tree	Poland	Ostafinska et al. 2018 (unpublished)
<i>Graphilbum</i> sp.	HG19	KX099637	n.a	n.a	<i>Pinus sylvestris</i> var. <i>mongolica</i>	China	Wang 2016 (unpublished)
<i>Graphilbum</i> sp. 1	55KFJD	KY568111	n.a	n.a	<i>Pityokteines curvidens</i>	Poland	Jankowiak et al. 2017
<i>Graphilbum</i> sp. 2	132aMFJD	KY568118	n.a	n.a	<i>Pissodes piceae</i>	Poland	Jankowiak et al. 2017
<i>Graphilbum</i> sp. 3	C2316	GU129997	n.a	n.a	<i>Hylurgus ligniperda</i>	USA	Kim et al. 2011
<i>Graphilbum</i> sp. 4	C2477	GU129987	n.a	n.a	<i>H. ligniperda</i>	USA	Kim et al. 2011
<i>Graphilbum</i> sp. 5	CMW 26258	EU785449	n.a	n.a	<i>Dendroctonus valens</i>	China	Lu et al. 2009
<i>Graphilbum</i> sp. 6	CMW 22829	DQ539535	n.a	n.a	<i>Pinus radiata</i>	Spain	Romon et al. 2007
<i>Graphilbum</i> sp. 7	C1496	DQ062977	n.a	n.a	<i>P. radiata</i>	New Zealand	Thwaites et al. 2005
<i>Gra. fragrans</i>	C1224	AF198248	n.a	n.a	Unknown	Sweden	Harrington et al. 2001
<i>Gra. kesiyae</i>	CMW41657	MG205668	n.a	n.a	<i>Pinus kesiya</i>	China	Chang et al. 2017
<i>Gra. microcarpum</i>	YCC612	GU134170	n.a	n.a	<i>Ips subelongatus</i>	Japan	Lin et al. 2009 (unpublished)
<i>Gra. nigrum</i>	CBS163.61	MH858010	n.a	n.a	Unknown	USA	Vu et al. 2019
<i>Gra. puerense</i>	CMW41942	MG205671	n.a	n.a	<i>P. kesiya</i>	China	Chang et al. 2017

<i>Gra. rectangulosporium</i>	TFM:FPH 7756	AB242825	n.a	n.a	<i>Abies</i> sp.	Japan	Ohtaka et al. 2006
<i>Gra. sparsum</i>	CBS405.77	MH861010	n.a	n.a	Unknown	USA	Vu et al. 2019
<i>Gra. tsugae</i>	UAMH 11701	NR137871	n.a	n.a	<i>Tsuga heterophylla</i>	Canada	Reid and Hausner 2015
<i>Graphium</i> sp.	UCR2162	n.a	n.a	KJ131241	<i>Persea americana</i>	USA	Twizeyimana et al. 2014 (unpublished)
<i>Graphium</i> sp.	UCR2163	n.a	n.a	KJ131242	<i>P. americana</i>	USA	Twizeyimana et al. 2014 (unpublished)
<i>Graphium</i> sp.	UCR2137	n.a	n.a	KJ131246	<i>P. americana</i>	USA	Twizeyimana et al. 2014 (unpublished)
<i>Graphium</i> sp.	UCR2140	n.a	n.a	KJ131247	<i>P. americana</i>	USA	Twizeyimana et al. 2014 (unpublished)
<i>Graphium</i> sp.	UCR2289	n.a	n.a	KM592360	<i>P. americana</i>	USA	Lynch et al. 2016
<i>Graphium</i> sp.	UCR2291	n.a	n.a	KM592361	<i>P. americana</i>	USA	Lynch et al. 2016
<i>Graphium</i> sp.	UCR2132	n.a	n.a	KM363259	unknown	USA	Twizeyimana et al. 2014 (unpublished)
<i>G. adansoniae</i>	CMW30617	n.a	n.a	HM630596	<i>Adansonia digitata</i>	South Africa	Cruywagen et al. 2010
	CMW30618	n.a	n.a	HM630598	<i>A. digitata</i>	South Africa	
	CMW30619	n.a	n.a	n.a	<i>A. digitata</i>	South Africa	
	CMW30620	n.a	n.a	HM630597	<i>A. digitata</i>	South Africa	
	CMW30621	n.a	n.a	n.a	<i>A. digitata</i>	South Africa	
	CMW30624	n.a	n.a	n.a	<i>A. digitata</i>	South Africa	
	CMW30622			HM630599	<i>A. digitata</i>	South Africa	

<i>G. basitruncatum</i>	JCM 9300	AB038427	n.a	KJ131248	Forest soil	Solomon Islands	Kumar et al. 2007
						Canada	
<i>G. carbonarium</i>	CMW12420	n.a	n.a	HM630603	<i>Salix babylonica</i> , <i>Pissodes sp</i>	China	Cruywagen et al. 2010; Paciura et al. 2010
	CMW12418	n.a	n.a	HM630602	<i>S. babylonica</i> , <i>Pissodes sp</i>	China	
<i>G. euwallaceae</i>	UCR2308	n.a	n.a	KM592363	<i>Acacia auriculiformis</i>	Vietnam	Lynch et al. 2016
	UCRFD97	n.a	n.a	KF534806	<i>Euwallacea sp.</i>	USA	
<i>G. fabiforme</i>	CMW30626	GQ200616	n.a	HM630592	<i>Adansonia rubrostipa</i>	Madagascar	Cruywagen et al. 2010
	CMW30627	GQ200617	n.a	HM630593	<i>A. rubrostipa</i>	Madagascar	
<i>G. fimbriisporum</i>	CMW5605	AY148177	n.a	HM630590	<i>Picea abies</i>	France	Cruywagen et al. 2010
	CMW5606	AY148180	n.a	HM630591	<i>P. abies</i>	Austria	
<i>G. kuroshium</i>	UCR4622	KX262285	n.a	KX262295	<i>Euwallacea sp</i>	USA	Na et al. 2018
	UCR4593	KX262276	n.a	KX262286	<i>P. americana</i>	USA	
<i>G. laricis</i>	CMW5603	AY148182	n.a	HM630589	<i>Larix decidua</i>	Austria	Cruywagen et al. 2010
	CMW5601	AY148183	n.a	HM630588	<i>L. decidua</i>	Austria	
<i>G. madagascariense</i>	CMW30628	GQ200619	n.a	HM630595	<i>Adansonia rubrostipa</i>	Madagascar	Cruywagen et al. 2010
	CMW30629	GQ200620	n.a	HM630594	<i>A. rubrostipa</i>	Madagascar	
<i>G. penicillioides</i>	CMW5292	n.a	n.a	HM630600	<i>Populus nigra</i>	Czech Republic	Cruywagen et al. 2010
	CMW5295	n.a	n.a	HM630601	<i>P. nigra</i>	Czech Republic	

