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Fungal associates of an invasive pine-infesting bark beetle, *Dendroctonus valens*, including seven new Ophiostomatalean fungi

S. Marincowitz[1], T.A. Duong[1], S.J. Taerum[1], Z.W. de Beer[1], M.J. Wingfield[1]

Key words

invasion biology
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seven new taxa
taxonomy

Abstract The red turpentine beetle (RTB; *Dendroctonus valens*) is a bark beetle that is native to Central and North America. This insect is well-known to live in association with a large number of Ophiostomatalean fungi. The beetle is considered a minor pest in its native range, but has killed millions of indigenous pine trees in China after its appearance in that country in the late 1990s. In order to increase the base of knowledge regarding the RTB and its symbionts, surveys of the beetle's fungal associates were initially undertaken in China, and in a subsequent study in its native range in North America. A total of 30 Ophiostomatalean species that included several undescribed taxa, were identified in these surveys. In the present study, seven of the undescribed taxa collected during the surveys were further characterised based on their morphological characteristics and multi-gene phylogenies. We proceeded to describe five of these as novel *Leptographium* spp. and two as new species of *Ophiostoma*. Four of the *Leptographium* spp. resided in the *G. galeiformis*-species complex, while one formed part of the *L. olivaceum*-species complex. One *Ophiostoma* sp. was a member of the *O. ips*-species complex, while the only new species from China was closely related to *O. floccosum*. Two of the previously undescribed taxa from North America were shown to be congeneric with *L. terebrantis*, implying that this species was most often isolated in association with the RTB in North America. The undescribed taxon from North America was identified as *O. ips*, and like *L. terebrantis*, this species was also not recognized during the initial North American survey. Resolving the identities of these taxa provides essential baseline information to better understand the movement of fungal pathogens with this beetle. This then enhances our ability to accurately assess and predict the risks of invasions by these and related fungi.

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INTRODUCTION

The red turpentine beetle (RTB), *Dendroctonus valens* (*Coleoptera*: *Curculionidae*, *Scolytinae*), is a bark beetle that occurs naturally throughout most regions of North and Central America. Its range extends from the Northwest Territories in Canada to Honduras, excluding the south-eastern USA (Owen et al. 2010). The beetle is commonly recognized as a secondary pest in its native range. This is because these insects primarily colonize the lower trunks and larger roots of stressed, dying or dead *Pinus* spp. (Smith 1971, Klepzig et al. 1995, Owen et al. 2010).

In the late 1990s, the RTB was reported in Shanxi province in northern China, where the insect was responsible for a tree-killing epidemic (Li et al. 2001, Yan et al. 2005). The affected trees were primarily Chinese red pine, *Pinus tabuliformis*, which was widely planted across the country as a major reforestation species (Li et al. 2001). Subsequent to the first outbreak, the beetles rapidly expanded their range throughout the region, killing more than 500 000 ha of pine trees (~10 M trees; Li et al. 2001, Miao et al. 2001, Zhang et al. 2002). Due to the severity of the RTB outbreak in China, various aspects of the invasion dynamics of RTB were studied (reviewed in Sun et al. 2013), including the ecology of the beetles (Yan et al. 2005), its yeast associates (Lou & Sun 2014), the bioactivities of microorganisms against host defensive monoterpenes (Xu et al. 2016), the survival and suitability of the beetles under a climate warming scenario (He et al. 2015), and the diversity of Ophiostomatalean fungi associated with the RTB (Lu et al. 2009a, b, 2010).

The Ophiostomatalean fungi is a group of ascomycete fungi that reside in the order *Ophiostomatales* (*Ascomycota: Sordariomycetidae*) (De Beer et al. 2013a). The group includes some of the most important pathogens of trees such as *Ophiostoma ulmi* and *O. novo-ulmi*, the causal agents of Dutch elm disease (Gibbs 1978, Brasier 1988, 1991), and *Leptographium wageneri* that causes black stain root disease of conifers (Cobb & Platt 1967, Goheen & Hansen 1978, Cobb 1988).

The majority of species in the *Ophiostomatales* are ecologically adapted for dispersal by scolytine bark beetles, such as RTB (Harrington 1988, Malloch & Blackwell 1993). In the past, many genera and species in the order were confused with species in the *Ceratocystidaceae* residing in the *Microascales* that share with them gross morphological characteristics and insect-related biology (Upadhyay 1981, Samuels 1993, De Beer et al. 2014). For this reason, the collective name 'ophiostomatoid fungi' has been used for the ecologically and morphologically similar, but phylogenetically unrelated, groups of fungi (De Beer et al. 2013a). For the purpose of the present study, we use the collective name Ophiostomatalean fungi, specifically not including species in the *Microascales*.

Dendroctonus valens was described in 1860 by LeConte and is the largest and most conspicuous species in the genus (Wood 1982). It is therefore remarkable that no systematic survey of the fungal associates of this beetle in its native range had been undertaken before the beetle became invasive in China. By 2005, a total of 13 fungal species had been reported from the beetle in North America and Mexico in a number of disjunct

[1] Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Private Bag X20, Pretoria 0028, South Africa;
corresponding author e-mail: seonju.marincowitz@fabi.up.ac.za.

Table 1 Ophiostomatoid fungi associated with *Dendroctonus valens* including the seven novel species (in **bold**) described in this study. Current species names are used as listed in the nomenclator by De Beer et al. (2013b).

Species	Identity confirmed with DNA sequences	China	USA & Mexico
Ceratocystiopsis collifera	✓	–	[1]Marmolejo & Butin (1990)
Graphilbum sp.	✓	Lu et al. (2009a) as *Ophiostoma rectangulosporium*-like	–
[2]*Graphium* sp.	✗	–	Owen et al. (1987)
Grosmannia sp. 1	✓	–	Taerum et al. (2013)
Grosmannia sp. 3	✓	–	Taerum et al. (2013)
G. aurea	✓	–	Taerum et al. (2013)
G. clavigera	✗	–	Six et al. (2003)
[3]*G. europhioides*	✗	–	Wright & Cain (1961), cited by Perry (1991) as *Ceratocystis europhioides*
G. huntii	✓	–	Taerum et al. (2013)
G. koreana	✓	Lu et al. (2009b) as *Leptographium koreanum*	Taerum et al. (2013)
[3]*G. piceiperda*	✗	–	Rumbold (1931) cited by Perry (1991) as *Ophiostoma piceaperdum*
G. radiaticola	✓	Lu et al. (2009b) as *Pesotum pini*	–
Leptographium sp.	✗	–	Davidson (1958)
Leptographium sp. 3	✓	–	Taerum et al. (2013)
L. alethinum	✓	Lu et al. (2009b)	–
***L. doddsii* sp. nov.**	✓	–	Taerum et al. (2013) as *Grosmannia* sp. 4
***L. gordonii* sp. nov.**	✓	–	Taerum et al. (2013) as *Grosmannia* sp. 2
***L. owenii* sp. nov.**	✓	–	Taerum et al. (2013) as *Grosmannia* sp. 7
L. pini-densiflorae	✓	Lu et al. (2009a)	–
L. pinicola	✓	Lu et al. (2009a) as *Hyalorhinocladiella pinicola*	–
[4]*L. procerum*	✓	Lu et al. (2009a, b)	Wingfield (1983), Harrington (1988), Klepzig et al. (1991), Jacobs et al. (2004), Taerum et al. (2013)
***L. raffai* sp. nov.**	✓	–	Taerum et al. (2013) as *Grosmannia* sp. 5
***L. seifertii* sp. nov.**	✓	–	Taerum et al. (2013) as *Grosmannia* sp. 6
L. sinoprocerum	✓	Lu et al. (2009b)	
[4]*L. terebrantis*	✓	–	Harrington & Cobb (1983), Wingfield (1983), Owen et al. (1987), Harrington (1988), Klepzig et al. (1991), Perry (1991), Fox et al. (1992), Six et al. (2003), Taerum et al. (2013) as *Leptographium* sp. 1 & *Leptographium* sp. 2
L. truncatum	✓	Lu et al. (2009a, b)	–
L. wageneri var. *ponderosae*	✓	–	Goheen (1976) and Goheen & Cobb (1978) as *Ceratocystis wageneri*, Harrington (1988) & Perry (1991) as *Ophiostoma wageneri*, Schweigkofler et al. (2005)
[5]*L. wageneri* var. *wageneri*	✗	–	Jacobs & Wingfield (2001)
L. wingfieldii	✓	–	Jacobs et al. (2004)
Ophiostoma floccosum	✓	Lu et al. (2009a)	Taerum et al. (2013)
***O. gilleteae* sp. nov.**	✓	–	Taerum et al. (2013) as *Ophiostoma* sp. 1
O. ips	✓	Lu et al. (2009a)	Upadhyay (1981), Owen et al. (1987), Klepzig et al. (1991), Perry (1991), Fox et al. (1992), Taerum et al. (2013) as *Ophiostoma* sp. 2
[6]*O. minus* (Europe)	✓	Lu et al. (2009a)	–
[6]*O. minus* (North America)	✓	–	Taerum et al. (2013)
O. piceae	✓	Lu et al. (2009a)	–
O. piliferum	✓	–	Perry (1991), Taerum et al. (2013)
***O. shanziensis* sp. nov.**	✓	Lu et al. (2009b) as *Pesotum aureum*	Taerum et al. (2013) as *Ophiostoma* sp. 3
Sporothix abietina	✓	Lu et al. (2009a) as *O. abietinum*	Taerum et al. (2013) as *O. abietinum*

[1] Plattner et al. (2009) produced DNA sequences of the ex-type isolate of *Ceratocystiopsis collifera* isolated from *D. valens* galleries by Marmolejo & Butin (1990), confirming its status as a distinct species in *Ceratocystiopsis*.

[2] This unnamed '*Graphium*' was most probably the synnematous asexual morph of an *Ophiostoma*, *Graphilbum* or *Leptographium* species, as no true *Graphium* (*Microascales*) species have been reported in any other study from the RTB.

[3] Perry (1991) listed these species as associates of *D. valens*, but neither of the original references cited by her mentioned *D. valens*. Therefore, we consider these associations questionable.

[4] Isolates labelled by Jacobs et al. (2004) as *L. terebrantis*, were shown to represent *L. procerum* by Lu et al. (2009a).

[5] Jacobs & Wingfield (2001) listed *D. valens* as an insect associate of *L. wageneri* var. *wageneri*, citing the following references (Goheen 1976, Harrington & Cobb 1983, Harrington 1988, Perry 1991). Goheen (1976) clearly referred to *L. wageneri* var. *ponderosae* as the study was focused on ponderosa pine. Harrington & Cobb (1983) referred to isolates from Douglas fir and ponderosa pine all as *Verticicladiella wageneri*, without distinguishing between them, and not clearly indicating the host tree from which they collected *D. valens* and the associated fungal isolates. It is thus not possible to conclude which variety of *L. wageneri* came from *D. valens*. Both Harrington (1988) and Perry (1991) linked *O. wageneri*, the sexual morph of *L. wageneri* var. *ponderosae*, with *D. valens*. It is thus clear that Jacobs & Wingfield (2001) were erroneous in citing these references as supporting their listing of *L. wageneri* var. *wageneri* as an associate of *D. valens*.

[6] *Ophiostoma minus* has been shown to represent two distinct phylogenetic species, one of European origin and the authentic *O. minus* of North American origin (Gorton & Webber 2000, Gorton et al. 2004, Lu et al. 2009a, Linnakoski et al. 2010). Due to unresolved typification issues, the European species remains to be renamed (De Beer et al. 2013b). For the purpose of this study, we distinguish between these taxa.

studies (Table 1). To date, the identity of only seven of these species has been confirmed with DNA sequences taken from the original material or new collections. Three of the species (*L. procerum*, *L. terebrantis* and *L. wageneri* var. *ponderosae*) are known from various host trees and beetles in North America (Cobb 1988, Jacobs & Wingfield 2001). One species, *Ceratocystiopsis collifera*, was isolated once from galleries of *D. valens* in Mexico (Marmolejo & Butin 1990) but has never again been reported. Two species (*O. ips*, *O. piliferum*) were collected recently in China and/or in the USA (Lu et al. 2009a, Taerum et al. 2013). The seventh species, *L. wingfieldii* is of European origin and was introduced into North America with its beetle vector, *Tomicus piniperda* (Jacobs et al. 2004). After the beetle established and spread throughout the north-eastern USA and the south-eastern Canada, *L. wingfieldii* was isolated from three beetle species native to North America, one of which was *D. valens* (Jacobs et al. 2004). The latter study illustrated clearly how fungal associates can be transferred from invasive to native beetle species (Wingfield et al. 2016, 2017).

The first systematic and thorough surveys of the Ophiostomatalean fungi associated with the RTB were conducted in the period between 2004 and 2006 in China, following its invasion there (Lu et al. 2008, 2009a, b). Collectively, 15 species were reported from China. These included two undescribed taxa, one of which was described as *L. sinoprocerum* (Lu et al. 2008), while the other was closely related to, but distinct from, *Graphilbum rectangulosporium* (Lu et al. 2009a, Taerum et al. 2013). A third species was reported as *Pesotum aureum* (Lu et al. 2009b), the name previously used for the asexual morph of *O. floccosum* (De Beer et al. 2013b). However, Taerum et al. (2013) showed that the Chinese species is distinct from *O. floccosum* (= *P. aureum*) and treated it as *Ophiostoma* sp. 3. The most dominant species in both Chinese surveys was *L. procerum*, representing 61 % of the total number of isolates obtained when the results of the two studies are combined (Lu et al. 2009a, b, Taerum et al. 2013).

In 2008 and 2009, Taerum et al. (2013) conducted the first extensive survey of the fungal associates of the RTB in North America. They reported 20 Ophiostomatalean species, 12 of which were apparently new to science, but these were not provided with names. In western North America (WNA), two seemingly undescribed taxa were dominant, with *Leptographium* sp. 1 representing 36.3 %, and *Ophiostoma* sp. 1 accounting for 33.1 % of the total number of isolates (Taerum et al. 2013). In eastern North America (ENA), *Leptographium* sp. 2 was the second most abundant species (15.6 %), after *L. procerum*. The two unnamed *Leptographium* spp. were closely related to each other and to *L. terebrantis*, while *Ophiostoma* sp. 1 formed part of the *O. ips*-species complex (Taerum et al. 2013). Similar to the situation in China, *L. procerum* was by far the most commonly encountered species in eastern North America, representing 45.6 % of all the isolates obtained. However, *L. procerum* was completely absent in western North America, the area from which the beetle was assumed to have been introduced into China (Taerum et al. 2013).

