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

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**Key words**

invasion biology  
phylogenetics  
*Scolytinae*  
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.

**Article info**

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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 et al. 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 monoterpens (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: *Sordaria-mycetidae*) (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
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 |
|---------------------------------|---------------------------------------|-------|-------------|
| Ceratocystis solani             | ✓                                     | –     | 1Marmolejo & Butin (1990) |
| Graphilbum sp.                  | ✓                                     | Lu et al. (2009a) as Ophiostoma rectangulosporum-like | – |
| ²Graphium sp.                   | x                                     | –     | 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                    | x                                     | –     | Six et al. (2003) |
| ³G. europhioides                | x                                     | –     | 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) |
| ³G. piceaperda                   | x                                     | –     | Rumbold (1931) cited by Perry (1991) as Ophiostoma piceaperdum |
| G. radiatricula                 | ✓                                     | Lu et al. (2009b) as Pesotum pini | – |
| Leptographium sp.               | x                                     | –     | 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. pinicola                     | ✓                                     | Lu et al. (2009a) as Hyalorhiocladia pinicola | – |
| ⁴L. 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) | – |
| ⁴L. terebrantis                 | ✓                                     | –     | Harrington & Cob (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, Schwiegkofler et al. (2005) |
| ³L. wageneri var. wageneri      | x                                     | –     | Jacobs & Wingfield (2001) |
| L. wingfieldii                  | ✓                                     | –     | Jacobs et al. (2004) |
| Ophiostoma floccosum            | ✓                                     | Lu et al. (2009a) | Taerum et al. (2013) |
| O. gilletteae 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 |
| ⁴O. minus (Europe)              | ✓                                     | Lu et al. (2009a) | – |
| ⁴O. 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 |
| Sporothrix abietina             | ✓                                     | Lu et al. (2009a) as O. abietinum | Taerum et al. (2013) as O. abietinum |

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

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) 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 as Verticiladiella 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. ponderosae 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. procercum, L. terebrantis and L. wageneri var. ponderosa) are known from various host trees and beetles in North America (Cobb 1988, Jacobs & Wingfield 2001). One species, Cerato-cystisopsis 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. wingfieldi is of European origin and was introduced into North America with its beetle vector, Tomiscus piniperda (Jacobs et al. 2004). After the beetle established and spread throughout the north-eastern USA and the south-eastern Canada, L. wingfieldi 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 Ophiostoma- talean 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. sinoprocercum (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. procercum, 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 Ophiostoma-talean 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. procercum. 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. procercum was by far the most commonly encountered species in eastern North America, representing 45.6 % of all the isolates obtained. However, L. procercum 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 morphologi- cal 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 For- estry 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), Roode- plaat, 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 steri- lized 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 Ni and SMZ18, mounted with a Nikon camera (DS-R2) 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 characteriza- tion. 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 Grossmannia 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. olivaceus-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
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 B2a together with B12o (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 1000 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, bumin 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 complexes.
complexes. Additionally, isolates representing a putatively new species grouped closely with *O. floccosum*, 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 Tauer et al. (2013), were included in the present study (Table 2). Datasets representing five different gene regions (ITS2-LSU, Act, βt, EF1-α and Anor) 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-epitope, 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 β and EF1-α datasets representing all known species and two isolates 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 β and EF1-α datasets representing all known species and two isolates 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).

**Fig. 4** Phylogeny resulting from the ML analysis of β 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).

**Fig. 5** Phylogenies resulting from the ML analysis of β 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).

**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 Ophiostomatidae. 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...
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 (Masoumi 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

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**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 (6.0 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. The conidia of ‘Leptographium sp. 2’ were 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.

**Grosmanina galeiformis**-species complex

Prior to this study, the G. galeiformis-species complex included three described spp., G. galeiformis (Zhou et al. 2004), G. radiatica (= *Pestonum pinii*) (Kim et al. 2005) and L. koraensis (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. Grosmanina galeiformis and G. radiatica 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.

**Type.** 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 doddsii 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 macroспорous, 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. Conidiospores pale brown, branched in 4–5 tiers, conidial droplets yellowish. Conidigenous 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 *Grosmanina* 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.

**Type.** USA, California, Sierra Nevada Mountains, *Dendroctonus valens*, Aug. 2009, M.J. Wingfield (holotype PREM 63068, culture ex-holotype CBS 143467 = CMW 34448).

**Diagnosis.** — This species has 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 ± 0.24) μm.

Asexual structures produced on pine twigs on surface of water agar. Sexual morph not observed. Conidiophores macroспорous, mononematous, synnematous. Synnematous conidiophores predominately oblong. Conidiogenous apparatus cylindrical to clavate, tapering towards apex, slightly constricted at base, hyaline, smooth, 8–12 × 1.5–2.5 (6.6 ± 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 submersed, 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.
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.
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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 macro-

nematous, 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, 218–1152 (627 ± 291.9) µm long, 7–13 (10.3 ± 1.64) 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 taper-

ing 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. Co-

nidia blastic, ellipsoidish, 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 (synne-

matous).

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).

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 63064, culture CBS 143468 = CMW 34451.

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

Sexual and asexual structures produced on pine twigs on the surface of water agar. Ascomata scarce; ascosmatal 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 macroconidial, 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. Monoconidial conidiophores hyalinorhinoocladiella-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 34445; 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 gigilleteae 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. gigilleteae, 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 1979).

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 (Hedcock 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.

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. CHN, 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 MUCJ 468456 = CMW 48329).
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) sporo-thrix-like; stipes upright, flexuous or straight, hyaline to sub-hyaline, 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).
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 proceraum 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. sinopercernum (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,
Because of its common occurrence in China, *L. procera* is considered by some researchers as a significant contributing factor to the death of *Pinus tabuliformis* (Lu et al. 2010, 2011). It has also been assumed that the insect, *L. procerum* is because of its common occurrence in China, (Taerum et al. 2013). 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 a beetle (*Taerum et al. 2013*). However, *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. oweni*, and *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. radiicolor* (= *Hylophloeospermum pinin*) 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 & Bilarski 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. oweni* 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 *Ophiostomatidae*. 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 some of these fungi are likely to have closer relationships with the beetles than others. It is also important to recognise that some of these fungi are likely 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 *Ophiostomatidae* fungi associated with the RTB, as well as the demonstrated ability of this insect to be invasive, presents a biosecurity risk. Several *Ophiostomatidae* 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 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 demonstrates 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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