<i>G. pseudormiticum</i>	CMW503	AY148186	n.a	HM630586	<i>Pinus</i> sp.	South Africa	Cruywagen et al. 2010
	CMW12285	FJ434981	n.a	HM630587	<i>Tsuga dumosa</i>	China	
<i>O. piliferum</i>	CBS129.32	AF221070	n.a	n.a	Unknown	Europe	Schroeder et al. 2001
<i>Sporothrix abietina</i>	CMW22310	n.a	HM067820	n.a	<i>Abies vejari</i>	Mexico	Linnakoski et al. 2010
<i>S. aemulophila</i>	CMW40381	n.a	KT192607	n.a	<i>Rapanea melanophloeos</i>	South Africa	Musvuugwa et al. 2015
	CMW40382	n.a	KT192608	n.a	<i>Rapanea melanophloeos</i>	South Africa	
<i>S. africanum</i>	CMW1104	n.a	DQ316162	n.a	<i>Protea caffra</i>	South Africa	Roets et al. 2006
<i>S. aurorae</i>	CMW19362	n.a	DQ396800	n.a	<i>Pinus elliotii</i>	South Africa	Zhou et al. 2006
<i>S. brasiliensis</i>	IPEC 16490	n.a	AM116946	n.a	Human	Brazil	Marimon et al. 2006
<i>S. cabralli</i>	CIEFAP456	n.a	KT381295	n.a	<i>Nothofagus</i> sp.	Patagonia	De Errasti et al. 2016
	CIEFAP458	n.a	KT381296	n.a	<i>Nothofagus</i> sp.	Patagonia	
<i>S. candida</i>	CMW26484	n.a	HM041874	n.a	<i>Eucalyptus cloezina</i>	South Africa	Kamgan Nkuekam et al. 2012
	CMW26485	n.a	HM041871	n.a	<i>E. cloezina</i>	South Africa	
	CMW26486	n.a	HM041872	n.a	<i>E. cloezina</i>	South Africa	
	CMW26483	n.a	HM041873	n.a	<i>E. cloezina</i>	South Africa	
<i>S. dentifunda</i>	CMW13016	n.a	AY495445	n.a	<i>Quercus</i> wood	Hungary	Aghayeva et al. 2005
<i>S. dimorphospora</i>	CMW12529	n.a	AY495439	n.a	Soil	Canada	Aghayeva et al. 2005
<i>S. fusiformis</i>	CMW9968	n.a	AY280461	n.a	<i>Populus nigra</i>	Azerbaijan	Aghayeva et al. 2004
<i>S. gemella</i>	CMW23057	n.a	DQ821554	n.a	<i>Protea caffra</i>	South Africa	Roets et al. 2006
<i>S. globosa</i>	FMR 8600	n.a	AM116966	n.a	Clinical	Spain	Marimon et al. 2006
<i>S. humicola</i>	CMW7618	n.a	EF139100	n.a	Soil	South Africa	De Beer et al. 2003

<i>S. inflata</i>	CMW12527	n.a	AY495437	n.a	Wheat-field soil	Germany	Aghayeva et al. 2005
<i>S. itsvo</i>	TM-2016a	n.a	KU639626	n.a	<i>Rapanea melanophloeos</i>	South Africa	Musvuugwa et al. 2016
	CMW40370	n.a	KU639625	n.a	<i>R. melanophloeos</i>	South Africa	
	CMW40326	n.a	KU639628	n.a	<i>R. melanophloeos</i>	South Africa	
<i>S. lunata</i>	CMW10565	n.a	AY280465	n.a	<i>Larix decidua</i>	Austria	Aghayeva et al. 2004
<i>S. luriei</i>	ATCC18616T	n.a	AM747289	n.a	Clinical	South Africa	Marimon et al. 2008
<i>S. mexicana</i>	CBS 120341	n.a	AM498344	n.a	Environmental	Mexico	Madrid et al. 2010
<i>S. pallida</i>	CMW40330	n.a	KT192610	n.a	<i>Olea capensis</i>	South Africa	Musvuugwa et al. 2015; De Meyer et al. 2008
	CMW40331	n.a	KT192611	n.a	<i>O. capensis</i>	South Africa	
	CMW40332	n.a	KT192612	n.a	<i>O. capensis</i>	South Africa	
	CBS150.87	n.a	EF139109	n.a	Sediment in water purification plant	Germany	
	CBS131.56	n.a	EF139110	n.a		Japan	
	CBS182.63	n.a	EF139111	n.a	<i>Stermonitis fusca</i> Garden soil	Netherlands	
<i>S. palmiculminata</i>	CMW20677	n.a	DQ316153	n.a	<i>Protea repens</i>	South Africa	Roets et al. 2006
<i>S. phasma</i>	CMW20676	n.a	n.a	n.a	<i>P. laurifolia</i>	South Africa	Roets et al. 2006
<i>S. protearum</i>	CMW1103	n.a	DQ316165	n.a	<i>P. caffra</i>	South Africa	Roets et al. 2006
<i>S. proteasedis</i>	CFR123	n.a	EU660466	n.a	<i>Protea</i> sp.	South Africa	Roets et al. 2009
<i>S. rapanae</i>	CMW40368	n.a	KU639623	n.a	<i>Rapanea melanophloeos</i>	South Africa	Musvuugwa et al. 2016
	CMW40369	n.a	KU639624	n.a	<i>R. melanophloeos</i>	South Africa	
	CMW40367	n.a	KU639622	n.a	<i>R. melanophloeos</i>	South Africa	

