



# *Ophiostoma* spp. associated with pine- and spruce-infesting bark beetles in Finland and Russia

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## Key words

bark beetle  
insect-fungus relationship  
*Ophiostoma*  
*Ophiostomatales*  
symbiosis

**Abstract** The timber and pulp industries of Finland rely heavily on importations from Russia as source of raw timber. These imports raise the risk of accidentally importing forest pests and pathogens, especially bark beetles and their associated fungi, into Finland. Although ophiostomatoid fungi have previously been reported from Finland and Russia, the risks of accidentally moving these fungi has prompted a first survey to compare the diversity of conifer-infesting bark beetles and associated fungi from boreal forests on both sides of the Finnish-Russian border. The aim of the present study was to identify and characterise *Ophiostoma* species isolated in association with 11 bark beetle species infesting *Pinus sylvestris* and *Picea abies* during this survey in the eastern parts of Finland and neighbouring Russia. Fungal isolates were grouped based on morphology and representatives of each morphological group were subjected to DNA sequence comparisons of the internal transcribed spaced region (ITS1, 5.8S, ITS2) and  $\beta$ -tubulin gene region. A total of 15 species of *Ophiostoma* were identified, including seven known species, five new species, and three species for which the identity remains uncertain. In the *O. piceae*-complex we identified *O. canum*, *O. floccosum*, *O. karelicum* and *O. rachisporum* sp. nov., and related to these, some isolates belonging to the European clade of *O. minus* in the *O. minus*-complex. *Ophiostoma bicolor* and *O. fuscum* sp. nov. were identified in the *O. ips*-complex, while *O. ainoae*, *O. brunneo-ciliatum*, *O. tapionis* sp. nov. and *O. pallidulum* sp. nov. were shown to group close to, but not in a strict monophyletic lineage with species of the *O. ips*-complex. Together with a single *O. abietinum*-like isolate, the only species that grouped close to the *Sporothrix schenckii*-*O. stenoceras* complex, was *O. saponiodorum* sp. nov.

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## INTRODUCTION

Global trade in untreated timber and wood products seriously raises the risk of introducing forest pathogens and pests into new environments (Tkacz 2002). One of the groups of wood infesting fungi that are easily transported through the movement of untreated wood products is the Ascomycete genus *Ophiostoma*. These fungi include many species that are agents of blue stain in timber (Seifert 1993) and some, such as the Dutch Elm Disease pathogens *O. ulmi*, *O. himal-ulmi* and *O. novo-ulmi*, are serious tree pathogens (Gibbs 1978, Brasier 1991). Bark beetles (*Coleoptera: Scolytinae*) are well-known vectors of *Ophiostoma* spp. and greatly facilitate the spread of these fungi (Wingfield et al. 1993, Kirisits 2004). The teleomorph and anamorph structures of these fungi are specifically adapted for insect dispersal (Malloch & Blackwell 1993), and many of the species are relatively specific symbionts of their insect vectors (Six 2003, Kirisits 2004).

The forestry, timber and paper industry contributes to approximately 10 % of the gross domestic product (GDP) of Finland and close to 30 % of total industrial output of the country (Finnish Forest Industries Federation 2010). Finnish forests cannot

supply the demand of the industry and the country thus relies heavily on Russia as source of raw timber. This raises concerns relating to the risks of introducing pests and pathogens on timber imported from Russia to Finland. For example, a large number of bark beetle species, including potential pest species not native to Finland, were identified by Siitonen (1990). However, studies on the fungal associates of these beetles in Finland and Russia are limited and the species identification was made at a time when DNA sequence comparisons were not commonly applied. Furthermore, we are not aware of any study where the bark beetles and their fungal flora in the two countries have been compared.

