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# Multigene phylogenies and morphological characterization of five new *Ophiostoma* spp. associated with spruce-infesting bark beetles in China

Mingliang Yin[a, b], Michael J. Wingfield[a], Xudong Zhou[a, b], Z. Wilhelm de Beer[a, *]

[a]Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, Gauteng Province, South Africa.

[b]China Eucalypt Research Centre (CERC), Chinese Academy of Forestry (CAF), Zhanjiang 524022, Guangdong Province, China.

*Corresponding author: Wilhelm de Beer, E-mail: wilhelm.debeer@fabi.up.ac.za

Tel: +27-124203938; Fax: +27-124203960

Xudong Zhou, present address: FuturaGene Biotechnology (Shanghai) Co., Ltd., 200233, Shanghai, China.

**ABSTRACT**

*Ophiostoma* spp. (Ophiostomatales, Ascomycota) are well-known fungi associated with bark beetles (Coleoptera: Scolytinae). Some of these are considered to be serious tree pathogens, while the majority is blue-stain agents of timber. In recent years, various bark beetle species have been reported as attacking spruce forests in Qinghai province, China, causing significant damage. Due to the limited number of studies about *Ophiostoma* spp. associated with bark beetles in China, a preliminary survey was done to explore the diversity of these fungi on spruce. The aims of the present study were firstly to identify and characterize new *Ophiostoma* spp. isolated from spruce-infesting bark beetles and their galleries in Qinghai Province, and secondly to resolve the phylogenetic relationships of some *Ophiostoma* spp. related to the Chinese isolates by using multigene phylogenetic analyses. Results obtained from four gene regions (ribosomal internal transcribed spacer regions, β-tubulin, calmodulin, translation elongation factor-1α) revealed five new *Ophiostoma* spp. from Qinghai. These included *O. nitidus* sp. nov., *O. micans* sp. nov., and *O. qinghaiense* sp. nov. in a newly defined *O. piceae* complex. The other two new species, *O. poligraphi* sp. nov. and *O. shangrilae* sp. nov., grouped in the *O. brunneo-ciliatum* complex. Based on DNA sequence and morphological comparisons, we also show that *O. arduennense* and *O. torulosum* are synonyms of *O. distortum*, while *O. setosum* is a synonym of *O. cupulatum*.

Key words: phylogeny, taxonomy, *Ophiostoma piceae* complex, *Ophiostoma brunneo-ciliatum* complex

**Introduction**

The genus *Ophiostoma* was first established by Sydow & Sydow (1919) with *O. piliferum* (previously *Sphaeria pilifera*) as type species, together with 11 other species causing bluestain on wood that were earlier described in the genera *Ceratostomella* and *Endoconidiophora* (Hedgcock 1906, Münch 1907). *Ophiostoma* spp. are generally characterized by short to long necked ascomata, crescent to allantoid shaped ascospores, and pesotum-, hyalorhinocladiella-, or sporothrix-like asexual morphs (De Beer & Wingfield 2013). Apart from the sporothrix-like asexual states, all the other spore-bearing structures are specifically adapted to produce spores in slimy droplets for dispersal by athropods. Bark beetles (Coleoptera: Scolytinae) are the most common vectors of *Ophiostoma* spp. (Six 2003, Kirisits 2004, Harrington 2005), although other insects like bark weevils (Viiri 2004, Jankowiak and Bilański 2013a, b), longhorn beetles (Jankowiak & Kolařík 2010), nitidulid beetles (Juzwik et al. 1998, Kamgan Nkuekam et al. 2012) and mites (Hofstetter et al. 2015, Roets et al. 2013) may also act as vectors. Most *Ophiostoma* spp. are considered to be agents of blue-stain on wood, rather than serious tree pathogens (Wingfield et al. 1993), except for *O. ulmi* (Schwarz 1928), *O. novo-ulmi* (Brasier 1991) and *O. himal-ulmi* (Brasier & Mehrotra 1995) that cause Dutch elm disease.

Under dual nomenclature system, *Ophiostoma* was considered a sexual genus. The name has been treated for long periods as synonym of other sexual genera such as *Ceratostomella* (Davidson 1942) and, more importantly, *Ceratocystis* (Bakshi 1951, Moreau 1952, Hunt 1956, Wright & Cain 1961, Griffin 1968, Olchowecki & Reid 1974, Upadhyay & Kendrick 1975, Upadhyay 1981). Although *Ophiostoma* spp. could be distinguished from *Ceratocystis* based on the differences in asexual state morphology, the composition of cell walls, and cycloheximide tolerance (Weijman & De Hoog 1975, Harrington 1981, De Hoog & Scheffer 1984), its taxonomic status remained unstable and confused until the first DNA sequence-based studies showed that *Ophiostoma* is a distinct genus in the Ophiostomatales, while *Ceratocystis* resided in the Microascales (Hausner et al. 1993, Spatafora & Blackwell 1994, De Beer et al. 2013). Zipfel et al. (2006) was the first to explore the subdivision of *Ophiostoma* after its divorce from *Ceratocystis*, but their study focused only on sexual species. They showed that *Grosmannia* and *Ceratocystiopsis,* previously considered synonyms of *Ophiostoma* (Wingfield 1993, Jacobs & Wingfield 2001), were distinct genera in the Ophiostomatales based on morphological differences supported by ribosomal large subunit (LSU) and β-tubulin sequence comparisons.

After the practice of dual nomenclature was abandoned in 2011 based on the one fungus one name principles (Hawksworth 2011, McNeill et al. 2012), the oldest genus name represented by its type species in any phylogenetically defined genus, has priority over all younger genus names, irrespective of the morph. Considering these changes, De Beer & Wingfield (2013) were the first to reassess the generic lineages within the Ophiostomatales including all available sequence data for both sexual and asexual species. Their analyses of the ribosomal LSU and internal transcribed spacer (ITS) gene regions showed four well-supported lineages representing *Raffaelea s. str., Ceratocystiopsis* (with *Hyalorhinocladiella* as synonym), *Fragosphaeria*, and *Graphilbum*. However, although species belonging to *Ophiostoma* and *Sporothrix* more or less grouped together, as did those of *Leptographium* and *Grosmannia*, these two major groups did not form well-supported monophyletic lineages. De Beer & Wingfield (2013) suggested that these two groups respectively should be treated as *Leptographium sensu lato* and *Ophiostoma sensu lato*. The authors showed that *Ophiostoma sensu lato* included, apart from a well-supported lineage defined as *Ophiostoma sensu stricto*, the *Sporothrix*

*schenckii-Ophiostoma stenoceras* complex with some other minor complexes and lineages. They suggested that the generic placement of these complexes and lineages be confirmed in future studies using more gene regions, and that until such time the current species names should be maintained to ensure nomenclatural stability.

According to the classification of De Beer & Wingfield (2013), *Ophiostoma s. str.* included 66 species, some of which grouped in the phylogenetically well-supported *O. ulmi*-, *O. pluriannulatum*-, and *O. ips* complexes. Although the remainder of the species in *Ophiostoma s. str.*, including the type species, *O. piliferum*, did not form part of well-defined species complexes, their inclusion within the genus as currently defined was confirmed. Among these species were *O. ulmi, O. introcitrinum* and *O. canum*, respectively the type species for three previously considered 'anamorph' genera, namely *Pesotum* (Crane & Schocknecht 1973), *Hyalopesotum* and *Pachnodium* (Upadhyay & Kendrick 1975). De Beer et al. (2013) thus listed the latter three genera as synonyms of *Ophiostoma s. str.* and provided new combinations in *Ophiostoma* for seven species, including four that are known only from their asexual states, namely *O. australiae, O. cupulatum*, and two ambrosial species, *O. tingens* and *O. macrosporum*. Prior to their study and in anticipation of the incorporation of the one fungus one name principles into the Code, Linnakoski et al. (2010) described two species, *O. fuscum* and *O. tapionis,* known only by their asexual states, in *Ophiostoma s. str.*

Over the years, by far the majority of taxonomic studies on the fungal associates of bark beetles belonging to *Ophiostoma s. str.* and other genera in the Ophiostomatales have been conducted in the Northern Hemisphere, especially Europe (Kirisits 2004, Jankowiak 2005, Linnakoski et al. 2008), North America (Hedgcock 1906, Davidson 1935, Rumbold 1936, Hunt 1956, Griffin 1968, Olchowecki & Reid 1974, Roe et al. 2010, Six et al. 2011), and Japan (Masuya et al. 2013). More recently, these fungi have gained research attention in Africa (Zhou et al. 2006, Kamgan Nkuekam et al. 2008, Roets et al. 2008, Grobbelaar et al. 2010) and China (Zhou et al. 2013), but in comparison to Europe, North America and Japan, much more work is needed in Africa, South America, Australasia, China, and the rest of Asia (Russia, India, South East Asia) to achieve a global perspective and understanding of these fungi.

In China, *Picea crassifolia* (Qinghai spruce) and *P. purpurea* (Purple cone spruce) are native species distributed in Qinghai, Gansu and Ningxia provinces in the northeastern escarpment of the Qinghai-Tibetan Plateau (Liu 2008, Sun et al. 2014). In recent years, bark beetles were reported attacking *P. crassifolia* and *P. purpurea* and causing mortality of trees in the Maixiu National Forest Park (infected area 1047 $ha^2$) and Xianmi National Forest Park (infected area 273 $ha^2$) in Qinghai province (Liu 2008). A survey of bark beetles and their associated fungi on spruce in these areas was conducted in 2010. Amongst others, five species belonging to *Ophiostoma s. str.* were isolated from four bark beetle species, *Dendroctonus micans*, *Ips nitidus*, *I. shangrilae*, and *Polygraphus poligraphus,* and their galleries. The aims of the present study were to accurately identify these *Ophiostoma* species, and secondly to resolve the phylogenetic relationships between the Chinese and closely related known species within the relevant species complexes.

**Materials and methods**

Isolates

All isolates included in the present study are listed in Table 1. Reference isolates were obtained from

the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Ex-type isolates of newly described species were deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands. Type specimens of new species were deposited in the National Collection of Fungi (PREM), Pretoria, South Africa. Taxonomic novelties were registered in MycoBank.