<i>S. schenckii</i>	CBS 359.36T	n.a	AM116911	n.a	Not known	Not known	Marimon et al. 2006
<i>S. splendens</i>	CMW20674	n.a	DQ316166	n.a	<i>Protea repens</i>	South Africa	Roets et al. 2006
<i>S. stenoceras</i>	CMW3202	n.a	AY280471	n.a	Pine pulp	South Africa	Aghayeva et al. 2004
<i>S. stylites</i>	CMW14543	n.a	EF139096	n.a	Pine utility pole	South Africa	De Meyer et al. 2008
<i>S. uta</i>	CMW40316	n.a	KU639616	n.a	<i>Rapanea melanophloeos</i>	South Africa	Musvuugwa et. al. 2016
					<i>R. melanophloeos</i>	South Africa	
					<i>R. melanophloeos</i>	South Africa	
<i>S. varieciabatus</i>	CMW23060	n.a	DQ821573	n.a	<i>Protea longifolia</i>	South Africa	Roets et al. 2008
<i>S. zambiensis</i>	CMW28604	n.a	EU660473	n.a	<i>P. caffra</i>	Zambia	Roets et al. 2009

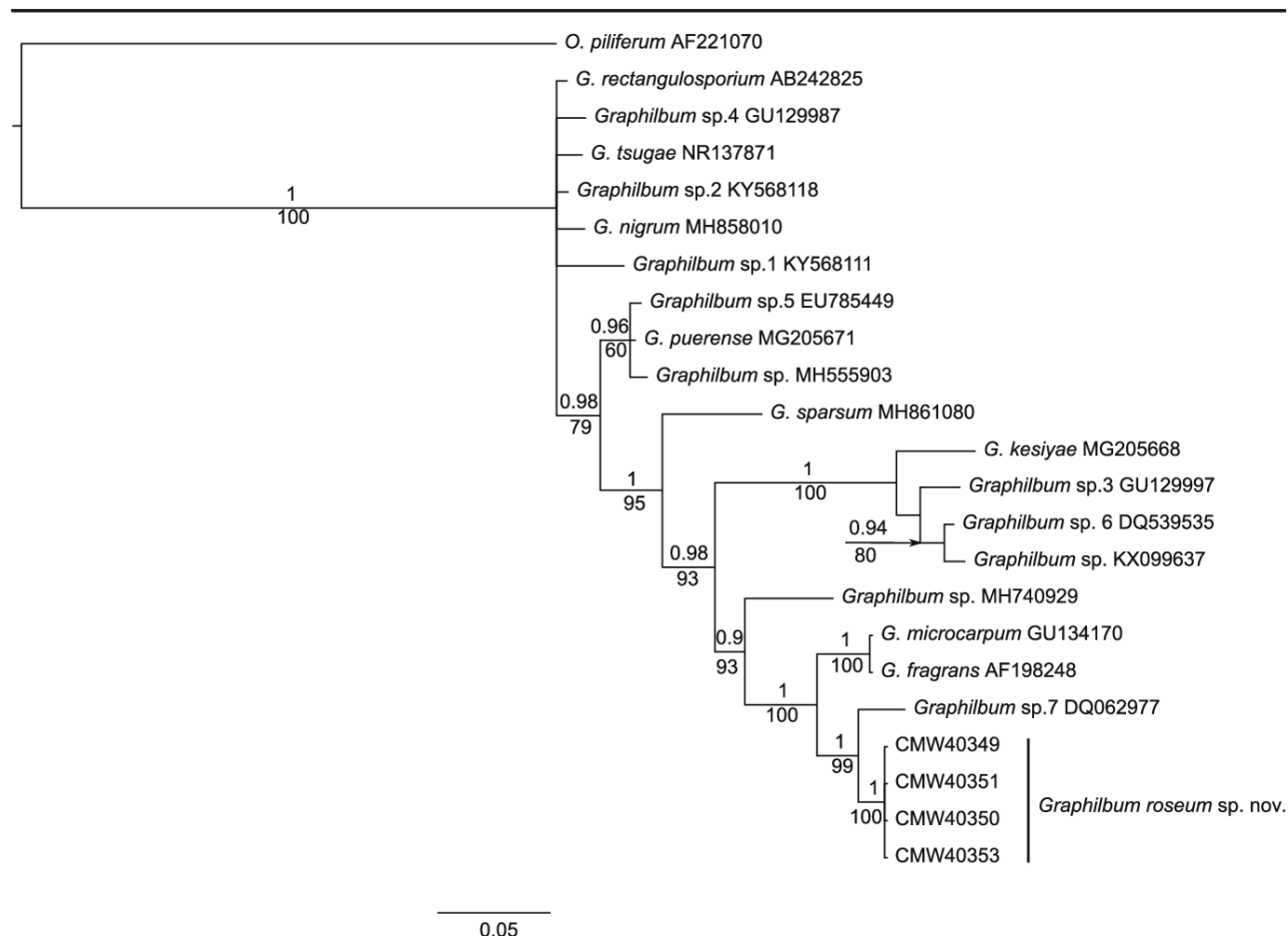


Fig. 1 Bayesian Inference consensus tree based on ITS sequence data for species of *Graphilbum*. Numbers of isolates obtained in the present study are printed in bold type. Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood analysis. The scale bar is in substitutions per site.

