The *Ophiostoma clavatum* species complex: a newly defined group in the *Ophiostomatales* including three novel taxa

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Abstract Two species of blue-stain fungi with similar morphologies, *Ophiostoma brunneo-ciliatum* and *O. clavatum*, are associates of bark beetles infesting *Pinus* spp. in Europe. This has raised questions whether they represent distinct taxa. Absence of herbarium specimens and contaminated or mistakenly identified cultures of *O. brunneo-ciliatum* and *O. clavatum* have accentuated the uncertainty regarding their correct identification. The aim of this study was to reconsider the identity of European isolates reported as *O. brunneo-ciliatum* and *O. clavatum* by applying DNA-based identification methods, and to provide appropriate type specimens for them. Phylogenetic analyses of the ITS, βT, TEF-1α and CAL gene sequences revealed that the investigated isolates represent a complex of seven cryptic species. The study confirmed that ITS data is insufficient to delineate species in some *Ophiostoma* species clusters. Lectotypes

and epitypes were designated for *O. clavatum* and *O. brunneo-ciliatum*, and three new species, *O. brunneolum*, *O. macroclavatum* and *O. pseudocatenulatum*, were described in the newly defined *O. clavatum*-complex. The other two species included in the complex are *O. ainoae* and *O. tapionis*. The results suggest co-evolution of these fungi in association with specific bark beetles. The results also confirm the identity of the fungus associated with the pine bark beetle *Ips acuminatus* as *O. clavatum*, while *O. brunneo-ciliatum* appears to be mainly associated with another pine bark beetle, *Ips sexdentatus*.

Keywords bark beetle-associated fungi; ophiostomatoid fungi; *Ophiostoma brunneolum*; *Ophiostoma macroclavatum*; *Ophiostoma pseudocatenulatum*

INTRODUCTION

Species of *Ophiostoma* Syd. (Ascomycota) include causal agents of blue-stain in timber as well as some important tree pathogens (Altenkirch et al. 2002; Kirisits 2013). These fungi produce spores in slimy droplets, which can attach to passing arthropods, especially bark beetles and mites (Wingfield et al. 1993; Kirisits 2004, 2013; Seifert et al. 2013). More than 130 species are currently recognised in the genus *Ophiostoma*, including a variety of sexual morphs and a continuum of asexual states ranging in complexity from sporothrix- and hyalorhinocladiella- to pesotum-like (De Beer et al. 2013). The genus includes several species complexes accommodating morphologically similar taxa. These complexes commonly include morphologically indistinguishable cryptic species, the boundaries of which can be resolved only by using multigene phylogenies (De Beer and Wingfield 2013).

In recent years, unprecedented outbreaks of the pine bark beetle *Ips acuminatus* (Gyll.), often accompanied by tree mortality, have been observed in Alpine *Pinus sylvestris* L. forests in Northern Italy, Switzerland and Southern Austria (Wermelinger et al. 2008; Krehan 2011; Colombari et al. 2012, 2013), and, unexpectedly, also in Southern Finland (Siitonen 2014). This is surprising because this bark beetle is generally known as one with low levels of aggressiveness, infesting mainly weakened, standing dead or fallen trees (Altenkirch et al. 2002). Recent outbreaks and the increasing importance of *I. acuminatus* as a pest have been attributed to hot and dry summers that have increased the susceptibility of pine trees to bark beetle infestation (Rebetez et al. 2004; Dobbertin et al. 2007; Wermelinger et al. 2008; Colombari et al. 2012; Siitonen 2014).

Several fungal species are known to be associated with *I. acuminatus* (Kirisits 2004). The fungus most consistently reported with this bark beetle is *Ophiostoma clavatum* Math. (Mathiesen 1950, 1951; Rennerfelt 1950; Mathiesen-Käärik 1953; Francke-Grosmann 1952, 1963a; Käärik 1975a, 1980). Another fungus, *O. brunneo-ciliatum* Math.-Käärik, has also been reported in a limited number of studies (Lieutier et al. 1991; Guérard et al. 2000; Villari et al. 2012) as an associate of *I. acuminatus*. However, the correctness of this association remains uncertain, because of the close morphological similarity between *O. brunneo-ciliatum* and *O. clavatum*.

Ophiostoma clavatum was originally described as an important associate of *I. acuminatus* infesting *Pinus sylvestris* in Sweden (Mathiesen 1951). Later, Mathiesen-Käärik (1953) also found this fungus associated with *Tomicus piniperda* L. Subsequently, *O. clavatum* has been reported only occasionally (Francke-Grosmann 1952; Francke-Grosmann 1963a; Aoshima 1965; Käärik 1975a) until the recognition of a fungus identical to *O. clavatum* consistently associated with *I. acuminatus* in the Alps (Villari 2012; Villari et al. 2012, 2013).

Ophiostoma brunneo-ciliatum, a species that is morphologically virtually identical to *O. clavatum*, was first described by Mathiesen-Käärik (1953) from *P. sylvestris* infested by the bark beetle *Ips sexdentatus* Boern. in Sweden. Mathiesen-Käärik (1953, 1960) distinguished *O. brunneo-ciliatum* from *O. clavatum* based on its ecology and physiology, and its larger ascocarps and conidiomata. Subsequent to its description, several studies have reported *O. brunneo-ciliatum* in association with *I. sexdentatus* infesting *Pinus* spp. (Lieutier et al. 1989, 1991; Kirschner 1998, 2001; Bueno et al. 2010; Jankowiak 2012). Some studies have claimed the occurrence of this fungus as an associate of *I. acuminatus* (Lieutier et al. 1991; Guérard et al. 2000). *Ophiostoma brunneo-ciliatum* has been also found as an important associate of the larch bark beetles *Ips cembrae* (Heer) infesting *Larix decidua* Mill. in Europe (Kirisits et al. 2000; Stauffer et al. 2001; Kirisits. 2001, 2004; Jankowiak et al. 2007) and *Ips subelongatus* Motschulsky (in some studies treated as conspecific with *I. cembrae*) infesting *L. kaempferi* (Lamb.) Carriére in Japan (Aoshima 1965; Yamaoka et al. 1998, 2009). Other studies have reported the fungus from various bark beetle species in Europe, including *Ips amitinus* Eichh., *I. typographus* L. and *Pityogenes chalcographus* L. on spruce (Kirisits et al. 2000; Kirisits 2001, 2004; Jankowiak et al. 2009; Linnakoski et al. 2010; Linnakoski 2011; Repe et al. 2013), and the pine shoot beetles *Tomicus minor* Hartig and *T. piniperda* (Linnakoski et al. 2010; Linnakoski 2011).

Ophiostoma ainoae H. Solheim, another species similar to *O. clavatum* and *O. brunneo-cilliatum* in having spirally coiled ostiolar hyphae, could have been confused with *O. clavatum*

and *O. brunneo-ciliatum* in past studies. This fungus is morphologically most similar to *O. clavatum* and is distinguished from that species only by the shape of ascospores and culture characteristics (Solheim 1986). In a recent study using DNA sequence-based identification methods, *O. ainoae* was surprisingly not collected from spruce infesting bark beetles in Poland (R. Jankowiak, unpublished data) despite its relatively frequent occurrence in previous years (Jankowiak 2005; Jankowiak et al. 2009).

In their original descriptions, type material was not designated for either *O. clavatum* or *O. brunneo-ciliatum* (Mathiesen 1951; Mathiesen-Käärik 1953). This lack of type material and the morphological similarities between the two species suggest that some of the specimens and isolates collected from *I. acuminatus* could have been misidentified. Moreover, it is probable that cryptic species have been overlooked in earlier studies where DNA methods were either not yet available or not applied. The aim of this study was thus to reconsider the identity and ecology of isolates from Europe previously reported as *O. clavatum* and *O. brunneo-ciliatum*, and to consider the phylogeny and taxonomy of these fungi together with other closely related species. Multigene DNA sequence analyses for four gene regions were conducted and accompanied with comparisons of morphological characteristics and ecology. This made it possible to resolve the problems relating to the typification of *O. brunneo-ciliatum* and *O. clavatum*, and to recognize and describe three novel taxa in the newly defined *O. clavatum* species complex.

MATERIALS AND METHODS

Fungal isolates and herbarium specimens

Examined material included living fungal isolates collected in previous studies, new isolates collected from Alpine forests (Fig. 1) and dried herbarium specimens (Table 1). Isolates that had not already been appropriately preserved in collections were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. The ex-type strains were also deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Some isolates of *O. clavatum* are also preserved in the culture collection of the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), University of Natural Resources and Life Sciences, Vienna (BOKU), Austria. Herbarium specimens were deposited in the Herbarium of the University of Turku, Finland (TUR), Finland. New names and typifications were registered in MycoBank (Robert et al. 2013).

DNA extraction, PCR and sequencing

Fungal isolates were grown on 2 % malt extract agar [MEA: 20 g malt extract l^{-1} (Biolab, Midrand, South Africa), 20 g Difco Bacto™ agar l^{-1} (Difco Laboratories, Detroit, MI, USA) and 1 l Milli-Q water] in 70 mm plastic Petri dishes for 1–2 weeks prior to DNA extraction. DNA was extracted using PrepMan Ultra Sample preparation reagent following the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA).

Gene regions investigated in the study were the internal transcribed spacer (ITS) regions (ITS1 and ITS2) including the 5.8S gene, as well as the partial β-tubulin (βT), the elongation factor 1-α (TEF-1α) and the calmodulin (CAL) genes. The ITS region was amplified using the primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). The partial βT gene was amplified using the primers T10 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995). The TEF-1α gene region was amplified using the primers EF2F (Marincowitz et al. 2015) and EF2R (Jacobs et al. 2004), which were replaced in some cases with the primer pair F-728F (Carbone and Kohn 1999) and EF2 (O'Donnell et al. 1998). The CAL gene was amplified using the primer pairs CL2F and CL2R (Duong et al. 2012) or CL3F and CL3R (Musvuugwa et al. 2015).

Amplification of the gene regions was performed in a 25 µl reaction mixture. The reaction mixture contained 0.15 µl of MyTaq™ DNA Polymerase (5 U/µl) (Bioline, Massachusetts, USA), 2.5 µl of MyTaq™ Reaction Buffer (5×) containing dNTPs, $MgCl_2$ and enhancers for the optimal performance, and 0.50 µL of each primer (for EF2F and CAL degenerate primers 20 mM stock concentration; for other primers 10 mM stock concentration) (Whitehead Scientific Ltd, Cape Town, South Africa). PCR reactions were performed using an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with the following conditions: an initial denaturation step at 95 ^{o}C for 2 min, followed by 35 cycles of 30 s at 94 ^{o}C, 30 s at 55 ^{o}C and 1 min at 72 ^{o}C, and a final chain elongation at 72 ^{o}C for 7 min. PCR products were visualised under UV light after staining 5 µL aliquots with 2 µL of GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA) and separation on a 1 % agarose gel. Successfully amplified products were purified using the Exo-SAP protocol: the remaining PCR product (20 µl) was mixed with 8 µl of Exo-SAP [5 µl of Exonuclease I (20 U/µL) (Fermentas, Vilnius, Lithuania) and 100 µL of Shrimp Alkaline Phosphatase (1 U/µL) (Roche Diagnostics, Indianapolis, USA) in a 1000 µL reaction mixture], and incubated at 37 °C for 15 minutes, followed by immediate incubation at 80 °C for 15 minutes.

The sequencing reactions were performed in a 12 µl reaction mixture. The reaction mixture contained 0.5 µL of BigDye® Terminator v3.1 Ready Reaction mixture (Perkin-Elmer Applied Biosystems, Warrington, UK), 2.1 µL of sequencing buffer, 1 µL of either the forward or reverse primer (10 or 20 mM stock concentration) and 2 µL of cleaned PCR product. The primers used for sequencing the ITS, the TEF-1α and CAL gene regions were the same as those used for PCR. For sequencing the partial βT gene, the T10 primer was replaced with the primer Bt2a (Glass and Donaldson 1995). The thermal cycling conditions for sequencing reactions were: 25 cycles of 10 s at 96 °C, 5 s at 55 °C and 4 min at 60 °C. Sequencing products were cleaned using ethanol/salt precipitation and dried in a laminar flow overnight. Sequencing was done on the ABI Prism 3100 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the DNA Sequencing Facility of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

Sequence analyses

Consensus sequences were determined using the program Geneious R6 (Biomatters Ltd, Auckland, New Zealand), after which a preliminary identification of the isolates was obtained using a BLAST search against the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov). The sequences were deposited in GenBank and their accession numbers are presented in Table 1.

Individual data sets for the ITS, the βT, the TEF-1α and the CAL gene regions were used for phylogenetic analyses. Data sets were compiled and edited in Molecular Evolutionary Genetic Analysis (MEGA) v6.06-mac (Tamura et al. 2013). Sequence alignments were performed using the online version of MAFFT v7 (Katoh and Standley 2013). The ITS data set was aligned using the FFT-NS-i strategy with a 200PAM/κ=2 scoring matrix, a gap opening penalty of 1.53 and an offset value of 0.00. The βT, the TEF-1α and the CAL data sets consisted of closely related DNA sequences and were thus aligned using the G-INS-i strategy with a 1PAM/κ=2 scoring matrix, a gap opening penalty of 1.53 and an offset value of 0.00. Aligned data sets of the protein-coding genes were compared to gene maps constructed by Yin et al. (2015) to determine the presence or absence of introns and confirm that introns and exons were appropriately aligned.

Phylogenetic analyses were performed for each of the data sets using three different methods: maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI). ML analyses were performed using RAxML v7.0.4 (Stamakis 2014) on the CIPRES Science

Gateway v3.3 (Miller et al. 2010) employing the GTR substitution matrix and a rapid bootstrap analysis (Stamakis et al. 2008) to search for the best-scoring ML tree. The number of bootstrap replicates was estimated using the boot stopping criterion implemented in RAxML (Pattengale et al. 2010). MP analyses were conducted using PAUP v. 4.0b10 (Swofford 2002). Gaps and missing data were included in the MP analyses as a fifth character. BI analyses based on a Markov Chain Monte Carlo (MCMC) simulation were carried out with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). The best fitting evolutionary models for each data set were determined using MrModeltest v2.3 (Nylander 2004) based on the Akaike Information Criterion (AIC). The MCMC chains were run for five million generations using a sample frequency of 100 (resulting in 50000 trees). Burn-in values were calculated for the respective data sets, and all sampled trees having lower than the burn-in values were discarded. The remaining trees were used to construct majority rule consensus trees.

Morphological characterization

Morphological characteristics for selected isolates and herbarium specimens chosen to represent the type specimens were examined. Cultures were grown on 2 % MEA and on 2 % oat meal agar (OA; 15 g oatmeal, 20 g Difco Bacto™ agar and 1 L Milli-Q water) with and without host tree twigs that in some cases induced conidiophore and ascocarp formation. Autoclaved twigs with bark were placed at the centres of 2% MEA and OA agar plates. In an attempt to obtain sexual structures for morphological descriptions of species revealed by the phylogenetic analyses, crosses between different isolates of the same species were made in all possible combinations on Petri dishes to which autoclaved host tree twigs had been added (Grobbelaar et al. (2010). To serve as controls, isolates were inoculated individually on Petri dishes. Cultures were incubated at 25ºC and inspected regularly for the presence of fruiting structures.

Morphological characteristics were examined by mounting the sexual and asexual fruiting structures in 80 % lactic acid on glass slides, and these were observed using a Zeiss Axioskop microscope (Carl Zeiss, Germany) with a AxioCam ICc3, and a Nikon Eclipse 50i microscope (Nikon Corporation Tokyo, Japan) with a Nikon DS-Fi1 camera system (Nikon Corporation, Tokyo, Japan) to capture photographic images. Measurements were made of 50 each of the taxonomically relevant structures where this was possible. Averages, ranges and standard deviations were computed for the measurements. The measurements are presented in the format '(min–)(mean–SD)–(mean+SD)(–max)'. For scanning electron microscopy (SEM), specimens were prepared and studied as described by Linnakoski et al. (2009).

Culture characteristics

Growth characteristics of isolates in pure culture were considered for three representative isolates (Table 1) of each of the studied species following the method described by Linnakoski et al. (2009). Five replicate plates per isolate were used for each temperature (5, 10, 15, 20, 25, 30 and 35 °C). Colony diameters (three measurements per plate) were determined 4, 6 and 8 d after inoculation. From the diameter measurements, radial growth rates in mm/day were calculated. Mean radial growth rates (± standard deviation) at each temperature were calculated as an average of readings for each species.

RESULTS

DNA sequence analyses

The amplified DNA fragments were approximately 600 bp long for the ITS gene regions, 500 bp long for the partial βT, 900 bp long for the TEF-1α, and 700 bp long for the CAL genes. The aligned data set for the ITS gene region included 84 taxa and 782 characters (with gaps). The βT data set consisted of 67 taxa and 333 characters (with gaps), and included partial intron 3, exon 4, intron 4, exon 5 (no intron 5) and partial exon 6. The TEF-1α data set consisted of 34 taxa and 809 characters (with gaps), including intron 3, exon 4 (no intron 4), and partial exon 5. The CAL data set consisted of partial intron 3, exon 4, intron 4, exon 5 (no intron 5), exon 6, and partial intron 6. Intron 4 included a highly variable microsatellite region that was excluded in further analyses. The final aligned CAL data set used in the analyses consisted of 34 taxa and 470 characters (with gaps). The BI, ML and MP analyses for each data set produced trees with similar topologies (Fig. 2–5). The best-fitting substitution model selected for BI analyses was GTR+I+G for all the data sets.