The aim of the present study was to confirm the identity of the unnamed taxa collected during previous surveys in North America and China (Lu et al. 2009b, Taerum et al. 2013). Where needed, a greater number of gene regions were sequenced for specific taxa based on available reference sequences for the species complexes to which they belong. Detailed morphological descriptions were made for those taxa that were found to represent novel species.

MATERIAL AND METHODS

Fungal isolates

Collection details for isolates included in the present study (Table 2) were described by Lu et al. (2009b) and Taerum et al. (2013). Isolates representing the single species from China were provided by Agro-food & Environmental fungal collection (MUCL), Earth and Life Institute, Belgium. The cultures from the USA are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. For this study, the isolates were plated on 2 % malt extract agar (MEA; 20 g BioLab malt extract, 20 g Difco agar, 1 L ionized water) and incubated at room temperature. Single hyphal tip cultures were prepared for DNA sequencing and morphological analyses. Pairing of isolates to induce putative sexual morphs was not attempted.

Dried cultures were deposited as holotype specimens in the South African National Collection of Fungi (PREM), Roodeplaat, South Africa. The ex-type isolates were deposited in the collection of the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, the Netherlands.

Microscopy and growth studies

Microscopic features were studied from isolates grown on sterilized pine twigs placed on the surface of 2 % water agar (WA; 20 g Difco agar, 1 L ionized water) in Petri dishes. Fungal structures were initially mounted in water, which was later replaced with 85 % lactic acid for visualization and measurements. Up to 50 fungal structures were measured when possible. Nikon microscopes (Nikon, Tokyo, Japan), Eclipse N*i* and SMZ18, mounted with a Nikon camera (DS-Ri2) were used for observation and capturing of images. Measurements for characteristic structures were made using the NIS Elements software (Nikon, Tokyo, Japan). Dimensions of the structures are presented as minimum–maximum (average ± standard deviation).

Culture characteristics were observed on isolates grown both on oatmeal agar (OA; 30 g oatmeal, 20 g Biolab malt extract, 1 L ionized water) and on 2 % MEA at an optimal temperature of each species.

To determine the growth rates of the fungi, a disc of agar (5 mm diam) cut from the actively growing margins of cultures was placed at the centre of 90 mm Petri dish containing 2 % MEA. Three replicate plates for each species were maintained in the dark at temperatures ranging from 10–35 °C in 5 °C intervals. Two measurements of colony diameter perpendicular to each other were made either when the mycelium reached the outer margin of the Petri dish or on the seventh day, after which the daily growth rates were calculated.

PCR, sequencing and phylogenetic analyses

Based on the culture morphology, representative isolates (Table 2) were selected for DNA sequence-based characterization. DNA was extracted from single hyphal tip cultures using the PrepMan Ultra Reagent (Applied Biosystems, USA), following the protocols described by Duong et al. (2012). Depending on reference sequences available from previous publications, different gene regions were amplified and sequenced for isolates residing in different species complexes. For the *Grosmannia clavigera*-species complex, ITS, beta-tubulin (*βt*), elongation factor 1-alpha (*EF1-α*), and actin (*Act*) regions, as well as an anonymous locus (*Anon*), were amplified and sequenced. For the *G. galeiformis*- and *L. olivaceum*-species complexes, ITS2-LSU, ITS, *βt* and *EF1-α* were amplified and sequenced. For the *Ophiostoma ips*-species complex and '*Pesotum aureum*', ITS, *βt* and *EF1-α* were amplified and sequenced. The primers used for PCR and sequencing of the various gene regions were

Table 2 List of isolates associated with *Dendroctonus valens* beetles or galleries. DNA sequences generated in this study are in **bold**. [T] indicates an ex-holotype isolate.

New name	Previous identification (Taerum et al. 2013)	Isolate no.		Origin (state/province)	Source	Genbank Accession numbers					
		CMW	Other			*βt*	*EF1-α*	*Act*	ITS2-LSU	ITS1-ITS2	*Anon*
L. doddsii sp. nov.	*Grosmannia* sp. 4	CMW 34479[T]	CBS 143470	California	Beetle in funnel trap	**MT637202**	**MT637205**	n/a	**MT637212**	**MT637215**	n/a
	Grosmannia sp. 4	CMW 34581	CBS 143475	Massachusetts	Gallery on *P. resinosa*	KF515856.1	KF515882.1	n/a	KF515913.1	**MT637216**	n/a
	Grosmannia sp. 4	CMW 34393	CBS 143463	California	Beetle in funnel trap	KF515855.1	KF515881.1	n/a	KF515916.1	**MT637225**	n/a
L. gordonii sp. nov.	*Grosmannia* sp. 2	CMW 34619[T]	CBS 143477	New Hampshire	Beetle on *P. resinosa*	**MT637203**	**MT637207**	n/a	**MT637213**	**MT637226**	n/a
L. owenii sp. nov.	*Grosmannia* sp. 7	CMW 34448[T]	CBS 143467	California	Beetle in funnel trap	KF515860.1	KF515884.1	n/a	KF515912.1	**MT637217**	n/a
L. raffai sp. nov.	*Grosmannia* sp. 5	CMW 34445	CBS 143466	California	Beetle in funnel trap	KF515857.	KF515879.1	n/a	KF515917.1	**MT637218**	n/a
	Grosmannia sp. 5	CMW 34451[T]	CBS 143468	California	Beetle in funnel trap	**MT637204**	**MT637206**	n/a	**MT637211**	**MT637219**	n/a
L. seifertii sp. nov.	*Grosmannia* sp. 6	CMW 34620[T]	CBS 143478	New Hampshire	Beetle on *P. resinosa*	KF515858.1	KF515885.1	n/a	KF515911.1	**MT637224**	n/a
	Grosmannia sp. 6	CMW 39393	CBS 143485	California	Beetle in funnel trap	KF515859.1	KF515886.1	n/a	KF515910.1	**MT637223**	n/a
L. terebrantis	*Leptographium* sp. 1	CMW 34391	CBS 143462	California	Beetle in funnel trap	KF515849.1	KF515877.1	**MT637190**	KF515902.1	n/a	**MT637195**
	Leptographium sp. 1	CMW 34406	CBS 143464	California	Beetle in funnel trap	**MT637198**	**MT637208**	**MT637191**	**MT637210**	n/a	**MT637196**
	Leptographium sp. 2	CMW 34531	CBS 143472	Maine	Gallery on *P. resinosa*	KF515850.1	KF515876.1	**MT637192**	KF515903.1	n/a	**MT637197**
	Leptographium sp. 2	CMW 34544	CBS 143474	Maine	Gallery on *P. resinosa*	**MT637199**	**MT637209**	**MT637193**	**MT637214**	n/a	**MT637194**
O. gilletteae sp. nov.	*Ophiostoma* sp. 1	CMW 30680	–	Washington	Beetle in funnel trap	KF515870.1	n/a	n/a	n/a	KF515894.1	n/a
	Ophiostoma sp. 1	CMW 30681[T]	CBS 143458	Washington	Beetle in funnel trap	**MT637200**	n/a	n/a	n/a	**MT637227**	n/a
	Ophiostoma sp. 1	CMW 34425	CBS 143465	California	Beetle in funnel trap	**MT637201**	n/a	n/a	n/a	**MT637220**	n/a
	Ophiostoma sp. 1	CMW 39390	–	Wisconsin	Beetle hand collected	KF515869.1	n/a	n/a	n/a	KF515895.1	n/a
O. ips	*Ophiostoma* sp. 2	CMW 30687	–	Arizona	Beetle in funnel trap	KF515868.1	n/a	n/a	n/a	KF515896.1	n/a
O. shanziensis sp. nov.	*Ophiostoma* sp. 3 / *P. aureum*	CMW 48329[T]	MUCL 46456	Shanxi, China	Gallery on *P. tabuliformis*	EU 502818	EU 502803	n/a	n/a	**MT637221**	n/a
	Ophiostoma sp. 3 / *P. aureum*	CMW 48330	MUCL 46632	Shanxi, China	Gallery on *P. tabuliformis*	EU 502819	EU 502804	n/a	n/a	**MT637222**	n/a

as follows: ITS1-F (Gardes & Bruns 1993) and ITS4 (White et al. 1990) for the ITS region; ITS3 and LR3 (White et al. 1990) for the ITS2-LSU region; T10 (O'Donnell & Cigelnik 1997) or Bt2a together with Bt2b (Glass & Donaldson 1995) for *βt* genes; EF2-F (Marincowitz et al. 2015) and EF2-R (Jacobs et al. 2004) were used for *EF1-α* genes; Lepact-F and Lepact-R (Lim et al. 2004) for *Act* genes; and UFM1_F and UFM1_R for Anonymous (*Anon*) locus introduced by Roe et al. (2010). Protocols for PCR amplification and sequencing were the same as described in Duong et al. (2012).

For phylogenetic analyses, separate datasets were compiled for each gene region and species complex and analysed independently. Reference sequences for all species residing in each species complex as well as outgroups were obtained from the NCBI nt database. Datasets were aligned using an online version of MAFFT v. 7 (Katoh & Standley 2013) and trimmed at both ends where necessary. All datasets were subjected to Gblocks v. 0.91b (Talavera & Castresana 2007) with less stringent options in order to remove any ambiguously aligned regions. Maximum likelihood (ML) analyses were conducted with IQ-TREE (Nguyen et al. 2015) with best-fit substitution models as determined by ModelFinder (Kalyaanamoorthy et al. 2017) and branch supports were assessed with 1 000 standard non-parametric bootstrap replicates. Bayesian inference (BI) analyses were conducted with MrBayes v. 3.2 (Ronquist et al. 2012) using the same model used for ML analysis. BI analysis was conducted with four independent runs, each with two chains, and runs were set to automatically stop when the average standard deviation of split frequencies across different runs is ≤ 0.01. Trees were sampled every 100th cycle, burnin value was set to 25 % of tree samples, and posterior probabilities were calculated from the remaining sampled trees.

RESULTS

Phylogenetic analyses

Previous phylogenetic analyses of the ITS1-ITS2 and ITS2-LSU gene regions (Taerum et al. 2013: f. 2–3) indicated that the unknown species included in this study reside in three previously defined species complexes of the Ophiostomatales, including the *L. olivaceum*-, *G. galeiformis*- and *O. ips*-species

Fig. 1 Phylogeny resulting from the ML analysis of the concatenated dataset of five gene regions (ITS2-LSU, *Act*, *βt*, *EF1-α* and *Anon*) of the *Grosmannia clavigera*-species complex. Nodes supports are presented as ML bootstrap (> = 75) / Bayesian posterior probabilities (> = 0.95). The dash '–' denotes no support for the corresponding analysis. Isolates previously identified as '*Leptographium* sp. 1' and '*Leptographium* sp. 2' in **bold** (Taerum et al. 2013) were confirmed to be *L. terebrantis* (T = ex-holotype).

complexes. Additionally, isolates representing a putatively new species grouped closely with *O. flocossum*, outside any of the currently defined species complexes. Based on the availability of sequence data from these complexes, different datasets were assembled and analysed separately for each species complex.

Four isolates from the USA, residing in the *G. clavigera*-species complex and identified as *Leptographium* sp. 1 and *Leptographium* sp. 2 by Taerum et al. (2013), were included in the present study (Table 2). Datasets representing five different gene regions (ITS2-LSU, *Act*, *βt*, *EF1-α* and *Anon*) were assembled and analysed for these species. Of the five gene regions, only *EF1-α* separated *Leptographium* sp. 1 and *Leptographium* sp. 2 from the other known species (data not shown), but this separation was not statistically supported. The other four gene regions grouped these isolates together with the type isolate of *L. terebrantis* (data not shown). The analysis of the concatenated dataset of all five gene regions (Fig. 1) indicated that the four isolates represented *L. terebrantis*.

Datasets for the *βt* and *EF1-α* gene regions were compiled and analysed for all known species currently residing in the *G. galeiformis*-species complex (Fig. 2), together with sequences for the isolates representing *Grosmannia* sp. 2, *Grosmannia* sp. 4,

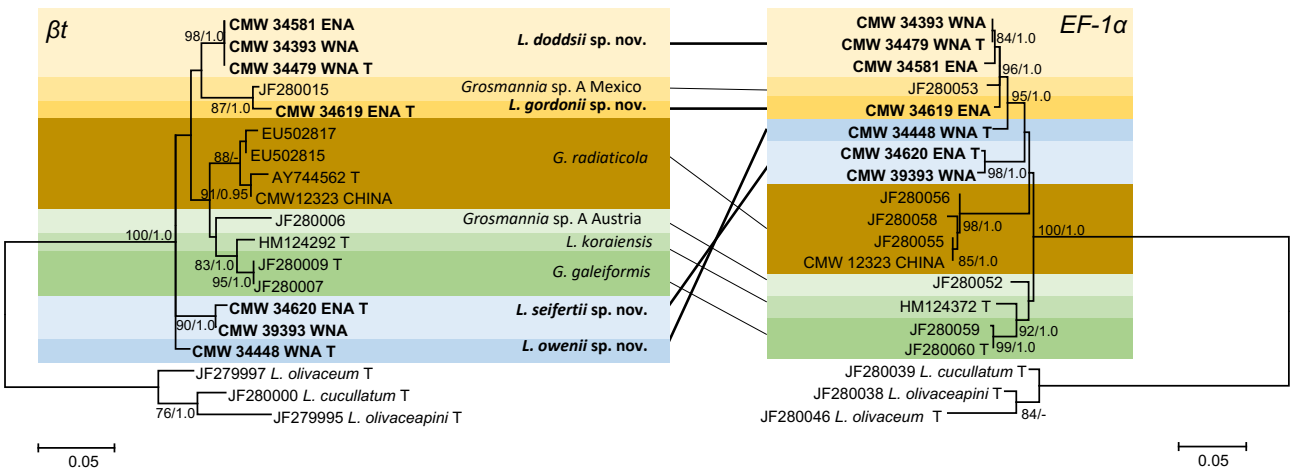

Fig. 2 Phylogenies resulting from the ML analysis of *βt* and *EF1-α* datasets of *Grosmannia galeiformis*-species complex. Representative species of *Leptographium olivaceum*-species complex were also included. Nodes supports are presented as ML bootstrap (> = 75) / Bayesian posterior probabilities (> = 0.95). The dash '–' denotes no support for the corresponding analysis (T = ex-holotype, ENA = Eastern North America, WNA = Western North America).