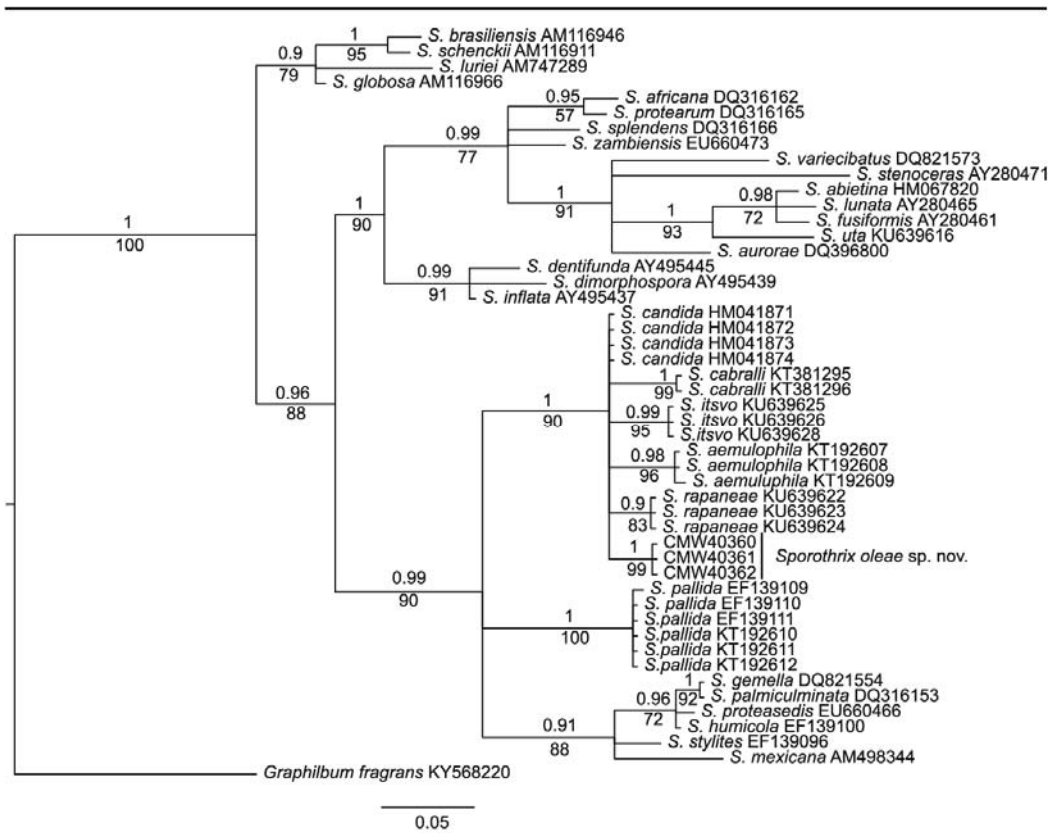


Fig. 2 Bayesian Inference consensus tree based on β T sequence data for species of *Sporothrix*. Numbers of isolates collected in the present study are printed in bold type. Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood analysis. The scale bar is in substitutions per site.

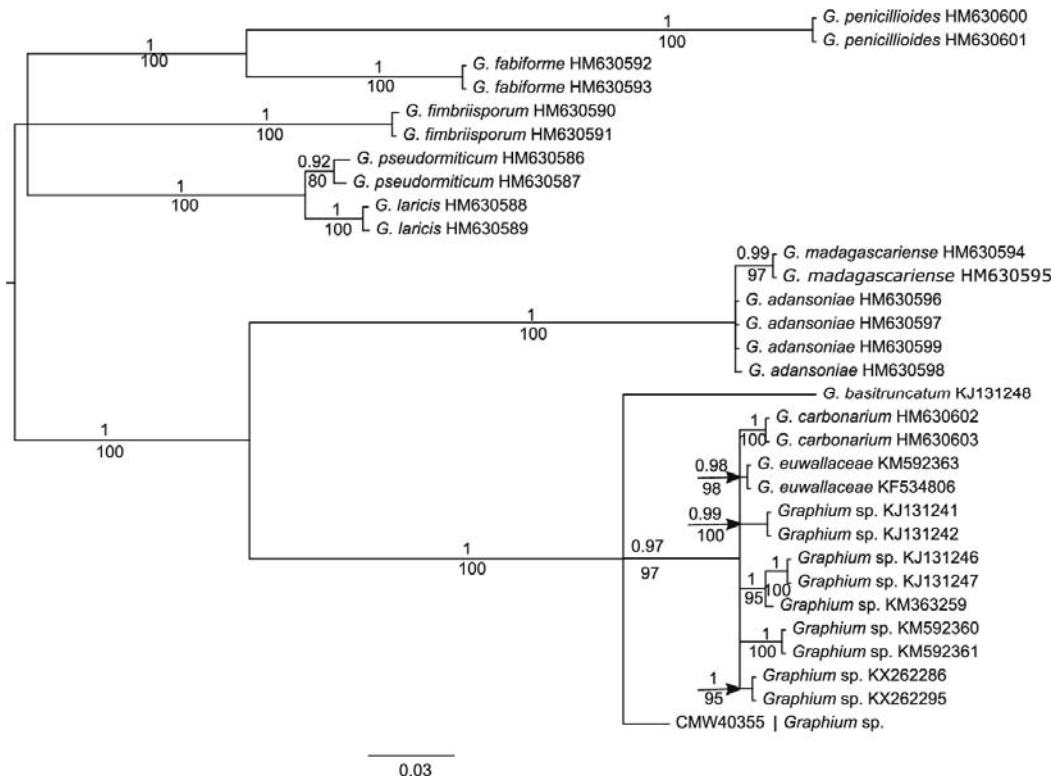


Fig. 3 Bayesian Inference consensus tree based on EF sequences of *Graphium* species. Numbers of isolates collected in the present study are printed in bold type. Values above nodes indicate posterior probabilities obtained through Bayesian Inference. Values below nodes indicate bootstrap values (1000 replicates) obtained from Maximum Likelihood analysis. The scale bar is in substitutions per site.