The ITS data did not distinguish clearly between the closely related species considered in this study, but was useful to assign isolates to species complexes (Fig. 2). Therefore, the ITS sequences were used to show the placement of the isolates within *Ophiostoma sensu lato* (Fig. 2). Although monophyly was not strongly supported, the phylogenetic analyses of the ITS gene region (Fig. 2) showed that the isolates considered and defined by the type strain of each species in the ITS tree form a distinct complex of species within *Ophiostoma sensu stricto.*

The partial βT gene was used to identify isolates to species level (Fig. 3). The βT data set included sequences for all the living isolates considered in this study (Table 1). The TEF-1α and the CAL data were used to further confirm the identities of the isolates to species level (Fig.

4–5). The sequences of the type isolates and sequences that represent the isolates with most variation in respect to their origin from different host trees, bark beetle vectors and locations were included in the TEF-1α and the CAL data sets. Variation found in the β-tubulin gene region was sufficient to distinguish between the different species in the complex. In the βT data set, 86/333 (25.8%) positions were variable. Excluding the outgroup species (*O. tapionis* Linnakoski, Z.W. De Beer & M.J. Wingf. and *O. ainoae*), intraspecific sequence variation was found in all species in the complex. Intraspecific variability of the βT gene was observed in 19 positions within *O. brunneo-ciliatum*, in two positions within *O. brunneolum*, in two positions within *O. clavatum*, in one position within *O. macroclavatum*, and in 10 positions within *O. pseudocatenulatum*.

The three protein-coding genes revealed better resolution for species level assignment than the ITS data. Based on the βT and the TEF-1α data, isolates resided in seven well-supported lineages (Figs. 3–4). The CAL gene complemented the other gene regions, but did not provide well-supported separation of the species in the complex (Fig. 5). Two of the taxonomically well-defined species, *Ophiostoma tapionis* and *O. ainoae*, formed distinct lineages that reside peripheral to the complex, and this was also supported based on the ITS sequence data (Figs. 2–5). However, both fungi are morphologically similar to other species investigated here, and thus meet the criteria for the species complex.

Morphological characteristics

Herbarium specimens and cultures of isolates examined had several morphological similarities, which justified treating them as a complex of species. When ascocarps were present, the most distinct characteristics common in the herbarium specimens and the isolates were the brown, spirally coiled ostiolar hyphae and ascospores with a cylindrical to rectangular shape in side view. The cultures on MEA were typically hyaline at first, later becoming dark-pigmented (brown to almost black). Irregularly arranged isolated patches of pesotum-like, tall macronematal conidiomata with brush-like heads were produced infrequently in most cultures. In addition, mononematous conidiophores were present in most cases. The dimensions of most morphological structures were highly variable and overlapped among species in the complex. For some species in the complex, information related to their ecology was taxonomically informative.

The colors of the colonies of some of the isolates (CBS 117572 = CMW 5212, CMW 5214, CBS 117591, CMW 41049 and CMW 41050; Table 1) were distinctly lighter (hyaline/white) than those of other isolates, which were generally brown. These relatively old isolates are likely

to have lost their ability to produce pigment due to long-term storage and frequent sub-culturing.

Crosses in culture for isolates of *O. clavatum*, *O. brunneo-ciliatum* and *O. tapionis* did not result in the formation of sexual structures, similar to previous findings for these taxa (Mathiesen 1951, Mathiesen-Käärik 1953, Linnakoski et al. 2010). This was also the case for *O. ainoae* and the one of the undescribed taxa (*O. brunneolum*). Sexual structures were found in crosses for the other two undescribed taxa (*O. macroclavatum* and *O. pseudocatenulatum*), but not in the control plates, suggesting that these species are heterothallic. Morphology of the sexual states of these two species are described in the diagnosis below.

Taxonomy

Based on the phylogenetic analyses for multiple gene regions, as well as the morphological characteristics and ecology, the isolates studied formed seven distinct lineages that represent discrete taxa. These taxa make up a distinct species complex within *Ophiostoma s. str.* (De Beer and Wingfield 2013) that is defined here as the *O. clavatum* complex, named after the first species to be described in the complex. Two of these taxa, *O. ainoae* and *O. tapionis*, are previously described and well-defined species that are now assigned to the *O. clavatum* complex. Two other lineages include isolates of *O. brunneo-ciliatum* and *O. clavatum*. Typification of both these species is resolved below by designating lectotypes and epitypes for them. The remaining three lineages represent novel taxa that are, herewith, formally described. A complete nomenclator is provided for all taxa in the species complex. Country, host, and insect associate reports that have been confirmed using comparisons of multigene DNA sequence data, are annotated with an asterisk (*).

Ophiostoma ainoae H. Solheim, Nord. J. Bot. 6:201 (1986). MB 103626.

Descriptions: Solheim (1986, p. 201, Fig. 1); Yamaoka et al. (1997, pp 1219–20, Fig. 12-17).

Phylogenetic data: Okada et al (1998); Hausner and Reid (2003); Gebhardt et al. (2005); Zipfel et al. (2006); Linnakoski et al. (2008, 2010); De Beer and Wingfield (2013); Repe et al. (2013).

Original reports in literature: Solheim (1986, 1992, 1993); Harding (1989); Pashenova et al. (1995); Yamaoka et al. (1997); Grubelnik (1998); Viiri (1997); Kirschner (1998, 2001); Kirisits et al. (2000); Jankowiak (2004, 2005); Viiri and Lieutier (2004); Sallé et al. (2005);

Jankowiak et al. (2009); Kirisits (2010); Linnakoski et al. (2010)*; Jankowiak and Kot (2011); Repe et al. (2013)*.

Type material: NORWAY, Akerhus, Ås, Slørstad, from *Picea abies* infested by *Ips typographus*, 11 June 1980, *H. Solheim*, **holotype** CBS H-3559, culture ex-holotype CBS 205.83 = CMW 1037 = NFRI 80-48/15 and 80-69/27 = IMI 285082.

Other specimens examined: NORWAY, Akerhus, Ås, from *Picea abies* infested by *Ips typographus*, 1980, *H. Solheim*, CBS 118672 = CMW 1903; RUSSIA, Ohtama, Lake Vodlajärvi, from *Picea abies* infested by *Ips typographus*, June 2004, *J. Ahtiainen*, CBS 128299 = CMW 23123.

Host trees: *Larix sibirica*, *Picea abies**, *Picea jezoensis*, *Pinus sylvestris*

Insect associates: *Crypturgus pusillus*, *Dryocoetes autographus*, *Hylurgops glabratus*, *Hylurgops palliatus*, *Ips cembrae*, *Ips typographus**, *Ips typographus japonicus*, *Ips sexdentatus*, *Orthotomicus laricis*, *Pityogenes chalcographus**, *Pityogenes bidentatus*, *Pityophthorus pityographus*, *Polygraphus poligraphus*

Known distribution: Austria, Denmark, Germany, Finland*, France, Japan, Norway*, Poland, Russia*, Slovenia*, Sweden

Notes: The species was first mentioned by Käärik (1975b) as '*Ceratocystis* gr. *clavata*'. Perithecia in *O. ainoae* isolates from spruce in Europe have been observed only rarely. Based on DNA sequence data, *O. ainoae* has been confirmed to occur only on *P. abies* and in association with the spruce-infesting bark beetles *I. typographus* and *P. chalcographus* (Linnakoski et al. 2010; Repe et al. 2013). Reports of this species from larch- and pine-infesting bark beetles will, therefore, require confirmation, as they may refer to other members in the *O. clavatum* species complex. Yamaoka et al. (1997) reported this fungus, based on morphology, as an associate of *I. typographus japonicus* Niijima infesting *Picea jezoensis* (Sieb. & Zucc.) Carr. Our assumption is that the Japanese isolates possibly represent an undescribed species, for which the identity needs to be confirmed based on DNA sequence comparisons. A species similar to *O. ainoae*, the taxonomic placement of which requires further study, has been reported from Bhutan in association with the bark beetle *Ips schmutzenhoferi* Holzschuh on *Picea spinulosa* (Griffith) A. Henry and *Pinus wallichiana* A. B. Jacks. (Kirisits et al. 2013).

Ophiostoma brunneo-ciliatum Math.-Käärik, Medd. Statens Skogsforskningsinst. 43:44 (1953). MB 302071. Fig. 6.

≡ *Ceratocystis brunneo-ciliata* (Math.-Käärik) J. Hunt, Lloydia 19:32 (1956).

Descriptions: Mathiesen-Käärik (1953, pp 41–45, Fig. 2); Hunt (1956, p. 32); Aoshima (1965); Upadhyay (1981, p. 74, Fig. 236–241); Yamaoka et al. (1998, p. 371, Fig. 11–15).

Phylogenetic data: Hausner and Reid (2003); Linnakoski et al. (2010); Jankowiak (2012); De Beer and Wingfield (2013).

Original reports in literature: Mathiesen-Käärik (1953); Redfern et al. (1987); Lieutier et al. (1989, 1991); Yamaoka et al. (1998, 2009), Kirisits et al. (2000); Stauffer et al. (2001); Jankowiak et al. (2007, 2009); Linnakoski et al. (2010)*, Jankowiak (2012)*; Repe et al. (2013)*.

Type material: SWEDEN, Lule Lappmark, Jokkmokk, Murjek, from *Pinus sylvestris* infested by *Ips sexdentatus*, 9 September 1952, *E. Rennerfelt*, **lectotype** (designated here, MBT 204694) UPS:BOT:F-130962; POLAND, Pateraki, from galleries of *Ips sexdentatus* infesting *Pinus sylvestris*, 20 May 2011, *R. Jankowiak*, **reference specimen** TUR 205571, reference culture CBS 141266 = CMW 39827.

Other specimens examined: SWEDEN, Lule Lappmark, Jokkmokk, Murjek, from *Pinus sylvestris* infested by *Ips sexdentatus*, 9 September 1952, *E. Rennerfelt*, UPS:BOT:F-130963, UPS:BOT:F-130964, UPS:BOT:F-130965, UPS:BOT:F-130967, BPI 595721; POLAND, Wierzchosławice, from galleries of *Ips cembrae* infesting *Larix decidua*, 20 May 2011, *R. Jankowiak*, CBS 141267 = CMW 39828; POLAND, Babimost, from galleries of *Ips sexdentatus* infesting *Pinus sylvestris*, 05 August 2010, *R. Jankowiak* CBS 141268 = CMW 39842.

Host trees: *Picea abies**, *Pinus sylvestris**, *Larix decidua**, *Larix kaempferi*

Insect vectors: *Ips acuminatus*, *Ips amitinus**, *Ips cembrae**, *Ips sexdentatus**, *Ips subelongatus*

Known distribution: Austria, Japan, Sweden, Poland*, Slovenia

Notes: *Ophiostoma brunneo-ciliatum* was originally described from *I. sexdentatus* infesting *P. sylvestris* in Sweden (Mathiesen-Käärik 1953). No type specimen was formally designated for the species. Mathiesen-Käärik (1953) studied the specimens collected by Rennerfelt in 1946 and 1952. The 1952 collection is preserved in the Museum of Evolution,

University of Uppsala, Sweden, and includes six specimens of *O. brunneo-ciliatum* (UPS:BOT:F-130962–130967). Hunt (1956) studied some of these specimens, deposited one of them (AM-K 12-1) in the U.S. National Fungus Collections (BPI 595721), and made a new combination in *Ceratocystis* for the species. In the present study, one of the UPS specimens is designated as a lectotype of *O. brunneo-ciliatum*. Villari (2012) showed that the only living culture from the original material (CBS 149.54 = CMW 1029) was contaminated. We obtained a fresh isolate (CBS 141266 = CMW 39827) from Poland, collected from the same host tree and the same bark beetle species as that linked to the original collection. The sexual state and synnematal asexual state of the latter material (Fig. 6 d–e) were observed only in the galleries of *I. sexdentatus* on *P. sylvestris*, never in culture. This is in agreement with the observations of Mathiesen-Käärik (1953). The mononematal morphology and culture characteristics of this isolate corresponded to the original description (Fig. 6, Table 2). Since the new isolate did not originate from the same country as the lectotype, it does not meet the requirements for epitypification suggested by Ariyawansa et al. (2014). We have, therefore, designated a dried culture of this isolate (TUR 205571) as a reference specimen for the species.

The host tree and beetle vector appear to provide important information in support of the taxonomic characteristics of *O. brunneo-ciliatum*. Isolates for which the identity was confirmed in this study using DNA sequence analysis originated either from *I. sexdendatus* infesting *P. sylvestris* or from *I. cembrae* infesting *L. decidua* (Table 1). Repe et al. (2013) reported *O. brunneo-ciliatum* from *I. amitinus* based on a sequence of the ITS gene region. These and other, solely morphology-based reports of *O. brunneo-ciliatum* in association with *I. amitinus* (Kirisits et al. 2000; Jankowiak et al. 2009) should be confirmed with comparisons for multiple gene regions, because the ITS data does not allow definitive species level identification in the complex. Likewise, *O. pseudocatenulatum* also occurs in association with *I. cembrae* on *L. decidua* (see below), raising doubts regarding the identity of isolates assigned to *O. brunneo-ciliatum* in previous studies on the mycobiota of this bark beetle species (Kirisits et al. 2000; Stauffer et al. 2001; Jankowiak et al. 2007). Yamaoka et al. (1998, 2009) reported *O. brunneo-ciliatum* as a dominant associate of *I. subelongatus* infesting *L. kaempferi*, but confirmation of species identity based on DNA sequence data is needed. Taking into account that substantial differences in the assemblages of ophiostomatoid fungi associated with *I. cembrae* in Europe and with *I. subelongatus* in Japan have been documented (Stauffer et al. 2001; Yamaoka et al. 2009; Masuya et al. 2013), the Japanese isolates could represent an undescribed species.

Isolates of *O. brunneo-ciliatum* showed considerable variation in culture color that ranged from light brown to almost black (Fig. 6 f). However, this variation could not be linked to

different host trees or bark beetle associates. In addition, micro-morphological comparisons showed no differences among the different isolates and together with the sequence data, it is clear that they belong to a single taxon. Measurements of the sexual and asexual structures also corresponded well with those in previous descriptions of *O. brunneo-ciliatum* (Fig. 6, Table 2).

Ophiostoma brunneolum Linnakoski, Z.W. De Beer & M.J. Wingf., **sp. nov**. MB 816636. Fig. 7.

Etymology: The epithet refers to the brown colony color of this species.

Sexual state not observed. *Conidiophores* micronematous. Hyalorhinocladiella-like asexual state present (Fig 7a–b). *Conidiogenous cells* arising directly from hyphae, (5–)14–28(–35) × 1–1.5 µm. *Conidia* hyaline, cylindrical, (3–)4–6(–7.5) × (1–)1.5–2(–2.5) µm, occasionally produced directly from hyphae. Secondary conidia occasionally produced. *Culture* characteristics: colonies on 2 % MEA hyaline at first, later becoming brown in the center, hyphae superficial, aerial mycelium sparse (Fig. 7c.). Optimal growth temperature at 25ºC. Culture growth rates 4.3 mm/d (± 0.2) at 20ºC and 5.6 mm/d (± 0.2) at 25ºC. No growth observed at 5ºC and at 35ºC.

Type material: RUSSIA, Ohtama, Vodlajärvi, from *Picea abies* infested with *Ips typographus*, September 2004, *J. Ahtiainen*, **holotype** TUR 205572, culture ex-holotype CBS 128227 = CMW 23143; RUSSIA, Ohtama, Vodlajärvi, from *Picea abies* infested with *Ips typographus*, September 2004, *J. Ahtiainen*, **paratype** TUR 205573, culture ex-paratype CBS 141078 = CMW 23142; RUSSIA, Ohtama, Vodlajärvi, from *Picea abies* infested with *Ips typographus*, September 2004, *J. Ahtiainen*, **paratype** TUR 205574, culture ex-paratype CBS 141269 = CMW 23144.

Host trees: *Picea abies**

Insect vectors: *Ips duplicatus**, *Ips typographus**

Known distribution: Czech Republic*, Russian Karelia*

Notes: Only a hyalorhinocladiella-like asexual state is currently known for *O. brunneolum*, which is similar that of *O. tapionis* that also resides in this complex. Isolates of this species were previously recognised as *O. brunneo-ciliatum* and treated as members of the *Ophiostoma ips* complex (Linnakoski et al. 2010).

Ophiostoma clavatum Math., Svensk Bot. Tidskr. 45:222 (1951). MB 302072. Figs 8–9.

≡ *Ceratocystis clavata* (Math.) J. Hunt, Lloydia 19:37 (1956).

Descriptions: Mathiesen (1950, p. 298, Fig. 10), Mathiesen (1951, pp 219–223, Fig. 5), Hunt (1956, pp 37–38), Upadhyay (1981, p. 137).