DNA extraction, PCR and sequencing

DNA extractions followed the protocol described by Yin et al. (2015). Four gene regions, including the ribosomal internal transcribed spacer (ITS) regions, and the beta-tubulin (βT), calmodulin (CAL) and translation elongation factor-1 alpha (TEF-1α) genes, were amplified for phylogenetic analyses. Primers used in this study were: ITS1-F (Gardes & Bruns 1993) and ITS4 (White et al. 1990) for ITS, T10 (O'Donnell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995) for βT, CL3F (5'-CCGARTWCAAGGAGGCSTTC-3') and CL3R (5'-TTCTGCATCATRAGYTGSAC-3') for CAL (designed in the present study), and EF2-F (Marincowitz et al. 2015) and EF2-R (Jacobs et al. 2004) for TEF-1α.

PCR reactions were conducted in 25 µL reaction mixtures containing 5 µL of Mytaq buffer (including $MgCl_2$, dNTPs and reaction buffer), 0.5 µL of Mytaq polymerase (Bioline, USA), 0.5 µL of each primer (10 µM), and 16.5 µL of PCR grade water. PCR conditions for these five gene regions were similar to those described by Yin et al. (2015). PCR products were purified with Sephadex G-50 columns (6%).

Sequencing PCRs were conducted with the same primer combinations used for PCR, together with the Big Dye Terminator 3.1 cycle sequencing premix kit (Applied Biosystems, Foster City, California, USA). A second purification step using the above-mentioned method was required after sequencing PCRs. Sequence analyses were done on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Consensus sequences were generated from forward and reverse sequences in the CLC Main Workbench 6.0 (CLC Bio, Aarhus, Denmark).

Phylogenetic analyses

Four single gene region datasets and one combined dataset were used for phylogenetic analyses. The ITS dataset included all sequences for reference species in *Ophiostoma s. str*. that were available from Genbank (Fig. 1) to show the placement of the Chinese isolates within *Ophiostoma s. str*. The outgroup taxa in the ITS dataset were *O. abietinum* and *O. stenoceras* that group peripheral to and do not form part of *Ophiostoma s. str.* (De Beer & Wingfield 2013). The three protein coding gene regions (βT, CAL and TEF-1α) were sequenced for 39 isolates in order to delineate closely related species (Table 1).

Alignments of related gene regions were conducted online in MAFFT 7.0 (Katoh et al. 2013). The data were then checked manually in MEGA 5.2 (Tamura et al. 2011) and compared with the *Grosmannia clavigera* gene maps (Yin et al. 2015) to ensure that introns and exons were aligned appropriately. Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI) were used for phylogenetic analyses.

MP analyses were conducted in PAUP* 4.0b10 (Swofford 2003). Gaps were treated as fifth state characters. One thousand bootstrap replicates were done to determine the branch node confidence.

Tree bisection and reconnection (TBR) was selected as the branch swapping option. The tree length (TL), Consistency Index (CI), Retention Index (RI), Homoplasy Index (HI) and Rescaled Consistency Index (RC) were recorded for each data set after generating the trees.

The best substitution models for the two likelihood methods (ML and BI analyses) were selected in jModelTest 2.1.1 (Posada 2008). MEGA 6 (Tamura et al. 2013) was used for ML analyses with the Nearest-Neighbor-Interchange (NNI) branch swapping option. Confidence intervals for nodes were determined using 1000 bootstrap replicates.

BI analyses were conducted in MrBayes 3.2 (Ronquist et al. 2012) by applying the Markov Chain Monte Carlo (MCMC) method. Four MCMC chains simultaneously and randomly started running for five million generations with the best substitution models determined in jModelTest 2.1.1. Trees were sampled every 100 generations. Burn-in values were determined in Tracer 1.4 (Rambaut & Drummond 2007). Trees sampled in the burn-in phase were discarded, and the remaining trees were used to construct majority rule consensus trees.

Morphology, growth studies and mating tests

Morphological characterization of new species were examined based on the structures of the ex-type isolates inoculated onto 2% water agar (WA, 20 g Difco agar and 1000 ml deionized water) with sterilized pine twigs. Culture characteristics were recorded on Oatmeal agar (OA, 30 g oatmeal, 20 g Difco Bacto$^{TM}$ malt extract [Becton, Dickinson & Company], and 1000ml deionized water), incubated at 25 ℃ for 14-21 d. Colour descriptions were done ba sed on the charts of Rayner (1970), and growth studies were done on 2 % Malt extract agar (MEA). All isolates of the new taxa were crossed against each other as described by Grobbelaar *et al*. (2010) in an attempt to induce the production of sexual structures.

**Results**

Phylogenetic analyses

Alignments of the ITS, βT, CAL, TEF-1α, and the combined datasets consisted respectively of 730, 446, 1040, 1089 and 3181 characters, including gaps. The exon/intron arrangement of the βT data included exons 3, 4, 5, and 6, interspersed with introns 3 and 4, but lacking intron 5. The aligned TEF-1α gene region consisted of exons 2, 3, 4, 5, 6, and 7, interspersed with introns 2, 3, 4, and 6, while lacking intron 5. The alignment of CAL dataset contained exons 3, 4, and 5, interspersed with introns 3 and 4 but intron 4 was lacking. The best evolutionary substitution models for all five datasets were GTR+G+I. The burn-in values in BI analyses for all data matrices were 300.

The three ITS trees (Fig. 1) were similar and showed the placement of the Chinese isolates within two groups (Groups A and B) in *Ophiostoma s. str.,* alongside some closely related known species. Group A included *O. piceae*, five other known species and three of the Chinese taxa. Almost all species in this group had identical ITS sequences. Group B had significant statistical support and included *O. brunneo-ciliatum*, *O. ainoae*, *O. tapionis* and Chinese taxa 4 and 5. Taxon 5 and *O. ainoae* had identical sequences. In Group C, the ITS gene region could not separate *O. distortum*, *O. torulosum* and *O. arduennense*. This was also true for *O. cupulatum* and *O. setosum*.

The BI, MP, ML analyses of the four protein-coding datasets resulted in similar tree topologies (Fig. 2).

Group A had good statistical support and all species in this group, including Taxa 1 to 3 from China, formed well-supported lineages in all gene regions. The only exception was *O. flexuosum* that had an identical CAL sequence with *O. piceae*, but the other two genes clearly separated the two species. In Group B all species were clearly separated in all data sets. Group C contained the ex-type and some additional isolates of three known species, *O. distortum, O. torulosum* and *O. arduennense*. In BT and TEF-1α, these species all had identical sequences, while *O. torulosum* had 1 bp difference from the other two species in CAL.

Morphology, growth studies and mating tests

Isolates of the five new taxa emerging from this study were similar in growth in culture, with colours initially hyaline, turning gray or olivaceous with age. Synnemata formed singly, and were abundant in culture. Hyphae were superficial on the agar. The conidia produced in droplets were initially hyaline, and later became yellowish. Morphological differences among these new taxa are listed in Table 2, and discussed in the *Notes* under the new species descriptions in the Taxonomy section. The optimal temperature for all new species was 25 ℃. None of the isolates grew at 30 ℃, except Taxon 5 (1.37 mm/d) and Taxon 4 (0.25 mm/d). None of the isolates grew below 5 ℃. No sexual state was observed for any of the new taxa, neither in single spore cultures used for DNA extractions, nor in crosses done between different isolates.

**Taxonomy**

Multilocus phylogenetic analyses of 43 isolates included in the present study revealed 22 distinct taxa in *Ophiostoma s. str.* Seventeen of these taxa represented known species. Our results showed that among these, *O. arduennense* and *O. torulosum* are synonyms of *O. distortum*, while *O. setosum* is a synonym of *O. cupulatum*. The remaining five taxa associated with bark beetles from conifers in China (Taxa 1-5) were distinct from any known taxa and are described here as new species. According to the recommendations of De Beer & Wingfield (2013), we name all new species described here in *Ophiostoma s. str.,* even though sexual states have not been observed for any of these species.

*Taxon 1*

***Ophiostoma nitidum*** M.L. Yin, Z.W. de Beer & M.J. Wingf., sp. nov.

Mycobank No.: MB 814781 (Fig. 3)

*Etymology* — The epithet reflects the species name of the bark beetle vector of this fungus, *Ips nitidus*.

*Sexual state* not observed. *Asexual state* pesotum-like. *Conidiophores* macronematous, synnematous, abundant in culture, *synnemata* occurring singly or in groups, expanding towards both the apex and the base, dark brown at base, becoming paler toward apex, (788-) 870-1050 (-1082) µm long including conidiogenous apparatus, (52-) 62-88 (-108) µm wide at base. *Conidiogenous cells* (26-) 28-31 (-33) × (0.9-) 1-1.1 (-1.3) µm. *Conidia* hyaline, 1-celled, smooth, clavate or obovoid, (3.3-) 3.6-4 (-4.3) × (1.4-) 1.5-1.7 (-1.8) µm. Synnematal asexual morphs usually common on different agar media with pine twigs.

Attached to substrate by brown rhizoid-like hyphae. *Culture characteristics*: Colonies on OA, hyaline at first, later becoming dark yellowish to light brown at centre. Hyphae submerged in agar with little aerial mycelium (2.3-) 2.6-4.0 (-4.2) µm in width. Colony margin smooth. Conidiophores form abundantly in clusters on OA. Colonies on 2% MEA flat, with optimal growth at 25 ℃, with radial growth rate 2.5 (± 0.5) mm/d, growth reduced below 10 ℃, no growth at 30 ℃ or above.

*Specimens examined*: **CHINA**, Qinghai, Maixiu National Forest Park, from *Picea crassifolia* infested by *Ips nitidus*, Aug. 2010, *X.D. Zhou* & *S. Taerum*, **holotype** PREM 60932, culture ex-holotype CBS 136525 = CMW 38907; from *P. crassifolia* infested by *I. nitidus*, Aug. 2010, *X.D. Zhou* & *S. Taerum*, **paratype** PREM 60933, culture ex-paratype CBS 136526 = CMW 38905.

Host trees: *Picea crassifolia*

Insect vector: *Ips nitidus*

Distribution: Qinghai Province, China

*Notes*: This species is most closely related to *O. rachisporum* (Linnakoski et al. 2010), *O. micans* (Taxon 2, this study) and *O. qinghaiense* (Taxon 3, this study). However, *O. rachisporum* can be distinguished from all these new species by its oblong conidia and much shorter synnemata. In comparison to the other species, Taxon 1 produces the most abundant number of synnemata on OA and grow slower on MEA.