Despite the occurrence of several native bark beetle species in Finland, previous studies (Table 1) have mainly focused on fungal associates of the spruce bark beetle, *Ips typographus* (Savonmäki 1990, Viiri 1997). The only *Ophiostoma* spp. from Finland of which identities have been confirmed by DNA sequences, are associates of the birch bark beetle, *Scolytus ratzeburgi* (Linnakoski et al. 2008, Kamgan et al. 2010). Reports of *Ophiostoma* species from Russia are more extensive, but apart from *O. karelicum* reported from *S. ratzeburgi* (Linnakoski et al. 2008), all identifications were based only on morphology (Table 1). Due to the relatively simple morphology and overlapping features between different species, it is often difficult to distinguish between these species and the accuracy of names that have been used in these studies is thus questionable. Thus, DNA sequence-based identification has become essential for reliable identification and the recognition of cryptic taxa amongst morphologically similar species of ophiostomatoid fungi (Gorton et al. 2004, Grobbelaar et al. 2009).

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**Table 1** *Ophiostoma* spp. previously reported from different beetles and/or host trees in Finland (F) and Russia (R). Identifications in all these studies were based on morphology, and only those marked with \* included DNA sequence comparisons.

Fungus	Beetle	Host tree	Origin	Reference
<i>O. ainoae</i>	<i>Ips acuminatus</i>	<i>Pinus sylvestris</i>	R	Pashenova et al. 2004
	<i>I. cembrae</i>	<i>Larix sibirica</i> , <i>P. sylvestris</i>	R	Pashenova et al. 1995
	<i>I. typographus</i>	–	R	Pashenova et al. 2001, 2004
	<i>I. typographus</i>	<i>Picea abies</i>	F	Viiri 1997
<i>O. bicolor</i>	<i>I. amitinus</i>	<i>P. abies</i> , <i>P. sylvestris</i>	F	Savonmäki 1990
	<i>I. cembrae</i>	<i>L. sibirica</i> , <i>P. sylvestris</i>	R	Pashenova et al. 1995, 2004
	<i>I. typographus</i>	<i>P. abies</i>	F	Savonmäki 1990, Viiri 1997
	<i>I. typographus</i>	<i>Picea obovata</i>	R	Pashenova et al. 2001, 2004
	<i>Pityogenes chalcographus</i>	<i>P. abies</i>	F	Savonmäki 1990
<i>O. borealis</i> *	<i>Scolytus ratzeburgi</i>	<i>Betula pendula</i>	F	Linnakoski et al. 2008, Kamgan et al. 2010
<i>O. curvicolle</i> (= <i>O. nigrum</i> )	<i>Monochamus urussovi</i>	<i>Abies sibirica</i>	R	Pashenova et al. 2007
	<i>Trypodendron lineatum</i>	<i>A. sibirica</i>	R	Pashenova et al. 2004
<i>O. fagi</i> (= <i>O. quercus</i> )	–	<i>Fagus orientalis</i>	R	Potlajczuk & Schekunova 1985
	–	<i>P. sylvestris</i>	R	Potlajczuk & Schekunova 1985
	–	<i>P. abies</i>	R	Potlajczuk & Schekunova 1985
<i>O. ips</i>	<i>I. acuminatus</i>	<i>P. sylvestris</i>	R	Pashenova et al. 2004
	<i>I. cembrae</i>	<i>P. sylvestris</i>	R	Pashenova et al. 1995
	<i>Pityogenes</i> sp.	<i>P. sylvestris</i>	R	Pashenova et al. 2004
<i>O. karelicum</i> *	<i>S. ratzeburgi</i>	<i>B. pendula</i>	F, R	Linnakoski et al. 2008
<i>O. kubanicum</i> (= <i>O. quercus</i> )	–	<i>Quercus robur</i>	R	Sherbin-Parfenenko 1953
	–	<i>Quercus</i> sp.	R	Potlajczuk 1957, Minkevich 1965, Kryukova & Terehova 1974, Kryukova & Plotnikova 1979a, b, Potlajczuk & Schekunova 1985, Selochnik 1998a, b
<i>O. minus</i>	<i>S. intricatus</i>	–	R	Ivanchenko 1957
	<i>I. acuminatus</i>	<i>P. sylvestris</i>	R	Pashenova et al. 2004
	<i>I. cembrae</i>	<i>L. sibirica</i> , <i>P. sylvestris</i>	R	Pashenova et al. 1995, 2004
	–	<i>P. abies</i> , <i>P. sylvestris</i>	R	Potlajczuk & Schekunova 1985