Phylogenetic data: no previous data available.

Original reports in literature: Mathiesen (1950, 1951); Rennerfelt (1950); Mathiesen-Käärik (1953); Francke-Grosmann (1952, 1963a); Aoshima (1965); Käärik (1975); Villari (2012)*; Villari et al. (2013)*.

Type material: SWEDEN, Dalarna, Hamra, from galleries of *Ips acuminatus* infesting *Pinus sylvestris*, 18 August 1950, *E. Rennerfelt*, **lectotype** (designated here, MBT 204695) UPS:BOT:F-130972; SWEDEN, Lunsen, Uppsala, from *Ips acuminatus* infesting *Pinus sylvestris*, 4 June 2009, *C. Villari*, **epitype** (designated here, MBT 204696) TUR 205575, culture ex-epitype CBS 141080 = CMW 37983.

Other specimens examined: ITALY, Val Venosta, *from Ips acuminatus* infesting *Pinus sylvestris*, 27 November 2008, *C. Villari*, CMW 37986; FRANCE, Var, from *Pinus sylvestris* infested with *Ips acuminatus*, 1997, *A. Yart*, herbarium specimen TUR 205576, culture CBS 141274 = CMW 37988; AUSTRIA, Carinthia, Bleiberg, from *Pinus sylvestris* infested with *Ips acuminatus*, 10 September 2012, *T. Kirisits & G. Hoch*, herbarium specimen TUR 205577, culture CBS 141183 = IFFF AC/1/IV/2 = CMW 41123.

Host trees: *Pinus sylvestris**, *Picea abies*

Insect vectors: *Ips acuminatus**, *Ips sexdentatus*, *Orthotomicus proximus*, *Tomicus piniperda*

Known distribution: Austria*, France*, Germany, Italy*, Japan, Norway*, Sweden*, former Yugoslavia

Notes: Rennerfelt (1950) first reported this species as *Ophiostoma* I from *P. sylvestris* infested by *I. acuminatus* in Sweden. Based on the material of Rennerfelt, Mathiesen (1951) provided a morphological description and named the fungus *O. clavatum*, but she did not designate a type specimen. The specimens of Mathiesen (who published later under the surnames Mathiesen-Käärik and Käärik) were originally stored in the herbarium of the Statens Skogforskningsinstitut, Experimentalfältet, Sweden. An *O. clavatum* specimen (AM-K 8-1) was studied (Table 3) by Hunt (1956) and Upadhyay (1981). Neither of these authors was able

to find sexual fruiting structures in this specimen, which could not be located for the present study. Nevertheless, Hunt (1956) considered the species valid and provided a new combination in *Ceratocystis*. Upadhyay (1981) considered the species as a *nomen dubium* due to the lack of sexual fruiting structures. De Beer et al. (2013) consequently suggested that the name *O. clavatum* should be resurrected by epitypification.

The original specimen of E. Rennerfelt (UPS:BOT:F-130972) is preserved in the herbarium of the Museum of Evolution, Uppsala. In the absence of a holotype, we have designated this specimen as a lectotype of *O. clavatum*. Unfortunately, the specimen lacks the pesotum-like asexual state of the fungus (Mathiesen 1951, p. 221, Fig. 5c). However, the line drawings of Mathiesen (1951), who studied this specimen, clearly depict the asexual state (Fig. 9). The original living cultures were deposited at the CBS, but the isolate deposited as *O. clavatum* at the CBS by Mathiesen-Käärik (CBS 135.51 = CMW 1028) has been identified as *Ophiostoma minus* (Hedgc.) Syd. (Villari 2012). Two isolates from the Norwegian Institute of Bioeconomy Research, Ås, Norway (CMW 41041 and CMW 41042) (Table 1) could represent original material from Mathiesen's (1951) description. Unfortunately, the labels of these cultures have been lost and, therefore, their origin remains uncertain. Sequences of these isolates are included in the phylogenetic analyses arising from the present study and they group with other *O. clavatum* isolates (Figs. 3–5). In the absence of other available authenticated material, we designate a recently collected, morphologically similar isolate originating from the same host tree species, associated bark beetle species and geographical location where the fungus was originally collected, as epitype of *O. clavatum*.

Subsequent to its original description, *O. clavatum* also has been reported from other pine-infesting bark beetles, including *I. sexdentatus*, *Orthotomicus proximus* Eichh. and *T. piniperda* (Käärik 1973, 1975a), as well as from *P. abies* (Käärik 1980). However, no material exists from these reports and the host information remains to be confirmed based on new isolations and DNA sequence data. For the present, *O. clavatum* should best be treated as an associate of only *I. acuminatus* infesting *P. sylvestris*. Ascomata develop only rarely in culture (Mathiesen-Käärik 1951), and in this study the production of the sexual stage was not observed. The sexual stage has been observed only on naturally infected wood. Isolates of *O. clavatum* produce both mononematous to synnematous conidiophores in culture. The typical tall, brush-like conidiomata were rare in most isolates growing on MEA and were more commonly produced on OA. Comparisons of cultures and morphological characteristics showed no differences among the isolates originating from different regions (Table 1). The cultures were dark brown

to almost black (Fig. 8). Measurements of the sexual and asexual structures corresponded well with those in previous descriptions of *O. clavatum* (Fig. 8, Table 3).

Ophiostoma macroclavatum Linnakoski, Z.W. De Beer & M.J. Wingf., **sp. nov.** MB 816637. Fig. 10.

Etymology: The epithet refers to the size and shape of the synnematous conidiophores that distinguish this species from the other species in the complex.

Sexual state rarely produced when two strains of opposite mating type are paired on agar with wood (sterilised spruce twigs). *Ascomata* superficial on media (Fig. 10a), bases dark, globose, (133–)158–195(-217) µm diam, sparsely ornamented with brown hyphal hairs (11–)17–35(–46) µm long, 1–1.5 µm wide at the apex, 1.5–2 µm wide at the base, necks black, straight or slightly curved, (467–)650–918(–1010) µm long, (29–)33–44(–52) µm wide at the base, (12–)14–19(–24) µm wide at the apex. Necks have pointed, knob-like protrusions near the base (Fig. 10a). *Ostiolar hyphae* absent or present (Fig. 10a,c), spirally coiled, septated, brown, becoming hyaline towards apex, (7–)9–11(–12) in number, (14–)19–33(–144) µm long, (1–)1.5–2.5(–3) µm wide at the base, 1–1.5(–2) µm wide at the apex. *Asci* not observed. *Ascospores* one-celled, cylindrical to rectangular in side view, (2–)3–3.5(–4) × 1–1.5 µm (Fig. 10b). *Conidiophores* macronematous, synnematous pesotum-like, spirally coiled (Fig. 8d–e), hyaline, stalk bases occasionally pale brown, (2184–)3117–5172(–6330) µm long including capitulum, (27–)58–111(–137) µm wide at the base; conidiophore heads (108–)223–577(–887) µm wide, composed of brush-like masses of hyphae. *Conidiogenous cells* (15–)22–34(–46) × 1–1.5 µm; conidia hyaline, one-celled, cylindrical, (3.5–)5–7(–8) × 1.5–2(–3) µm (Fig. 10f), aggregating into a cream-colored mucilaginous mass surrounding the conidiophore heads (Fig. 10d). Synnematal asexual state rare on malt extract agar, more commonly produced on oatmeal agar. Conidiophores first formed in the center of colony, later found in irregularly arranged isolated patches. Hyalorhinocladiella-like micronematal asexual state present (Fig 10g). Conidiogenous cells arising directly from hyphae, (7–)11–21(–26) × 1–1.5) µm; conidia hyaline, cylindrical, 4–5.5(–7) × 1–1.5(–2) µm. *Culture characteristics*: Colonies on 2 % MEA first hyaline, later becoming olivaceous black (Fig. 10h), mycelium superficial on agar, aerial mycelium sparse. Growth rates 4.7 mm/d (± 1.0) at 20°C and 6.3 mm/d (± 1.0) at 25°C. Optimal growth at 25°C. No growth observed at 5°C and 35°C.

Type material: RUSSIA, Lisino-Korpus, from *Pityogenes chalcographus* infesting *Pinus sylvestris*, February 2006, *R. Linnakoski*, **holotype** TUR 205578 (dried culture obtained from

cross between CMW 23115 × CMW 23118), culture ex-type CBS 141081 = CMW 23115; RUSSIA, Lisino-Korpus, from *Pityogenes chalcographus* infesting *Picea abies*, February 2006, *R. Linnakoski*, **paratype** TUR 205579, culture ex-paratype CBS 141082 = CMW 23116; RUSSIA, Lisino-Korpus, from *Picea abies* infested with *Ips typographus*, February 2006, *R. Linnakoski*, **paratype** TUR 205580, culture ex-paratype CBS 141085 = CMW 23120.

Other specimens examined: RUSSIA, Lisino-Korpus, from *Pityogenes chalcographus* infesting *Pinus sylvestris*, February 2006, *R. Linnakoski*, culture CBS 141084 = CMW 23118

Host trees: *Picea abies**, *Pinus sylvestris**

Insect vectors: *Ips amitinus**, *Ips duplicatus**, *Ips typographus**, *Pityogenes chalcographus**

Known distribution: Czech Republic*, Estonia*, Finland*, Poland*, Russian Karelia*

Notes: Isolates of this species were previously treated as *O. brunneo-ciliatum* in the *Ophiostoma ips* complex by Linnakoski et al. (2010). It is probable that this taxon was also treated as *O. ainoae* or *O. brunneo-ciliatum* in earlier publications (e.g., Kirisits et al. 2000; Jankowiak 2005; Jankowiak et al. 2009; Repe et al. 2013). In an unpublished study of fungi associated with *I. amitinus* in Finland and Estonia more than half of the individuals were carrying a fungus tentatively named *O. ainoae* (H. Solheim, unpublished data). Two isolates from this survey that were included in the present study were both *O. macroclavatum*.

Ophiostoma pseudocatenulatum Jankowiak, R. Linnakoski & Z.W. De Beer, **sp. nov**. MB 816638. Fig. 11.

Etymology: The specific epithet refers to the ascospores that often accumulate in a formation resembling a chain.

Sexual state often produced on wood when the two mating types are paired on MEA with sterilised larch twigs (Fig. 11a), rarely produced on MEA. *Ascomata* superficial on media (Fig. 11b), bases dark, globose, (155–)202–256(–279) µm diam, sparsely ornamented with brown hyphal hairs (11–)15–55(–11) µm long, 0.5–1.5 µm wide at the apex, 1.5–2.5 µm wide at the base, necks black, straight or slightly curved, (442–)599–859(–1064) µm long, (30–)35–54(–77) µm wide at the base, (12–)14–19(–25) µm wide at the apex. *Ostiolar hyphae* absent or present (Fig. 10c), light brown with lighter and blunt apex, spirally coiled, aseptate, (3–)9–14(–15) in number, (16–)25–42(–55) µm long, (1–)1.5–2.5(–3) µm wide at the base, (0.5–)0.5–1(–

1.5) µm wide at the apex. *Asci* not observed. *Ascospores* one-celled, cylindrical or ovoid (when ascospores have a hyaline sheath) in side view, straight, (3–)3.5–4(–5) × (1–)1.5–2 (–2) µm, sometimes with hyaline sheath, 0.5–1 µm thick, released from neck in chains (Fig. 11d). *Conidiophores* macronematous, synnematous, pesotum-like, aggregated in small groups resembling sporodochia (Fig. 11e–f), hyaline, stalk bases rarely pale brown, (1366–)1931–3696(–4534) µm long including capitulum, (18–)58–144(–320) µm wide at the base; conidiophore heads (124–)178–948(–1641) µm wide, composed of brush-like masses of hyphae. *Conidiogenous cells* (13–)17–22(–46) × 0.5–1.5 µm; conidia hyaline, one-celled, cylindrical, (3.5–)4.5–7(–9.5) × (0.5)1–1.5(–2) µm (Fig. 10g–h), aggregating into a cream-colored mucilaginous mass surrounding the conidiophore heads (Fig. 11e). Synnematal anamorph rare on malt extract agar, more commonly produced on agar with larch twigs. Hyalorhinocladiella-like micronematal asexual state present (Fig 11i). Conidiogenous cells arising directly from hyphae, (7–)11–21(–26) × 1–1.5) µm; conidia hyaline, cylindrical, obovate, 4–5.5 (–7) × 1–1.5(–2) µm (Fig. 10j). *Culture characteristics*: Colonies on 2 % MEA first hyaline, later becoming olivaceous gray (Fig. 11k), mycelium superficial on agar, aerial mycelium quite abundant, floccose. Growth rates 5.8 mm/d (± 0.8) at 20ºC and 8.0 mm/d (± 0.9) at 25ºC. Optimal growth at 30ºC (8.1 mm/d; ± 1.0). Growth observed at 5ºC (1.3 mm/d) and 35ºC (4.4 mm/d).

Type material: POLAND, Rudziniec, from galleries of *Ips cembrae* infesting *Larix decidua*, 26 June 2014, *R. Jankowiak*, **holotype** TUR 205581 (dried culture obtained from cross between CMW 43103 × CMW 43106), culture ex-type CBS 141276 = CMW 43103; POLAND, Rudziniec, from adult *Ips cembrae* infesting *Larix decidua*, 26 June 2014, *R. Jankowiak*, **paratype** TUR 205582, culture ex-type CMW 43097; POLAND, Rudziniec, from galleries of *Ips amitinus* infesting *Larix decidua*, 17 June 2014, *R. Jankowiak*, **paratype** TUR 205583, culture ex-type CBS 141278 = CMW 43106.

Host tree: *Larix decidua**

Insect vectors: *Ips amitinus**, *Ips cembrae**

Known distribution: Austria*, Czech Republic*, Poland*, Scotland*

Notes: Isolates of this species were recognised as *O. brunneo-ciliatum* in previous publications (Kirisits et al. 2000; Stauffer et al. 2001; Jankowiak et al. 2007; Linnakoski et al. 2010; Villari 2012; Villari et al. 2013). This fungus (and not *O. brunneo-ciliatum*) is likely the most frequent fungal associate of the larch bark beetle *I. cembrae* (Kirisits et al. 2000; Stauffer et al. 2001;

Jankowiak et al. 2007); however, *O. brunneo-ciliatum* has also been confirmed in association with this insect (see above). It is likely that isolates from *I. amitinus* in previous studies (Kirisits et al. 2000; Jankowiak et al. 2009; Repe et al. 2013) also represent this fungus or/and *O. macroclavatum* (see above).

Ophiostoma tapionis Linnakoski, Z.W. De Beer & M.J. Wingf., Persoonia 25:84 (2010). MB 518882.

Descriptions: Linnakoski et al. (2010)

Phylogenetic data: Linnakoski et al. (2010); De Beer and Wingfield (2013)

Original reports in literature: Linnakoski et al. (2010)*

Type material: FINLAND, Southern Savonia, Punkaharju, from *Hylurgops palliatus* infesting *Picea abies*, February 2006, *R. Linnakoski*, **holotype** KUO 021872, culture ex-holotype CBS 128120 = CMW 23265; FINLAND, Southern Savonia, Punkaharju, from *Hylastes brunneus* infesting *Pinus sylvestris*, February 2006, *R. Linnakoski*, **paratype** KUO 021873, culture ex-paratype CBS 128122 = CMW 23266; RUSSIA, Lisino-Korpus, from *Hylurgops palliatus* infesting *Pinus sylvestris*, February 2006, *R. Linnakoski*, **paratype** KUO 021875, culture ex-paratype CBS 128121 = CMW 23269.

Host trees: *Picea abies**, *Pinus sylvestris**

Insect vectors: *Hylastes brunneus**, *Hylastes cunicularius**, *Hylurgops palliatus**, *Ips typographus**, *Pityogenes chalcographus**, *Tomicus minor**

Known distribution: Finland*, Norway, Poland*, Russian Karelia*

Notes: Only a hyalorhinocladiella-like asexual state is currently known for this fungus. In the original description, isolates of this species were recognised as members of the *Ophiostoma ips* complex (Linnakoski et al. 2010). This species has been recently found as an associate of *H. palliatus* in Norway (H. Solheim, unpublished data) and *Hylastes cunicularius* in Poland (R. Jankowiak, unpublished data).

DISCUSSION

Results of this study provide robust support for a newly recognised and phylogenetically clearly defined complex in *Ophiostoma sensu stricto*. The *O. clavatum* complex defined here currently accommodates seven morphologically similar species. The species boundaries in the complex are redefined and descriptions have been provided for three new *Ophiostoma* species. Lectotypes and epitypes have also been designated for *O. clavatum* and *O. brunneo-ciliatum*.