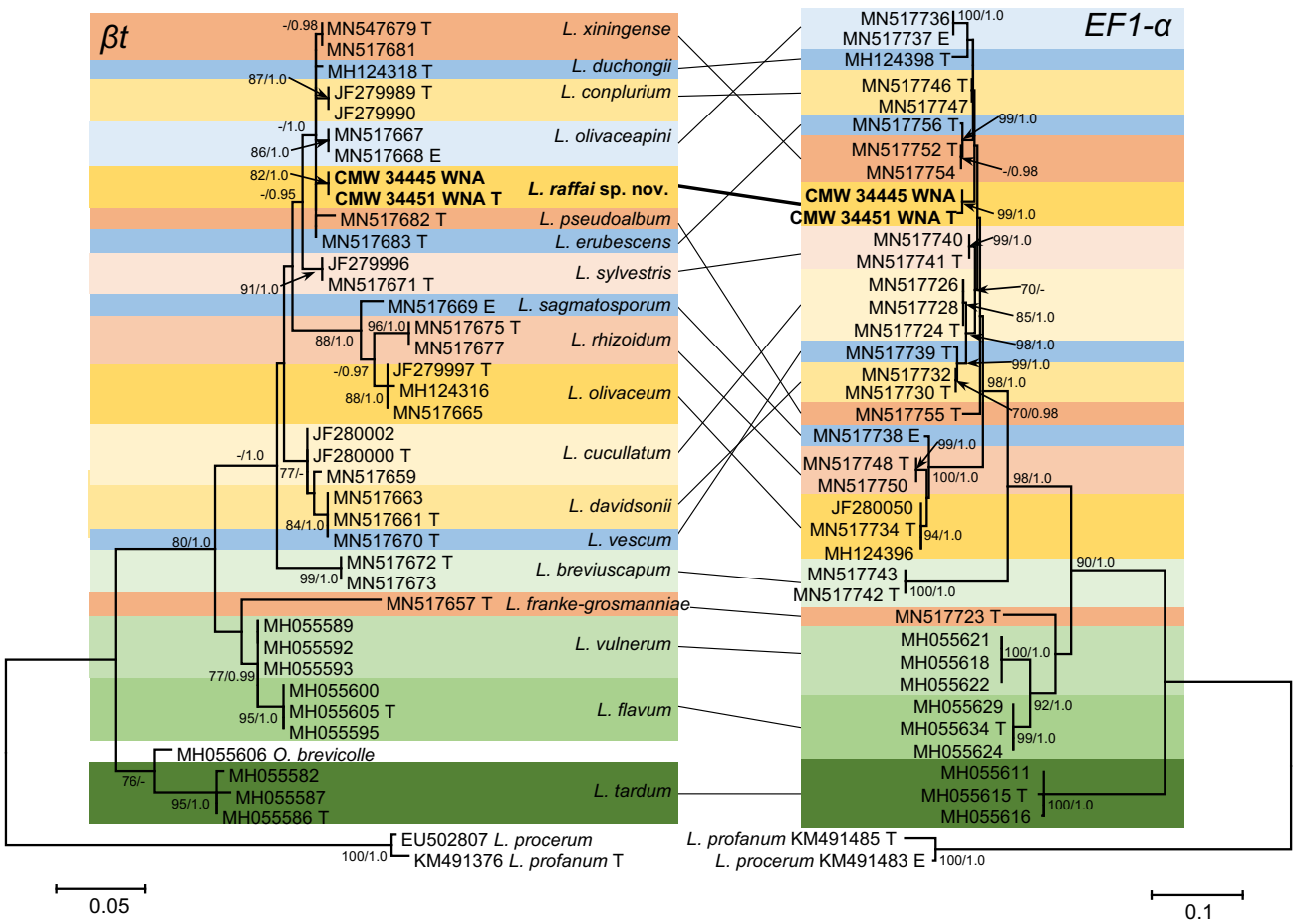

Fig. 3 Phylogenies resulting from the ML analysis of *βt* and *EF1-α* datasets of *Leptographium olivaceum*-species complex. Nodes supports are presented as ML bootstrap (> = 75) / Bayesian posterior probabilities (> = 0.95). The dash '–' denotes no support for the corresponding analysis (T = ex-holotype, E = ex-epitype, WNA = Western North America).

Grosmannia sp. 6 and *Grosmannia* sp. 7 as defined by Taerum et al. (2013). Selected species in the *L. olivaceum*-species complex were included as outgroup. Both ML and BI analyses confirmed the novelty of the four species as defined by Taerum et al. (2013) (Table 2).

For the *L. olivaceum*-species complex, the *βt* and *EF1-α* datasets representing all known species and two isolates representing *Grosmannia* sp. 5 as defined by Taerum et al. (2013), were assembled and analysed. Sequences of *L. procerum* and *L. profanum* were included as outgroup taxa. The ML and BI analyses of both datasets confirmed that *Grosmannia* sp. 5 represented a novel species (Fig. 3).

For the *O. ips*-species complex, the *βt* sequences for all known species and from the isolates representing *Ophiostoma* sp. 1 and *Ophiostoma* sp. 2 as defined by Taerum et al. (2013) was compiled and analysed. Both ML and BI analyses confirmed that *Ophiostoma* sp. 1 represented a novel species (Fig. 4), while the *Ophiostoma* sp. 2 isolate belonged to *O. ips*.

Two isolates from the RTB in China treated as *Pesotum aureum*, the asexual morph of *O. floccosum*, by Lu et al. (2009b) were also considered in this study. Currently, *O. floccosum* does not group in any of the defined species complexes in *Ophiostoma*. It resides peripheral to the *O. piceae*-species complex (De Beer & Wingfield 2013) and close to, but not in a monophyletic lineage with, *O. ainoae*, *O. brunneociliatum*, *O. shangrilae*, *O. poligraphi* and *O. tapionis* (Yin et al. 2016). Thus, the datasets consisting of both *βt* and *EF1-α* sequences from representative isolates of *O. floccosum* and the related species mentioned above, as well as the two '*P. aureum*' isolates, were compiled and analysed. Both ML and BI analyses (Fig. 5) indicated that the latter two isolates represented a novel species closely related to, but clearly distinct from *O. flocossum*.

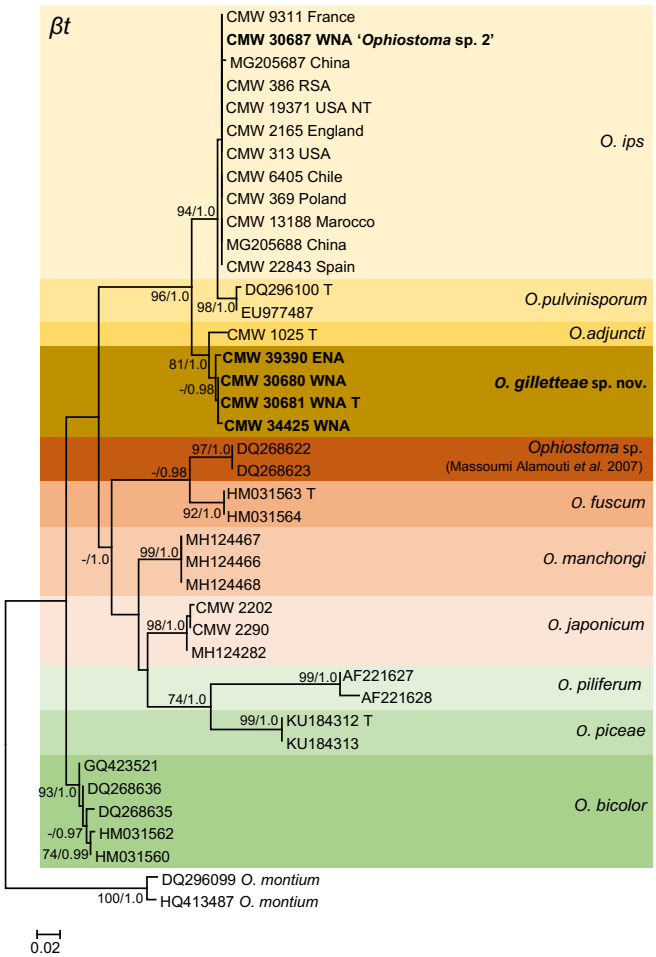

Fig. 4 Phylogeny resulting from the ML analysis of *βt* dataset of *Ophiostoma ips*-species complex. Nodes supports are presented as ML bootstrap (> = 75) / Bayesian posterior probabilities (> = 0.95). The dash '–' denotes no support for the corresponding analysis. Isolates previously identified as '*Ophiostoma* sp. 2' in **bold** (Taerum et al. 2013) was confirmed to be *O. ips* (T = ex-holotype, NT = ex-neotype, ENA = Eastern North America, WNA = Western North America).

Taxonomy

The novel nature of six species reported by Taerum et al. (2013) and the species reported as *Pesotum aureum* by Lu et al. (2009b), was confirmed based on our multi-gene phylogenetic analyses. However, our data also showed that some of the taxa treated by Taerum et al. (2013) as distinct species, were known species. *Leptographium* sp. 1 and *Leptographium* sp. 2 of Taerum et al. (2013) represent *L. terebrantis*, and *Ophiostoma* sp. 2 is *O. ips*.

De Beer & Wingfield (2013) introduced and defined 18 species complexes in the *Ophiostomatales*. This was to accommodate groups of species that share common morphological and/or ecological features and that are supported by robust phylogenetic data. Six of the seven novel species recognized in the present study resided in three of these species complexes. Four of these taxa grouped in the *Grosmannia galeiformis*-species complex, and one each in the *Leptographium olivaceum*- and the *Ophiostoma ips*-species complexes. One novel species did not belong to any of the previously defined complexes, but clearly resided in *Ophiostoma*. The descriptions of the novel

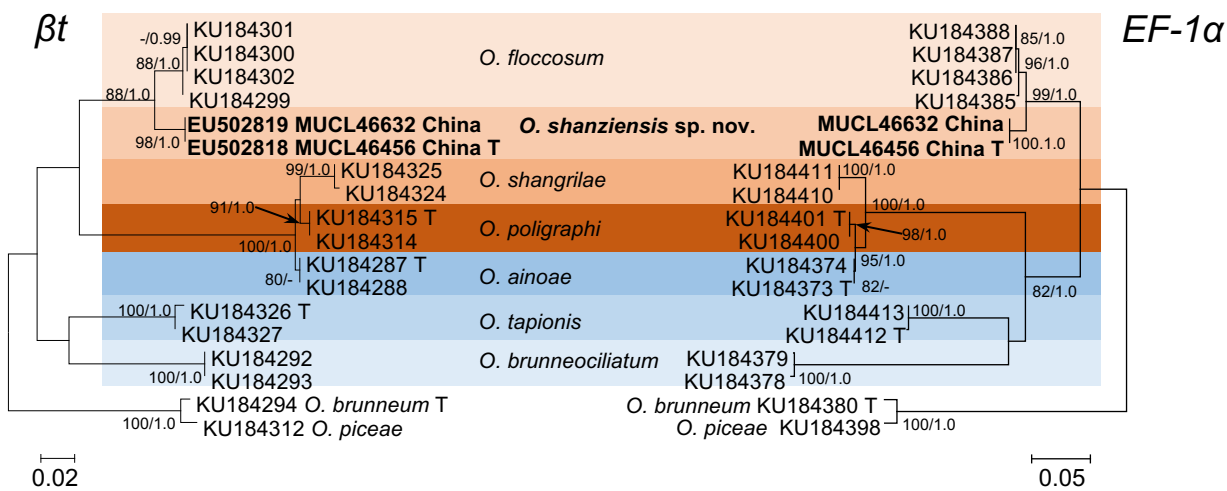

Fig. 5 Phylogenies resulting from the ML analysis of *βt* and *EF1-α* datasets of *Ophiostoma floccosum* and closely related species. Node supports are presented as ML bootstrap (> = 75) / Bayesian posterior probabilities (> = 0.95). The dash '–' denotes no support for the corresponding analysis (T = ex-holotype).

taxa are presented below within the context of the species complexes in which they reside.

Six of the novel taxa are described as species of *Leptographium* within complexes where many of the related species remain treated in *Grosmannia*. The delineation of these two genera is not clear at present. We consequently followed the recommendation of De Beer & Wingfield (2013) that all new species, which they had defined in *Leptographium* s.lat., and excluding those forming part of the *Grosmannia penicillata*-species complex, should be treated as *Leptographium.* This is regardless of the presence or absence of a sexual morph. A similar situation has existed between *Ophiostoma* and *Sporothrix* (De Beer & Wingfield 2013). However, in a subsequent study these two genera were shown to be distinct and were redefined (De Beer et al. 2016). The two species described in the present study as *Ophiostoma* fit all the criteria for the genus as defined by De Beer et al. (2016).