	<i>Pityogenes</i> sp.	<i>P. sylvestris</i>	R	Pashenova et al. 2004
<i>O. novo-ulmi</i>	<i>Tomicus piniperda</i>	<i>P. sylvestris</i>	R	Pashenova et al. 2004
	–	–	R	Storozhenko et al. 2000
<i>O. piceae</i>	Scolytid beetles	<i>Ulmus</i> spp.	R	Brasier 1990
	<i>Hylurgops palliatus</i>	<i>P. abies</i> , <i>P. sylvestris</i>	F	Savonmäki 1990
	<i>I. amitinus</i>	<i>P. abies</i> , <i>P. sylvestris</i>	F	Savonmäki 1990
	<i>I. typographus</i>	<i>P. abies</i>	F	Savonmäki 1990, Viiri 1997
	<i>M. urussovi</i>	<i>A. sibirica</i>	R	Pashenova et al. 2007
	–	–	F, R	Wegelius 1938, Meyer 1946, Storozhenko et al. 2000
	–	<i>P. abies</i> , <i>Pinus</i> sp.	R	Potlajczuk & Schekunova 1985
	<i>P. chalcographus</i>	<i>P. abies</i>	F	Savonmäki 1990
	<i>T. piniperda</i>	<i>P. sylvestris</i>	F	Savonmäki 1990
	<i>T. lineatum</i>	<i>P. abies</i> , <i>P. sylvestris</i>	F	Savonmäki 1990
<i>O. piliferum</i>	<i>I. cembrae</i>	<i>L. sibirica</i> , <i>P. sylvestris</i>	R	Pashenova et al. 1995, 2004
	<i>I. typographus</i>	<i>P. abies</i>	F	Savonmäki 1990
	–	–	R	Rodjgin 1947
	–	<i>Quercus</i> sp.	R	Liubarski 1937
	<i>T. piniperda</i>	<i>P. sylvestris</i>	R	Pashenova et al. 2004
<i>O. quercus</i> *	<i>S. ratzeburgi</i>	<i>B. pendula</i>	F	Linnakoski et al. 2008
<i>O. roboris</i> (= <i>O. quercus</i> )	<i>Mesosa myops</i>	<i>Quercus</i> sp.	R	Minkevich 1965
	–	<i>Betula</i> sp.	R	Minkevich 1965
	–	<i>Malus domestica</i>	R	Potlajczuk & Schekunova 1985
	–	–	R	Storozhenko et al. 2000
	–	<i>Quercus robur</i>	R	Selochnik 1998a,b
	–	<i>Quercus</i> sp.	R	Sherbin-Parfenenko 1953, Potlajczuk 1957, Potlajczuk & Schekunova 1985, Vorontsov 1986
	<i>S. intricatus</i>	<i>Quercus</i> sp.	R	Ivanchenko 1957, Minkevich 1965
<i>O. tetropii</i>	<i>I. typographus</i>	<i>P. abies</i>	F	Savonmäki 1990, Viiri 1997
	<i>P. chalcographus</i>	<i>P. abies</i>	F	Savonmäki 1990
<i>O. ulmi</i>	–	<i>Fraxinus</i> sp.	R	Potlajczuk & Schekunova 1985
	–	–	R	Storozhenko et al. 2000
	–	<i>Quercus</i> sp.	R	Vorontsov 1986
	–	<i>Ulmus</i> sp.	R	Dudina 1936, 1938, Potlajczuk & Schekunova 1985
	Scolytid beetles	<i>Ulmus</i> spp.	R	Brasier 1990
<i>O. valachicum</i>	–	<i>Q. robur</i>	R	Selochnik 1998a, b
	–	<i>Quercus</i> sp.	R	Sherbin-Parfenenko 1953, Potlajczuk 1957, Potlajczuk & Schekunova 1985
	<i>S. intricatus</i>	<i>Quercus</i> sp.	R	Ivanchenko 1957

A large number of ophiostomatoid fungi have been reported from mainland Europe and Scandinavia that exist in symbiosis with bark beetle species occupying different niches (Kirisits 2004). Consistent with other studies, it is likely that the species diversity for these fungi is more extensive in the boreal forests of Finland and Russia than has previously been recognised. Consequently, an extended survey of bark beetle-associated fungi is currently being undertaken at 12 different sites in the boreal forests of Karelia, on both the Finnish and Russian sides of the border. As part of this survey, fungal collections

have been made from the most common bark beetle species infesting the two dominant conifer species in the boreal forests, Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*). The aims of this study were to identify all species of *Ophiostoma* collected during this survey, and to describe new species found among them. These identifications were based on morphological characteristics and DNA sequence comparisons for the internal transcribed spacer (ITS) regions of the ribosomal RNA and part of the  $\beta$ -tubulin gene.