Species assigned to the *O. clavatum* complex share several similarities that distinguish them from the other species within *Ophiostoma s. str.* (De Beer and Wingfield 2013). The oldest known species in the complex, *O. clavatum*, together with four other species that have known sexual stages (*O. ainoae*, *O. brunneo-ciliatum*, *O. macroclavatum* sp. nov. and *O. pseudocatenulatum* sp. nov.), are characterised by brown, spirally coiled ostiolar hyphae and cylindrical to rectangular ascospores, sometimes covered by a thin sheath. The asexual stages of these species range from mononematous hyalorhinocladiella- to synnematous pesotum-like forms. The pesotum-like conidiophores represent two different types: synnematous conidiophores of *O. ainoae* are more similar to e.g. those of *O. ulmi* (Upadhyay 1981; De Beer and Wingfield 2013), and large synnematous, clavate, 'brush-like' conidiomata are found in *O. brunneo-ciliatum*, *O. clavatum*, *O. macroclavatum* sp. nov. and *O. pseudocatenulatum* sp. nov. These are unique morphological characteristics not known to occur in other species in *Ophiostoma s. str*. The full-grown cultures in agar plates are typically darkly pigmented. However, cultures lose their pigmentation over time when they are sub-cultured. All species in the complex are associates of conifer-infesting bark beetles, most notably of beetles in the genus *Ips*.

Results of this study confirmed the identity of *O. clavatum* isolates collected from *I. acuminatus* infesting *P. sylvestris* in Europe. It appears that the fungus has a relatively specific association with this bark beetle species and its host tree. This apparently strict association has also been reported in several previous studies (Mathiesen 1950, 1951; Rennerfelt 1950; Mathiesen-Käärik 1953; Francke-Grosmann 1952, 1963a; Käärik 1975a, 1980; Villari 2012; Villari et al. 2013). Although the majority of publications support the view that *O. clavatum* is the primary associate of *I. acuminatus*, some of the previous reports of fungi associated with this beetle species raise questions. For example, an isolate collected from *I. acuminatus* (CMW 37988) that had previously been treated as *O. brunneo-ciliatum* (Lieutier et al. 1991; Guérard et al. 2000) was identified as *O. clavatum* in the present study. This finding most likely excludes the association of *O. brunneo-ciliatum* with *I. acuminatus*. Although *O. clavatum* has been reported from other pine-infesting bark beetles (Käärik 1973, 1975a) as

well as from spruce (Käärik 1980), no material was available from these studies. We could thus not confirm an association between *O. clavatum* and other bark beetles or host trees.

Ophiostoma brunneo-ciliatum was originally isolated from *P. sylvestris* infested by *I. sexdentatus* in Sweden (Mathiesen-Käärik 1953). Unfortunately, recently collected isolates are not available from the same vector beetle and tree host in the same geographic area where the first collections of the fungus were made. At the time of Mathiesen-Käärik's (1953) study, *I. sexdentatus* was a common bark beetle in Sweden (Voolma et al. 2004). However, reports of *I. sexdentatus* in Nordic countries have declined in recent decades, and the beetle is currently included in the national Red Lists in Finland, Norway and Sweden (ArtDatabanken 2015; Kålås et al. 2010; Rassi et al. 2010). Based on the present study, isolates identified as *O. brunneo-ciliatum* are found in association with *I. sexdentatus* infesting *P. sylvestris* and *I. cembrae* infesting *L. decidua*. This observation is consistent with previous studies, in which the fungus has been reported from *I. sexdentatus* infesting *Pinus* spp. (Mathiesen-Käärik 1953, 1960; Lieutier et al. 1989, 1991; Bueno et al. 2010; Jankowiak 2012) as well from *I. cembrae* infesting *L. decidua* in Europe (Kirisits et al. 2000; Stauffer et al. 2001; Jankowiak et al. 2007). However, *O. brunneo-ciliatum* appears to be a frequent associate of mainly *I. sexdentatus*, while *I. cembrae* most likely vectors this species only occasionally, and *O. pseudocatenulatum* may be the dominant fungus associated with the latter insect.

There have been numerous previous reports of *O. brunneo-ciliatum* in Europe, suggesting that the fungus is associated with various different bark beetles and host trees (Lieutier et al. 1989, 1991; Kirisits et al. 2000; Kirisits 2001, 2004; Bueno et al. 2010; Stauffer et al. 2001; Jankowiak et al. 2007, 2009; Linnakoski et al. 2010; Linnakoski 2011; Jankowiak 2012; Repe et al. 2013). For this reason, we re-examined some isolates from our previous studies identified as *O. brunneo-ciliatum* (Linnakoski et al. 2010; Linnakoski 2011; Villari et al. 2013; R. Jankowiak, B. Strzałka and B. Kacprzyk 2014, unpublished data). The multigene phylogenetic analyses provided robust evidence that none of these isolates represent *O. brunneo-ciliatum*. Although morphological characteristics and ecology overlap, substantial differences in phylogenetic data clearly supports the treatment of these isolates as novel species, described here as *O. brunneolum* sp. nov., *O. macroclavatum* sp. nov. and *O. pseudocatenulatum* sp. nov.

Ophiostoma brunneolum was found in association with *I. typographus* and *I. duplicatus* in Russia and the Czech Republic. Only the hyalorhinocladiella-like asexual stage of this fungus is currently known. This species appears to be relatively rare in the investigated habitats, at least in comparison to *O. macroclavatum*. *Ophiostoma macroclavatum* differs from the other species in the complex by its apparently wider occurrence in association with several spruce- and pine-

infesting bark beetles, and also based on its morphological characteristics. The most distinct feature of *O. macroclavatum* is its exceptionally large pesotum-like conidiomata. The third novel species, *O. pseudocatenulatum*, has a distinct ecology as an associate of the bark beetles *I. cembrae* and *I. amitinus* on *L. decidua* in Europe. Unlike the other novel species, *O. pseudocatenulatum* has ascospores that emerge from perithecial necks in chains. In addition, it has a substantially more rapid growth on MEA and a higher optimal temperature for growth in culture than any other species in the *O. clavatum* complex. These features may be connected to the ecology of *L. decidua*. As a light-demanding tree species, *L. decidua* often colonizes disturbed forest sites and open land, and is exposed to strong solar insolation. For this reason, fungal associates of bark beetles infesting larch would need to be able to colonize strongly heated sapwood and phloem of trees.

Identification of species residing in the *O. clavatum* complex has previously relied strongly on morphological characteristics but more recently on sequence data for the ITS region. Results of the present study show that reliable delimitation of these cryptic species requires multigene DNA sequence data and phylogenetic analyses. Of the four gene regions used in this study, only the ITS data failed to provide sufficient resolution for species delineation within the *O. clavatum* complex. Therefore, ITS sequence data should be used with caution for species level identification purposes. Furthermore, isolates of species identified in previous studies could be the same as the novel cryptic taxa described here, or they could represent additional novel species in the *O. clavatum* complex. In line with other investigations delineating cryptic species within the ophiostomatoid fungi, the present study implies that the species in the *O. clavatum* complex are more specifically associated with particular bark beetles than has been recognised previously, suggesting close co-evolution between the insects and their specific fungal associates.

Very little is known regarding potential pathogenicity of the species residing in the *O. clavatum* complex. The species have generally been considered as weak or non-aggressive pathogens that cause rapidly spreading blue-stain in sapwood of *P. sylvestris* (Mathiesen 1951; Käärik 1980; Lieutier et al. 1991; Guérard et al. 2000). However, there is evidence that some of these fungi might be involved in exhausting their host plant defenses (Lieutier et al. 1991; Guérard et al. 2000; Villari et al. 2012). The large number of recently emerging and severe outbreaks of beetles vectoring species in this complex suggests that these fungi could become increasingly important as agents of blue-stain or forest pathogens. This underpins the need for correct identification of these fungi, and further studies are clearly required to better understand their distribution, ecology and pathogenicity.

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REFERENCES

Altenkirch W, Majunke C, Ohnesorge B (2002) Waldschutz auf ökologischer Grundlage. Verlag Eugen Ulmer, Stuttgart, Germany

Aoshima K (1965) Studies on wood-staining fungi of Japan. Dissertation, University of Tokyo [in Japanese]

ArtDatabanken (2015) Rödlista 2010. ArtDatabanken, SLU, Uppsala, Sweden. Available from http://www.slu.se/artdatabanken (accessed April 2015) [in Swedish]

Ariyawansa HA, Hawksworth DL, Hyde KD, Jones EBG, Maharachchikumbura SSN, Manamgoda DS, Thambugala KM, Udayanga D, Camporesi E, Daranagama A, Jayawardena R, Liu J-K, McKenzie EHC, Phookamsak R, Senanayake IC, Shivas RG, Tian Q, Xu JC (2014) Epitypification and neotypification: guidelines with appropriate and inappropriate examples. Fungal Divers 69:57–91

Bueno A, Diez JJ, Fernández MM (2010) Ophiostomatoid fungi transported by *Ips sexdentatus* (Coleoptera; Scolytidae) in *Pinus pinaster* in NW Spain. Silva Fenn 44:387–397

Carbone I, Kohn LM (1999) A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91:553–556

Colombari F, Battisti A, Schroeder LM, Faccoli M (2012) Life-history traits promoting outbreaks of the pine bark beetle *Ips acuminatus* (Coleoptera: Curculionidae, Scolytinae) in the south-eastern Alps. Eur J Forest Res 3:553–561. doi:10.1007/s10342-011-0528-y

Colombari F, Schroeder M, Battisti A, Faccoli M (2013). Spatio-temporal dynamics of *Ips acuminatus* outbreak and implications for management. Agr Forest Entomol 15:34−42. doi:10.1111/j.1461-9563.2012.00589.x

De Beer ZW, Seifert KA, Wingfield MJ (2013) A nomenclator for ophiostomatoid genera and species in the *Ophiostomatales* and *Microascales*. In: Seifert KA, De Beer ZE, Wingfield MJ (eds) Ophiostomatoid fungi: expanding frontiers. CBS Biodiv Ser 12:261–268

De Beer ZW, Wingfield MJ (2013) Emerging lineages in the *Ophiostomatales*: In: Seifert KA, De Beer ZE, Wingfield MJ (eds) Ophiostomatoid fungi: expanding frontiers. CBS Biodiv Ser 12:21–46

Dobbertin M, Wermelinger B, Bigler C, Bürgi M, Carron M, Foster B, Gimmi U, Rigling M (2007) Linking increasing drought stress to Scots pine mortality and bark beetle infestations. Sci World J 7:231–239. doi:10.1100/tsw.2007.58

Duong TA, De Beer ZW, Wingfield BD, Wingfield MJ (2012) Phylogeny and taxonomy of species in the *Grosmannia serpens* complex. Mycologia 104:715–732. doi:10.385/11–109

Francke-Grosmann H (1952) Über die Ambrosiazucht der beiden Kiefernborkenkäfer *Myelophilus minor* Htg. und *Ips acuminatus* Gyll. Medd Statens Skogsforskningsinst 41:1–52

Francke-Grosmann H (1963a) Die Übertragung der Pilzflora bei dem Borkenkäfer *Ips acuminatus* Gyll.: Ein Beitrag zur Kenntnis der Ipiden-Symbiosen. Z Angew Entomol 52:355–361

Francke-Grosmann H (1963b) Some new aspects in forest entomology. Ann Rev Entomol 8:415–438

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for Basidiomycetes – application to the identification of mycorrhiza and rusts. Mol Ecol 2:113–118

Gebhardt H, Weiss M, Oberwinkler F (2005) *Dryadomyces amasae*: a nutritional fungus associated with ambrosia beetles of the genus *Amasa* (Coleoptera: Curculionidae, Scolytinae). Mycol Res 109:687–696

Glass NL, Donaldson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Appl Environ Microbiol 61:1323–1330

Grobbelaar J, de Beer ZW, Bloomer P, Wingfield MJ, Wingfield BD (2010) *Ophiostoma tsotsi* sp. nov., a wound- infesting fungus of hardwood trees in Africa. Mycopathol 169:413–423. doi:10.1007/s11046-009-9267-8

Grubelnik RJ (1998) Untersuchungen über die Zusammensetzung der Mycoflora von *Ips typographus* L. auf ausgewählten Wald-Standorten unter besonderer Berücksichtigung der pathogenen Art *Ceratocystis polonica*. Master/Diploma Thesis, Universität für Bodenkultur Wien (BOKU)

Guérard N, Dreyer E, Lieutier F (2000) Interactions between Scots pine, *Ips acuminatus* (Gyll.) and *Ophiostoma brunneo-ciliatum* (Math.): estimation of the critical thresholds of attack and

inoculation densities and effects on hydraulic properties in the stem. Ann For Sci 57:681–690. doi:10.1051/forest:2000149

Harding S (1989) The influence of mutualistic blue-stain fungi on bark beetle population dynamics. Dissertation, Royal Veterinary and Agricultural University Copenhagen

Hausner G, Reid J (2003) Notes on *Ceratocystis brunnea* and some other *Ophiostoma* species based on partial ribosomal DNA sequence analysis. Can J Bot 81:865–876. doi:10.1139/b03-080

Hunt J (1956) Taxonomy of the genus *Ceratocystis*. Lloydia 19:1–59

Jacobs K, Bergdahl DR, Wingfield MJ, Halik S, Seifert KA, Bright DE, Wingfield BD (2004) *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. Mycol Res 108:411–418. doi:10.1017/S0953756204009748

Jankowiak R (2004) Ophiostomatoid fungi associated with the spruce bark beetle (*Ips typographus*) new for Poland: occurrence and morphology. Phytopathol Pol 33:5–21

Jankowiak R (2005) Fungi associated with *Ips typographus* on *Picea abies* in southern Poland and their succession into the phloem and sapwood of beetle-infested trees and logs. Forest Pathol 35:37–55

Jankowiak R (2012) Ophiostomatoid fungi associated with *Ips sexdentatus* on *Pinus sylvestris* in Poland. Dendrobiology 68:43–54

Jankowiak R, Kot M (2011) Ophiostomatoid fungi associated with bark beetles (Coleoptera: Scolytidae) colonizing branches of *Pinus sylvestris* in Southern Poland. Pol Bot J 56:287–293

Jankowiak R, Rossa R, Místa K (2007) Survey of fungal species vectored by *Ips cembrae* to European larch trees in Raciborskie forests (Poland). Czech Mycol 59:227–239

Jankowiak R, Kacprzyk M, Młynarczyk M (2009) Diversity of ophiostomatoid fungi associated with bark beetles (Coleoptera: Scolytidae) colonizing branches of Norway spruce (*Picea abies*) in southern Poland. Biologia 64:1170–1177. doi:10.2478/s11756-009-0188-2

Katoh K, Standley D (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 30:772–780. doi:10.1093/molbev/mst010

Kirisits T (2001) Studies on the association of ophiostomatoid fungi with bark beetles in Austria with special emphasis on *Ips typographus* and *Ips cembrae* and their associated fungi *Ceratocystis polonica* and *Ceratocystis laricicola*. Dissertation, Universität für Bodenkultur Wien (BOKU)

Kirisits T (2004) Fungal associates of European bark beetles with emphasis on the Ophiostomatoid fungi. In: Lieutier F, Day KR, Battisti A, Grégoire J-C, Evans H (eds) Bark and wood boring insects in living trees in Europe, a synthesis. Kluwer Academic Publishers, Dordrecht, pp 181–235

Kirisits T (2010) Fungi isolated from *Picea abies* infested by the bark beetle *Ips typographus* in the Białowieża forest in north-eastern Poland. Forest Pathol 40:100–110. doi:10.1111/j.1439-0329.2009.00613.x

Kirisits T (2013) Dutch elm disease and other *Ophiostoma* diseases. In: Gonthier P, Nicolotti, G (eds) Infectious forest diseases. CABI, Wallingford, pp 256–282

Kirisits T, Grubelnik R, Führer E (2000) Die ökologische Bedeutung von Bläuepilzen für rindenbrütende Borkenkäfer. In: Müller F (ed) Mariabrunner Waldbautage 1999. Umbau sekundärer Nadelwälder. FBVA- Berichte, Schriftenreihe der Forstlichen Bundesversuchsanstalt, Wien 111, pp 117–137. http://bfw.ac.at/040/pdf/1348_02.pdf (accessed November 2015)

Kirisits T, Konrad H, Wingfield MJ, Chhetri DB (2013) Ophiostomatoid fungi associated with the Eastern Himalayan spruce bark beetle, *Ips schmutzenhoferi*, in Bhutan and their pathogenicity to *Picea spinulosa* and *Pinus wallichiana*. In: Seifert KA, De Beer ZE, Wingfield MJ (eds) Ophiostomatoid fungi: expanding frontiers. CBS Biodiv Ser 12:99–112

Kirschner R (1998) Diversität mit Borkenkäfern azzoziierter filamentöser Mikropilze. Dissertation, Eberhard-Karls-Universtität, Tübingen

Kirschner R (2001) Diversity of filamentous fungi in bark beetle galleries in Central Europe. In: Misra JK, Horn BW (eds) *Trichomyces* and other fungal groups. Robert W. Lichtwardt Commemoration Volume. Science Publishers, Inc, Enfield, Plymouth, pp 175–196

Kålås J, Viken Å, Henriksen S, Skjelseth S. (eds) (2010). The 2010 Norwegian Red List for Species. Norwegian Biodiversity Information Centre, Norway

Käärik A (1973) The succession of blueing fungi in insect galleries in roundwood during storage. Research Note No R 83. Royal Coll. For. Dep. Forest Products, Stockholm, Sweden, pp 1–20

Käärik A (1975a) Insekter och blånad. Uppsatser/Sveriges Lantbruksuniversitet, Institutionen för Virkeslära, 0349-8913; 54 Skogs- och Virkesskydd: 170–187