***Leptographium* spp.**

***Grosmannia clavigera*-species complex**

The *G. clavigera*-species complex includes eight species (Fig. 1), one of which remains to be formally described (Massoumi Alamouti et al. 2011, Six et al. 2011, De Beer & Wingfield 2013). Taerum et al. (2013) reported two undescribed taxa, *Leptographium* sp. 1 and *Leptographium* sp. 2, from the RTB. Our sequence data showed that these isolates belong to *Leptographium terebrantis.* It was interesting that the isolates from California in the western USA ('*Leptographium* sp. 1') and those collected from Maine, Massachusetts and New Hampshire in the eastern USA ('*Leptographium* sp. 2') were genetically distinct from each other and from the ex-type isolate from Louisiana and another isolate from Minnesota. Yet, when analysed together, they all formed a well-supported lineage. The isolates from the eastern and western USA showed some morphological variation (Fig. 6–7). '*Leptographium* sp. 1' had optimum growth

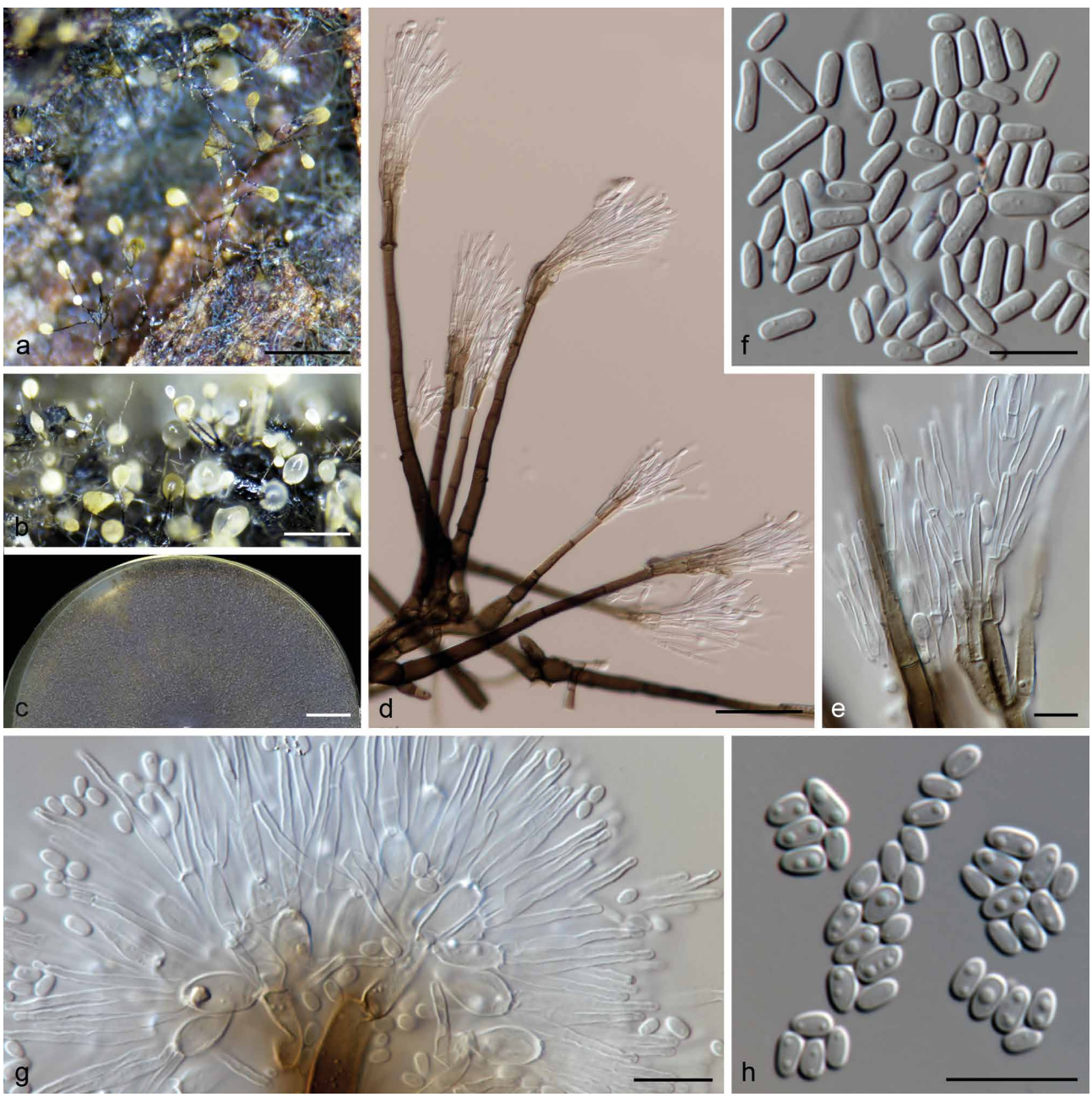

Fig. 6 Morphological features of *Leptographium terebrantis* ('*Leptographium* sp. 2', CBS 143472 = CMW 34531) from Maine in the eastern USA. a–b. Conidiophores with spore droplets on pine twig; c. colony on 2 % MEA at 25 °C in the dark for 5 d; d–f. conidiophores and conidiogenous apparatus producing ellipsoidal to cylindrical-shaped conidia (f); g–h. conidiogenous apparatus producing ellipsoidal conidia (h), also showing inflated cells. — Scale bars: a–b = 250 µm; c = 10 mm; d = 50 µm; e–h = 10 µm.

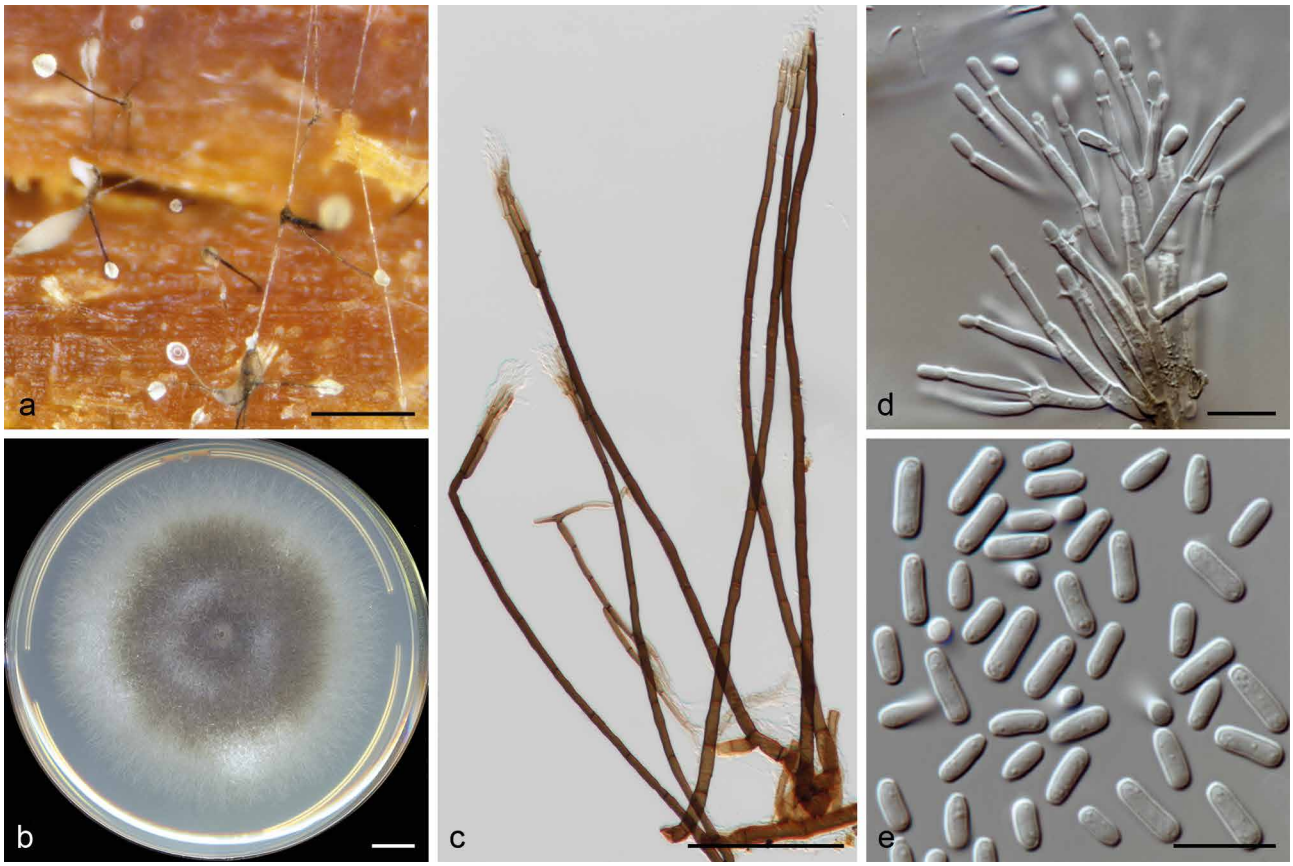

Fig. 7 Morphological features of *Leptographium terebrantis* (‘*Leptographium* sp. 1’, CBS 143462 = CMW 34391) from California in the western USA. a. Conidiophores with slimy spore droplets on pine twig; b. colony on 2 % MEA for 7 d at 25 °C in the dark; c. conidiophores; d. conidiogenous cells; e. conidia. — Scale bars: a = 250 µm; b = 10 mm; c = 100 µm; d–e = 10 µm.

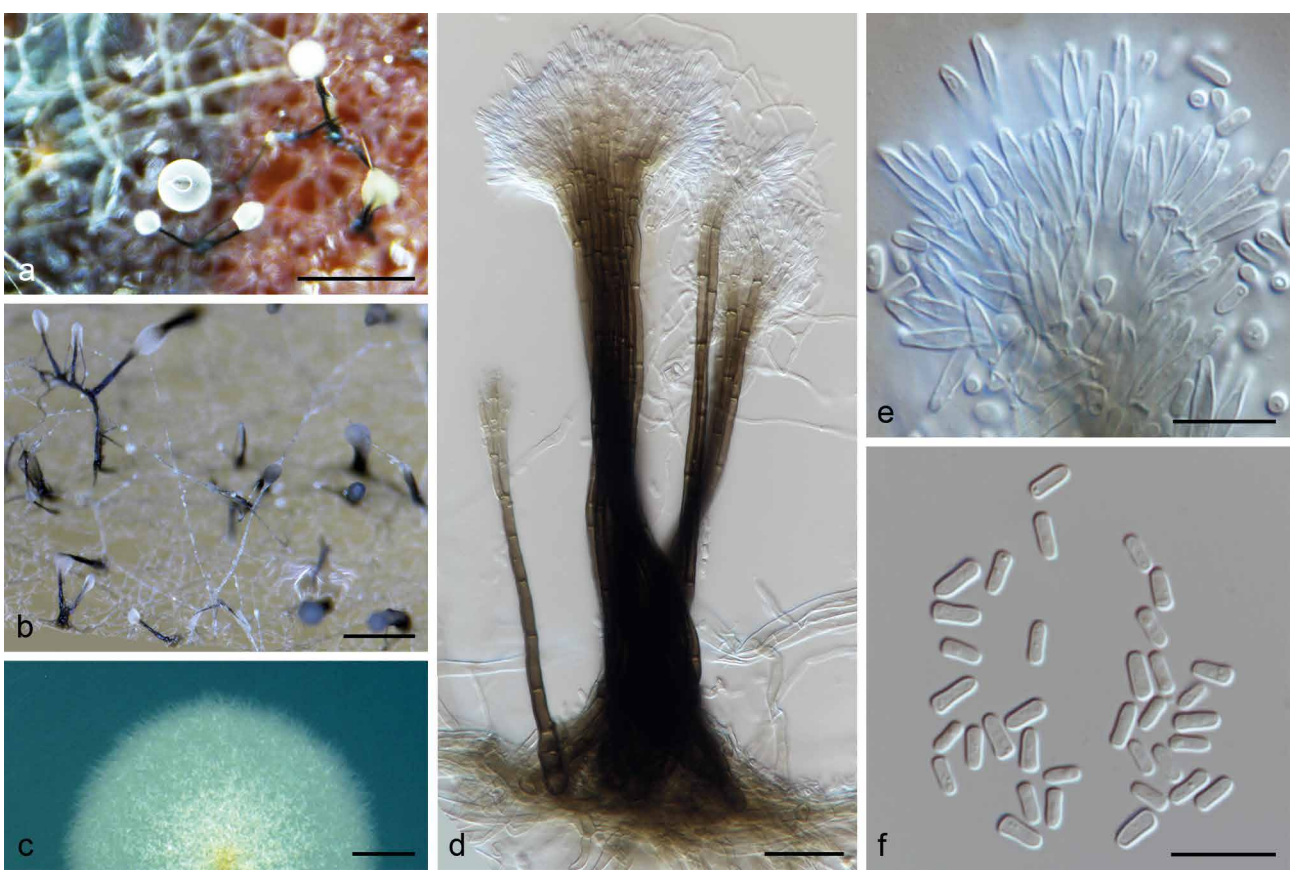

Fig. 8 Morphological features of *Leptographium doddsii* sp. nov. (ex-holotype, CBS 143470 = CMW 34479). a–b. Conidiophores with spore droplets formed on pine twig (a) and on 2 % MEA (b); c. colony on 2 % MEA at 25 °C in the dark for 7 d; d. conidiophores; e. conidiogenous cells; f. conidia. — Scale bars: a–b = 250 µm; c = 5 mm; d = 25 µm; e–f = 10 µm.

at 20 °C (5.0 mm/d) whereas '*Leptographium* sp. 2' grew best at 25 °C (7.2 mm/d). The conidia of '*Leptographium* sp. 1' were ellipsoidal to oblong with pointed bases and inflated apices and ranged in 3.5–9 × 2–3 µm. In contrast, the conidia of '*Leptographium* sp. 2' was variable ranging from ellipsoidal (3–5 × 1.5–2.5 µm) to elongated or oblong (3–10 × 1.5–3.5 µm) gradually tapering towards the base. This finding supports the suggestion of Massoumi Alamouti et al. (2011) that the delineation of species such as *L. terebrantis* and *G. clavigera* requires further study including larger numbers of isolates than previously considered.

Grosmannia galeiformis-species complex

Prior to this study, the *G. galeiformis*-species complex included three described spp., *G. galeiformis* (Zhou et al. 2004), *G. radiaticola* (= *Pesotum pini*) (Kim et al. 2005) and *L. koraiensis* (Chang et al. 2019). Linnakoski et al. (2012) and Chang et al. (2019) labelled two isolates from Mexico and Austria respectively, as '*G. galeiformis* A' recognizing that these probably represent a distinct taxon. They grouped together without support. In our analyses (Fig. 2) these isolates separated. In addition, the isolates from the USA collected by Taerum et al. (2013) were shown to represent four new species. *Grosmannia galeiformis* and *G. radiaticola* are known to be heterothallic (Zhou et al. 2004), all the new species described here are known only from their asexual morphs, as is characteristic for the other species in the complex.

Leptographium doddsii Marinc., Z.W. de Beer, M.J. Wingf., *sp. nov*. — MycoBank MB835953; Fig. 8

Etymology. Named for Dr Kevin Dodds of USDA Forest Service, Durham, New Hampshire, who assisted in the collection of RTB specimens from which isolates were obtained for the present study.

Typus. USA, California, Sierra Nevada Mountains, *Dendroctonus valens*, Aug. 2009, *M.J. Wingfield* (holotype PREM 63065, culture ex-holotype CBS 143470 = CMW 34479).

Diagnosis — This species is phylogenetically distinct from the others based on beta-tubulin and elongation factor 1-alpha and its conidial shape is predominantly oblong.