Based on ITS data, isolates of the *Graphilbum* grouped as a distinct taxon (Fig. 1) sister to an unnamed *Graphilbum* species previously collected from *Pinus radiata* D. Don in Australia (Thwaites et al. 2005). The *Sporothrix* isolates resolved within the *S. candida* species complex with strong support when both ITS (Appendix 1) and β T data (Fig. 2) were analyzed. Based on the β T data set, isolates of this taxon formed monophyletic clade, but resolution for taxa in the *S. candida* species complex remain poor (Fig. 2). Isolates of the *Graphium* taxon grouped in a clade containing *G. basitruncatum* (Matsush.) Seifert & G. Okada, *G. carbonarium* Paciura, Z.W. de Beer, X.D. Zhou & M.J. Wingf., *G. euwallaceae* Twizeyim., S.C. Lynch & Eskalen, *G. jumulu* P.A. Barber & Crous and various underdescribed taxa based on ITS (Appendix 2) and EF sequences (Fig. 3). For ITS, rooting of the tree containing *Graphilbum* followed de Beer et al. (2016) and *Ophiostoma piliferum* (Fr.) Syd. & P. Syd. was used as the outgroup, and the *Sporothrix* ITS data set was rooted using *Graphilbum rectangulosporium*

(Ohtaka, Masuya & Yamaoka) Z.W. de Beer & M.J. Wingf.. *Graphilbum fragrans* (Math.-Käärik) Z.W. de Beer, Seifert & M.J. Wingf. was used as outgroup for the *Sporothrix* BT data set. The *Graphium* ITS data set was rooted using *Scedosporium* and *Parascedosporium* as outgroups following Lackner and De Hoog (2011). For the more limited *Graphium* EF data set, the tree was rooted as to be maximally consistent with the ITS tree and was rooted using *Graphium penicillioides* Corda as outgroup.

TAXONOMY

The *Sporothrix* and *Graphilbum* taxa collected from wounds on native Afromontane forest trees were recognised as new species based on a combination of evidence from phylogenetic analyses and micro-morphological characteristics. These are described below. A detailed morphological description of the *Graphium* taxon isolated in the present study is also provided to aid future comparative studies. This taxon may also represent an undescribed species, but currently there is insufficient morphological and molecular data to support this hypothesis.

Sporothrix oleae Musvuugwa, L.L. Dreyer & F. Roets, sp. nov. Figures 4a–f.
Mycobank MB 822536

Etymology: The epithet *oleae* refers to the genus name of the host (*Olea capensis* ssp. *macrocarpa*).

Ascomata embedded in or superficial on host substrate ellipsoidal to subglobose, black, covered with pigmented hyphae in culture, 90–150 µm diam (150–240 µm diam in culture); necks black, upright, 320–580 µm long (up to 1.6 mm long in culture), 17–32 µm wide at the base, 4–8 µm wide at the apex; ostiolar hyphae divergent, hyaline, often septate, straight, tapering towards the apex, 15–43 µm long. *Asci* not observed. *Ascospores* kidney-shaped (almost triangular) in side view with pointed ends, aseptate, hyaline, sheaths not observed, 2.5–4.5 × 1.5–2 µm (avg. 3.4 × 1.8 µm), accumulating in transparent sticky droplets at tips of necks, milky white when masses are dry. *Conidiophores* in culture one-celled or occasionally branched and bearing several conidiogenous cells, either borne on vegetative hyphae or on upright hyphae. Conidiogenous cells blastic, cylindrical, tapering towards the apex, denticulate, distally often with inflated clusters of denticles, 15–25 µm long, 1–2 µm wide near the base. *Conidia* hyaline, aseptate, ellipsoidal with a pointed base, 3–5.5 × 2–3 µm (avg. 4.2 × 2.5 µm). *Colonies* pale yellow (19" f), fluffy towards the centre, circular with entire edge, odourless. Colony diameter reaching 42 mm after 10 d on 2% MEA at optimal growth temperature of 25°C. No growth below 10°C or above 35°C.