Käärik A (1975b) Succession of microorganisms during wood decay. In: Biological transformation of wood by microorganisms. In: Liese W (ed) Proceedings of the Sessions on Wood Products Pathology at the 2nd International Congress of Plant Pathology, 10–12 September 1973, Minneapolis, USA. Springer-Verlag, Berlin, pp 39–51

Käärik, A (1980) Fungi causing sap stain in wood. The Swedish University of Agricultural Sciences, Department of Forest Products. Report Nr R 114

Krehan H (2011) Borkenkäferprobleme bei Kiefern in einem Steinschlagschutzwald in Kärnten. Forstschutz Aktuell 53:2–4. http://bfw.ac.at/400/pdf/fsaktuell_53_1.pdf (accessed November 2015)

Lieutier F, Yart A, Garcia J, Ham MC, Morelet M, Levieux J (1989) Champignons phytopathogènes associés à deux coléeoptères scolytidae du pin sylvestre (*Pinus sylvestris* L.) et étude préliminaire de leur agressivité envers l'hote. Ann Sci For 46:201–216

Lieutier F, Garcia J, Yart A, Vouland G, Pettinetti M, Morelet M (1991) Ophiostomatales (Ascomycetes) associated with *Ips acuminatus* Gyll. (Coleoptera, Scolytidae) in Scots pine (*Pinus sylvestris* L.) in South-Eastern France, and comparison with *Ips sexdentatus* Boern. Agronomie 11:807–817 [in French]

Linnakoski R (2011) Bark beetle-associated fungi in Fennoscandia with special emphasis on species of *Ophiostoma* and *Grosmannia*. Dissertationes Forestales 119. doi:10.14214/df.119

Linnakoski R, De Beer ZW, Rousi M, Solheim H, Wingfield MJ (2009). *Ophiostoma denticiliatum* sp. nov. and other *Ophiostoma* species associated with the birch bark beetle in southern Norway. Persoonia 23:9–15. doi:10.3767/003158509X46803

Linnakoski R, De Beer ZW, Rousi M, Niemelä P, Pappinen A, Wingfield MJ (2008) Fungi, including *Ophiostoma karelicum* sp. nov., associated with *Scolytus ratzeburgi* infesting birch in Finland and Russia. Mycol Res 112:1475–1488. doi:10.1016/j.mycres.2008.06.007

Linnakoski R, De Beer ZW, Ahtiainen J, Sidorov E, Niemelä P, Pappinen A, Wingfield MJ (2010) *Ophiostoma* spp. associated with pine- and spruce-infesting bark beetles in Finland and Russia. Persoonia 25:72–93. doi:10.3767/003158510X550845

Marincowitz S, Duong TA, De Beer ZW, Wingfield MJ (2015) *Cornuvesica*: A little known mycophilic genus with a unique biology and unexpected new species. Fungal Biol 119:615–630. doi:10.1016/j.funbio.2015.03.007

Masuya H, Yamaoka Y, Wingfield MJ (2013). Ophiostomatoid fungi and their associations with bark beetles in Japan. In: Seifert KA, De Beer ZE, Wingfield MJ (eds) Ophiostomatoid fungi: expanding frontiers. CBS Biodiv Ser 12:77–90

Mathiesen A (1950) Über einige mit Borkenkäfern assoziierte Bläuepilze in Schweden. Oikos 2:275–308

Mathiesen A (1951) Einige neue *Ophiostoma*-Arten in Schweden. Sven Bot Tidskr 45:203–232

Mathiesen-Käärik A (1953) Eine Übersicht über die gewöhnlichsten mit Borkenkäfern assoziierten Bläuepilze in Schweden und einige für Schweden neue Bläupilze. Medd Statens Skogsforskningsinst 43:1–74

Mathiesen-Käärik A (1960) Studies on the ecology, taxonomy and physiology of Swedish insect-associated blue stain fungi, especially the genus *Ceratocystis*. Oikos 11:1–25

Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Sciences Gateway for inference of large phylogenetic trees. In: Proceedings of the Gateway Computing Environments Workshop (GCE). New Orleans, LA, USA. November 14, 2010, pp 1–8

Musvuugwa T, De Beer ZW, Duong TA, Dreyer LL, Oberlander KC, Roets F (2015) New species of Ophiostomatales from Scolytinae and Platypodinae beetles in the Cape Floristic Region, including the discovery of the sexual state of *Raffaelea*. A Van Leeuw J Microb 108:933–950. doi:10.1007/s10482-015-0547-7

Nylander JAA (2004) MrModeltest v.2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Sweden

O'Donnel K, Cigelnik E (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol Phylogenet Evol 7:103–116. doi:10.1006/mpev.1996.0376

O'Donnell K, Kistler HC, Cigelnik E, Ploetz RC (1998) Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. PNAS 95:2044–2049

Okada G, Seifert KA, Takematsu A, Yamaoka Y (1998) A molecular phylogenetic reappraisal of the *Graphium* complex based on 18S rDNA sequences. Can J Bot 76:1495–1506. doi:10.1139/b98-089

Pashenova NV, Vetrova V P, Matrenina RM, Sorokina E N (1995) *Ophiostoma* fungi in the galleries of the larch bark beetle. Lesovedenie 6:62–68

Pattengale ND, Alipour M, Bininda-Emonds OR, Moret BM, Stamakis A (2010) How many bootstrap replicates are necessary? J Comput Biol 17:337–354. doi:10.1089/cmb.2009.0179

Rassi P, Hyvärinen E, Juslén A, Mannerkoski I. (eds.) (2010) The 2010 Red List of Finnish Species. Ympäristöministeriö & Suomen ympäristökeskus, Helsinki

Redfern DB, Stoakley JT, Steele H, Minter DW (1987) Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae*. Plant Pathol 36:467–480

Rennerfelt E (1950) Über den Zusammenhang zwischen dem Verblauen des Holzes und den Insekten. Oikos 2:120–137

Repe A, Kirisits T, Piškur, de Groot M, Kump B, Jurc M (2013) Ophiostomatoid fungi associated with three spruce-infesting bark beetles in Slovenia. Ann For Sci 70:717–727. doi:10.1007/s13595-013-0311-y

Rebetez M, Dobbertin M (2004) Climate change may already threaten Scots pine stands in the Swiss Alps. Theor Appl Climatol 79:1–9. doi:10.1007/s00704-004-0058-3

Robert V, Vu D, Amor AB, van de Wiele N, Brouwer C, Jabas B, Szoke S, Dridi A, Triki M, Ben Daoud S, Chouchen O, Vaas L, de Cock A, Stalpers JA, Stalpers D, Verkley GJ, Groenewald M, Dos Santos FB, Stegehuis G, Li W, Wu L, Zhang R, Ma J, Zhou M, Gorjon SP, Eurwilaichitr L, Ingsriswang S, Hansen K, Schoch C, Robbertse B, Irinyi L, Meyer W, Cardinali G, Hawksworth DL, Taylor JW, Crous PW (2013). MycoBank gearing up for new horizons. IMA Fungus 4:371–379. doi:10.5598/imafungus.2013.04.02.16

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574. doi:10.1093/bioinformatics/btg180

Sallé A, Monclus R, Yart A, Garcia J, Romary P, Lieutier F (2005) Fungal flora associated with *Ips typographus*: frequency, virulence, and ability to stimulate the host defence reaction in relation to insect population levels. Can J For Res 35:365–373

Seifert K.A., De Beer Z.W, Wingfield M.J. (eds) (2013) Ophiostomatoid fungi: Expanding frontiers. Utrecht, CBS-KNAW Fungal Biodiversity Centre. CBS Biodiversity Series 12

Siitonen, J. (2014) *Ips acuminatus* kills pines in southern Finland. Silva Fenn 48: article id 1145. doi:10.14214/sf.1145

Solheim H (1986) Species of Ophiostomataceae isolated from *Picea abies* infested by the bark beetle *Ips typographus*. Nord J Bot 6:199–207

Solheim H (1992) The early stages of fungal invasion in Norway spruce infested by the bark beetle *Ips typographus*. Can J Bot 70:1–5. doi:10.1139/b92-001

Solheim H (1993) Fungi associated with the spruce bark beetle *Ips typographus* in an endemic area in Norway. Scan J Forest Res 8:118–122. doi:10.1080/02827589309382760

Stamakis A (2014) RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312–1313. doi:10.1093/bioinformatics/btu033

Stamakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML web-servers. Syst Biol 57:758–771. doi:10.1080/10635150802429642

Stauffer C, Kirisits T, Nussbaumer C, Pavlin R, Wingfield MJ (2001) Phylogenetic relationships between the European and Asian eight spined larch bark beetle populations (Coleoptera, Scolytidae) inferred from DNA sequences and fungal associates. Eur J Entomol 98:99–105

Swofford DL (2002) PAUP* 4.0: phylogenetic analysis using parsimony (* and other methods). Sinauer Associates, Sunderland

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30:2725–2729. doi:10.1093/molbev/mst197

Upadhyay HP (1981) A monograph of *Ceratocystis* and *Ceratocystiopsis*. The University of Georgia Press, Georgia, USA

Viiri H (1997) Fungal associates of the spruce bark beetle *Ips typographus* L. (Col. Scolytidae) in relation to different trapping methods. J Appl Entomol 121:529–533

Viiri H, Lieutier F (2004) Ophiostomatoid fungi associated with the spruce bark beetle, *Ips typographus*, in three areas in France. Ann For Sci 61:1–5

Villari C (2012) Fungi associated with the pine engraver beetle *Ips acuminatus* and their interactions with the host tree. Dissertation, Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente, Universita' Degli Studi Di Padova

Villari C, Battisti A, Chakraborty S, Michelozzi M, Bonello P, Faccoli M (2012) Nutritional and pathogenic fungi associated with the pine engraver beetle trigger comparable defenses in Scots pine. Tree Physiol 37:867–879. doi:10.1093/treephys/tps056

Villari C, Tomlinson JA, Battisti A, Boonham N, Capretti P, Massimo F (2013) Use of loop-mediated isothermal amplification for detection of *Ophiostoma clavatum*, the primary blue stain fungus associated with *Ips acuminatus*. Appl Environ Microbiol 79:2527–2533. doi:10.1128/AEM.03612-12

Voolma K, Mandelshtam MJ, Shcherbakov AN, Yakovlev EB, Õunap H, Süda I, Popovichev BG, Sharapa TV, Galasjeva TV, Khairetdinov RR, Lipatkin VA, Mozolevskaya EG (2004) Distribution and spread of bark beetles (Coleoptera: Scolytidae) around the Gulf of Finland: a comparative study with notes on rare species of Estonia, Finland and North-Western Russia. Entomol Fenn 15:198–210

Wermelinger B, Rigling A, Schneider Mathis D, Dobbertin M (2008) Assessing the role of bark- and wood-boring insects in the decline of Scots pine (*Pinus sylvestris*) in the Swiss Rhone valley. Ecol Entomol 33:239−249. doi:10.1111/j.1365-2311.2007.00960.x

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, California, pp 315–321

Wingfield M.J., Seifert K.A., Webber J.F. (eds) (1993) *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. APS Press, St Paul, MN

Yamaoka Y, Wingfield MJ, Takahashi I, Solheim H (1997) Ophiostomatoid fungi associated with the spruce bark beetle *Ips typographus* f. *japonicus* in Japan. Mycol Res 101:1214–1227

Yamaoka Y, Wingfield MJ, Ohsawa M, Kuroda Y (1998) Ophiostomatoid fungi associated with *Ips cembrae* in Japan and their pathogenicity to Japanese larch. Mycoscience 39:367–378

Yamaoka Y, Chung W-H, Masuya, H, Hizai, M (2009) Constant association of ophiostomatoid fungi with the bark beetle *Ips subelongatus* invading Japanese larch logs. Mycoscience 50:165–172

Zipfel RD, De Beer ZW, Jacobs K, Wingfield BD, Wingfield MJ (2006) Multi-gene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. Stud Mycol 55:75–97. doi:10.3114/sim.55.1.133

Fig. 1 a Exposed galleries of *Ips acuminatus* on the sapwood surface of *Pinus sylvestris* and **b-c** extensive blue-stain development in the sapwood of *Pinus sylvestris* caused by its fungal associate *Ophiostoma clavatum*. *Scale bars* **a, c** 1 cm

Fig. 2 Phylogenetic tree obtained from ML analyses of the ITS data set. Isolates used for DNA sequencing in this study are printed in **bold** type. Bootstrap support values above 70 % for ML/MP are presented at the nodes. Posterior probabilities (above 70 %) obtained from BI are indicated by bold lines at the relevant branching points. * = bootstrap values < 70 %. T = ex-type isolates. *Scale bar* = total nucleotide difference between taxa. *S.* = *Sporothrix*

Fig. 3 Phylogenetic tree obtained from ML analyses of the βT data set of the *Ophiostoma clavatum* complex isolates. Isolates used for DNA sequencing in this study are printed in **bold** type. Bootstrap support values above 70 % for ML/MP are presented at the nodes. Posterior probabilities (above 70 %) obtained from BI are indicated by bold lines at the relevant branching points. * = bootstrap values < 70 %. T = ex-type isolates. *Scale bar* = total nucleotide difference between taxa

Fig. 4 Phylogenetic tree obtained from ML analyses of the TEF-1α data set of the *Ophiostoma clavatum* complex isolates. Isolates used for DNA sequencing in this study are printed in **bold** type. Bootstrap support values above 70 % for ML/MP are presented at the nodes. Posterior probabilities (above 70 %) obtained from BI are indicated by bold lines at the relevant branching points. * = bootstrap values < 70 %. T = ex-type isolates. *Scale bar* = total nucleotide difference between taxa

Fig. 5 Phylogenetic tree obtained from ML analyses of the CAL data set of the *Ophiostoma clavatum* complex isolates. Isolates used for DNA sequencing in this study are printed in **bold** type. Bootstrap support values above 60 % for ML/MP are presented at the nodes. Posterior probabilities (above 60 %) obtained from BI are indicated by bold lines at the relevant branching points. * = bootstrap values < 60 %. T = ex-type isolates. *Scale bar* = total nucleotide difference between taxa

Fig. 6 Morphological characteristics of the *Ophiostoma brunneo-ciliatum* lectotype specimen (UPS:BOT:F-130972) (**a–c**), as well as of structures in the *Ips sexdentatus* galleries on *Pinus sylvestris* and living cultures (CMW 39827, CMW 39828, CMW 39842) (**d–f**). **a** Ascoma. **b**

Spirally coiled, brown ostiolar hyphae. **c** Rectangular ascospores. **d** Pesotum-like conidioma (found only in the bark beetle galleries). **e**. Brush-like conidioma (found only in the bark beetle galleries). **f** Cultures on MEA. *Scale bars* **a, d–e** 100 µm, **b–c** 10 µm

Fig. 7 Morphological characteristics of *Ophiostoma brunneolum* (CMW 23143). **a** Hyalorhinocladiella-like asexual state. **b** Conidia. **c** Culture on MEA. *Scale bars* **a–b** 10 µm

Fig. 8 Morphological characteristics of the lectotype (UPS:BOT:F-130972) of *Ophiostoma clavatum* (**a–c**), as well as of living cultures (CMW 37983, CMW 37988) (**d–f**). **a** Ascoma. **b** Spirally coiled, brown ostiolar hyphae. **c** Rectangular ascospores. **d** Brush-like conidioma. **e** Hyalorhinocladiella-like asexual state. **f** Cultures on MEA. *Scale bars* **a, d** 100 µm, **b–c, e** 10 µm