Asexual structures produced on pine twigs on surface of water agar. Sexual morph not observed. *Conidiophores* single or gregarious, macronematous, mononematous or synnematous. *Synnematous conidiophores* consisting of 2–many stipes loosely compacted, 168–288 (215 ± 30.0) µm long, 5–35 (14.9 ± 8.60) µm wide near base, 6–25 (9.9 ± 5.17) µm wide below conidiogenous apparatus. *Mononematous conidiophores* gregarious, stipes smooth, uniformly pigmented, brown, upright, slightly bulging at apex, 45–146 (111 ± 33.8) µm long, 4–7 (4.6 ± 0.84) µm wide at base, tapering to 3–4 (3.5 ± 0.31) µm wide near apex, with 3–7 septa. *Basal cells* bulbous or rhizoid. *Conidiogenous apparatus* branched in 3–5 tiers, unevenly layered, 44–155 (78.7 ± 30.34) µm long. *Conidiogenous cells* cylindrical to clavate, tapering towards apex, slightly constricted at base, hyaline, smooth, 8–12 × 1–2.5 (9.6 ± 0.90 × 1.76 ± 0.23) µm. *Conidia* blastic, hyaline, oblong, often abruptly tapering to base, 3–6 × 1.5–2.5 (4.5 ± 0.76 × 2.0 ± 0.27) µm.

Culture characteristics — Colonies optimum growth at 25 °C (1.4 mm/d), followed by at 20 °C (1.3 mm/d), no growth at 30 °C and 35 °C, growing in circular mode with smooth margins. Mycelia submerged, flat, sparse. Colony morphology uniform at all temperatures.

Additional materials examined. USA, California, Sierra Nevada Mountains, *Dendroctonus valens*, Aug. 2009, *M.J. Wingfield*, cultures CBS 143463 = CMW 34393; Massachusetts, State forestry land near Ware, *Dendroctonus valens*, Nov. 2009, *M.J. Wingfield*, PREM 63066, culture CBS 143475 = CMW 34581.

Notes — *Leptographium doddsii* was represented by 20 isolates collected from New Hampshire, Massachusetts and California, labelled as *Grosmannia* sp. 4 in the study of Taerum et al. (2013). The fungus had loosely arranged synnemata that were typical of species in the *G. galeiformis*-species complex.

Leptographium gordonii Marinc., Z.W. de Beer, M.J. Wingf., *sp. nov*. — MycoBank MB835954; Fig. 9

Etymology. Named for Prof. Tom Gordon of the University of California, Davis, an exceptional plant pathologist who provided support and accommodation for MJW during sabbatical leave and during which many of the fungi described in this study were collected.

Typus. USA, New Hampshire, White Mountain National Forest, isolated from *Dendroctonus valens* in *Pinus resinosa*, Nov. 2009, *M.J. Wingfield* (holotype PREM 63067, culture ex-holotype CBS 143477 = CMW 34619).

Diagnosis — *Leptographium gordonii* is closely related to *L. doddsii*. Their conidial dimensions are similar but the shape of the conidia showed differences. *Leptographium doddsii* has oblong conidia with abruptly tapering bases, whereas *L. gordonii* has ellipsoidal to cylindrical conidia.

Asexual structures produced on pine twigs on surface of water agar. Sexual morph not observed. *Conidiophores* macronematous, synnematous, consisting of a few to many compact stipes, rarely mononematous, upright, single or gregarious, 330–700 (572 ± 83.5) µm long, 12–85 (41.5 ± 19.0) µm wide near base, 9–60 (31 ± 13.7) µm wide below conidiogenous apparatus; *stipes* brown, gradually becoming paler towards apex. *Conidiogenous apparatus* pale brown, branched in 4–5 tiers, conidial droplets yellowish. *Conidiogenous cells* blastic, hyaline, oblong, tapering towards apex, showing percurrent growth, 10–32 (22.4 ± 6.0) µm long, 1.5–2.5 (2.0 ± 0.21) µm wide near base, 1–2 (1.3 ± 0.15) µm near apex. *Conidia* hyaline, oblong to ellipsoidal, gradually tapering to base, 3.5–5 × 2–2.5 (4.3 ± 0.37 × 2.1 ± 0.17) µm.

Culture characteristics — Colonies showing optimum growth at 25 °C (2 mm/d), followed by at 20 °C (1.9 mm/d), no growth at 30 °C and 35 °C, growing in circular mode with smooth margins, above and reverse colour transparent. Mycelia flat, submerged, sparse.

Notes — *Leptographium gordonii* was represented by eight isolates collected from New Hampshire, labelled as *Grosmannia* sp. 2 in the study of Taerum et al. (2013).

Leptographium owenii Marinc., Z.W. de Beer, M.J. Wingf., *sp. nov*. — MycoBank MB835955; Fig. 10

Etymology. Named after Dr Don Owen who spent much of his career working on the RTB, contributing considerable knowledge to our understanding of this important bark beetle and who assisted MJW in collecting many fungi described in this study.

Typus. USA, California, Sierra Nevada Mountains, *Dendroctonus valens*, Aug. 2009, *M.J. Wingfield* (holotype PREM 63063, culture ex-holotype CBS 143467 = CMW 34448).

Diagnosis — This species had ellipsoidal to oblong conidia, similarly shaped to those of *L. gordonii*, but unlike the other two new species in the complex of which conidial shape is predominantly oblong. It also has shorter conidial dimensions when compared to the other species: *L. doddsii* 3–6 × 1.5–2.5 (4.5 ± 0.76 × 2.0 ± 0.27) µm; *L. gordonii* 3.5–5 × 2–2.5 (4.3 ± 0.37 × 2.1 ± 0.17) µm; *L. seifertii* 4–7 × 2–3 (5.7 ± 0.78 × 2 ± 0.24) µm.

Asexual structures produced on pine twigs on surface of water agar. Sexual morph not observed. *Conidiophores* macronematous, mononematous, synnematous. *Synnematous conidiophores* 316–441 (372 ± 44.21) µm long from basal cell to just below conidiogenous apparatus, 7–82.5 (33.4 ± 21.6) µm wide near base. *Mononematous conidiophores* two kinds: 1) mostly, stout, dark brown and tall; 2) occasionally present, short, brown

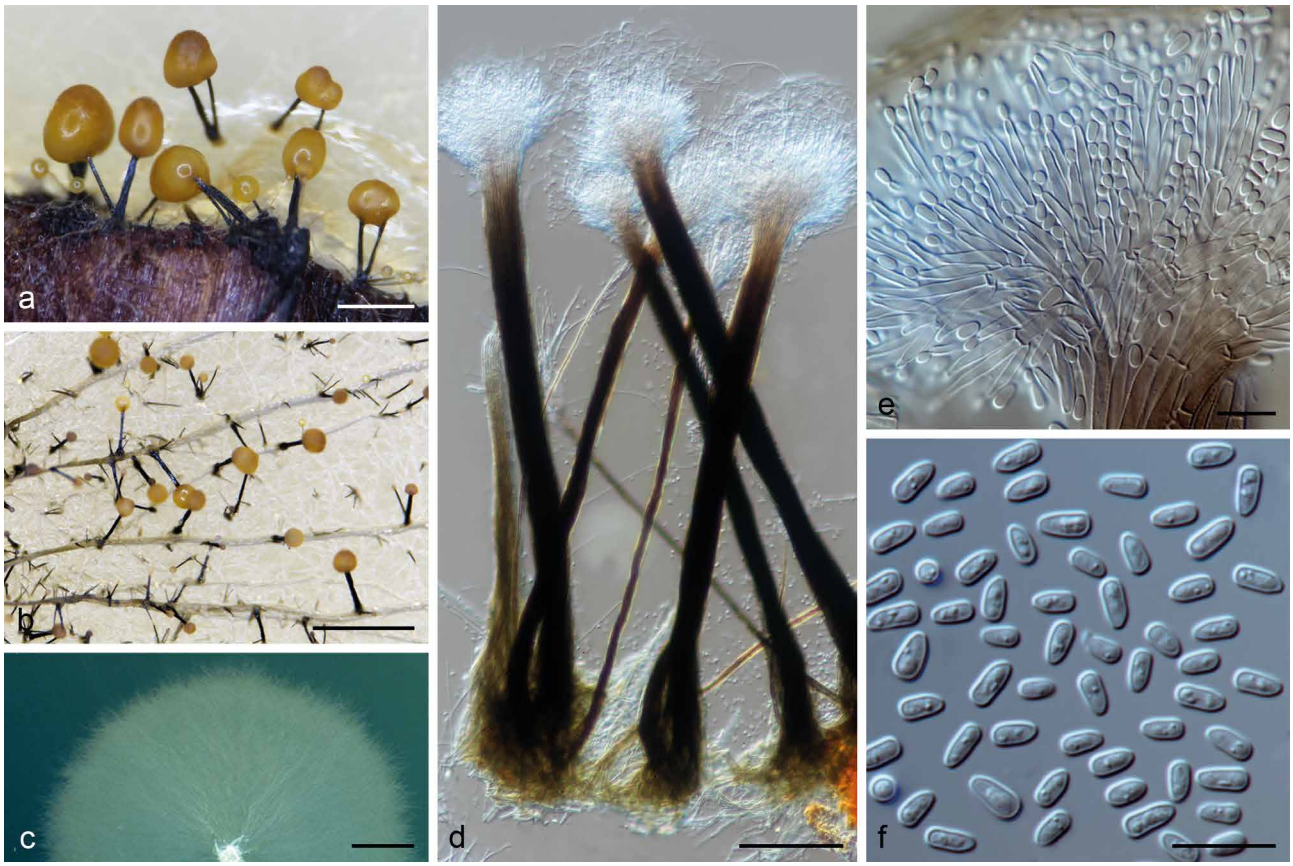

Fig. 9 Morphological features of *Leptographium gordonii* sp. nov. (ex-holotype, CBS 143477 = CMW 34619). a–b. Conidiophores with slimy spore mass on pine twig (a) and on water agar (b); c. colony on 2 % MEA at 25 °C for 7 d in the dark; d. conidiophores; e. conidiogenous apparatus; f. conidia. — Scale bars: a = 500 µm; b = 1 mm; c = 5 mm; d = 100 µm; e–f = 10 µm.

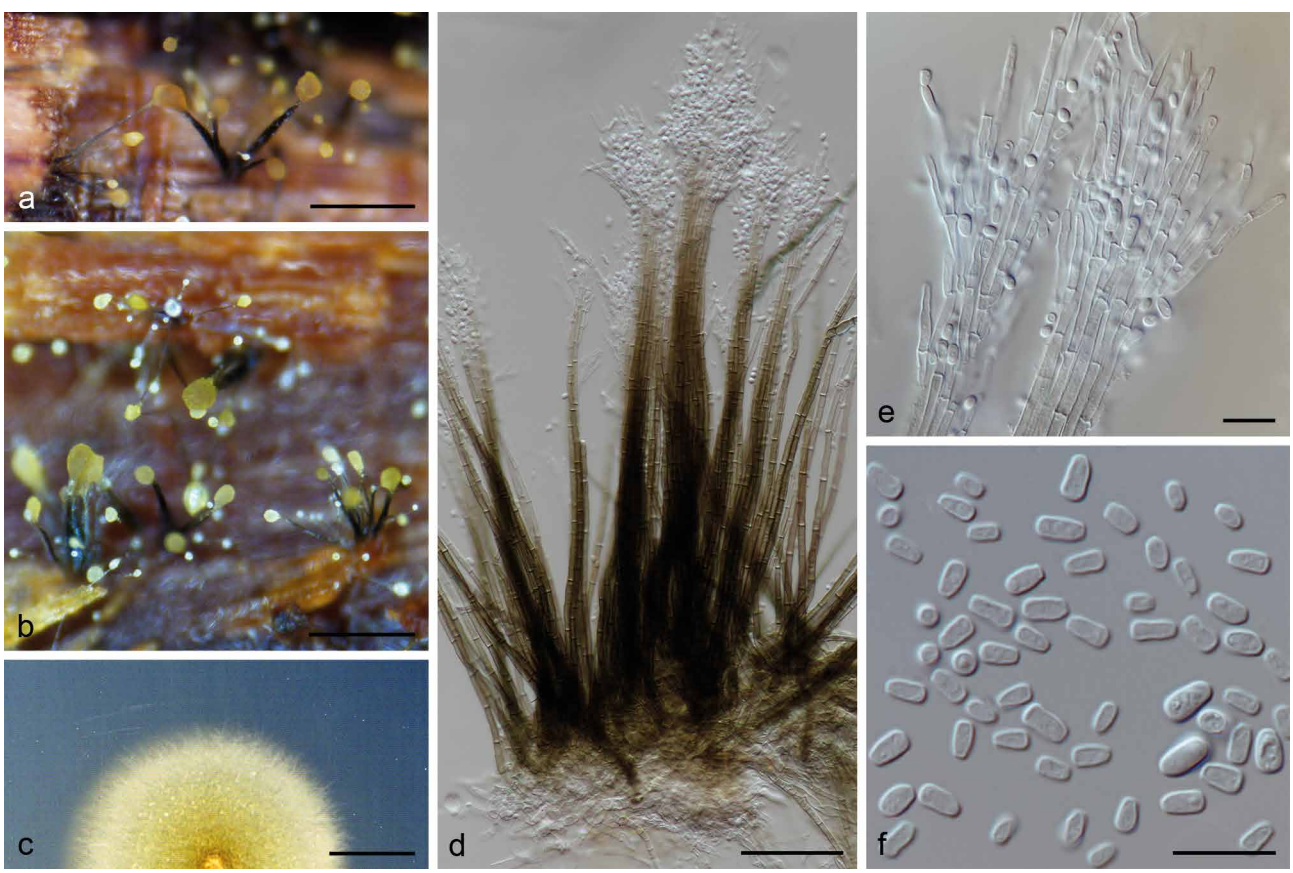

Fig. 10 Morphological features of *Leptographium owenii* sp. nov. (ex-holotype, CBS 143467 = CMW 34448). a–b. Conidiophores with slimy spore mass on pine twig; c. colony on 2 % MEA for 7 d at 20 °C in the dark; d. conidiophores; e. conidiogenous cells; f. conidia. — Scale bars: a–b = 250 µm; c = 5 mm; d = 50 µm; e–f = 10 µm.

and short, closely located with synnematous ones, rarely secondary conidiophores developing from branches; *stipes* smooth, uniformly pigmented, brown, upright or occasionally mildly undulated, slightly bulging at apex, 218–1152 (627 ± 291.9) µm long, 7–13 (10.3 ± 1.64) µm wide near base, 5–10 (7.8 ± 1.02) µm wide near apex, with 4–11 septa; *basal cells* morphology vary from rhizoid-like to foot-like. *Conidiogenous apparatus* branched in 3–6 tiers, 61.5–164 (99.7 ± 23.26) µm long. *Conidiogenous cells* cylindrical or clavate, gradually tapering towards apex, often slightly constricted at base, hyaline, smooth, tip showing sympodial growth with production of new conidium, 15–26 (19.9 ± 2.58) µm long, 1–2.5 (1.9 ± 0.37) µm wide near base, 1–2 (1.4 ± 0.20) µm wide near apex. *Conidia* blastic, ellipsoidal, gradually tapering to truncated base, hyaline, 3–4.5 × 1.5–2.5 (3.5 ± 0.35 × 2 ± 0.17) µm (mononematous), 3–5 × 2–2.5 (4.0 ± 0.35 × 2.2 ± 0.25) µm (synnematous).