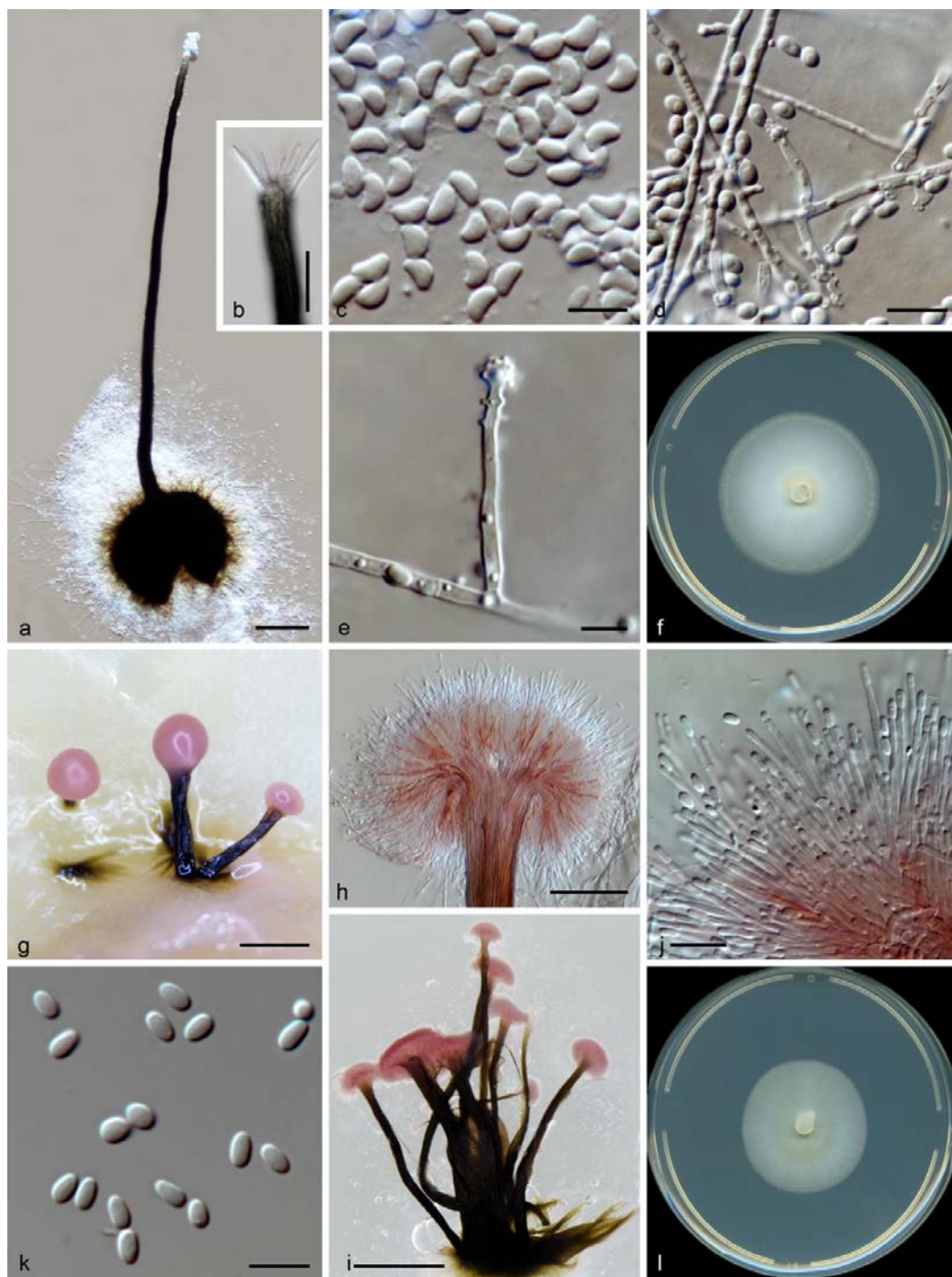


Fig. 4 Microscopic features of *Sporothrix oleae* sp. nov. and *Graphilbum roseum* sp. nov.; a–f. *S. oleae* (ex-holotype, CMW 40362 = CBS 142082). a. Ascoma produced in culture. b. Diverging ostiolar hyphae. c. Ascospores. d. Conidiogenous cells and conidia, e. Conidiogenous cell with an inflated cluster of denticles at the apex. f. Colony grown in the dark at 25 °C for 10 d; g–l *G. roseum* (ex-holotype, CMW 40349 = CBS 141074). g–i. Synnemata produced in culture with slimy conidial mass at the top. j. Conidiogenous cells. k. Conidia. l. Colony grown in the dark at 25 °C for 10 d. Scale bars: μm , a = 100 μm , b = 25 μm , c, e, k = 5 μm , g, i = 500, h = 50 μm , j = 10 μm

Typification: South Africa, Western Cape Province, Gouldveld. *Olea capensis* ssp. *macrocarpa* wound on tree trunk covered with ascomata, October 2011, T. Musvuugwa, **holotype** PREM 61674; culture ex-holotype CMW 40362 = CBS 142082. Same location, damaged tree trunk of different tree individual of same species, PREM 61672 (**paratype**); culture ex-paratype CMW 40360 = CBS 142026. PREM 61673 (**paratype**); culture ex-paratype CMW 40361 = CBS 142027.

Notes. *Sporothrix oleae* grouped with *S. aemulophila*, *S. cabralii*, *S. candida*, *S. itsvo* and *S. rapanae* within the *S. candida* species complex, all of which produce sexual state except for *S. cabralii*. These species share some common features such as optimal growth temperature (25–30°C) and the presence of ostiolar hyphae. Among these closely-related species, *S. oleae* can be easily distinguished from the rest by its nearly triangular ascospores. Even though ascospores of *S. itsvo* and *S. candida* were described in their original descriptions as reniform and kidney-shaped, respectively, judging from the images provided, their ascospores are rather allantoid to curved oblong (Kamgan Nkuekam et al. 2012; Musvuugwa et al. 2016a). Ascospore dimensions separate *S. oleae* (2.5–4.5 × 1.5–2 µm) from *S. itsvo* (1.3–2.4 × 0.3–0.5 µm) but *S. oleae* shares similar ranges with *S. aemulophila* (2–5.5 × 0.2–1.8 µm), *S. rapanae* (2.8–4.7 × 0.3–0.6 µm) and *S. candida* (3.5–5 × 1–1.7 µm).

Graphilbum roseum Musvuugwa, L.L. Dreyer & F. Roets, sp. nov. Figures 4g–l.
Mycobank MB 822537

Etymology: The epithet *roseum* (Latin for pink or rosy) refers to the pink conidial masses produced by synnemata.

Ascomata not observed. Asexual sporulation from *synnemata* determinate with terminal, slimy conidial masses formed on the surface of host substrate and also on 2% MEA incubated for 4 weeks in the dark at 25°C, up to 2.2 mm long including conidiogenous cells; stipes black, subapically dark to pale brown, 14–150 µm wide at base, 19–110 µm wide apically. Heads of synnemata branched in 4–5 tiers, up to 390 µm wide, conidial mass hyaline when young, becoming pink with age and red when dry. *Conidiogenous cells* annellidic, hyaline, cylindrical, tapering towards the apex, 14.5–19 µm long, 1–2 µm wide at the base, 1–1.5 µm wide near the apex. *Conidia* hyaline, aseptate, smooth, oblong to ovoidal, 2–3.5 × 1–2 µm (avg. 2.9 × 1.6 µm). *Colonies* pale yellow (19''f) with hyaline edge, becoming light to moderate yellowish brown (17'''b) at centres, firm in texture at edges. Colony diameter reaching 29.5 mm

after 10 d on 2% MEA at optimal growth temperature of 25°C. No growth below 10°C or above 30°C.

Typification: South Africa, Western Cape Province, Gouna. *Curtisia dentata* wound on branch covered with synnemata, October 2011, T. Musvuugwa, **holotype** PREM 61337; culture ex-holotype CMW 40349 = CBS 141074. Same location, damaged branch of a different tree of same species, PREM 61338 (**paratype**); culture ex-paratype CMW 40350 = CBS 141075. South Africa, Western Cape Province, Groenkop. *Pterocelastrus tricuspidatus* wound on trunk covered with synnemata, October 2011, T. Musvuugwa, PREM 61339 (**paratype**); culture ex-paratype CMW 40351 = CBS 141076.