*Taxon 2*

***Ophiostoma micans*** M.L. Yin, Z.W. de Beer & M.J. Wingf., sp. nov.

Mycobank No.: MB 814782 (Fig. 4)

*Etymology* — The epithet reflects the species name of the bark beetle vector of this fungus, *Dendroctonus micans*.

*Sexual state* not observed. Asexual state pesotum-like. *Conidiophores* macronematous, synnematous, abundant in culture, *synnemata* occurring singly or in groups, expanding towards both the apex and the base, dark brown at base, becoming paler toward apex, (583-) 620-745 (-761) µm long including conidiogenous apparatus, (27-) 39-68 (-85) µm wide at base. *Conidiogenous cells* (28-) 33-38 (-44) × (1.1-) 1.2-1.5 (-1.7) µm. *Conidia* hyaline, 1-celled, smooth, oblong, clavate or obovoid, (3.2-) 3.5-4.2 (-4.4) × (1.4-) 1.5-1.6 (-1.7) µm. Synnematal asexual morphs usually common on different agar media with pine twigs. Attached to substrate by brown rhizoid-like hyphae. *Culture characteristics*: Colonies on OA, hyaline at first, later becoming light brown at centre. Hyphae submerged in agar with little aerial mycelium, (1.6-) 1.9-3.9 (-4.4) µm in width. Colony margin smooth. Conidiophores rarely occurring on OA, but abundantly superficial on twigs on WA. Colonies on 2% MEA, with optimal growth at 25 ℃, with radial growth rate 2.8 (± 0.5) mm/d, growth reduced below 10 ℃, no growth at 30 ℃ or above.

*Specimens examined*: **CHINA**, Qinghai, Maixiu National Forest Park, from *Picea crassifolia* infested by *Dendroctonus micans*, Aug. 2010, *X.D. Zhou* & *S. Taerum*, **holotype** PREM 60930, culture ex-holotype CBS 136523 = CMW 38903; from *P. crassifolia* infested by *D. micans*, Aug. 2010, *X.D. Zhou* & *S. Taerum*, **paratype** PREM 60931, culture ex-paratype CBS 136524 = CMW 38909.

Host trees: *Picea crassifolia*

Insect vector: *Dendroctonus micans*

Distribution: Qinghai Province, China

*Notes*: *Ophiostoma micans* is most closely related to *O. rachisporum* (Linnakoski et al. 2010), *O. nitidum* (Taxon 1, this study) and *O. qinghaiense* (Taxon 3, this study). This species has shorter and wider synnemata and the colony colour is lighter than those of the latter two species. Its conidia are wider than those of *O. rachisporum* (Linnakoski et al. 2010).

*Taxon 3*

***Ophiostoma qinghaiense*** M.L. Yin, Z.W. de Beer & M.J. Wingf., sp. nov.

Mycobank No.: MB 814783 (Fig. 5)

*Etymology* — The epithet reflects Qinghai Province in China where it was first collected.

*Sexual state* not observed. Asexual state pesotum-like. *Conidiophores* macronematous, synnematous, abundant in culture, *synnemata* occurring singly or in groups, expanding towards both the apex and the base, dark brown at base, becoming paler toward apex, (1060-) 1110-1081 (-1287) µm long including conidiogenous apparatus, (91-) 104-129 (-153) µm wide at base. *Conidiogenous cells* (17-) 23-29 (-37) × (1.5-) 1.9-2.4 (-2.8) µm. *Conidia* hyaline, 1-celled, smooth, clavate or obovoid, (3.3-) 3.7-4.2 (-4.5) × (1.2-) 1.4-1.7 (-1.9) µm. Synnematal asexual morphs usually common on different agar media with pine twigs. Attached to substrate by brown rhizoid-like hyphae. *Culture characteristics*: Colonies on OA, hyaline at first, later becoming light brown to dark brown at centre. Hyphae submerged in agar with some aerial mycelium, (2.5-) 2.6-3.9 (-4.0) µm in width. Colony margin smooth. Conidiophores form abundantly on OA and on twigs on WA. Colonies on 2% MEA, with optimal growth at 20 ℃, with radial growth rate 3 (± 0.5) mm/d, g rowth reduced below 10 ℃, no growth at 30 ℃ or above.

*Specimens examined*: **CHINA**, Qinghai, Maixiu National Forest Park, from *Picea crassifolia* infested by *Polygraphus poligraphus*, Aug. 2010, *X.D. Zhou & S. Taerum*, **holotype** PREM 60928, culture ex-holotype CBS 136521 = CMW 38902; from *P. crassifolia* infested by *Dendroctonus micans*, Aug. 2010, *X.D. Zhou & S. Taerum*, **paratype** PREM 60929, culture ex-paratype CBS 136522 = CMW 38904; from *P. crassifolia* infested by *P. poligraphus*, Aug. 2010, *X.D. Zhou & S. Taerum*, culture CMW 38906.

Host tree: *Picea crassifolia*

Insect vectors: *Polygraphus poligraphus, Dendroctonus micans*

Distribution: Qinghai Province, China

*Notes*: This species is most closely related to *O. rachisporum* (Linnakoski et al. 2010), *O. nitidum* (Taxon 1, this study) and *O. micans* (Taxon 2, this study). *Ophiostoma qinghaiense* has much longer synnemata and larger conidia than those of *O. rachisporum*, and its darker colony colour distinguishes it from *O. nitidum* and *O. micans*.

*Taxon 4*

***Ophiostoma shangrilae*** M.L. Yin, Z.W. de Beer & M.J. Wingf., sp. nov.

Mycobank No.: MB 814784 (Fig. 6)

*Etymology* — The epithet reflects the species name of the bark beetle vector of this fungus, *Ips shangrila*.

*Sexual state* not observed. Asexual state pesotum-like. *Conidiophores* macronematous, synnematous, abundant in culture, *synnemata* occurring singly or in groups, expanding towards both the apex and the base, dark brown at base, becoming paler toward apex, (723-) 763-870 (-925) µm long including conidiogenous apparatus, (58-) 70-83 (-89) µm wide at the base. *Conidiogenous cells* (21-) 23-28 (-32) × (1.5-) 1.7-2 (-2.3) µm. *Conidia* hyaline, 1-celled, smooth, clavate or obovoid, (3.3-) 3.5-4.4 (-5.1) × (1.2-) 1.5-1.8 (-2) µm. Synnematal asexual morphs usually common on different agar media with pine twigs. Attached to substrate by brown rhizoid-like hyphae. *Culture characteristics*: Colonies on OA, hyaline at first, later becoming dark olivaceous to brown at centre. Hyphae submerged in agar with some aerial mycelium, (2.1-) 2.3-4.5 (-4.9) µm in width. Colony margin smooth. Conidiophores not observed on OA but forms abundantly in clusters on twigs on WA. Colonies on 2% MEA with optimal growth at 25 ℃ and radial growth rate 3.3 (± 0.5) mm/d, growth reduced below 10 ℃ and at 30 ℃, no growth at 35 ℃ or above.

*Specimens examined*: **CHINA**, Qinghai, Xianmi forest park, from *Picea purpurea* infested by *Dendroctonus micans*, Aug 2010, *X.D. Zhou & S. Taerum*, **holotype** PREM 60926, culture ex-holotype CBS 136519 = CMW 38901; from *P. purpurea* infested by *Ips shangrila*, Aug 2010, *X.D. Zhou & S. Taerum*, **paratype** PREM 60927, culture ex-paratype CBS 136520 = CMW 38900.

*Host tree*: *Picea purpurea*

*Insect vectors*: *Ips shangrila, Dendroctonus micans*

*Distribution*: Qinghai Province, China

*Notes*: *Ophiostoma shangrilae* has longer synnemata and slightly smaller conidia when compared to related species such as *O. ainoae* (Solheim 1986) and *O. brunneo-ciliatum* (Mathiesen-Käärik 1953, Hunt 1956). Moreover, this species can be easy distinguished from *O. poligraphi* (Taxon 5, this study) by its unique colony morphology, more synnemata formed on twigs, longer synnemata and smaller conidia.

*Taxon 5*

***Ophiostoma poligraphi*** M.L. Yin, Z.W. de Beer & M.J. Wingf., sp. nov.

Mycobank No: MB 814785 (Fig. 7)

*Etymology* — The epithet reflects the species name of the bark beetle vector of this fungus, *Polygraphus poligraphus*.

*Sexual state* not observed. Asexual state pesotum-like. *Conidiophores* macronematous, synnematous, abundant in culture, *synnemata* occurring singly or in groups, expanding towards both the apex and the base, dark brown at base, becoming paler toward apex, (484-) 501-668 (-796) µm long including conidiogenous apparatus, (51-) 55-64 (-70) µm wide at the base. *Conidiogenous cells* (16-) 19-29 (-34) × (1.3-) 1.7-2.1 (-2.3) µm. *Conidia* hyaline, 1-celled, smooth, clavate or obovoid, (5.9-) 6.2-8 (-10) × (1.6-) 1.7-2 (-2.5) µm. Synnematal asexual morphs usually common on different agar media with birch twigs. Attached to the substrate by brown rhizoid-like hyphae. *Culture characteristics*: Colonies on OA, hyaline at first, later becoming dark olivaceous to brown. Snake-like hyphae submerged in agar with

little aerial mycelium, (3.3-) 3.5-4.0 (-4.4) µm in width. Colony margin smooth. Conidiophores not found on OA and rarely forms on twigs on WA. Colonies on 2% MEA flat, with optimal growth at 25 ℃, with radial growth rate 4.1 (± 0.5) mm/d, growth reduced below 10 ℃ and at 30 ℃, no growth at 35 ℃ or above.

*Specimens examined*: **CHINA**, Qinghai, Maixiu National Forest Park, from *Picea crassifolia* infested by *Polygraphus poligraphus*, Aug. 2010, *X.D. Zhou* & *S. Taerum*, **holotype** PREM 60925, culture ex-holotype CBS 136517 = CMW 38899; from *P. crassifolia* infested by *Dendroctonus micans*, Aug. 2010, *X.D. Zhou* & *S. Taerum*, **paratype** PREM 609324, culture ex-paratype CBS 136518 = CMW 38898.