Culture characteristics — Colonies showed optimum growth at 20 °C (0.8 mm/d) followed by 25 °C (0.5 mm/d), no growth at 30 °C and 35 °C. Colony morphology uniform at all temperatures, growing in circular mode with smooth edges, above and reverse colour pale luteous with scattered asexual fruiting structures. Mycelia flat, mostly submerged.

Notes — *Leptographium owenii* was represented by three isolates from California. It corresponds with *Grosmannia* sp. 7 in the study of Taerum et al. (2013). *Leptographium gordonii*, *L. doddsii* and *L. owenii* showed optimum growth at 20–25 °C. However, *L. gordonii* (1.9–2 mm/d) and *L. doddsii* (1.3–1.4 mm/d) grew more rapidly than *L. owenii* (0.5–0.8 mm/d).

Leptographium seifertii Marinc., Z.W. de Beer, M.J. Wingf., *sp. nov*. — MycoBank MB835956; Fig. 11

Etymology. Named for Dr Keith Seifert who has contributed greatly to fungal taxonomy throughout his career. Amongst others, he resolved the generic placement of many ophiostomatoid species previously aggregated in the genus *Graphium*.

Typus. USA, New Hampshire, White Mountain National Forest, *Dendroctonus valens* on *Pinus resinosa*, Nov. 2009, *M.J. Wingfield* (holotype PREM 63068, culture ex-holotype CBS 143478 = CMW 34620).

Diagnosis — Within the *G. galeiformis*-species complex, *L. seifertii* could be distinguished by its large conidial dimensions (4–7.5 × 1.5–3 µm).

Asexual structures produced on pine twigs on the surface of water agar. Sexual morph not observed. *Conidiophores* macronematous, mostly synnematous, occasionally a branch growing out becoming a secondary conidiophore or sterile hypha. *Synnematous conidiophores* consisting of 2–many stipes, loosely packed, gregarious, 213–525 (317 ± 78.5) µm long, 9–45 (23 ± 10.3) µm wide near base, 5–64.5 (22 ± 12.4) µm wide. *Mononematous conidiophores* scattered, upright, 169–805 (588 ± 121.1) µm long, 6.5–13 (11 ± 1.5) µm wide near base, 7–10 (9 ± 0.8) µm wide near apex; *individual stipes* smooth, brown to dark brown, becoming paler towards apex, upright, slightly bulging at apex, with 3–10 septa; *basal cells* bulbose. *Conidiogenous apparatus* branched in 4–8 tiers, 78–161 (100 ± 21.1) µm long, branch cells often wider at apex, brown becoming paler to apex. *Conidiogenous cells* blastic, cylindrical, gradually tapering towards apex, base slight constricted, hyaline, smooth, 13–24 (16 ± 2.5) µm long, 1–2 (1.7 ± 0.23) µm wide near base, 1–2 (1.2 ± 0.15) µm wide near apex. *Conidia* hyaline, oblong, gradually tapering to base, 4–7 × 2–3 (5.7 ± 0.78 × 2.0 ± 0.24) µm.

Fig. 11 Morphological features of *Leptographium seifertii* sp. nov. (ex-holotype, CBS 143478 = CMW 34620). a–b. Conidiophores with spore droplets on pine twig; c. colony on 2 % MEA at 20 °C for 7 d in the dark; d. conidiophores; e. conidiogenous cells; f. conidia. — Scale bars: a–b = 500 µm; c = 5 mm; d = 50 µm; e–f = 10 µm.

Culture characteristics — Colonies optimum growth at 20 °C (0.7 mm/d), followed by at 25 °C (0.6 mm/d), no growth at 30 °C and 35 °C, growing in circular mode with smooth to undulate margins, sparse, above and reverse colour pale luteous. Colony morphology more or less uniform but at 25 °C more aerial, cottony and white mycelium produced.

Additional material examined. USA, California, Sierra Nevada Mountains, El Dorado National Forest, *Dendroctonus valens* on *Pinus* sp., Nov. 2009, *M.J. Wingfield*, PREM 63069, culture CBS 143485 = CMW 39393.

Notes — *Leptographium seifertii* was represented by two isolates from New Hampshire and 10 from California. It corresponds to *Grosmannia* sp. 6 in the study of Taerum et al. (2013).

Leptographium olivaceum-species complex

Linnakoski et al. (2012) was the first to define this species complex as the *Grosmannia olivacea*-species complex, including five species. De Beer & Wingfield (2013) added an additional species. The complex was recently revised by Yin et al. (2019), who included eight known species and described six new species in the complex. They treated all species in the complex as *Leptographium*, providing new combinations for seven species, including *L. olivaceum*. All species in the complex are characterized by synnematous asexual morphs, and those with known sexual morphs all produce ascomata with almost cylindrical necks and abundant ostiolar hyphae. In our analyses (Fig. 3), the species from the RTB described below clustered with *L. conplurium* (Yin et al. 2019), *L. sylvestris* (Yin et al. 2019), *L. olivaceapini* (Davidson 1971) and *L. cucullatum* (Solheim 1986).

Leptographium raffai Marinc., Z.W. de Beer, M.J. Wingf., *sp. nov*. — MycoBank MB835957; Fig. 12

Etymology. Named for Prof. Ken Raffa, University of Wisconsin forest entomologist for his career-long and extensive contributions to the study of conifer-infesting bark beetles including their ecology and association with fungi.

Typus. USA, California, Sierra Nevada Mountains, isolated from *Dendroctonus valens*, Aug. 2009, *M.J. Wingfield* (holotype PREM 63064, culture ex-holotype CBS 143468 = CMW 34451).

Diagnosis — This species shared morphological similarities such as synnematous conidiophores and ellipsoidal to cylindrical conidia with the related species mentioned above. However, *L. raffai* had smaller conidia (3–5.5 × 1–2 µm) compared to *L. olivaceapini* (2–7 × 1–2 µm) and *L. cucullatum* (3.5–8.2 × 1.7–3.5 µm). It also showed differences in its growth characteristics: *L. conplurium* and *L. raffai* grew the fastest at 25 °C with a similar growth rate (*L. conplurium* at 2.5 mm/d, *L. raffai* at 2.7 mm/d). The optimum growth temperature of *L. sylvestris* was 30 °C.

Asexual structures produced on pine twigs on the surface of water agar. Sexual morph not observed. *Conidiophores* scarcely produced, macronematous, synnematous, upright, straight or curved, scattered, seldom split at base, consisting of compactly packed stipes, 110–377 (281 ± 72.8) µm high, 11–40 (23.5 ± 8.19) µm wide near base, gradually tapering to 8.5–32 (16.7 ± 6.49) µm wide towards apex, brown, gradually less pigmented towards apex, smooth or verruculose; *conidiogenous apparatus* branched in 3–5 tiers, 41–83 (62.3 ± 15.22) µm long, 1° and 2° branches pale brown. *Conidiogenous cells* blastic, hyaline, cylindrical, gradually tapering towards apex, constricted at base, smooth, showing percurrent growth, 11–18 × 1–2 (15.1 ± 1.77 × 1.7 ± 0.23) µm. *Conidia* hyaline, ellipsoidal to cylindrical, tapering to truncated base, 3–5.5 × 1–2 (4.1 ± 0.55 × 1.8 ± 0.21) µm.

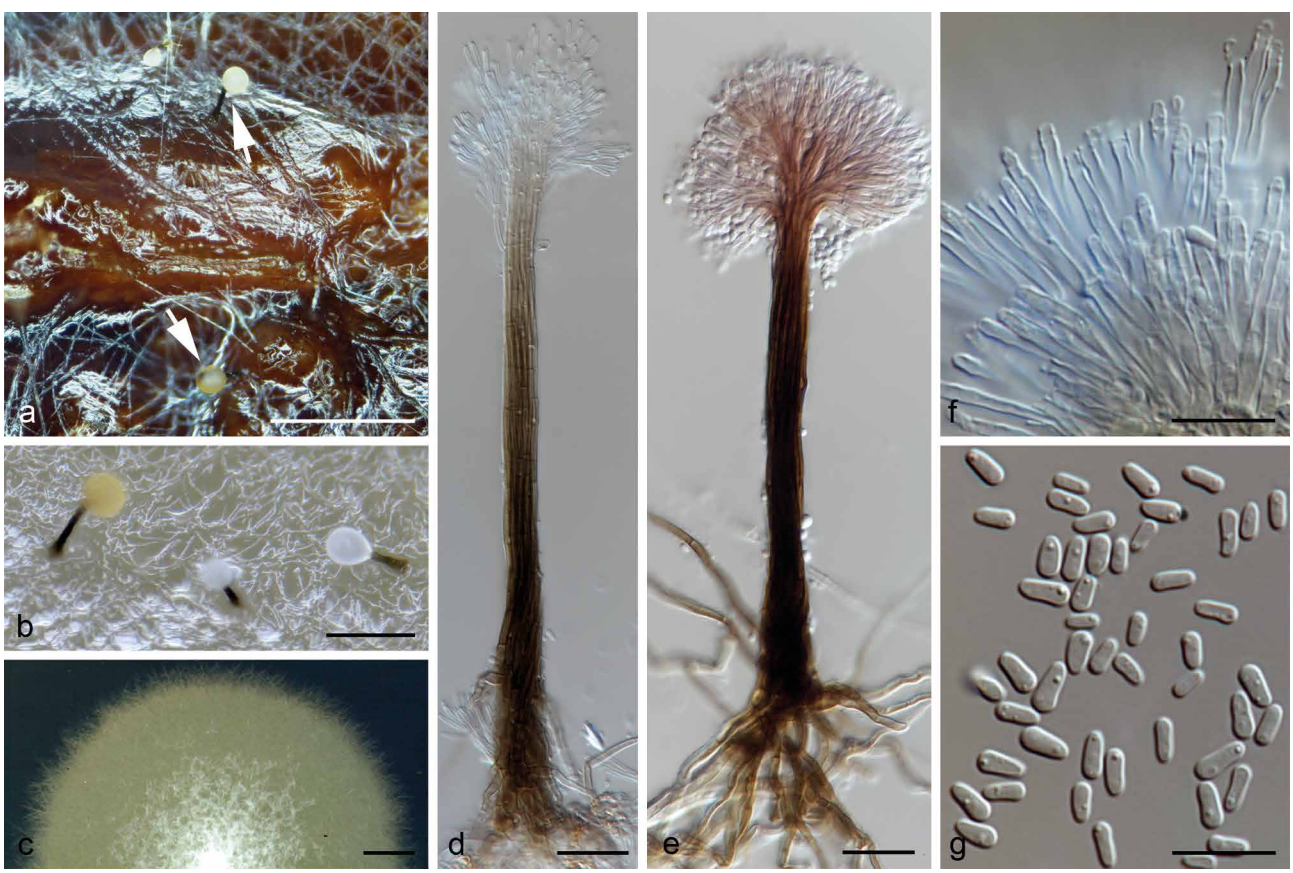

Fig. 12 Morphological features of *Leptographium raffai* sp. nov. (ex-holotype, CBS 143468 = CMW 34451). a–b. Conidiophores (arrows) with spore droplets on pine twig (a) and on water agar (b); c. colony on 2 % MEA at 25 °C for 7 d in the dark; d–e. conidiophore on pine twig (d) and on MEA (e); f. conidiogenous cells; g. conidia. — Scale bars: a = 500 µm; b = 250 µm; c = 5 mm; d–e = 25 µm; f–g = 10 µm.

Culture characteristics — Cultures showing optimum growth at 25 °C (2.7 mm/d) followed by at 20 °C (2.2 mm/d), no growth at 35 °C, growing in circular mode with smooth margins, above and below translucent in colour. Mycelia mostly submerged.

Additional material examined. USA, California, Sierra Nevada Mountains, isolated from *Dendroctonus valens*, Aug. 2009, *M.J. Wingfield*, PREM 63062, culture CBS 143466 = CMW 34445.

Notes — *Leptographium raffai* was represented by two isolates collected from California. It corresponds to *Grosmannia* sp. 5 in the study of Taerum et al. (2013).

Ophiostoma spp.

Ophiostoma ips-species complex

The *O. ips*-species complex as defined by Linnakoski et al. (2010) and De Beer & Wingfield (2013) is one of the best recognised species complexes in the *Ophiostomatales*. The sexual morphs consist of long-necked ascomata producing cylindrical ascospores surrounded by pillow-shaped to rectangular sheaths. The complex includes at least eight known species as well as some undescribed lineages. The multigene analyses of De Beer & Wingfield (2013) show that it is in need of revision.

Ophiostoma gilletteae Marinc., Z.W. de Beer, M.J. Wingf., *sp. nov*. — MycoBank MB835958; Fig. 13

Etymology. Named for Dr Nancy Gillette, formally of the USDA Forest Service who provided great enthusiasm for, and facilitated surveys in, the western USA during which many specimens included in this study were collected.

Typus. USA, Washington State, Colville Reservation, Pendleton Canyon, *Dendroctonus valens*, May 2008, *N. Gillette* (holotype PREM 63060, culture ex-holotype CBS 143458 = CMW 30681).