Notes. *Graphilbum roseum* grouped closest to *Graphilbum* sp. 7, one of several undescribed taxa in the genus (De Beer and Wingfield 2013). *Graphilbum roseum* is known only from its asexual state, but its synnematous *Pesotum*-like conidiophores with conidia in slimy masses resemble other species in the genus (De Beer et al. 2013a). No other *Graphilbum* species have been reported to produce pink conidial masses. Rather they usually produce cream coloured conidia (e.g. Mathiesen-Käärik 1953). In comparison with *G. fragrans*, an apparently closely related species to *G. roseum*, conidiogenous cells for *G. fragrans* are 10-43 µm long and 1-1.5 µm wide (Mathiesen-Käärik 1953), while those of *G. roseum* are 14.5-19 µm long, 1-2 µm wide. For *G. fragrans*, conidia are 3-6 x 1-2 µm, while for *G. roseum* they are 2-3.5 x 1-2 µm. *Graphilbum roseum*, provisionally called *G. roseus* in Musvuugwa (2014), was also isolated from various mite species associated with wounds on the host trees, suggesting that they may be intimately involved in the ecology of this fungus (Musvuugwa 2014; Musvuugwa et al. 2016b).

***Graphium* sp. (Fig. 5)**

Based on sequence data, the *Graphium* species grouped with *G. basitruncatum*, *G. carbonarium*, *G. euwallaceae* and numerous undescribed taxa. All of these species produce both conidia and chlamydospores (Lynch et al. 2016) that vary little in size and shape between different taxa. The taxon isolated in the present study differs from *G. basitruncatum* in that the conidia of the latter are more slender (Matsushima 1971). All isolates of this taxon were obtained from conidiophore-covered wounds on branches of *Ilex mitis* at Assegaibosch, Stellenbosch, Western Cape Province, South Africa.

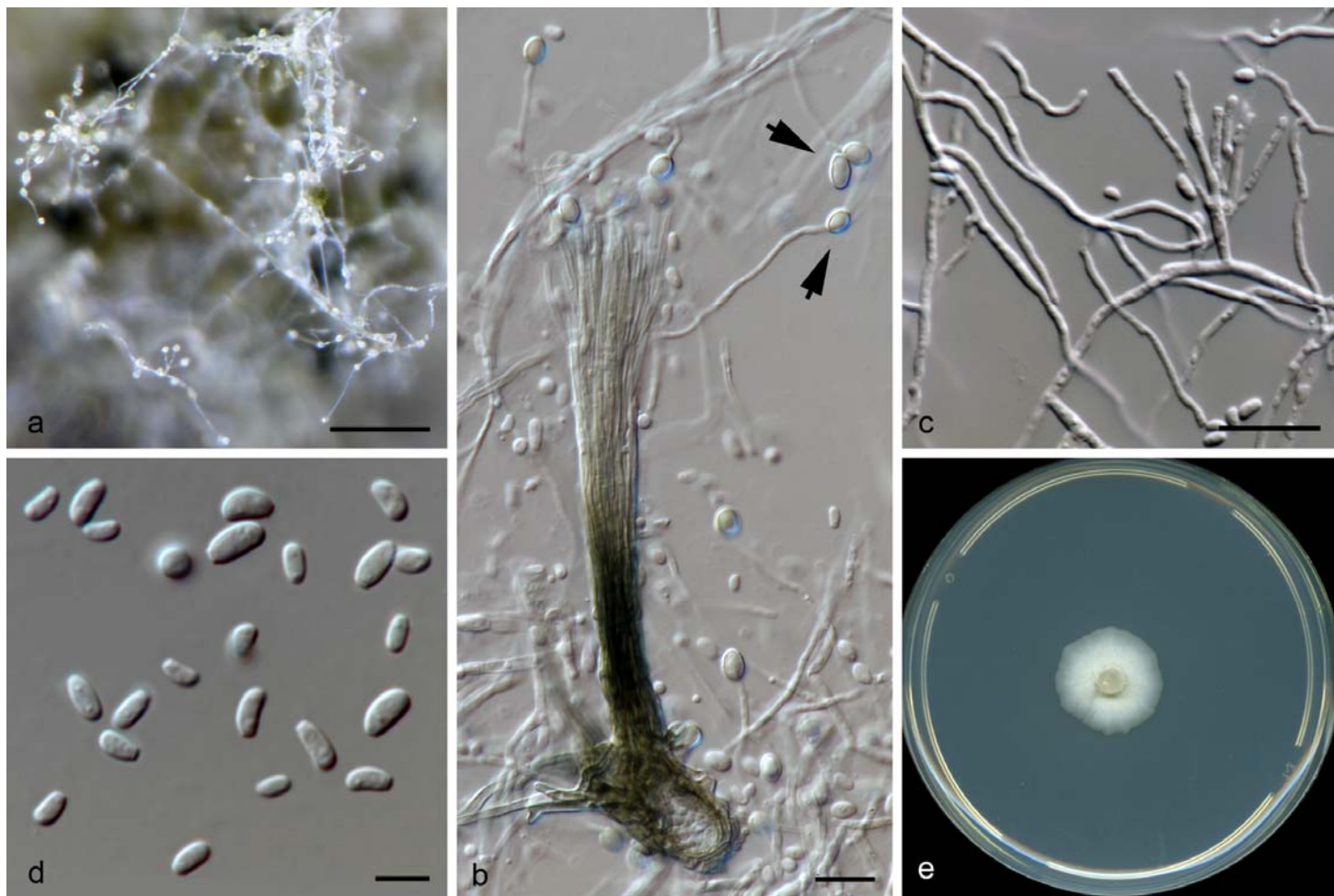


Fig. 5 Microscopic features of *Graphium ilicis* sp. nov. (ex-holotype CMW 40355 = CBS 141071). a. Conidiophores. b. Synnematus conidiophore and chlamydospores (arrows). c. Mononematous conidiophores. d. Conidia. e. Colony grown in the dark at 25 °C for 10 d. Scale bars: a = 100 μ m, b = 10 μ m, c = 50 μ m, d = 5 μ m