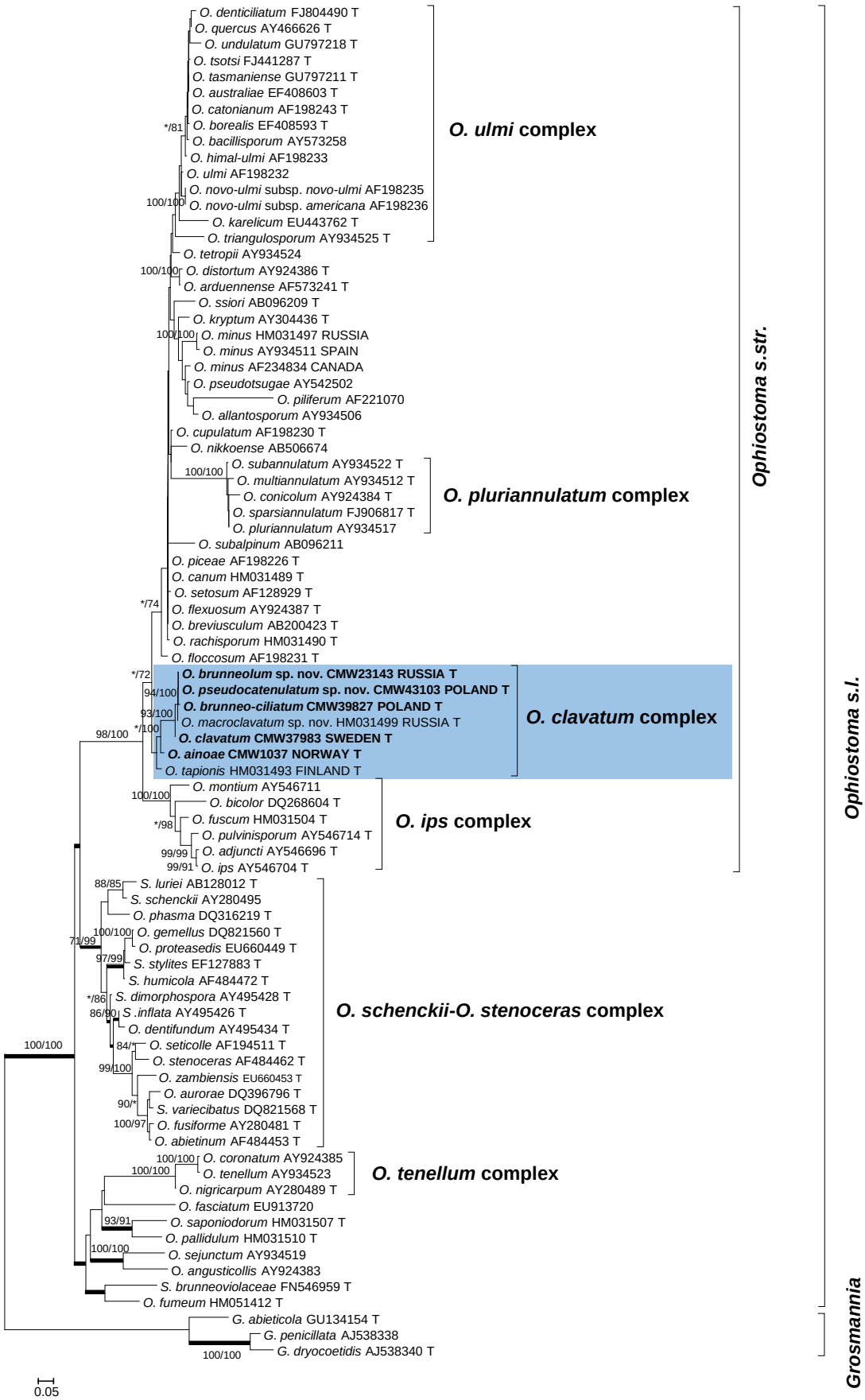
Fig. 9 Line drawings of *Ophiostoma clavatum* from Mathiesen (1951, p. 221, Fig. 5c). **a** Ascoma. **b** Ascospores. **c** Brush-like conidioma. **d** Spirally coiled, brown ostiolar hyphae. **e** Hyalorhinocladiella-like conidiophore. **f** conidia. *Scale bars* **a, c** 300 µm, **b, f** 10 µm, **d** 40 µm, **e** 30 µm.

Fig. 10 Morphological characteristics of *Ophiostoma macroclavatum* sp. nov. (CMW 23115) **a** Ascoma formed on spruce twigs (CMW 25115 × CMW 23118). **b** Rectangular ascospores. **c** Spirally coiled, brown ostiolar hyphae. **d–e** Brush-like conidioma. **f** Conidia. **g** Hyalorhinocladiella-like conidiophore. **h** Culture on MEA. *Scale bars* **a, e** 100 µm, **b–c** 10 µm, **d** 1000 µm, **f–g** 10 µm

Fig. 11 Morphological characteristics of *Ophiostoma pseudocatenulatum* sp. nov. (CMW 43103). **a** Ascoma formed on larch twigs (CMW 43103 × CMW 43106). **b** Ascoma. **c** Rectangular ascospores. **d**. Spirally coiled, brown ostiolar hyphae. **e–f** Brush-like conidioma. **g** Conidia. **h.** Hyalorhinocladiella-like conidiophore and conidia. **i** Culture on MEA. *Scale bars* **b, f** 250 µm, **d** 25 µm, **c, g, h** 10 µm

Figure 1

Figure 2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O. catonianum AF198243 T
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O. borealis EF408593 T
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O. himal-ulmi AF198233
O. ulmi AF198232
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O. novo-ulmi subsp. novo-ulmi AF198235
O. novo-ulmi subsp. americana AF198236
O. karelicum EU443762 T
O. triangulosporum AY934525 T
O. ulmi complex
O. tetropii AY934524
100/100
O. distortum AY924386 T
O. arduennense AF573241 T
O. ssiori AB096209 T
O. kryptum AY304436 T
100/100
O. minus HM031497 RUSSIA
O. minus AY934511 SPAIN
O. minus AF234834 CANADA
O. pseudotsugae AY542502
O. piliferum AF221070
O. allantosporum AY934506
O. cupulatum AF198230 T
O. nikkoense AB506674
100/100
O. subannulatum AY934522 T
O. multiannulatum AY934512 T
O. conicolum AY924384 T
O. sparsiannulatum FJ906817 T
O. pluriannulatum AY934517
O. pluriannulatum complex
O. subalpinum AB096211
O. piceae AF198226 T
O. canum HM031489 T
O. setosum AF128929 T
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O. breviusculum AB200423 T
O. rachisporum HM031490 T
O. floccosum AF198231 T
Ophiostoma s.str.
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O. pseudocatenulatum sp. nov. CMW43103 POLAND T
93/100
O. brunneo-ciliatum CMW39827 POLAND T
*/100
O. macroclavatum sp. nov. HM031499 RUSSIA T
98/100
O. clavatum CMW37983 SWEDEN T
O. ainoae CMW1037 NORWAY T
O. tapionis HM031493 FINLAND T
O. clavatum complex
100/100
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O. bicolor DQ268604 T
*/98
O. fuscum HM031504 T
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99/99
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99/91
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O. phasma DQ316219 T
100/100
O. gemellus DQ821560 T
71/99
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S. stylites EF127883 T
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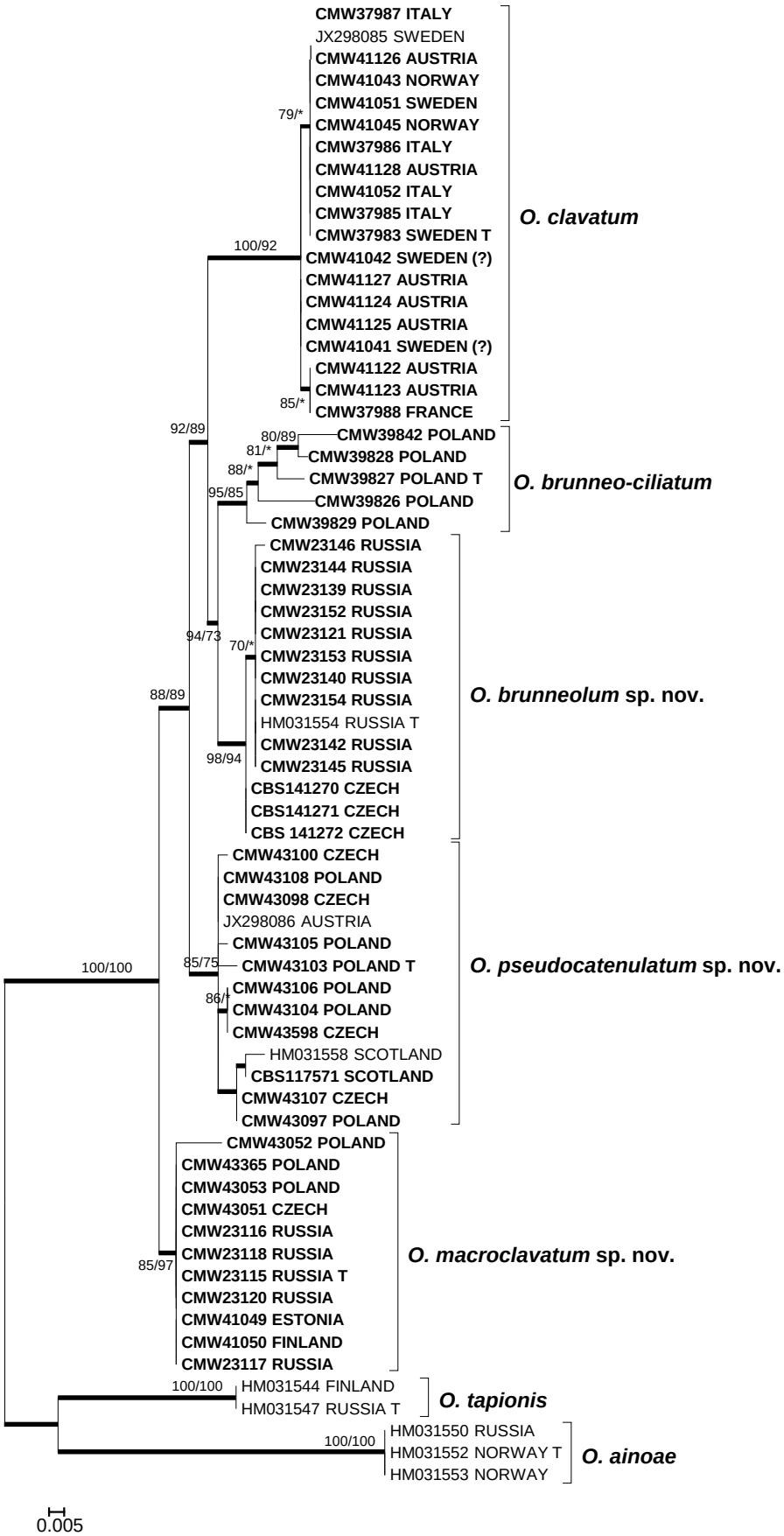
Figure 3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CMW37988 FRANCE
O. clavatum
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CMW39828 POLAND
CMW39827 POLAND T
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CMW39829 POLAND
O. brunneo-ciliatum
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CMW23144 RUSSIA
CMW23139 RUSSIA
CMW23152 RUSSIA
CMW23121 RUSSIA
CMW23153 RUSSIA
CMW23140 RUSSIA
CMW23154 RUSSIA
HM031554 RUSSIA T
CMW23142 RUSSIA
CMW23145 RUSSIA
CBS141270 CZECH
CBS141271 CZECH
CBS 141272 CZECH
O. brunneolum sp. nov.
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JX298086 AUSTRIA
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CMW43598 CZECH
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CBS117571 SCOTLAND
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CMW43051 CZECH
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CMW23118 RUSSIA
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CMW23120 RUSSIA
CMW41049 ESTONIA
CMW41050 FINLAND
CMW23117 RUSSIA
O. macroclavatum sp. nov.
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HM031547 RUSSIA T
O. tapionis
HM031550 RUSSIA
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O. ainoae
79/*
100/92
85/*
92/89
80/89
81/*
88/*
95/85
94/73
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85/97
100/100
100/100
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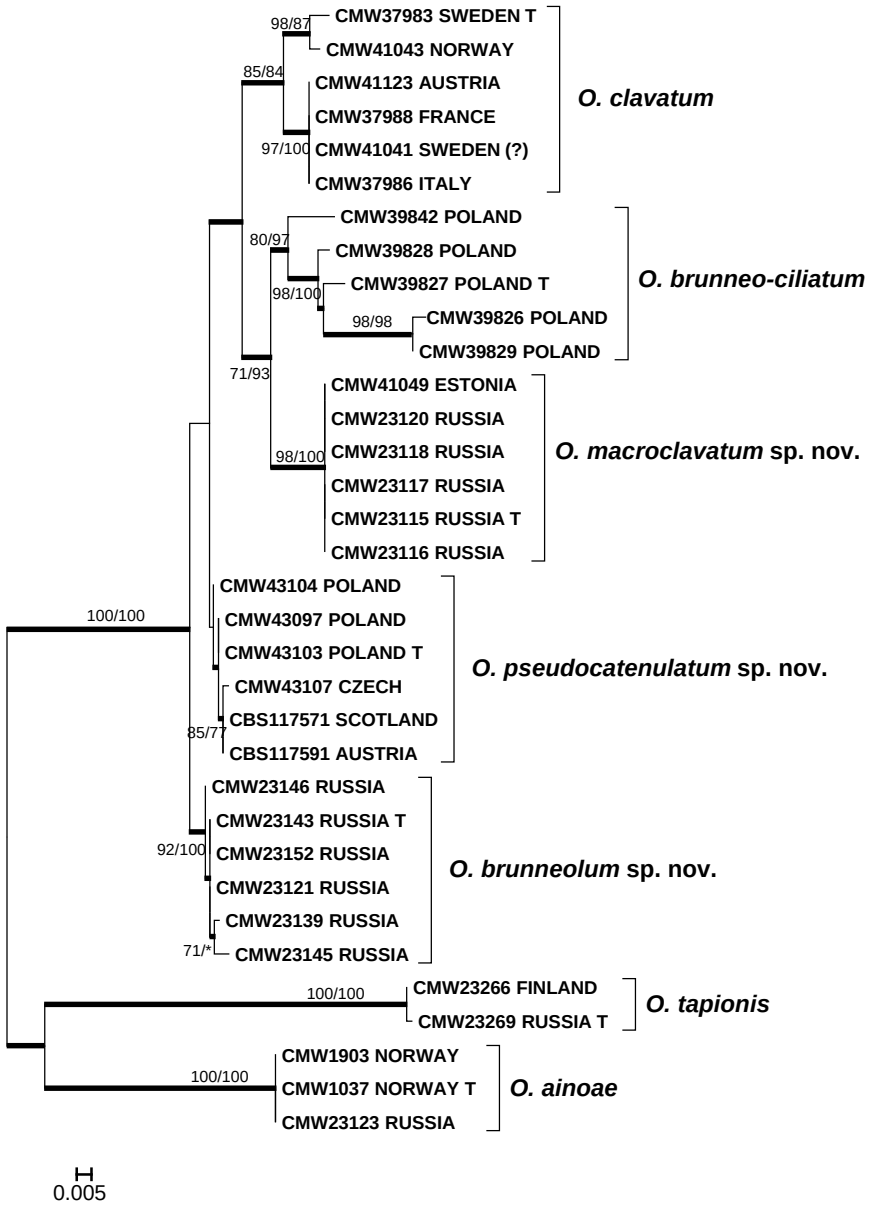
Figure 4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80/97
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98/100
98/98
CMW39826 POLAND
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O. brunneo-ciliatum
71/93
CMW41049 ESTONIA
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98/100
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100/100
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85/77
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92/100
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71/*
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100/100
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O. tapionis
100/100
CMW1903 NORWAY
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O. ainoae
0.005

Figure 5


CMW39829 POLAND
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66/*
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98/100
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O. pseudocatenulatum sp. nov.
100/100
CMW23269 RUSSIA T
CMW23266 FINLAND
O. tapionis
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100/100
CMW1903 NORWAY
CMW1037 NORWAY T
O. ainoae
0.005