Diagnosis — *Ophiostoma gilletteae* grouped closest to *O. adjuncti*. The two fungi shared similar colony morphology on 2 % MEA and had similar conidial dimensions (5–7 × 2–3.5 µm in *O. adjuncti*, 2.5–7.5 × 1.5–3 µm in *O. gilletteae*). Other than being phylogenetically distinct, they differed in their optimal growth temperatures: *O. adjuncti* at 20 °C and *O. gilletteae* at 25 °C, ascospore dimensions (4–5.5 × 1.8–2.2 µm in *O. adjuncti*, 3–4 × 2 µm in *O. gilletteae*) and synnematous conidiophores (absent in *O. adjuncti*, present in *O. gilletteae*) (Davidson 1978).

Sexual and asexual structures produced on pine twigs on the surface of water agar. *Ascomata* scarce; *ascomatal base* ellipsoidal, dark brown, covered with minute pigmented hyphae, 182–296 (233 ± 38.5) µm high, 169–266 (204 ± 32.7) µm wide; *ascomatal necks* straight or curved, brown and becoming paler towards apex, 359–1195 (758 ± 201) µm long, 35–115 (68 ± 22) µm wide at base, becoming narrower towards apex, 12–18 (14 ± 1.7) µm wide near apex. *Ascospores* hyaline, oblong, covered with rectangular-shaped gelatinous sheath, 3–4 × 2 (3.4 ± 0.32 × 1.9 ± 0.15) µm including sheath. *Conidiophores* macronematous, mono- or synnematous. *Synnematous conidiophores* pesotum-like, in tufts, loosely compacted, upright, 145–760 (399 ± 331) µm high, brown, uniformly pigmented throughout; *conidiogenous apparatus* hyaline to slightly pigmented, branched in a few tiers; *conidiogenous cells* blastic, mostly in whorls, cylindrical, often gradually tapering towards apex, hyaline, smooth, showing percurrent growth, sporogenous part mostly limited to upper half but occasionally throughout entire cell, 16–39 (27 ± 6.6) µm long, 2 (1.8 ± 0.2) µm wide at base, 1–2 (1.3 ± 0.2) µm wide near apex. *Mononematous conidiophores* hyalorhinocladiella-like, upright, hyaline to slightly pigmented, 35–68 (53 ± 13.3) µm long. *Conidia* hyaline, oblong, often tapering to truncated base, often with inflated or round apex when borne on mononematous stipes, 3–5 × 2 (4.0 ± 0.49 × 2 ± 0.13) µm (mononematous), 4–7 × 2–3 (5.5 ± 0.63 × 2.0 ± 0.27) µm (synnematous).

Culture characteristics — Colonies optimum growth at 25 °C (3.7 mm/d), followed by at 20 °C (2.9 mm/d), no growth at 35 °C. Colonies showing circular growth mode with smooth margins, above almost colourless and reverse olivaceous brown in inner circle. Mycelia mostly submerged, sparse.

Additional materials examined. USA, California, Sierra Nevada Mountains, from *Dendroctonus valens*, Aug. 2009, *M.J. Wingfield*, PREM 63061, culture CBS 143465 = CMW 34425; Wisconsin, Wood County, Plum Creek timber, *Dendroctonus valens*, 2008, *K.R. Raffa*, culture CMW 39390; Washington State, Colville Reservation, Pendleton Canyon, *Dendroctonus valens*, May 2008, *N. Gillette*, culture CMW 30680.

Notes — *Ophiostoma gilletteae* was represented by 69 isolates labelled as *Ophiostoma* sp. 1 in the study of Taerum et al. (2013). These included isolates from both the eastern and western USA. This was the species most often isolated in the survey considered by Taerum et al. (2013), representing 33.1 % of the isolates collected in the western North America, and 10.9 % of isolates from the eastern North America. All the species in the complex are associated with pine-infesting beetles. *Ophiostoma adjuncti*, the closest relative of *O. gilletteae*, is a staining fungus isolated from the wood tissue of *Pinus ponderosa* killed by *Dendroctonus adjunctus*, as well as from adult beetles in New Mexico (Davidson 1978).

The uncertain placement of *Ophiostoma floccosum*

Harrington et al. (2001) included nine species in their broadly defined *O. piceae*-species complex, including *O. floccosum* and *O. quercus*. They also treated *Pesotum aureum* (Hedgcock 1906) as an asexual name of *O. floccosum* (Mathiesen 1951). After the one fungus one name principles were adopted (Hawksworth et al. 2011), *P. aureum* became a formal synonym of *O. floccosum* (De Beer et al. 2013b). Linnakoski et al. (2010) also adopted a broad concept for the *O. piceae*-species complex that included a hardwood and conifer clade, but *O. floccosum* grouped distinct from these clades. De Beer & Wingfield (2013) defined the hardwood clade as the *O. ulmi*-species complex, but did not find phylogenetic support for the conifer species and chose not to define an *O. piceae*-species complex. Also, *O. floccosum* did not group with *O. piceae* and the other 'conifer clade' species in their analyses. Yin et al. (2016) proceeded to redefine the *O. piceae*-species complex, but restricted it to a monophyletic clade including five known species (*O. piceae*, *O. flexuosum*, *O. canum*, *O. brunneum*, *O. rachisporum*) and three novel species from China. In their analyses, *O. floccosum* formed a monophyletic lineage distinct from all other species, but still clearly residing in *Ophiostoma*. For our analyses (Fig. 5), we included *Ophiostoma* spp. relatively closely related to *O. floccosum* and the new taxon.

De Beer & Wingfield (2013) named and defined species complexes when three or more species formed a lineage with significant statistical support in at least one gene region. In addition, their view was that these species should share morphological and/or ecological characters. Although *O. floccosum* and its novel sister species constitute a well-defined lineage, it is inordinately early to define this as a species complex based on the guidelines of De Beer & Wingfield (2013).

Ophiostoma shanziensis Marinc., Z.W. de Beer, M.J. Wingf., *sp. nov*. — MycoBank MB835959; Fig. 14

Etymology. Name refers to Shanxi, the province of China where the fungus was collected.

Typus. China, Shanxi province, Guailingdi, from phloem adjacent to *Dendroctonus valens* gallery in *Pinus tabuliformis*, Jan. 2004, *Q. Lu & C. Decock* (holotype PREM 63070, culture ex-holotype MUCL 46456 = CMW 48329).

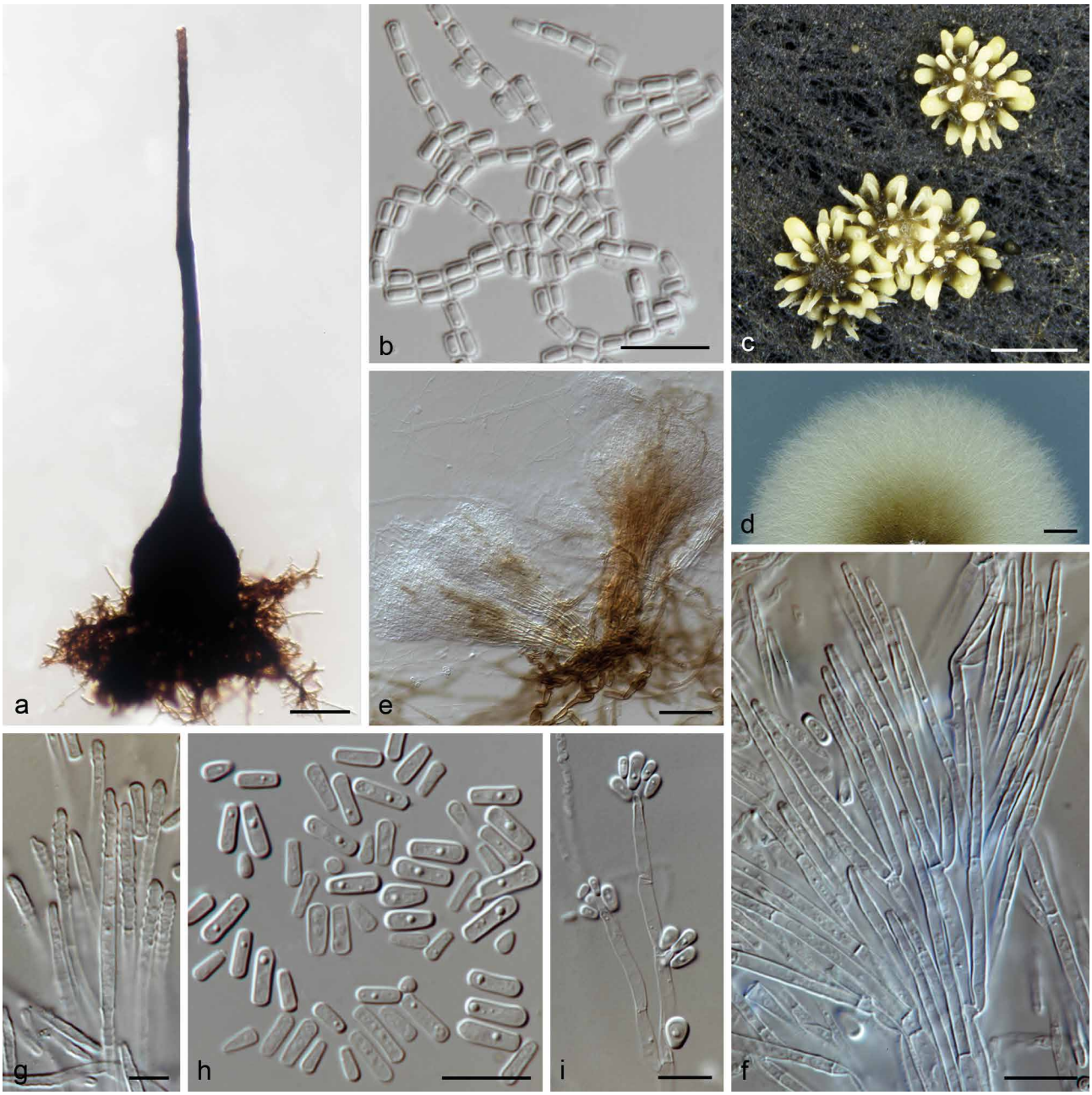

Fig. 13 Morphological features of *Ophiostoma gilletteae* sp. nov. (ex-holotype, CBS 143458 = CMW 30681). a. Ascoma; b. ascospores enclosed with rectangular-shaped gelatinous sheath; c. conidiophores on water agar; d. colony on 2 % MEA at 25 °C for 7 d in the dark; e–h. synnematous conidiophores, conidiogenous apparatus, conidiogenous cells, and conidia of pesotum-like morph; i. mononematous conidiophores and conidia of hyalorhinocladiella-like morph. — Scale bars: a = 100 µm; b, f, h = 10 µm; c = 1 mm; d = 5 mm; e = 50 µm; g, i = 5 µm.

Diagnosis — Similar to *O. floccosum*, two distinct types of conidiophores are commonly produced in *O. shanziensis*. A rounded protuberance at the side of synnemata that Harrington et al. (2001) observed in *O. floccosum* isolates, was absent in *O. shanziensis*. The original description of *O. floccosum* (Mathiesen 1951) mentioned two conidial dimensions, 6.8 × 3.6 µm for mononematous conidia and 4.0 × 2.2 µm for synnematous conidia. We were not able to distinguish between conidia arising from synnematous and mononematous conidiophores in *O. shanziensis*, and the average dimensions (3–4.5 × 1–2 µm, av. 3.8 × 1.5 µm) were smaller than those presented in the study of Mathiesen (1951).

Asexual structures produced on pine twigs on the surface of water agar. Sexual morph not observed. *Conidiophores* mostly formed on aerial hyphal strings, single, grouped, conidiophores in range from micro- to macronematous and from mononematous to synnematous. *Synnematous conidiophores*: *stipes* flexuous, consisting of tightly compact hyphae, 162–1205 (583 ± 298) µm long, 6–34 (17.1 ± 5.77) µm wide near base, 5–39 (19.1 ± 7.93) µm wide before conidiogenous apparatus, individual hypha smooth, pale brown, uniformly pigmented; *conidiogenous apparatus* branched in 4–5 tiers, 54–377 (112 ± 65.4) µm long; *conidiogenous cells* hyaline, flexuous. *Mononematous conidiophores* in two types: 1) sporothrix-like; *stipes* upright, flexuous or straight, hyaline to subhyaline, occasionally branched, septate; *conidiogenous cells* blastic, hyaline, cylindrical, 7–66 (34.2 ± 15.56) µm long, 1–2 (1.6 ± 0.34) µm wide near base, gradually tapering towards apex, 1–1.5 (1.2 ± 0.19) µm wide before fertile region, smooth, upper fertile region, 2–49 (16.7 ± 13.12) µm long, denticulate, showing sympodial growth; 2) leptographium-like, less common, possibly derivates of synnematous conidiophores; *stipes* flexuous or straight, upright hyaline to pale brown, occasionally branched, septate; *conidiogenous apparatus* branched; *conidiogenous cells* blastic, hyaline. *Conidia* in slimy heads or droplets, whitish or creamy with age, hyaline, ellipsoidal, slightly curved in side view, tapering to base, 3–4 × 1–2 µm (3.8 ± 0.26 × 1.5 ± 0.09 µm).