*Host tree*: *Picea crassifolia*

*Insect vectors*: *Polygraphus poligraphus, Dendroctonus micans*

*Distribution*: Qinghai Province, China

*Notes*: *Ophiostoma poligraphi* is most closely related to *O. ainoae* (Solheim 1986) and *O. shangrilae* (Taxon 4, this study), and has shorter synnemata and larger conidia than those species. Furthermore, this species can be distinguished from *O. shangrilae* by its unique colony morphology, very few sporulating structures on twigs, shorter synnemata, and larger conidia. This species also differs from *O. shangrilae* in growth faster on MEA.

***Ophiostoma distortum*** (R.W. Davidson) de Hoog & Scheffer, Mycologia 76: 297. 1984.

Synonyms: *Ceratocystis distorta* R.W. Davidson, Mycologia 63: 10. 1971.

*Ceratocystis torulosa* Butin & G. Zimm., Phytopathol. Z. 74: 284. 1972.

*Ophiostoma torulosum* (Butin & G. Zimm.) Hausner, J. Reid & Klassen, Can. J. Bot. 71: 1264. 1993.

*Ophiostoma arduennense* F.X. Carlier, Decock, K. Jacobs & Maraite, Mycol. Res. 110: 805. 2006.

*Specimens examined*: Of *O. distortum*: **United States**, Alaska, from *Abies concolor* infested by ambrosia beetles, 23 Jul. 1964, Davidson R. W., **holotype** BPI 595730 (not seen), culture ex-holotype CBS 429.82 = DSMZ 4897 = RWD 575-C, from *Picea engelmannii* infested by ambrosia beetles.

Of *C. torulosa*: **Germany**, from *Fagus sylvatica* infested by *Xyloterus domesticus*, Nov. 1970, G. Zimmerman, **Isotype** CBS H-6825 (not seen), culture ex-isotype CBS 770.71 = ATCC 26401 = DSM 1507. **Austria**, Upper Austria Region, Zell am Moos, from the sapwood of *Fagus sylvatica*, Feb. 1994, T. Kirisits, culture CMW 10574.

Of *O. arduennense*: **Belgium**, Walloon Region, Luxemburg Province, Chiny Forest, sapwood of *Fagus sylvatica* infested by *Xyloterus domesticus*, 9 Jan. 2002, F.X. Carlier & T. Defrance, **holotype** dry culture of MUCL 44866 grown on 2% MEA for three months, culture ex-holotype MUCL 44866 = CMW 40266; other cultures MUCL 44869, MUCL 44870, MUCL 45367.

***Ophiostoma setosum*** Uzunovic, Seifert, S.H. Kim & C. Breuil, Mycol. Res. 104: 490. 2000.

Synonyms: *Pesotum cupulatum* McNew & Harrington, Mycologia 93: 121. 2001.

*Ophiostoma cupulatum* (McNew & Harrington) Z.W. de Beer, Seifert & M.J. Wingf., *In K.A. Seifert, Z.W. de Beer, M.J. Wingfield, eds., The Ophiostomatoid Fungi, Expanding Frontiers, p. 252. 2013.*

*Specimens examined*: Of *O. setosum*: **Canada**, British Columbia, Vancouver, from *Tsuga heterophylla* logs, Aug. 1997, J. Clark, **holotype** DAOM 225944 (AU160-38×AU160-53, on sterilized *Pseudotsuga menziesii*), cultures ex-holotype CMW 27833 = AU160-38, CMW 27834 = AU160-53.

Of *P. cupulatum*: **United States**, Washington, Aberdeen, from stained wood of *Pseudotsuga menziesii*, Dec. 1997, *S. John & D.L. McNew*, **holotype** BPI 746441, culture ex-holotype CBS 102358 = CMW 37441.

**Discussion**

In the present study, multigene phylogenies (ITS, βT, CAL, TEF-1α) of 45 isolates revealed five new species of *Ophiostoma s. str.* associated with four spruce-infesting bark beetles in China. Our analyses also resolved the phylogenetic relationships of several species within the *O. piceae*- and *O. brunneo-ciliatum* complexes.

In our analyses, the first three species from China (Taxa 1 to 3) described in the present study formed part of a larger lineage (Group A) that was well-supported in all three protein-coding genes. Six known species were also included: *O. brunneum*, *O. breviusculum*, *O. canum*, *O. flexuosum, O. rachisporum,* and the one that was first described, *O. piceae* (Münch 1907). This was the name-bearing species for the *O. piceae* complex, defined by Harrington et al. (2001) based on ITS sequences. In addition to *O. piceae*, they included eight species producing synnematous asexual states in the complex, with one species, *O. ips* as outgroup. Although they stated that the group was monophyletic, the monophyly was not statistically supported and two major clades could be recognized, one representing the conifer-inhabiting species (*O. piceae*, *O. canum*, *O. floccosum* and *O. setosum*), and the other the hardwood-inhabiting species (*O. quercus, O. catonianum*, *O. ulmi*, *O. novo-ulmi*, and *O. himal-ulmi*). In subsequent studies, the latter lineage was shown to have good support and it was referred to as the 'hardwood clade' of the *O. piceae* complex (Grobbelaar et al. 2009, 2010, Linnakoski et al. 2010), or the *O. quercus* complex (Kamgan Nkuekam et al. 2011). De Beer & Wingfield (2013) included 15 species and suggested that it be named the *O. ulmi* complex (see Fig. 1) after the first species in the complex that was described (Schwarz 1922). However, in the ITS and LSU analyses of De Beer & Wingfield (2013) the other, conifer-inhabiting species previously included in the *O. piceae* complex (Harrington et al. 2001, Linnakoski et al. 2010), did not form a monophyletic lineage. De Beer & Wingfield (2013) thus refrained from applying the term '*O. piceae* complex' altogether. Although our ITS analyses did not reveal a strongly supported lineage for the conifer-inhabiting, synnematous species, these species grouped together (Fig. 1, Group A) as they all had identical ITS sequences, with the exception of *O. rachisporum*. However, in all three the individual gene trees as well as the tree based on concatenated data (Fig. 2), these species formed a monophyletic lineage with good support. In addition, the species with known sexual morphs are all characterized by unsheathed, allantoid ascospores, while most produced pronounced pesotum-like synnemata and sporothrix-like asexual morphs. We believe that there is enough support and recommend that Group A in our analyses can be referred to as a newly defined *O. piceae* complex.

In the studies of Linnakoski et al. (2010) and De Beer & Wingfield (2013), *O. brunneo-ciliatum* and *O. ainoae* always grouped close to each other, with *O. tapionis* slightly peripheral to the other two (Figs 1,

2). The ascomata of the first two species are unique among the Ophiostomatales in the spiralling coiled ostiolar hyphae that they produce (Mathiesen-Käärik 1953, Solheim 1986). The cylindrical ascospores with rectangular sheaths broadly resemble those of species in the *O. ips* complex, although the latter tend to have sheaths that are more pointed, sometimes referred to as pillow-shaped (De Beer & Wingfield 2013). However, the *O. ips* complex is one of the best supported species complexes in *Ophiostoma s. str.*, and *O. brunneo-ciliatum* and *O. ainoae* always group outside this complex, despite the morphological similarities (Zipfel et al. 2006, Linnakoski et al. 2010, De Beer & Wingfield 2013). De Beer & Wingfield (2013) defined a lineage as a species complex when it contained three or more species with statistical, morphological or ecological support. To date, *O. brunneo-ciliatum* and *O. ainoae* thus have not met these requirements. In the present study, two new species from China, Taxa 4 and 5, and *O. tapionis* grouped with these two species, in a clade that had good support in the ITS and CAL trees, but less support in the βT and TEF-1α (Figs 1, 2). However, if *O. tapionis* is considered as outside, the other four species group together with good support in ITS, CAL, and TEF-1α *O. ips,* enabling us to define the *O. brunneo-ciliatum* species complex.

Group C contained three species *O. distortum* (Davidson 1958)*, O. torulosum* (Butin & Zimmermann 1972)*,* and *O. arduennense* (Carlier et al. 2006) (Figs 1, 2, Table 3). *Ophiostoma arduennense* and *O. torulosum* originated from the same ambrosia beetle species, *Xyloterus domesticus* (now *Trypodendron domesticum*), in the same hardwood host, *Fagus sylvatica,* in Europe (Carlier et al. 2006, Butin & Zimmermann 1972). *Ophiostoma distortum* is from an unknown ambrosia beetle attacking various conifers in the USA (Davidson 1971). Our DNA sequence results, supported by morphological similarities between the three taxa (Table 3), suggest that these three species are synonymous.

A fourth lineage consisting of two species, *Ophiostoma setosum* and *O. cupulatum*, forms Group D (Figs 1, 2, Table 3). The two species were described almost simultaneously: *O. setosum* (with a sexual morph) from *Tsuga heterophylla* in Canada (Uzunovic et al. 2000), and *Pesotum cupulatum* (presenting only the asexual morph) from *Pseudotsuga* and *Tsuga* in the USA (Harrington et al. 2001). Based on mating compatibility, Harrington et al. (2001) treated *P. cupulatum* as anamorph of *O. setosum.* De Beer et al. (2013) treated the two species distinct as a result of differences in the ITS sequences generated in different studies, and provided a new combination for *P. cupulatum* in *Ophiostoma* following the one fungus one name principles. We re-sequenced the ex-type isolates of both species and showed that they had identical sequences in the ITS, βT, and TEF-1α gene regions, but differed 9 bp in the CAL gene region. Based on these results and morphological similarities (Table 3), we follow the suggestion of Harrington et al. (2001) that the two names are synonyms, with *O. setosum* taking preference as the older name.

To date the majority of studies on *Ophiostoma* spp. in Asia has been conducted in Japan and Korea, with only a few published reports from China. Our results revealed five new *Ophiostoma* spp. from China. The country has diverse environments and abundant forest resources that are ideal habitats for bark beetles and ophiostomatalean fungi. Our results suggest that many more of these fungal species remain to be discovered and described from those forests.