Figure 6

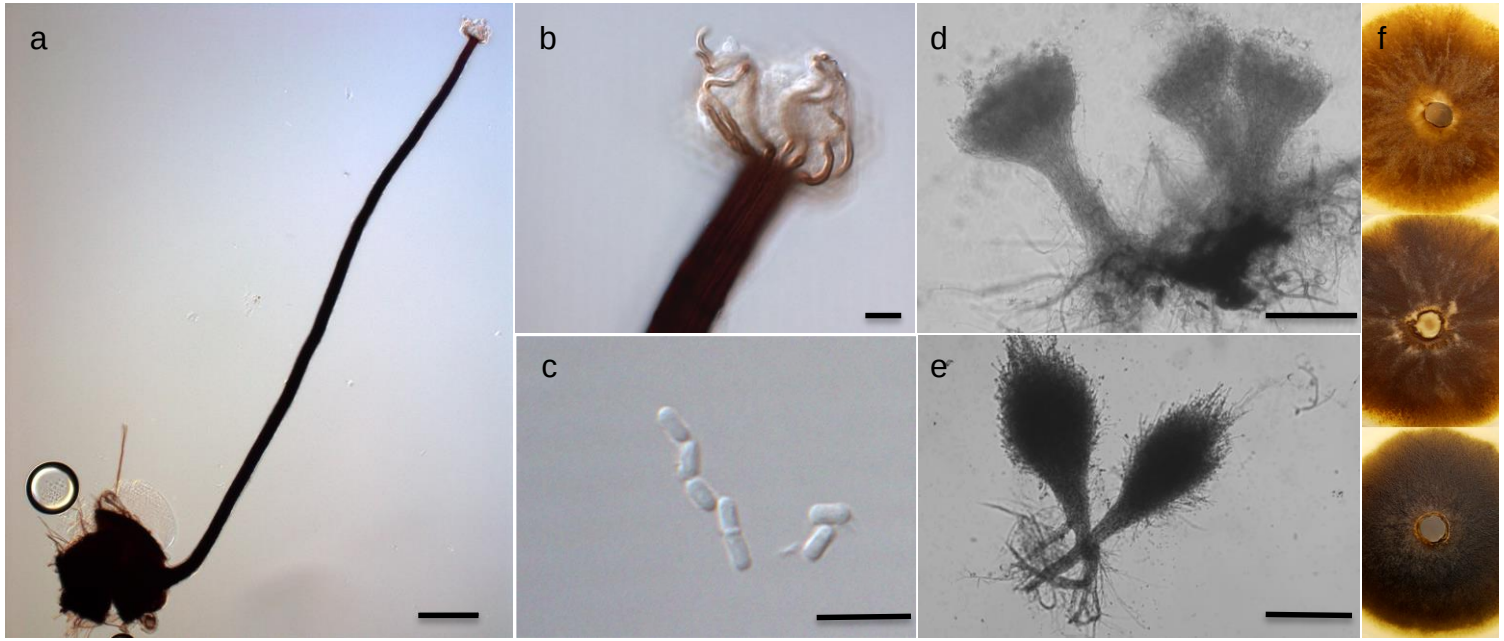

Figure 7

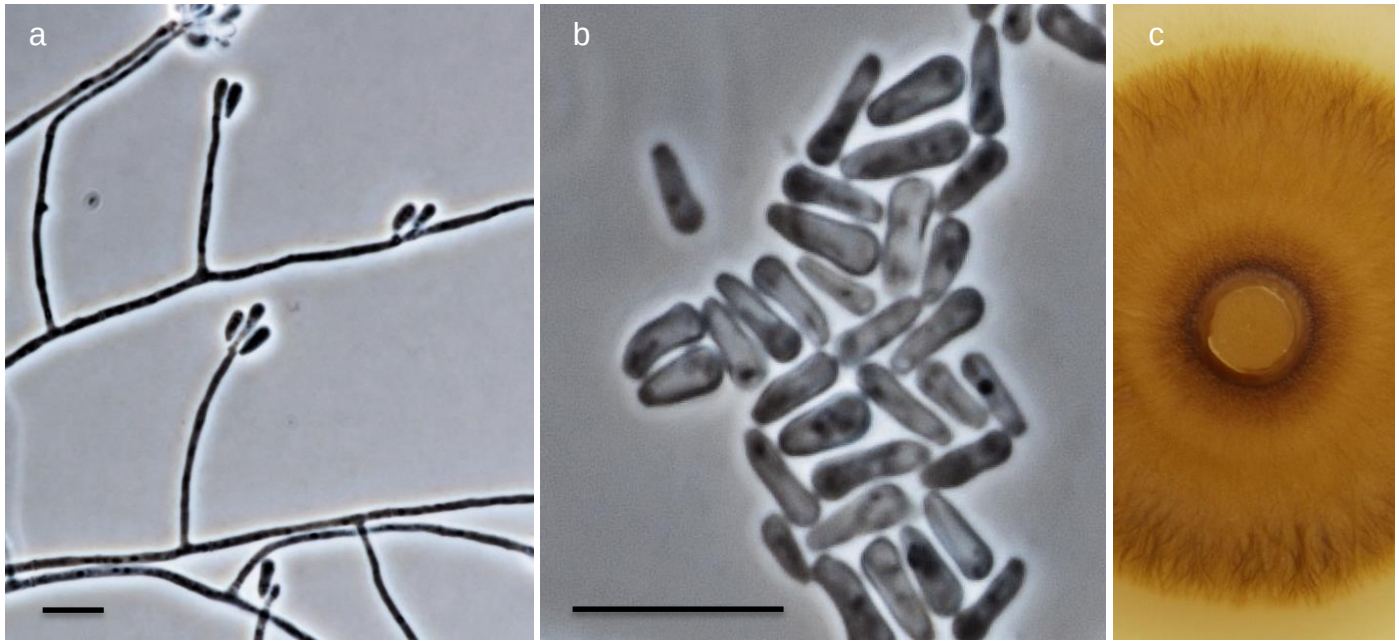

Figure 8

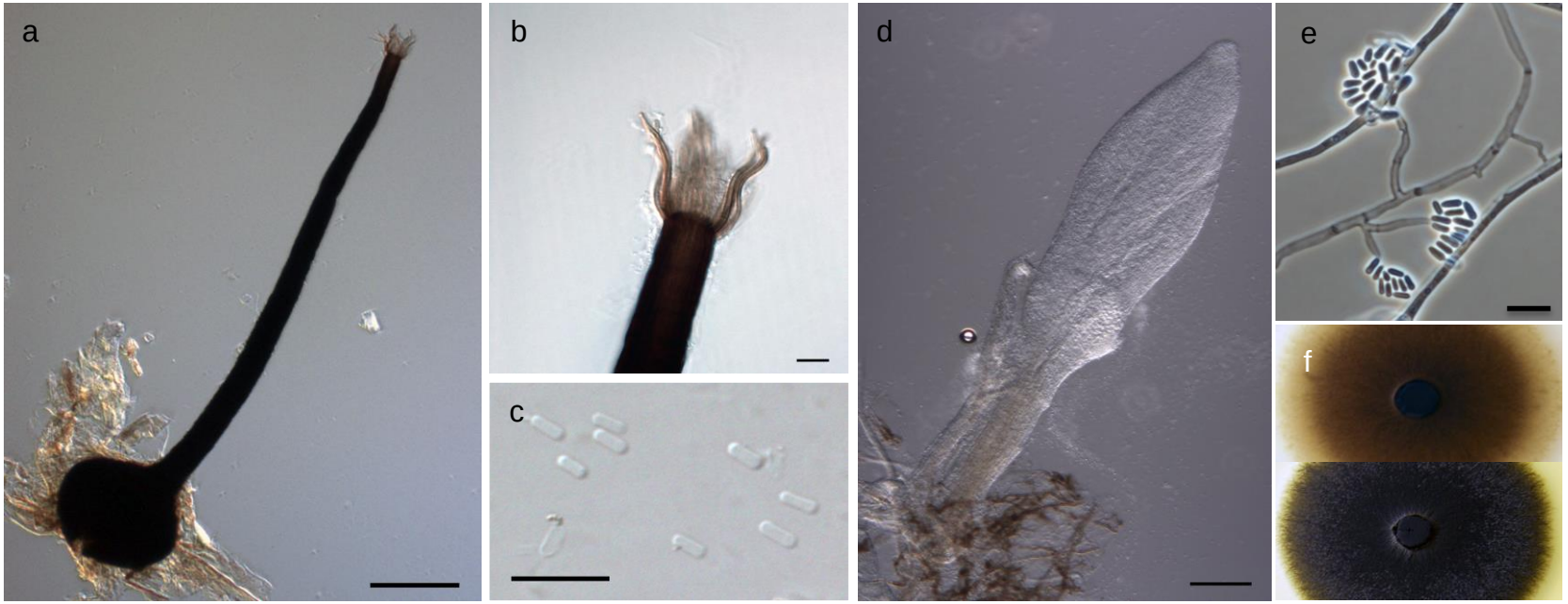

Figure 9

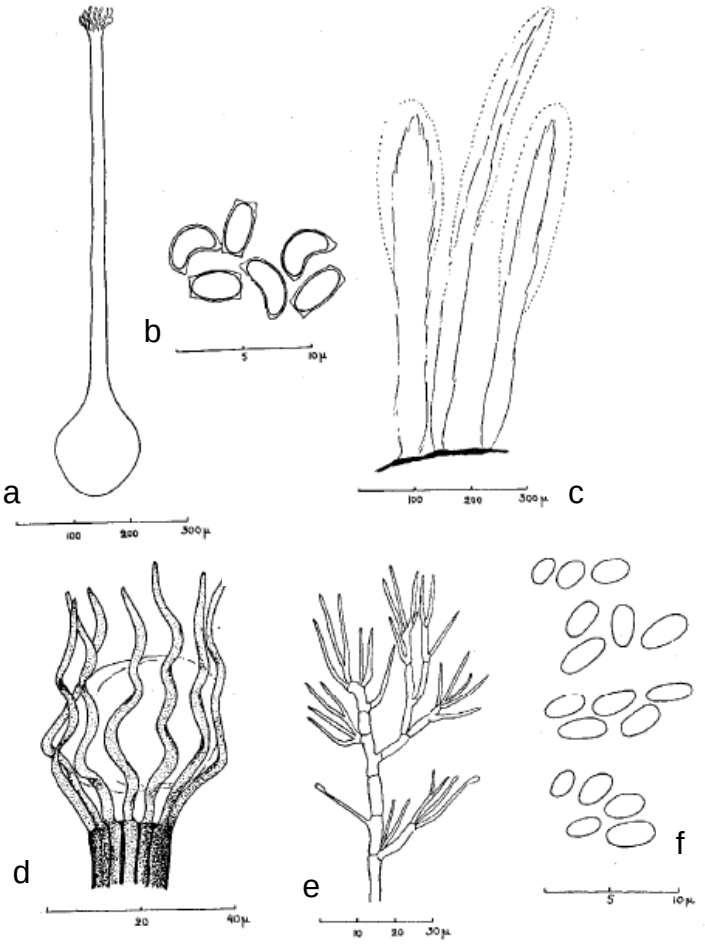

Figure 10

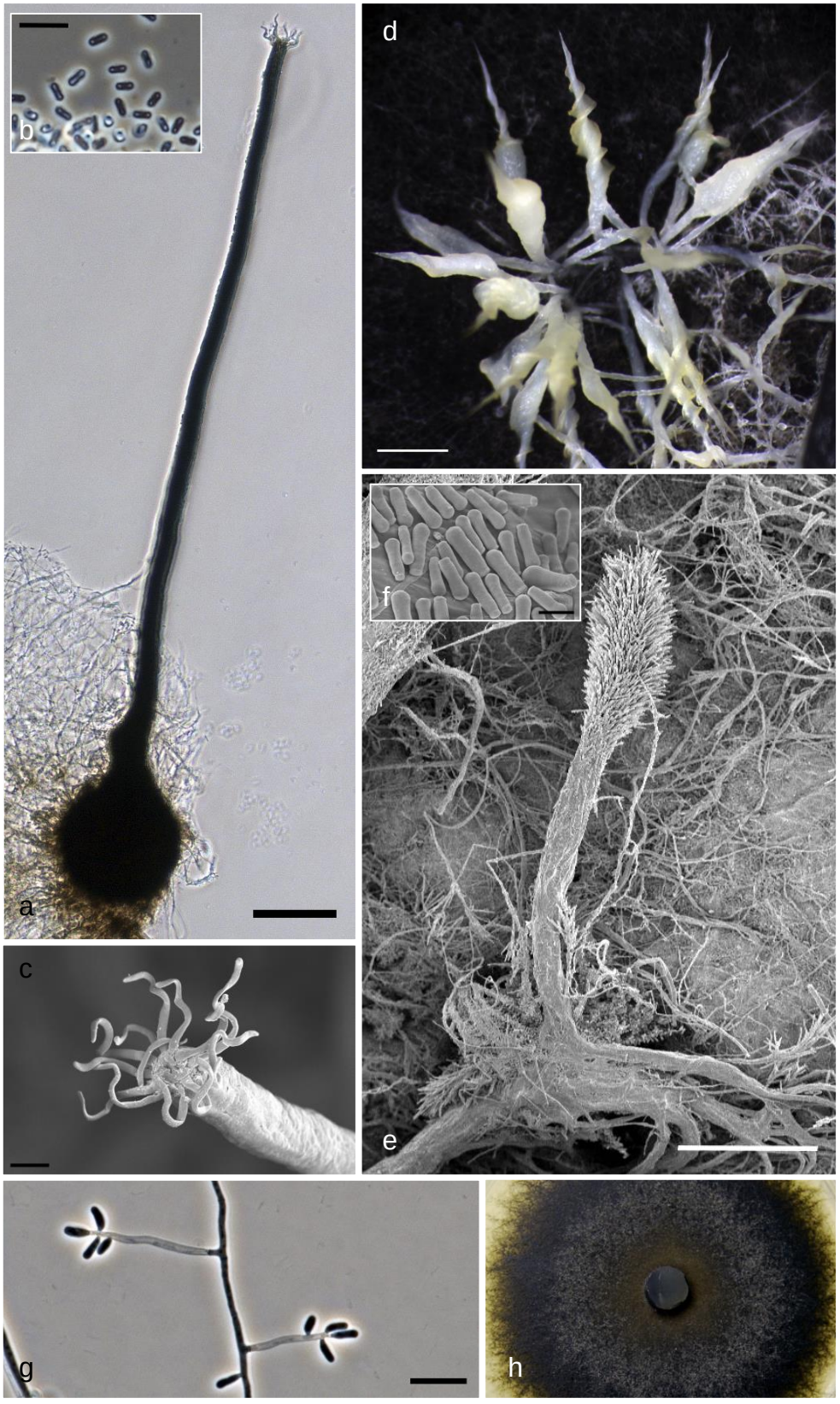

Figure 11

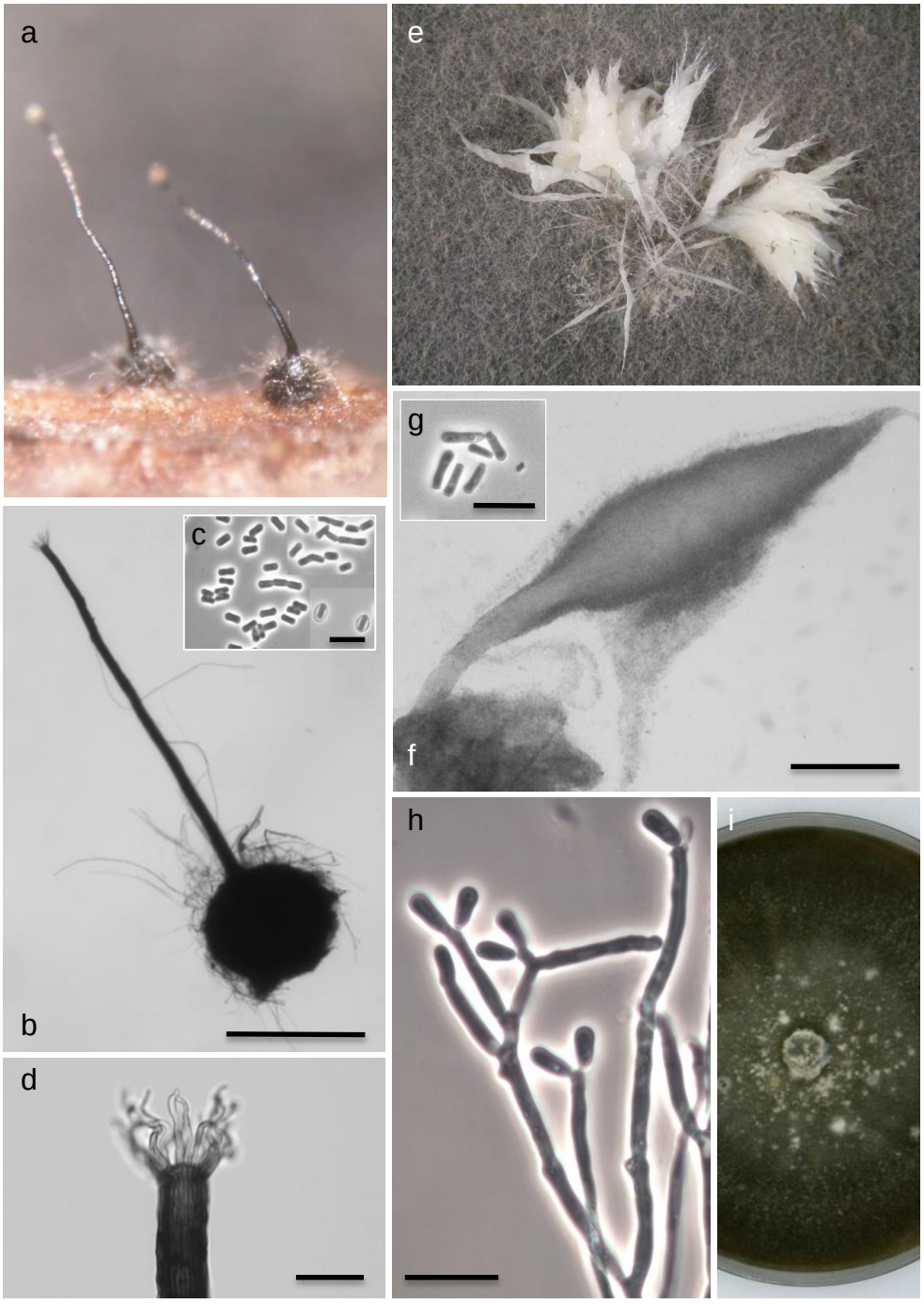

Table 1. *Ophiostoma clavatum* complex isolates and herbarium specimens examined in this study.