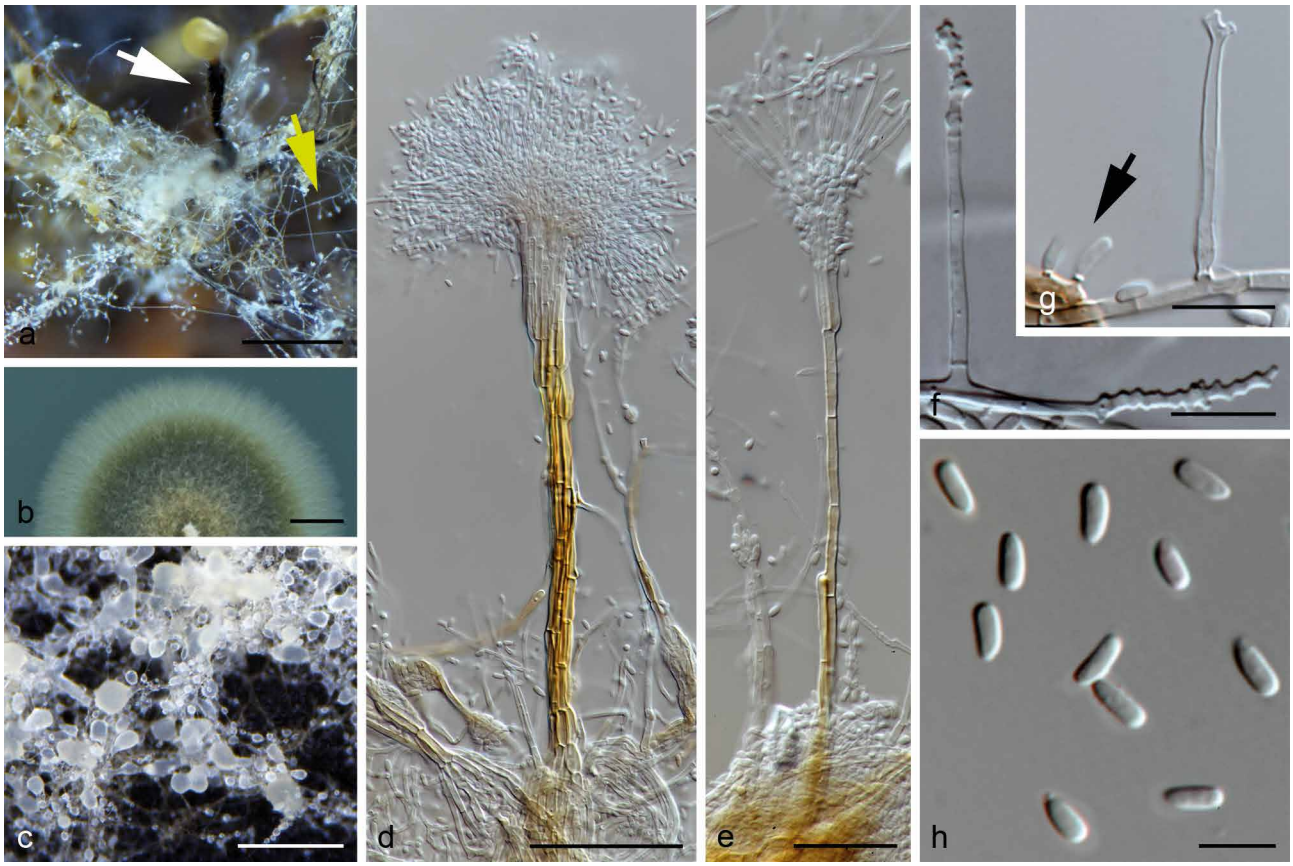

Fig. 14 Morphological features of *Ophiostoma shanziensis* sp. nov. (ex-holotype, MUCL 46456 = CMW 48329). a. Synnematous (white arrow) and mononematous (yellow arrow) conidiophores produced on aerial hyphae on pine twig; b. colony on 2 % MEA at 25 °C for 7 d in the dark; c. conidial masses on MEA; d. synnematous conidiophore; e. mononematous conidiophores (rare), leptographium-like; f. mononematous conidiophores showing a stretch of denticles to which conidia was attached; g. conidiogenous cells and conidia directly borne on vegetative hyphae (arrow); h. conidia. — Scale bars: a = 500 µm; b = 5 mm; c = 250 µm; d = 50 µm; e = 25 µm; f–g = 10 µm; h = 5 µm.

Culture characteristics — Colonies optimum growth at 25 °C (1.8 mm/d), followed by at 20 °C (1.4 mm/d), no growth shown at 35 °C, above and reverse colour umber to brown vinaceous inner circle with 5 mm margin of colourless to buff, showing flat, growing in circular mode with smooth margins. Mycelia mostly submerged with sparse fluffy aerial hyphae. Colony morphology similar at all temperatures.

Additional material examined. China, Shanxi province, Guailingdi, from phloem adjacent to *Dendroctonus valens* gallery in *Pinus tabuliformis*, Jan. 2004, *Q. Lu & C. Decock*, PREM 63071, culture MUCL 46632 = CMW 48330.

Notes — This species was initially identified as *Pesotum aureum* (asexual morph of *O. floccosum*) by Lu et al. (2009b) and labelled as *Ophiostoma* sp. 3 by Taerum et al. (2013). However, additional isolates and analyses of sequences for a greater number of gene regions in the present study showed that the fungus resides in a distinct clade as a sister taxon to *O. floccosum*. *Ophiostoma shanziensis* did not produce a sexual morph in this study.

DISCUSSION

Seven novel species of Ophiostomatalean fungi collected in association with *D. valens* (RTB) in previous studies have been described based on morphology and multi-gene phylogenetic analyses. Six of these taxa originated in the USA and the seventh was from an earlier study conducted in China. Furthermore, our analyses showed that two taxa tentatively recognised as novel by Taerum et al. (2013), represented *L. terebrantis*, and a third was of *O. ips*. Collectively, 32 species of Ophiostomatalean fungi have now been reported in association with the RTB, of which four remain unnamed (Table 1). Three of the unnamed taxa treated by Taerum et al. (2013) for which the original isolates had been lost, were excluded from the present study, as was an unnamed *Graphilbum* sp. from the study of Lu et al. (2009a).

Resolving the identity of the fungal taxa associated with the RTB enhances our ability to better understand new invasions and potential risks of range expansions by this beetle. A total of 500 isolates were obtained in the three surveys (Lu et al. 2009a, b, Taerum et al. 2013), but the relative numbers of isolates per species obtained from different areas should be interpreted with care. This is because different sampling biases could have occurred during different collections, the collection and isolation methods used by different collectors varied, the age of beetles and galleries varied, and isolation success can be influenced by numerous different factors. Nevertheless, certain important trends were clear from this study. For example, 461 of the 500 isolates belonged to one of five species complexes in the Ophiostomatales, four in *Leptographium* and one in *Ophiostoma*.

The greatest number of isolates obtained during the three surveys (Lu et al. 2009a, b, Taerum et al. 2013), which formed the basis for this study, resided in the *Leptographium procerum*-species complex. In a recent revision of this complex, Yin et al. (2015) recognized nine species, while Chang et al. (2019) described a tenth species. All species in the species complex excluding two are associated with pine-infesting beetles, mostly from East Asia (Yin et al. 2015). Two of the species from RTB, *L. sinoprocerum* (Lu et al. 2008, 2009b) and *L. pini-densiflorae* (Lu et al. 2009a), were isolated in low numbers. The latter species has previously been reported from *Tomicus piniperda* and other pine-infesting bark beetles in Japan (Masuya et al. 2000,

2009), Thailand (Yamaoka et al. 2007) as well as in Korea (Hong et al. 2015), while *L. sinoprocerum* has been reported only from China. *Leptographium procerum* was by far the most commonly isolated species in the RTB surveys, where 125 of the 192 isolates were from China and 67 from the eastern USA (Taerum et al. 2013).

Because of its common occurrence in China, *L. procerum* is considered by some researchers as a significant contributing factor to the death of *Pinus tabuliformis* trees in that country (Lu et al. 2010, 2011). It has also been assumed that the insect, *D. valens* (RTB), was of North American origin and that *L. procerum* entered China with the RTB (Lu et al. 2011, Sun et al. 2013, Taerum et al. 2013). However, it is also known to occur in the absence of RTB in several countries of Europe (Taerum et al. 2017). In the survey of Taerum et al. (2013), *L. procerum* could not be isolated from any RTB specimens collected in the western USA, the area that was suggested to be the source of the RTB invasion in China (Cai et al. 2008, Cognato et al. 2005, Taerum et al. 2016). A population genetics study of the fungus by Taerum et al. (2017) revealed that the European populations had the highest genetic diversity. These authors, consequently, hypothesized that *L. procerum* most likely had a European origin and probably arrived independently of the beetle in China, where it then adopted the beetle as a vector.

The second largest number of isolates (99 in total) included in this study represented four species in the *G. clavigera*-species complex (Taerum et al. 2013). The first of these species, *Grosmannia aurea*, was represented by 13 isolates from the western North America where it is known to be associated with the mountain pine beetle, *Dendroctonus ponderosae* (Jacobs & Wingfield 2001, Roe et al. 2011). Five isolates of an undescribed species (*Grosmannia* sp. 1) in the species complex, originating from the eastern North America and included in the study of Taerum et al. (2013), had unfortunately been lost and could not be further characterized. The third species residing in this complex that was not collected during the three RTB surveys is *L. wingfieldii* (Lu et al. 2009a, b, Taerum et al. 2013). This fungus of European origin was found by Jacobs et al. (2004) in the galleries of *D. valens* on two *Pinus* spp. in Vermont, where the beetle co-occurs with the invasive *Tomicus piniperda*.

The largest number of isolates (81) from the North American survey (Taerum et al. 2013) were those of the fourth member of the *G. clavigera*-species complex, *L. terebrantis*, with 58 isolates from California and 23 from Maine, Massachusetts and New Hampshire. *Leptographium terebrantis* was first described from *D. terebrantis* infesting *Pinus taeda* in Louisiana and Mississippi (Barras & Perry 1971). This fungus is also commonly associated with various pine-infesting weevils (*Curculionidae*: *Curculioninae*) in the north, central and eastern USA where it commonly occurs together with *L. procerum* (Wingfield 1983, 1986). The latter fungus has been implicated in causing a disease known as white pine root decline (Lackner & Alexander 1982, Jacobs & Wingfield 2001), but this view has been contested in studies showing that it has low levels of pathogenicity in comparison to *L. terebrantis* (Wingfield 1986, Jacobs & Wingfield 2001).

Given the reasonably common occurrence of *L. terebrantis* in California, it seems fortunate that the fungus has not yet been found in China. In this regard, it is remarkable that none of the species in the *G. clavigera*-species complex have been found in China. Of these fungi, only *L. wingfieldii* has been reported from *Tomicus piniperda* in Japan (Masuya et al. 1998, 2009).

The third highest number of isolates in this study (77) belonging to a single species complex resided in the *O. ips*-species complex. By far the majority of these (69 isolates) were those of *O. gilletteae*. This was also the only species present in all nine states of the USA that were surveyed. *Ophiostoma gilletteae* was, however, absent from China, where seven isolates of *O. ips* were found (Lu et al. 2009a). It was also surprising that only one isolate of *O. ips* was collected by Taerum et al. (2013) in the USA. Similar to *O. ips*, *O. gilletteae* might be of North American origin considering its abundance and omnipresence in pine habitats in that country (Zhou et al. 2007). All eight described species in the *O. ips*-species complex have a strong association with pine-infesting bark beetles (Six et al. 2011, De Beer & Wingfield 2013, Chang et al. 2019). However, Chang et al. (2017) suggested that *O. ips*, and perhaps some of the other species, might have a stronger association with mites than with beetles.

The *G. galeiformis*-species complex included 38 isolates representing four species, namely, *L. doddsii*, *L. gordonii*, *L. owenii*, *L. seifertii*. Prior to this study, the complex included only two described species, *G. galeiformis* from northern Europe (Bakshi 1951, Linnakoski et al. 2010), and *G. radiaticola* (= *Hyalopesotum pini*) from Chile, Finland, New Zealand, Poland, Russia, South Africa, Korea (Hutchison & Reid 1988, Zhou et al. 2004, Kim et al. 2005, Linnakoski et al. 2010, Jankowiak & Bilański 2013), and a few undescribed cryptic species from Mexico, Austria and the USA (Linnakoski et al. 2012, De Beer & Wingfield 2013). *Leptographium gordonii* was found exclusively in the eastern state of New Hampshire, while *L. owenii* was found in the western state of California. *Leptographium doddsii* and *L. seifertii* were found in both California and some of the eastern States (New Hampshire in the case of both species and Maine for *L. doddsii*). Species in this complex are typically associated with conifer-infesting bark beetles and are responsible for the staining of freshly cut timber (Kim et al. 2011, Linnakoski et al. 2012).

The remaining species collected from RTB during the surveys that formed the basis of the present study, resided in a diverse array of species complexes and lineages in the *Ophiostomatales*. One of these that was not encountered in the recent surveys but is worth mentioning is *Leptographium wageneri* var. *ponderosae*. This fungus is one of three varieties of *L. wageneri*, which are important conifer pathogens, and the causal agents of a serious disease known as black stain root disease (Harrington & Cobb 1983, Cobb 1988). *Leptographium wageneri* var. *ponderosae* has been associated with the galleries of *D. valens* (see also notes with Table 1), and a sexual morph of the fungus was suggested to occur in this niche (Goheen & Cobb 1978). There is, however, no evidence that the RTB is a vector of *L. wageneri* var. *ponderosae* and the results of the present study support this view.

The RTB does not have specialized internal structures in which fungal symbionts are carried. The associates of this insect are external symbionts and the nature of their relationship with the beetles must vary depending on various external factors. Some of these fungi are likely to have closer relationships with the beetles than others. It is also important to recognise that phoretic mites carried by bark beetles such as the RTB, are known to play an important role in vectoring fungi associated with these beetles (Chang et al. 2017). Thus, some of the fungi found in this study are most likely more closely associated with mites than with the beetles themselves. These interactions are clearly complex and they deserve more intensive study as has recently also been highlighted by Biedermann et al. (2019).

The high species diversities of Ophiostomatalean fungi associated with the RTB, as well as the demonstrated ability of this insect to be invasive, presents a biosecurity risk. Several Ophiostomatalean fungi have been accidentally introduced into new areas of the world (Wingfield et al. 2017). Some of these species have emerged as damaging in their invasive ranges

where they have encountered naïve hosts. In addition, the formation of new vector-symbiont associations presents an added threat, as novel combinations of insects and associated symbionts can be highly destructive (Wingfield et al. 2016). The association between RTB and *L. procerum* presents an interesting example of this threat, as there is some evidence that RTB may be destructive in China in part because of its association there with *L. procerum* (Lu et al. 2010, 2011). It is possible that other associates of RTB, including fungi that have been described in this study, may emerge as important pathogens if they are accidentally introduced into new environments or encounter naïve hosts.

This study has considerably improved the existing knowledge of RTB and its associated fungi. Yet our understanding of the Ophiostomatalean associates of RTB remains limited compared with other bark beetles such as the aggressive *Ips typographus* (Reid et al. 1967, Owen et al. 1987, Raffa 1988). This is in part due to the fact that the RTB is a relatively minor pest in its native environment. However, the unexpected outbreak of this insect in China has highlighted the fact that minor pests in one part of the world can cause substantial damage elsewhere. The threat is emphasised by globalization and global warming that expands the boundaries of the native habitats of pests (Burgess & Wingfield 2016, Hurley et al. 2016). The present study provides a challenge to extend our surveys of fungi and other organisms that live in association with the RTB. This should be both in its native and invasive ranges, thus providing a deeper understanding of its ecology and relative importance.

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