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**Table 1** Isolates of *Ophiostoma* spp. included in this study.

| Species | Isolate [1, 2] | | Origin | Host | Insect | GenBank accession no. [3] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CMW no. | Other no. | | | | ITS | βT | CAL | TEF-1α |
| *Ophiostoma ainoae* | 1037[H] | CBS 205.83 | Norway | *Picea abies* | *Ips typographus* | KU184416 | KU184287 | KU184330 | KU184373 |
| | 23123 | CBS 128299 | Russia | *P. abies* | *I. typographus* | KU184417 | KU184288 | KU184331 | KU184374 |
| *O. araucariae* | 40665[H] | CBS 114.68 | Chile | *Araucaria araucana* | - | KU184418 | KU184289 | KU184332 | KU184375 |
| *O. arduennense* | 40266[H] | MUCL 44866 | Belgium | *Fagus sylvatica* | *Xyloterus signatus* | KU184419 | KU184290 | KU184333 | KU184376 |
| | 40267 | MUCL 44869 | Belgium | *F. sylvatica* | *X. signatus* | KU184420 | KU184291 | KU184334 | KU184377 |
| *O. breviusculum* | - | JCM 11980 | Japan | *Larix kaempferi* | *Dryocoetes baicalicus* | AB200422 | AB200428 | - | - |
| | - | JCM 12500[H] | Japan | *L. kaempferi* | *D. baicalicus* | AB200421 | AB200427 | - | - |
| *O. brunneo-ciliatum* | 5212 | - | Scotland | *Larix* sp. | *Ips cembrae* | KU184422 | KU184293 | KU184336 | KU184379 |
| | - | CBS 117571 | Scotland | *Larix decidua* | *I. cembrae* | KU184421 | KU184292 | KU184335 | KU184378 |
| *O. brunneum* | 1027[H] | CBS 161.61 | USA | *Abies lasiocarpa* | - | KU184423 | KU184294 | KU184337 | KU184380 |
| *O. canum* | 29495 | CBS 124499 | Norway | *Betula pendula* | *Scolytus ratzeburgi* | KU184424 | KU184295 | KU184338 | KU184381 |
| *O. cupulatum* | 37441[H] | CBS 102358 | USA | *Pseudotsuga menziesii* | - | KU184425 | KU184296 | KU184339 | KU184382 |
| *O. distortum* | 40668[H] | CBS 429.82 | USA | *Abies concolor* | Ambrosia beetle | KU184426 | KU184297 | KU184340 | KU184383 |
| *O. flexuosum* | 907[H] | CBS 208.83 | Norway | *P. abies* | *I. typographus* | KU184427 | KU184298 | KU184341 | KU184384 |
| *O. floccosum* | 12623 | - | Austria | *Pinus sylvestris* | - | KU184428 | KU184299 | KU184342 | KU184385 |
| | 23287 | - | Russia | *P. abies* | *I. typographus* | KU184429 | KU184300 | KU184343 | KU184386 |
| | 23288 | - | Finland | *P. sylvestris* | *Hylurgops palliatus* | KU184430 | KU184301 | KU184344 | KU184387 |
| | 34182 | CBS 799.73 | Sweden | - | - | KU184431 | KU184302 | KU184345 | KU184388 |
| *O. nikkoense* | 17193[H] | JCM 11728 | Japan | *Abies mariesii* | *Polygraphus proximus* | KU184434 | KU184305 | KU184348 | KU184391 |
| | 17194 | JCM 11729 | Japan | *Abies homolepis* | *P. proximus* | KU184435 | KU184306 | KU184349 | KU184392 |
| *O. piceae* | 8093 | CBS 119678 | Canada | - | *Tetropium* sp. | KU184442 | KU184313 | KU184356 | KU184399 |
| | 13239 | CBS 819.85 | Canada | *Betula papyrifera* | - | KU184438 | KU184309 | KU184352 | KU184395 |
| | 13241 | CBS 426.94 | Austria | *P. abies* | - | KU184439 | KU184310 | KU184353 | KU184396 |
| | 13243 | CBS 102356 | USA | *P. menziesii* | - | KU184440 | KU184311 | KU184354 | KU184397 |
| | 25034[H] | CBS 108.21 | Germany | - | - | KU184441 | KU184312 | KU184355 | KU184398 |
| *O. rachisporum* | 23272[H] | CBS 128119 | Finland | *P. sylvestris* | *Trypodendron lineatum* | KU184448 | KU184319 | KU184362 | KU184405 |
| | 23273 | - | Finland | *P. sylvestris* | *Hylurgops palliatus* | KU184449 | KU184320 | KU184363 | KU184406 |
| | 23274 | CBS 128123 | Finland | *P. sylvestris* | *T. lineatum* | KU184450 | KU184321 | KU184364 | KU184407 |
| *O. setosum* | 27833[H] | - | Canada | *Tsuga heterophylla* | - | KU184451 | KU184322 | KU184365 | KU184408 |
| | 27834[H] | - | Canada | *T. heterophylla* | - | KU184452 | KU184323 | KU184366 | KU184409 |

| Species | Isolate [1, 2] | | Origin | Host | Insect | GenBank accession no. [3] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CMW no. | Other no. | | | | ITS | βT | CAL | TEF-1α |
| *O. tapionis* | 23265[H] | CBS 128120 | Finland | *Picea abies* | *H. palliatus* | KU184455 | KU184326 | KU184369 | KU184412 |
| | 23269 | CBS 128121 | Russia | *P. sylvestris* | *H. palliatus* | KU184456 | KU184327 | KU184370 | KU184413 |
| *O. torulosum* | 10574 | - | Austria | *F. sylvatica* | - | KU184458 | KU184329 | KU184371 | KU184415 |
| | 40670[H] | CBS 770.71 | Germany | *F. sylvatica* | *Xyloterus domesticus* | KU184457 | KU184328 | KU184372 | KU184414 |
| Taxon 1 *O. nitidum* sp. nov. | 38905[P] | CBS 136526 | China | *Picea crassifolia* | *I. nitidus* | KU184436 | KU184307 | KU184350 | KU184393 |
| | 38907[H] | CBS 136525 | China | *P. crassifolia* | *I. nitidus* | KU184437 | KU184308 | KU184351 | KU184394 |
| Taxon 2 *O. micans* sp. nov. | 38903[H] | CBS 136523 | China | *P. crassifolia* | *Dendroctonus micans* | KU184432 | KU184303 | KU184346 | KU184389 |
| | 38909[P] | CBS 136524 | China | *P. crassifolia* | *D. micans* | KU184433 | KU184304 | KU184347 | KU184390 |
| Taxon 3 *O. qinghaiense* sp. nov. | 38902[H] | CBS 136521 | China | *P. crassifolia* | *Polygraphus poligraphus* | KU184445 | KU184316 | KU184359 | KU184402 |
| | 38904[P] | CBS 136522 | China | *P. crassifolia* | *D. micans* | KU184446 | KU184317 | KU184360 | KU184403 |
| | 38906 | - | China | *P. crassifolia* | *D. micans* | KU184447 | KU184318 | KU184361 | KU184404 |
| Taxon 4 *O. shangrilae* sp. nov. | 38900[P] | CBS 136520 | China | *Picea purpurea* | *Ips shangrila* | KU184453 | KU184324 | KU184367 | KU184410 |
| | 38901[H] | CBS 136519 | China | *P. purpurea* | *I. shangrila* | KU184454 | KU184325 | KU184368 | KU184411 |
| Taxon 5 *O. poligraphi* sp. nov. | 38898[P] | CBS 136518 | China | *P. crassifolia* | *P. poligraphus* | KU184443 | KU184314 | KU184357 | KU184400 |
| | 38899[H] | CBS 136517 | China | *P. crassifolia* | *P. poligraphus* | KU184444 | KU184315 | KU184358 | KU184401 |

[1] CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; JCM = Japan Collection of Microorganisms, RIKEN BioResource Center, Tsukuba, Ibaraki, Japan; MAFF = Culture Collection of the National Institute of Agrobiological Sciences, Japan; MUCL = Mycothèque de l'Université Catholique de Louvain, a founding partner of the Belgian Co-ordinated Collections of Microorganisms (BCCM), Belgium.

[2] [H] = ex-holotype isolate, [P] = ex-paratype isolate.

[3] ITS = internal transcribed spacer region of the nuclear ribosomal DNA gene; βT = Beta-tubulin; CAL = Calmodulin; TEF-1α = Translation elongation factor 1-alpha.

**Table 2** Morphological comparisons of closely related species from Groups A.

| **Species** | ***O. rachisporum*** | **Taxon 1** | **Taxon 2** | **Taxon 3** |
|---|---|---|---|---|
| **Group** | A | A | A | A |
| **Sexual state** | Present (Homothallic) | unknown | unknown | unknown |
| **Synnematal length (μm)** | (135–)200–365(–547) | (788-) 870-1050 (-1082) | (583-) 620-745 (-761) | (1060-) 1110-1081 (-1287) |
| **Conidial shape** | oblong | clavate or obovoid | clavate or obovoid | clavate or obovoid |
| **Conidial size (μm)** | (2–)3–4(–4.5) | (3.3-) 3.6-4 (-4.3) | (3.2-) 3.5-4.2 (-4.4) | (3.3-) 3.7-4.2 (-4.5) |
| | × 1–1.5 | × (1.4-) 1.5-1.7 (-1.8) | × (1.4-) 1.5-1.6 (-1.7) | × (1.2-) 1.4-1.7 (-1.9) |
| **Growth rate (mm/d)** | - | 2.5 (± 0.5) | 2.8 (± 0.5) | 3 (± 0.5) |
| **Host** | *Picea abies, Pinus sylvestris* | *Picea crassifolia** | *P. crassifolia** | *P. crassifolia** |
| **Insect** | *Trypodendron lineatum* | *Ips nitidus** | *Dendroctonus micans** | *Polygraphus poligraphus** |
| | *Hylurgops palliatus* | | | *D. micans** |
| **Distribution** | Finland, Russia | Qinghai, China* | Qinghai, China* | Qinghai, China* |
| **Reference** | Linnakoski et al. 2010 | This study | This study | This study |

* Hosts, insect vectors and countries from which the identity of isolates was confirmed based on DNA sequences.