Species	Isolate numbers[1]		Herbarium	Origin	Host	Insect vector	Collector	GenBank accession no.			
	CMW	CBS						ITS	β-tubulin	TEF 1-α	CAL
O. ainoae	1037[T]	205.83[T]	CBS H-3559[H]	Norway	*Picea abies*	*Ips typographus*	H. Solheim	KU094682	HM031552	KU094745	KU094779
	1903	118672		Norway	*P. abies*	*I. typographus*	H. Solheim	HM031495	HM031553	KU094746	KU094780
	23123	128299		Ohtama, Russia	*P. abies*	*I. typographus*	J. Ahtiainen	HM031496	HM031550	KU094747	KU094781
O. brunneo-ciliatum	39826	141265		Wierzchosławice, Poland	*Larix decidua*	*Ips cembrae*	R. Jankowiak		KU094687	KU094748	KU094782
	39827[a,b]	141266	TUR 205571	Pateraki, Poland	*Pinus sylvestris*	*I. sexdentatus*	R. Jankowiak	KU094683	KU094688	KU094749	KU094783
	39828[a]	141267		Wierzchosławice, Poland	*L. decidua*	*Ips cembrae*	R. Jankowiak		KU094689	KU094750	KU094784
	39829			Wierzchosławice, Poland	*L. decidua*	*I. cembrae*	R. Jankowiak		KU094690	KU094751	KU094785
	39842[a]	141268		Babimost, Poland	*P. sylvestris*	*I. sexdentatus*	R. Jankowiak		KU094691	KU094752	KU094786
			UPS:BOT:F-130962[L]	Jokkmokk, Sweden	*P. sylvestris*	*I. sexdentatus*	E. Rennerfelt				
			BPI 595721	Jokkmokk, Sweden	*P. sylvestris*	*I. sexdentatus*	A. Mathiesen-Käärik				
			UPS:BOT:F-130963	Jokkmokk, Sweden	*P. sylvestris*	*I. sexdentatus*	E. Rennerfelt				
			UPS:BOT:F-130964	Jokkmokk, Sweden	*P. sylvestris*	*I. sexdentatus*	E. Rennerfelt				

			UPS:BOT:F-130965	Jokkmokk, Sweden	*P. sylvestris*	*I. sexdentatus*	E. Rennerfelt				
			UPS:BOT:F-130967	Jokkmokk, Sweden	*P. sylvestris*	*I. sexdentatus*	E. Rennerfelt				
O. brunneolum sp. nov.	23121			Lisino-Korpus, Russia	*P. abies*	*I. typographus*	Z.W. de Beer		KU094692	KU094753	KU094787
	23139			Ohtama, Russia	*P. abies*	*I. typographus*	J. Ahtiainen		KU094693	KU094754	KU094788
	23140			Ohtama, Russia	*P. abies*	*I. typographus*	J. Ahtiainen		KU094694		
	23142[P,a,b]	141078[P]	TUR 205573[P]	Ohtama, Russia	*P. abies*	*I. typographus*	J. Ahtiainen		KU094695		
	23143[T,a,b]	128127[T]	TUR 205572[H]	Ohtama, Russia	*P. abies*	*I. typographus*	J. Ahtiainen	KU094684	HM031554	KU094755	KU094789
	23144[P,a]	141269[P]	TUR 205574[P]	Ohtama, Russia	*P. abies*	*I. typographus*	J. Ahtiainen		KU094696		
	23145			Ohtama, Russia	*P. abies*	*I. typographus*	J. Ahtiainen		KU094697	KU094756	KU094790
	23146			Ohtama, Russia	*P. abies*	*I. typographus*	J. Ahtiainen		KU094698	KU094757	KU094792
	23152			Ohtama, Russia	*P. abies*	*I. typographus*	J. Ahtiainen		KU094699	KU094758	KU094792
	23153			Ohtama, Russia	*P. abies*	*I. typographus*	J. Ahtiainen		KU094700		
	23154	141079		Ohtama, Russia	*P. abies*	*I. typographus*	J. Ahtiainen		KU094701		
		141272		Hlubočec, Czech Republic	*P. abies*	*Ips duplicatus*	R. Jankowiak		KU094702		
		141270		Hlubočec, Czech Republic	*P. abies*	*I. duplicatus*	R. Jankowiak		KU094703		
		141271		Pustá Polom, Czech Republic	*P. abies*	*I. dublicatus*	R. Jankowiak		KU094704		
O. clavatum	37983[E,a,b]	141080[E]	TUR 205575[E]	Lunsen, Sweden	*P. sylvestris*	*Ips acuminatus*	C. Villari	KU094685	KU094705	KU094759	KU094793
	37984	141273		Lunsen, Sweden	*P. sylvestris*	*I. acuminatus*	C. Villari		JX298085		

	37985			Val Dogna, Italy	*P. sylvestris*	*I. acuminatus*	C. Villari		KU094706		
	37986[a]			Val Venosta, Italy	*P. sylvestris*	*I. acuminatus*	C. Villari		KU094707	KU094760	KU094794
	37987			Val Camonica, Italy	*P. sylvestris*	*I. acuminatus*	C. Villari		KU094708		
	37988[a]	141274	TUR 205576	Var, France	*P. sylvestris*	*I. acuminatus*	A. Yart		KU094709	KU094761	KU094795
	41041*			Sweden (?)	–	–	–		KU094710	KU094762	KU094796
	41042*			Sweden (?)	–	–	–		KU094711		
	41043			Buskerud, Norway	*P. sylvestris*	*I. acuminatus*	H. Solheim		KU094712	KU094763	KU094797
	41045			Buskerud, Norway	*P. sylvestris*	*I. acuminatus*	H. Solheim		KU094713		
	41051			Sweden	–	–			KU094714		
	41052			San Vito, Italy	*Pinus* sp.	–	B. Långström		KU094715		
	41122	141182		Bleiberg, Austria	*P. sylvestris*	*I. acuminatus*	T. Kirisits, G. Hoch		KU094716		
	41123[a,b]	141183	TUR 205577	Bleiberg, Austria	*P. sylvestris*	*I. acuminatus*	T. Kirisits, G. Hoch		KU094717	KU094764	KU094798
	41124	141184		Bleiberg, Austria	*P. sylvestris*	*I. acuminatus*	T. Kirisits, G. Hoch		KU094718		
	41125	141185		Bleiberg, Austria	*P. sylvestris*	*I. acuminatus*	T. Kirisits, G. Hoch		KU094719		
	41126	141186		Bleiberg, Austria	*P. sylvestris*	*I. acuminatus*	T. Kirisits, G. Hoch		KU094720		
	41127	141187		Bleiberg, Austria	*P. sylvestris*	*I. acuminatus*	T. Kirisits, G. Hoch		KU094721		
	41128	141188		Bleiberg, Austria	*P. sylvestris*	*I. acuminatus*	T. Kirisits, G. Hoch		KU094722		
			UPS:BOT:F-F130972[L]	Dalarna, Sweden	*P. sylvestris*	*I. acuminatus*	E. Rennerfelt				
O. macroclavatum sp. nov.	23115[T,a,b]	141081[T]	TUR 205578[H]	Lisino-Korpus, Russia	*P. sylvestris*	*Pityogenes chalcographus*	Z.W. de Beer	HM031499	KU094723	KU094765	KU094799
	23116[PT,b]	141082[P]	TUR 205579[P]	Lisino-Korpus, Russia	*P. abies*	*P. chalcographus*	Z.W. de Beer		KU094724	KU094766	KU094800
	23117	141083		Lisino-Korpus, Russia	*P. abies*	*I. typographus*	Z.W. de Beer		KU094725	KU094767	KU094801

	23118[a]	141084		Lisino-Korpus, Russia	*P. sylvestris*	*P. chalcographus*	Z.W. de Beer		KU094726	KU094768	KU094802
	23120[PT,a]	141085[P]	TUR 205580[P]	Lisino-Korpus, Russia	*P. abies*	*I. typographus*	Z.W. de Beer		KU094727	KU094769	KU094803
	41049			Märjamaa, Estonia	–	*Ips amitinus*	H. Solheim		KU094728	KU094770	KU094804
	41050			Punkaharju, Finland	–	*I. amitinus*	H. Solheim		KU094729		
	43051			Opava, Czech Republic	*P. abies*	*I. duplicatus*	R. Jankowiak		KU094730		
	43052			Węgierska Górka, Poland	*P. abies*	*I. amitinus*	R. Jankowiak		KU094731		
	43053			Węgierska Górka, Poland	*P. abies*	*P. chalcographus*	R. Jankowiak		KU094732		
	43365			Węgierska Górka, Poland	*P. abies*	*I. amitinus*	R. Jankowiak		KU094733		
O. pseudocatenulatum sp. nov.	5212	117571		Atholl, Scotland	*L. decidua*	*I. cembrae*	T. Kirisits, D. B. Redfern, M. J. Wingfield	HM031500	KU094734	KU094771	KU094805
	5214			Scotland	*L. decidua*	*I. cembrae*	T. Kirisits, D. B. Redfern, M. J. Wingfield	HM031501	HM031558		KU094806
		117591		Kindberg, Austria	*L. decidua*	*I. cembrae*	T. Kirisits		JX298086	KU094772	KU094807
	43097[P,a,b]		TUR 205582[P]	Rudziniec, Poland	*L. decidua*	*I. cembrae*	R. Jankowiak		KU094735	KU094773	KU094808
	43098	141275		Albrechtice, Czech Republic	*L. decidua*	*I. cembrae*	R. Jankowiak		KU094736		
	43100			Otín, Czech Republic	*L. decidua*	*I. cembrae*	R. Jankowiak		KU094737		
	43103[T,a,b]	141276[T]	TUR 205581[H]	Rudziniec, Poland	*L. decidua*	*I. cembrae*	R. Jankowiak	KU094686	KU094738	KU094774	KU094809
	43104	141277		Rudziniec, Poland	*L. decidua*	*I. amitinus*	R. Jankowiak		KU094739	KU094775	KU094810
	43105			Rudziniec, Poland	*L. decidua*	*I. amitinus*	R. Jankowiak		KU094740		

	43106[P,a,b]	141278[PT]	TUR 205583[P]	Rudziniec, Poland	*L. decidua*	*I. amitinus*	R. Jankowiak		KU094741		
	43107			Albrechtice, Czech Republic	*L. decidua*	*I. cembrae*	R. Jankowiak		KU094742	KU094776	KU094811
	43108			Rudziniec, Poland	*L. decidua*	*I. cembrae*	R. Jankowiak		KU094743		
	43598	141279		Albrechtice, Czech Republic	*L. decidua*	*I. cembrae*	R. Jankowiak		KU094744		
O. tapionis	23265[T]	128120[T]	KUO 021872[H]	Lisino-Korpus, Russia	*P.sylvestris*	*Hylurgops palliatus*	Z.W. de Beer	HM031494	HM031545	KU094777	KU094812
	23266[P]	128122[P]	KUO 021873[P]	Punkaharju, Finland	*P. sylvestris*	*Hylastes brunneus*	Z.W. de Beer	HM031493	HM031544	KU094778	KU094813

[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; TUR: Herbarium, Centre for Biodiversity, University of Turku, Finland; KUO: Kuopio Museum of Natural History, Kuopio, Finland; UPS: the Museum of Evolution, University of Uppsala, Sweden; BPI: U.S. National Fungus Collections, USDA, Beltsville, USA

[a] Isolates used in growth studies; [b] Isolates used in morphological descriptions; [T] Type/Ex-type; [E] Epitype/Ex-epitype; [P] Paratype/Ex-paratype; [H] Holotype; [L] Lectotype

* Isolates might represent original material from Mathiesen (1951)

Table 2. Morphological comparisons of the lectotype specimen, the reference culture, and the previous descriptions of *Ophiostoma brunneo-ciliatum**.

Character	**Lectotype** **UPS F-130962**	**Reference culture** **CMW 39827**	**Mathiesen-Käärik 1953**	**Hunt 1956**	**Upadhyay 1981**
Perithecia		Not observed			
Color	Black		Black	Black	Dark brown to black
Base diameter	176–(198–271(–290) µm		(192–)246(–283) µm	160–220 µm	(150–)200–350(–380) µm
Ornamentation	Sparsely ornamented with (24–)57–125(140) × 1.5–2(–2.5) µm long brown hyphae		Up to 200 µm long, greyish hyphae	Sparsely ornamented with brown, branched hyphae	Mid brown to brown unbranched or branched hyphae present
Neck length	(608–)794–1129(–1136) µm		(850–)1330(–1760) µm, neck has often pointed, knob-like protrusions	Up to 1400 µm	(550–)590–1250(–1700) µm
Neck base width	(32–)36–48(–55) µm		(34–)45(–47) µm	30–40 µm	30–45(–57.5) µm
Neck apex width	(15–)16–20 µm		(17–)20(–23) µm	19–24 µm	15–25(–30) µm
Ostiolar hyphae	(37–)38–52(–63) µm long, brown, spirally curved		(12–)18(–20) in number, 28–43 µm long, brown, spirally curved	Numerous, up to 70 µm long, pale brown, shaped like a corkscrew	Absent or 22–57,5 (–80) µm when present, brown to pale brown, spirally curved
Ascospores	Rectangular, (2–)3.5–4.5(–5.5) × 1.5–2(–2.5) µm		Rectangular, 4 × 1.8 µm	Rectangular, 4–5 × 2 µm	Rectangular, 3.5–5 × 1.5–2.5(–3) µm
Asexual state					
Synnematal	Not observed	Not observed	Graphium (brush-like); synnemata 800–1500(–2500) µm long, 16–60 µm wide, black; conidia (4–)5.7(–6.8) ×	Graphium (brush-like); synnematal stipes 1500 × 80 µm, brown, heads up to 100 µm in diameter; conidia 4.5–6 ×	Pesotum (brush-like); synnemata 100–3600(–4000) µm including conidiogenous apparatus, 15–70 wide, brown

			(1.2–)1.5(–1.9) µm	1–2 µm	to dark brown or black at the base; conidia (2.5–)3–6.5(–8.5) × 1–2(–2.5) µm
Mononematal	Not observed	Hyalorhinocladiella-like; conidiogenous cells (6–)11–23.5(–35) µm long, 1–1.5 µm wide; conidia (3–)3.5–4.5(–5) µm × 1–1.5(–2) µm	Cephalosporium-like; conidia (4.2–)6.8(–8.9) × (2.5–)3.1(–4.3) µm	Cephalosporium-like; conidiophores 10–30 µm long	Not reported
Cultures					
Growth rate		6.15 (± 0.3) mm/day (20ºC), 8.9 (± 0.2) mm/day (25ºC)			6.3 mm/day (22 ºC)
Color		Hyaline at first, becoming dark brown to black	Hyaline at first, becoming dark brown to black	Hyaline at first, becoming brownish black to black	Hyaline at first, becoming gray olive to black
Origin	Sweden	Poland	Sweden	Sweden	Sweden
Insect vector and host	*I. sexdentatus* on *P. sylvestris*	*I. sexdentatus* on *P. sylvestris*	*I. sexdentatus* on *P. sylvestris*	*I. sexdentatus* on *P. sylvestris*	*I. sexdentatus* on *P. sylvestris*

* The species has also been described by Yamaoka et al. (1998) and Aoshima (1965).

Table 3. Morphological comparisons of the lectotype specimen, ex-epitype culture, and the previous descriptions of *Ophiostoma clavatum*.

Character	**Lectotype** **UPS F-130972**	**Ex-epitype** **CMW 37983**	**Mathiesen 1951**	**Hunt 1956**
Perithecia		Not observed		Not observed*
Color	Black		Brownish black	
Base diameter	121–148 µm		(127–)177–238 µm	
Ornamentation	Absent		Absent	
Neck length	559–591 µm		(453–)566–(830) µm	
Neck base width	34–43 µm		(28.3–)35.9(–45.3) µm	
Neck apex width	15–19 µm		(11.3–)15.8(–19.8) µm	
Ostiolar hyphae	12–13 in number, (16–)24–40(–44) µm long, spirally curved		9–12 in number, (14–)52.2(–81.2) µm long, spirally curved	
Ascospores	Cylindrical to rectangular, (3–)3.5–4(–6.5) × (1–)1.5(–2) µm		Long-elliptic or orange section shaped, 3.3 × 1.4 µm	
Asexual state				
Synnematal	Not observed	Pesotum (brush-like); synnemata rarely produced in culture, (959–)1123–1515(–1636) µm long, (32–)41–64(–78) µm wide at base, hyaline to brown; conidia cylindrical to ellipsoid, (3.5–)4–5(–5.5) × 1.5–2 µm	Pesotum (brush-like); synnemata 150–500 µm long, 40–200 µm wide, brown; conidia ellipsoid, (2.7–)3.2(–3.5) × (1.7–)1.9(–2.1) µm	Pesotum (brush-like); synnemata rarely produced in culture, 500 µm long, 50 µm wide, brown; conidia cylindrical to ellipsoid, 4–6 × 2–2.5 µm
Mononematal	Not observed	Hyalorhinocladiella-like; conidiogenous cells (2–)7.5–21(–35) long, (1–)1.5–2(–3.5) µm wide; conidia	Cephalosporium-like; conidia (5.1–)6.3.(–7.4) × (2.2–)2.9(–4.2) µm	Conidiophores up to 35 µm long and 2 µm wide; conidia as in synnematal anamorph

		cylindrical to ellipsoid, 4–5.5(–6.5) × (1–)1.5–2(–2.5) µm; secondary conidia occasionally formed, 2.5–3.5(–5) × 1–1.5 µm		
Cultures				
Growth rate		4 (±0.8) mm/day (20ºC), 6 (±0.3) mm/day (25ºC)		
Color		Hyaline at first, becoming dark brown to black	Hyaline at first, becoming dark green to dark brown	Hyaline at first, becoming dark olive green to black
Origin	Sweden	Sweden	Sweden	Sweden
Insect vector and host	*I. acuminatus* on *P. sylvestris*	*I. acuminatus* on *P. sylvestris*	*I. acuminatus* on *P. sylvestris*	*I. acuminatus* on *P. sylvestris*

* Hunt (1956) used Mathiesen's (1951) measurements for the description of the ascomatal characteristics.