Description: *Conidiophores* on host tissues and in culture variable, micronematous to macronematous, mononematous, simple or branched, occasionally synnematous in old cultures. *Conidiogenous* cells blastic, hyaline, cylindrical, straight or flexuous, 6–60 x 1–2 µm. *Conidia* hyaline, aseptate, oblong to obovate with a truncate base, straight or slightly curved, 3–5.5 x 1.5–3 µm (avg. 4.4 x 2.1 µm). *Chlamydospores* subhyaline to slightly pigmented, thick-walled, aseptate, ellipsoidal, 4.5–7 x 2.5–4.5 µm (avg. 5.7 x 3.8 µm). *Colonies* white, greyish in the centre, fluffy appearance, with edges crenated; optimal temperature for growth is at 25°C on MEA, with colony diameter reaching 29.1 mm after 10 days. No growth at or below 10°C. Causes black staining of host wood. *Ascomata* not observed.

Discussion

In previous CFR-based assessments of native host trees, a diverse array of ophiostomatoid fungi were found associated with wounds (Kamgan Nkuekam et al. 2008; Musvuugwa et al. 2016a, b) and subcortical beetles (Musvuugwa et al. 2015). Here, two species belonging to the Ophiostomatales and one species belonging to the Microascales were isolated from wounds on native trees growing in the Afromontane forests of the Cape Floristic Region (CFR), of which two are described in separate genera.

Sporothrix oleae is a member of the *S. candida* complex (De Beer et al. 2016). Several recently described species, isolated from these forests, also belong to this complex (Musvuugwa et al. 2015, 2016a). Until the recent isolation of the beetle-associated *Sporothrix pallida* and *Raffaelea vaginata* (Musvuugwa et al. 2015), no other ophiostomatoid fungus had been recorded from *O. capensis* ssp. *macrocarpa*. Based on our analyses, *S. oleae* is closely related to *S. candida sensu stricto*, a known associate of Cerambycidae beetles that were isolated from wounds on *Eucalyptus cloeziana* F. Muell in South Africa (Kamgan Nkuekam et al. 2012), *S. rapanae* from *Xyleborinus aemulus* and its galleries on *Rapanea melanophloeos* (Musvuugwa et al. 2015), and *S. itsvo* and *S. aemulophila* from damaged wood and inner bark of *R. melanophloeos* (Musvuugwa et al. 2016a). The only species in the complex that does not originate from South Africa is *S. cabralii* de Errasti & Z.W. de Beer from galleries of *Gnathotrupes ambrosia* beetles on dead wood of *Nothofagus pumilio* (Poepp. & Endl.) Krasser in Argentina (De Errasti et al. 2016). All species in the complex thus target hardwoods in the Southern Hemisphere, and although not confirmed, most probably are all vectored by arthropods.

Graphilbum roseum belongs to a genus that groups basal to other genera in the Ophiostomatales (De Beer and Wingfield 2013). The genus comprises ten named and several unnamed species (De Beer and Wingfield 2013, Reid and Hausner 2015) collected from various parts of the world, including Canada, Europe, America and Australia (Geldenhuis et al. 2004; Kim et al. 2005). *Graphilbum* sp. 7, the closest relative of *G. roseum*, was initially reported as an unnamed *Pesotum* J.L. Crane & Schkn. species from sapstain on *Pinus radiata* in Australia (De Beer and Wingfield 2013; Thwaites et al. 2005). Although *G. roseum* was isolated from wounds, some of the other species in the genus are associated with conifer-infesting bark beetles (De Beer et al. 2013a; Reid and Hausner 2015). *Graphilbum fragrans* (Mathiesen-Käärik) Z.W. de Beer, Seifert & M.J. Wingf., for example, has been isolated from *Ips sexdentatus* Börner infesting *Pinus sylvestris* L. in Sweden (Mathiesen-Käärik 1953). In South Africa, the same species was associated with *Hylastes angustatus* Herbst infesting *Pinus patula* Schiede ex Schldl. Cham. in Mpumalanga (Zhou et al. 2006). Unlike the other two species described in this study, *G. roseum* was isolated from several host trees, including *Curtisia dentata*, *Halleria lucida*, *Pterocelastrus tricuspidatus*, *Trichocladus crinitus* and *Olea capensis* ssp. *macrocarpa*. It is known to be pathogenic to indigenous (e.g. *Curtisia dentata* and *Rapanea melanophloeos*) and exotic (*Acacia mearnsii* and *Eucalyptus grandis*) hardwood trees and is likely dispersed by mites and other wound-associated arthropods (Musvuugwa et al. 2016b).

Graphium belongs to the Microascales. The species most closely related to the taxon isolated in the present study based on molecular studies seems to be *G. basitruncatum*, which was first isolated from forest soil in the Solomon Islands (Matsushima 1971). It has also been isolated as an opportunistic human pathogen from a patient with leukaemia in Canada (Deepali et al. 2007). *Graphium carbonarium*, a member in the clade that is sister to *G. ilicis* and *G. basitruncatum*, was first described from a *Pissodes* Germar weevil infesting *Salix babylonica* L. in China (Paciura et al. 2010), but this species, and all other taxa in this clade, seem to be commonly associated with *Euwallaceae* sp. ambrosia beetles (Scolytinae) from the USA (invaded habitats) and Asia (putative origin) (Lynch et al. 2016). This represents a major difference between the taxon collected in the present study and most other species in the genus in that it is wound-associated (De Beer et al. 2013b; Geldenhuis et al. 2004; Lynch et al. 2016; Okada et al. 2000; Paciura et al. 2010). Other *Graphium* species isolated from South Africa include *G. adansoniae* Cruywagen, Z.W. de Beer & Jol. Roux isolated from *Adansonia digitata* L. (Cruywagen et al. 2010) and *G. pseudormiticum* from a bark beetle on exotic pine trees (Mouton et al. 1994). There

are no sexual stages known for species in this genus (De Beer et al. 2013b). To the best of our knowledge, no other ophiostomatoid fungus has to date been isolated from *Ilex mitis*.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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