**Table 3** Morphological comparisons of closely related species from Groups B

| Species | *O. ainoae* | *O. brunneo-ciliatum* | Taxon 4 | Taxon 5 |
|---|---|---|---|---|
| **Group** | B | B | B | B |
| **Sexual state** | present | present | unknown | unknown |
| **Synnematal length (μm)** | about 150-250 | 800-1500 | (723-) 763-870 (-925) | (484-)501-668(-796) |
| **Conidial shape** | clavate | cylindrical | clavate or obovoid | clavate or obovoid |
| **Conidial size (μm)** | 2.5-9.0 x 1.7-3.2 | 4.5-6 × 1-2 | (3.3-) 3.5-4.4 (-5.1) × (1.2-) 1.5-1.8 (-2) | (5.9-) 6.2-8 (-10) × (1.6-) 1.7-2 (-2.5) |
| **Growth rate (mm/d)** | - | - | 3.3 (± 0.5) | 4.1 (± 0.5) |
| **Host** | *Picea abies** | *Pinus sp., Larix decidua** | *Picea purpurea** | *P. crassifolia** |
| **Insect** | *Ips typographus** | *Ips sexdentatus, I. cembrae** | *Ips shangrila* ,D. micans** | *P. poligraphus* , D. micans** |
| **Distribution** | Norway*, Russia* | Sweden, Scotland* | Qinghai, China* | Qinghai, China* |
| **Reference** | Solheim 1986; Linnakoski et al. 2010 | Mathiesen-Käärik 1953, Hunt 1956 | This study | This study |

* Hosts, insect vectors and countries from which the identity of isolates was confirmed based on DNA sequences.

**Table 4** Morphological comparisons of closely related species in Group C, as well as Group D.

| Species | *O. arduennense* | *O. torulosum* | *O. distortum* | *O. cupulatum* | *O. setosum* |
|---|---|---|---|---|---|
| **Group** | C | C | C | D | D |
| **Sexual state** | present | present | present | unknown | present |
| **Ascomatal neck length (µm)** | (240–) 600 (–960) | 500-700 | 400-600 | - | 550-2400 |
| **Ascospore shape** | reniform | reniform | elongate orange-section | - | orange-section |
| **Ascospore size (µm)** | 3-5 × 1 | 3.5-4.4 × 1.5-2 | 3.5-5 × 1-1.5 | - | 2.5-3.5 × 1-1.5 |
| **Asexual state** | unknown | sporothrix-like | sporothrix-like | pesotum-like | pesotum-like |
| **Conidial shape** | - | ovoid to obclavate | cylindrical to curved elongate | cylindrical to obovoid | oblong-ellipsoidal to slightly ovate |
| **Conidial size (µm)** | - | 3-4.5 × 1.6-2.1 | 2.5-8 × 1.2-2 | 3-5.5 × 1-2 | (2-) 3-5 × 1-1.5 |
| **Host** | *Fagus sylvatica** | *Fagus sylvatica** | *Abies concolor** | *Pseudotsuga menziesii** | Hemlock sp.* |
| | | | *Abies balsamea* | *Tsuga* sp. | *Pinus contorta* |
| | | | *Picea engelmannii* | *Pinus radiata* | *Picea glauca* |
| | | | *Pinus contorta* | | *Tsuga heterophylla* |
| **Insect** | *Xyloterus domesticus** | *Xyloterus domesticus** | Ambrosia beetle spp.* | Unknown | Unknown |
| | *X. signatus* | | *Pityokteines sparsus* | | |
| | *Xyleborus dispar* | | | | |
| | *Hylecoetus dermestoides* | | | | |
| **Distribution** | Belgium* | Germany*, Austria | USA*, Canada | USA*, New Zealand | Canada* |
| **Reference** | Carlier et al. 2006 | Butin & Zimmermann 1972 | Davidson 1971 | Harrington et al. 2001 | Uzunovic et al. 2000 |

* Hosts, insect vectors and countries from which the identity of isolates was confirmed based on DNA sequences.

Figure legends

**Fig. 1** ML tree of *Ophiostoma sensu stricto* generated from the ITS DNA sequence data. Sequences generated from this study are printed in bold type. Bold branches indicate posterior probabilities values ≥0.95. Bootstrap values ≥ 75% are recorded at nodes as ML/MP. * Bootstrap values < 75%. T = ex-holotype isolate

**Fig. 2** ML trees of selected species in *Ophiostoma sensu stricto* generated from DNA sequences of three protein gene regions as well as their combined datasets. Bold branches indicate posterior probabilities values ≥ 0.95. Bootstrap values ≥ 75% are recorded at nodes as ML/MP. * Bootstrap values < 75%. H = ex-holotype isolate, P = ex-paratype isolate.

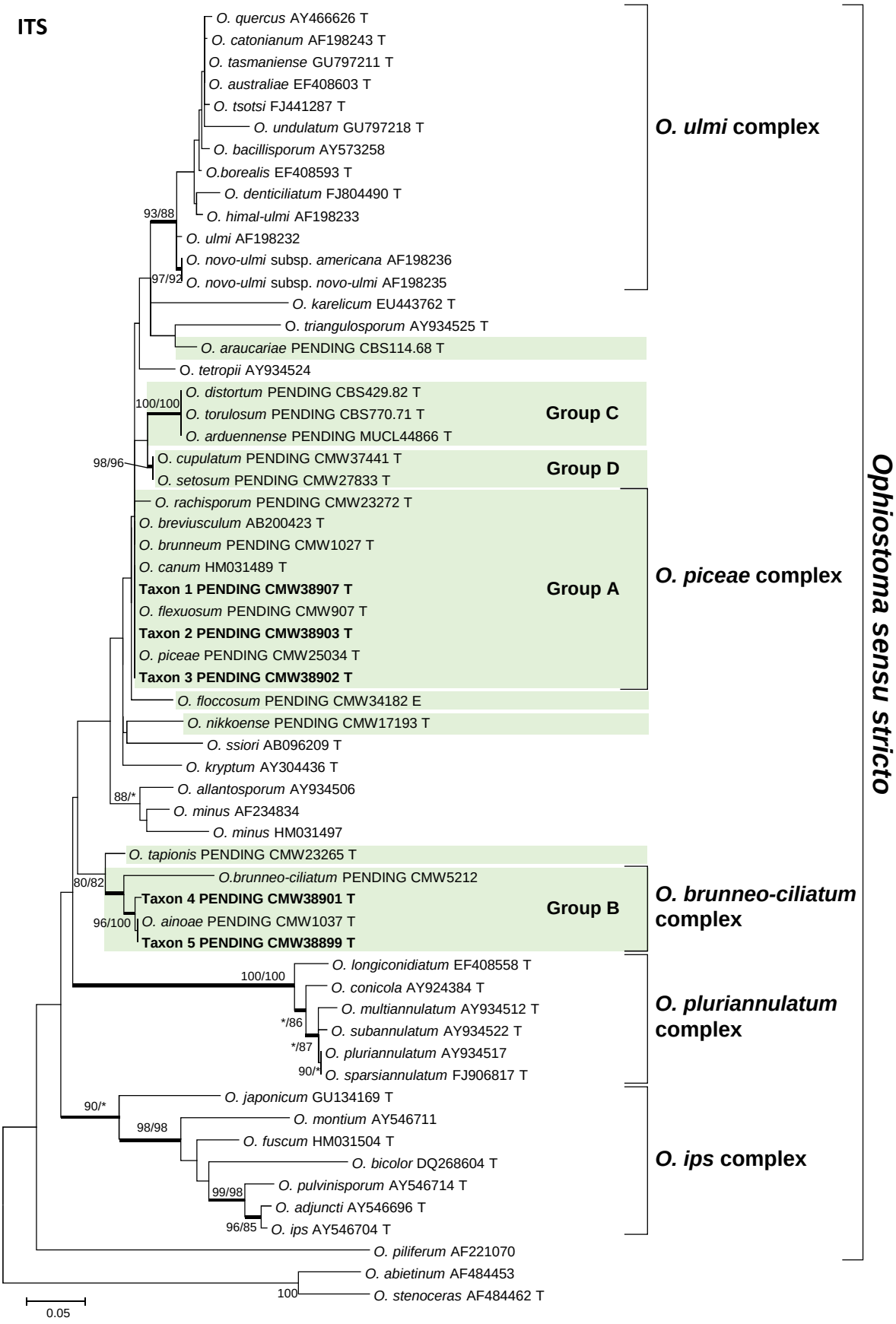
**Fig. 3** Morphological characters of *Ophiostoma nitidum* sp. nov. (CMW 38907, Taxon 1) a. Fourteen days old culture on OA; b. synnematous asexual state on wood tissue on WA; c. conidiophore; d. conidiogenous apparatus; e. conidiogenous cells; f. conidia. Scale bars: b = 200 µm, c = 100 µm, d = 50 µm, e = 10 µm, f = 5 µm

**Fig. 4** Morphological characters of *Ophiostoma micans* sp. nov. (CMW 38903, Taxon 2) a. Fourteen days old culture on OA; b. synnematous asexual state on wood tissue on WA; c. conidiophore; d. conidiogenous apparatus; e. conidiogenous cells; f. conidia. Scale bars: b = 200 µm, c = 100 µm, d = 50 µm, e = 20 µm, f = 5 µm

**Fig. 5** Morphological characters of *Ophiostoma qinghaiense* sp. nov. (CMW 38902, Taxon 3) a. Fourteen days old culture on OA; b. synnematous asexual state on wood tissue on WA; c. conidiophore; d. conidiogenous apparatus; e. conidiogenous cells; f. conidia. Scale bars: b = 200 µm, c = 100 µm, d = 50 µm, e = 10 µm, f = 5 µm

**Fig. 6** Morphological characters of *Ophiostoma shangrilae* sp. nov. (CMW 38901, Taxon 4) a. Fourteen days old culture on OA with black background; b. synnematous asexual state on wood tissue on WA; c-d. conidiophore; e. conidiogenous cells; f. conidia. Scale bars: b = 200 µm, c = 100 µm, d = 50 µm, e = 10 µm, f = 5 µm

**Fig. 7** Morphological characters of *Ophiostoma poligraphi* sp. nov. (CMW 38899, Taxon 5) a. Fourteen days old culture on OA with black background; b. synnematous asexual state on wood tissue on WA; c. conidiophore; d. conidiogenous apparatus; e. conidiogenous cells; f. conidia. Scale bars: b = 200 µm, c = 50 µm, d = 25 µm, e = 10 µm, f = 5 µm


ITS
O. quercus AY466626 T
O. catonianum AF198243 T
O. tasmaniense GU797211 T
O. australiae EF408603 T
O. tsotsi FJ441287 T
O. undulatum GU797218 T
O. bacillisporum AY573258
O.borealis EF408593 T
O. denticiliatum FJ804490 T
O. himal-ulmi AF198233
O. ulmi AF198232
O. novo-ulmi subsp. americana AF198236
O. novo-ulmi subsp. novo-ulmi AF198235
O. karelicum EU443762 T
O. triangulosporum AY934525 T
O. araucariae PENDING CBS114.68 T
O. tetropii AY934524
O. distortum PENDING CBS429.82 T
O. torulosum PENDING CBS770.71 T
O. arduennense PENDING MUCL44866 T
O. cupulatum PENDING CMW37441 T
O. setosum PENDING CMW27833 T
O. rachisporum PENDING CMW23272 T
O. breviusculum AB200423 T
O. brunneum PENDING CMW1027 T
O. canum HM031489 T
Taxon 1 PENDING CMW38907 T
O. flexuosum PENDING CMW907 T
Taxon 2 PENDING CMW38903 T
O. piceae PENDING CMW25034 T
Taxon 3 PENDING CMW38902 T
O. floccosum PENDING CMW34182 E
O. nikkoense PENDING CMW17193 T
O. ssiori AB096209 T
O. kryptum AY304436 T
O. allantosporum AY934506
O. minus AF234834
O. minus HM031497
O. tapionis PENDING CMW23265 T
O.brunneo-ciliatum PENDING CMW5212
Taxon 4 PENDING CMW38901 T
O. ainoae PENDING CMW1037 T
Taxon 5 PENDING CMW38899 T
O. longiconidiatum EF408558 T
O. conicola AY924384 T
O. multiannulatum AY934512 T
O. subannulatum AY934522 T
O. pluriannulatum AY934517
O. sparsiannulatum FJ906817 T
O. japonicum GU134169 T
O. montium AY546711
O. fuscum HM031504 T
O. bicolor DQ268604 T
O. pulvinisporum AY546714 T
O. adjuncti AY546696 T
O. ips AY546704 T
O. piliferum AF221070
O. abietinum AF484453
O. stenoceras AF484462 T
93/88
97/92
100/100
98/96
88/*
80/82
96/100
100/100
*/86
*/87
90/*
90/*
98/98
99/98
96/85
100
0.05
Group C
Group D
Group A
Group B
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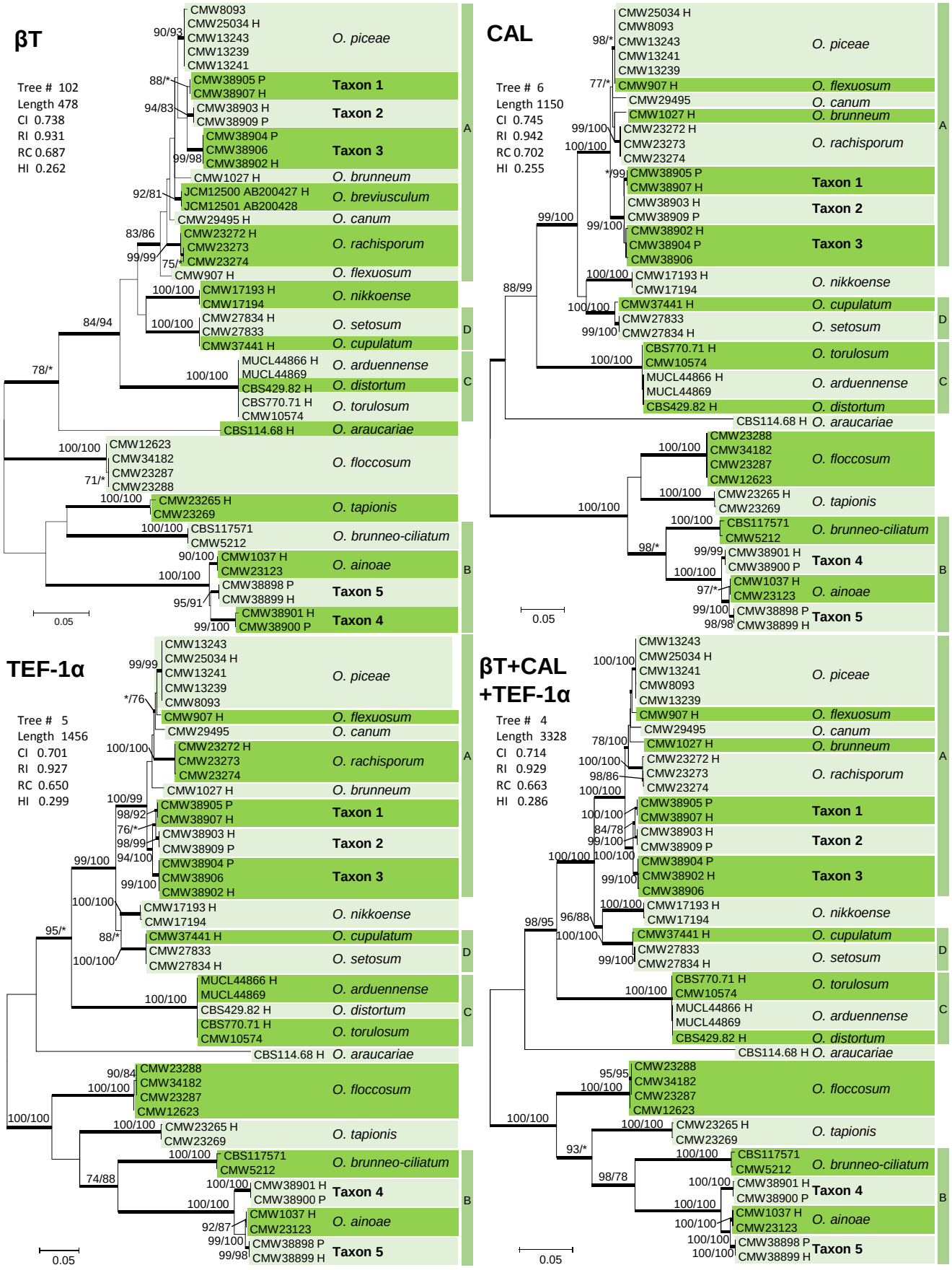
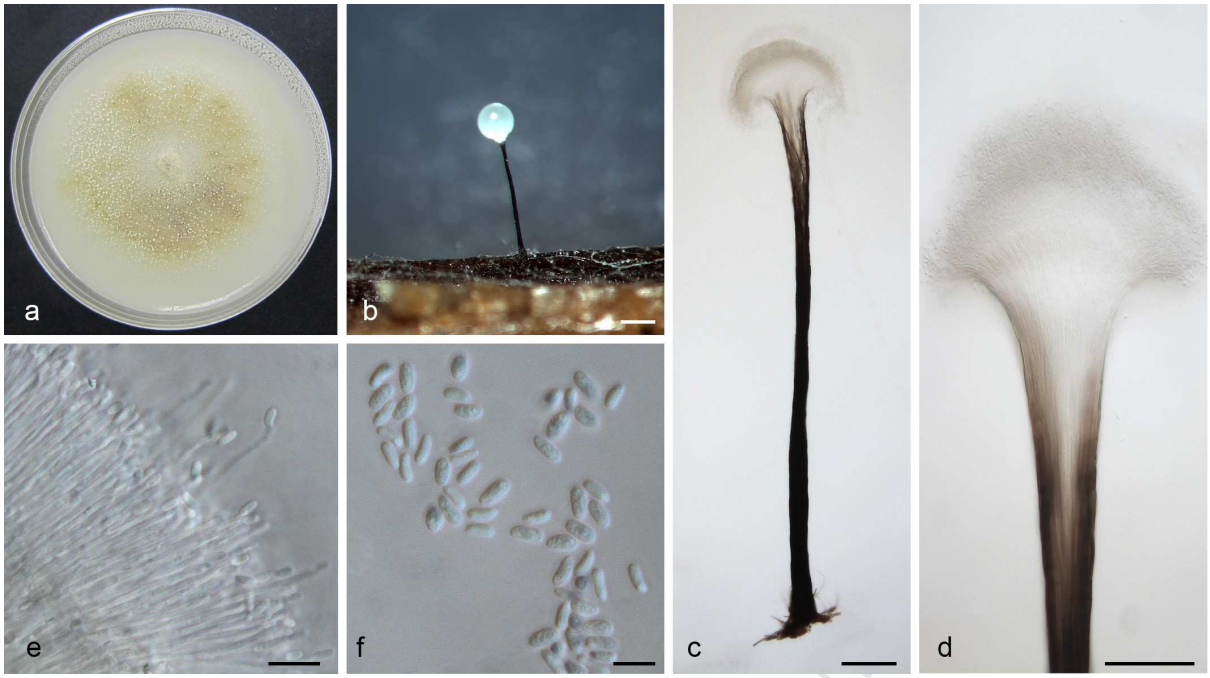
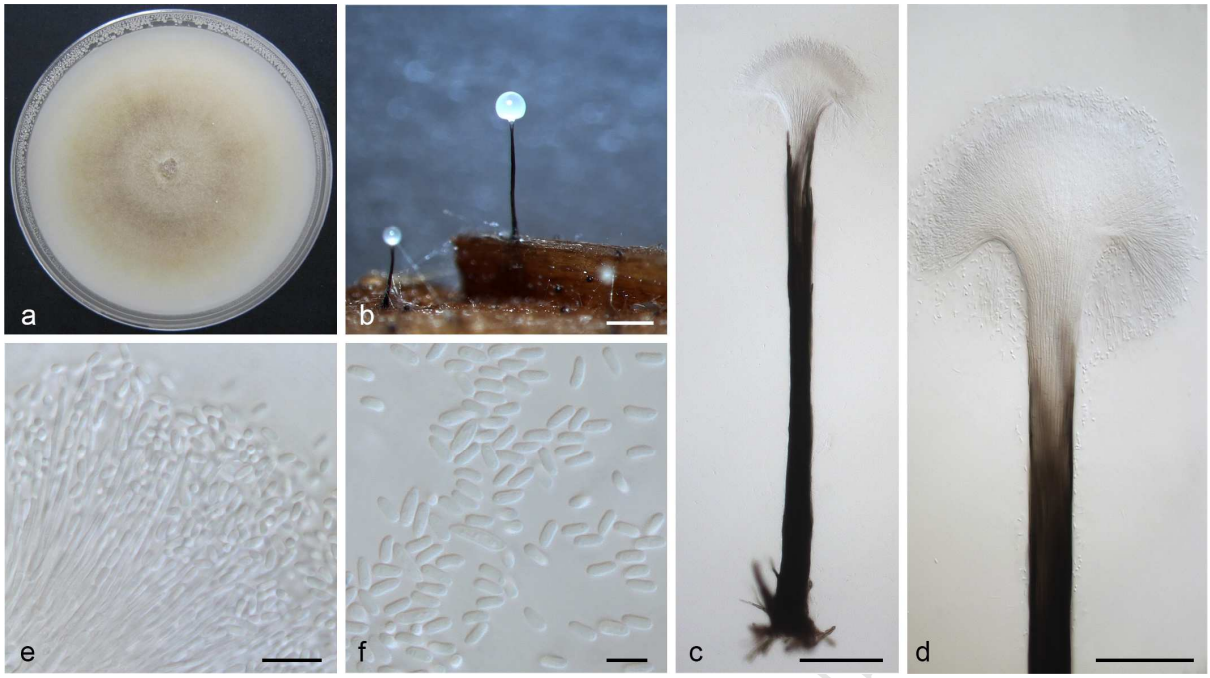
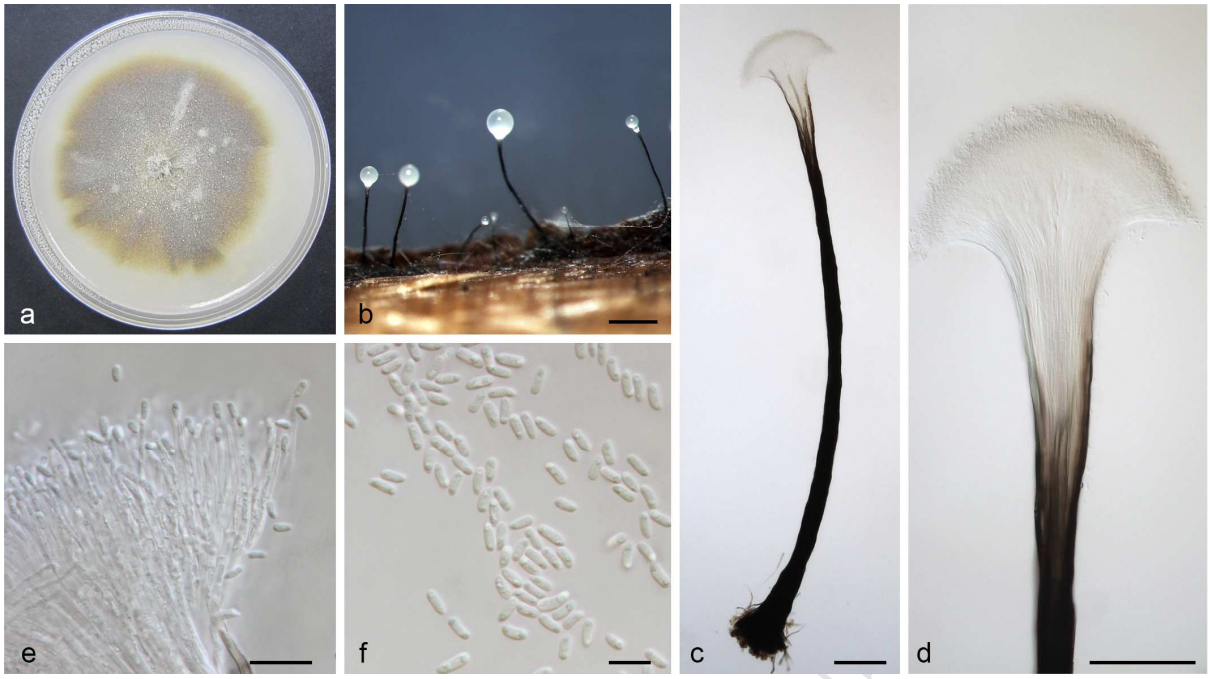
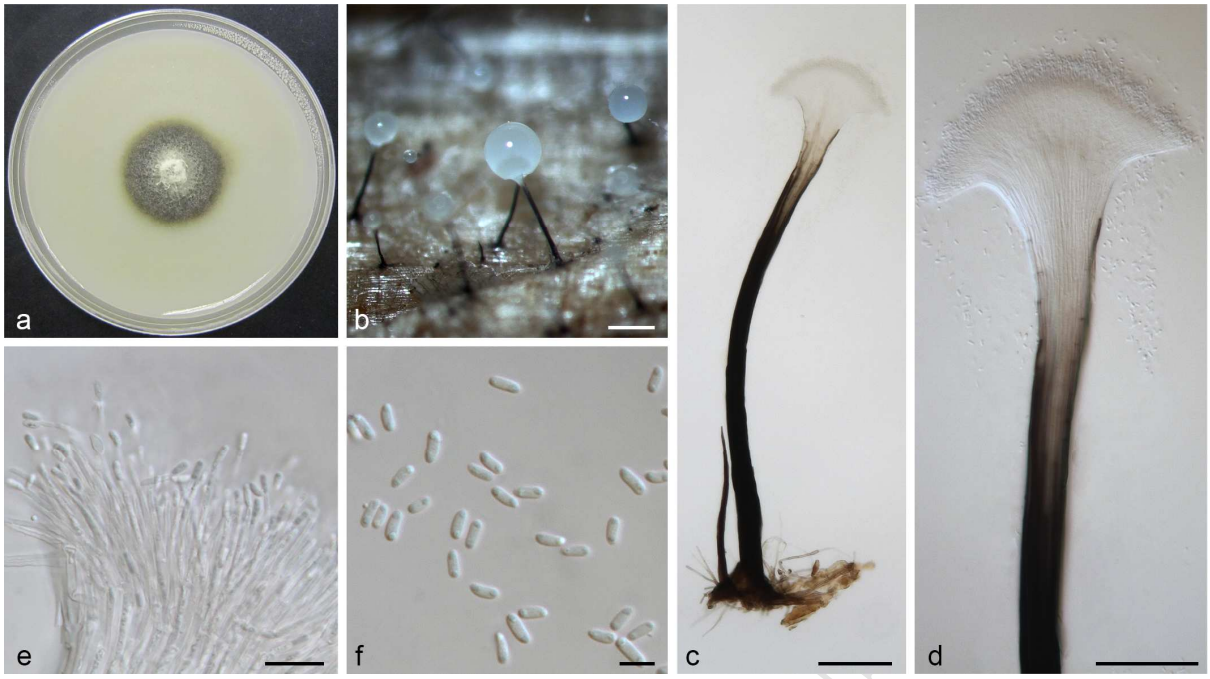
O. piceae complex
O. brunneo-ciliatum complex
O. pluriannulatum complex
O. ips complex
Ophiostoma sensu stricto

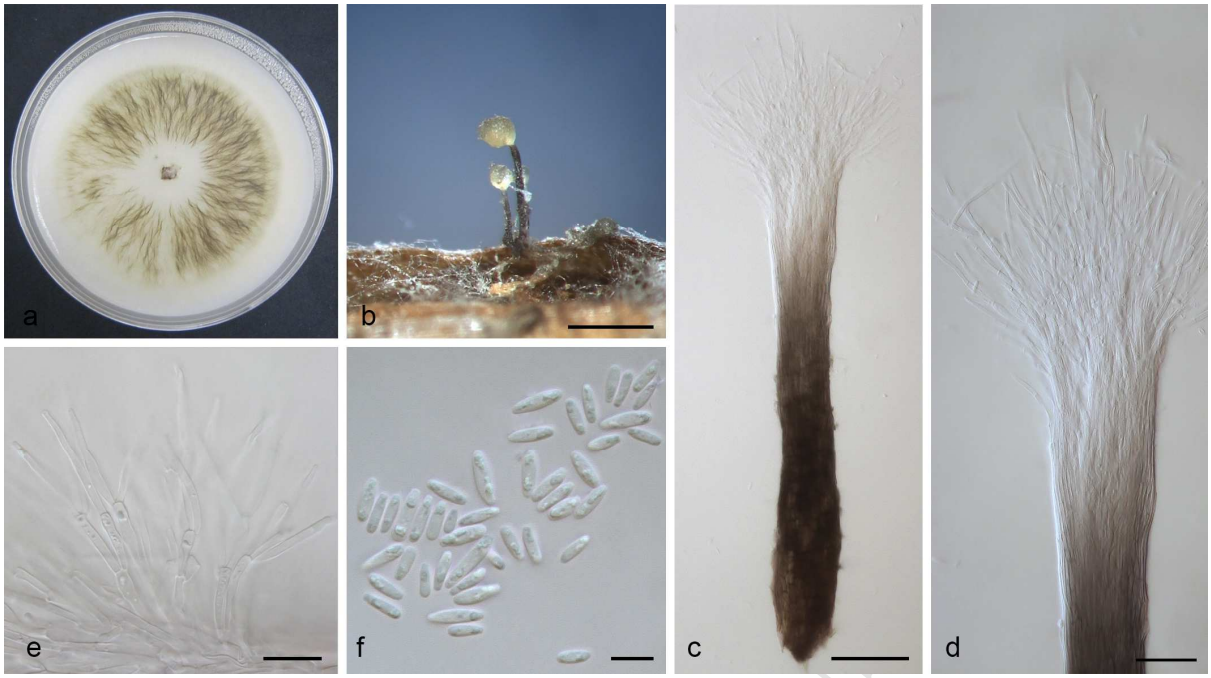












A survey was done on fungal associates of spruce-infesting beetles in Qinghai, China.

DNA sequences of four gene regions were used to identify and classify isolates.

Five new *Ophiostoma* species were discovered and described.

These taxa grouped in the *O. piceae* and *O. brunneo-ciliatum* species complexes.