<i>O. tapionis</i> sp. nov.	F	128 120 128 122	23265 <sup>a,b,T</sup> 23266 <sup>a,b</sup> 23263 34940 23264 23269 <sup>a,b</sup> 23194 28030	D-101406 D-101407	021872 021873 021874	Punkaharju, Finland Punkaharju, Finland Punkaharju, Finland Punkaharju, Finland Punkaharju, Finland Lisino-Corpus, Russia Punkaharju, Finland Kivernapa, Russia	<i>P. abies</i> <i>P. sylvestris</i> <i>P. abies</i> <i>P. abies</i> <i>P. sylvestris</i> <i>P. sylvestris</i> <i>P. abies</i> <i>P. sylvestris</i>	<i>H. palliatus</i> <i>H. brunneus</i> <i>H. palliatus</i> <i>H. palliatus</i> <i>T. minor</i> <i>H. palliatus</i> <i>Dyocoetes autographus</i> <i>T. minor</i>	de Beer Linnakoski Linnakoski Linnakoski Linnakoski Linnakoski de Beer Linnakoski	— HM031493 — — — HM031494 HM031502 HM031511	HM031545 HM031544 HM031546 HM031543 HM031548 HM031547 HM031549 HM031568
<b>Isolates of reference species</b>											
<i>O. abietinum</i>		125.89 <sup>T</sup> 205.83 <sup>T</sup>	22310 1037	—	—	Mexico Norway	<i>Abies vejarii</i> <i>P. abies</i>	<i>Pseudohylesinus</i> sp. <i>I. typographus</i>	Marmolejo Solheim	AF484453 <sup>2</sup> —	HM067820 HM031552
<i>O. ainoae</i>		118672	1903	—	—	Norway	<i>P. abies</i>	—	Olsen	HM031495	HM031553
<i>O. bicolor</i>		—	5031	—	—	Austria	<i>Pinus</i> sp.	<i>I. amitinus</i>	Kirisits	HM031506	—
<i>O. brevisulcum</i>		—	—	JCM 12500 <sup>T</sup>	—	Japan	<i>Larix kaempferi</i>	<i>D. baicalicus</i>	Yamaoka	—	HM031516
<i>O. brunneo-ciliatum</i>		—	—	JCM 12501 <sup>T</sup>	—	Japan	<i>L. kaempferi</i>	<i>D. baicalicus</i>	Yamaoka	AB200423 <sup>3</sup>	HM031517
<i>O. canum</i>		133.51 <sup>A</sup>	5212	—	—	Scotland	<i>Larix</i> sp.	—	Kirisits	HM031500	HM031559
<i>O. narclissi</i>		138.50 <sup>T</sup>	5214	—	—	Sweden	<i>Larix</i> sp.	—	Kirisits	HM031501	HM031558
<i>O. rostrorornatum</i>		434.77 <sup>A</sup>	1096 456	—	—	Netherlands USA	<i>P. sylvestris</i> <i>Narcissus</i> sp. pulpwood chips	— — —	Mathiesen Limber Eslin	HM031489 AF484451 <sup>2</sup> AY194509 <sup>4</sup>	HM031518 HM067821 HM067822

<sup>1</sup> CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMW: Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; VT: Culture Collection of the Technical Research Centre of Finland, Espoo, Finland; JCM: Japan Collection of Micro-organisms, Saitama, Japan; KUO: Kuopio Museum of Natural History, Kuopio, Finland.

<sup>2</sup> From the study of de Beer et al. (2003a), <sup>3</sup> Chung et al. (2006), and <sup>4</sup> Jacobs et al. (2003).

<sup>a</sup> Isolates used in growth studies; <sup>b</sup> Isolates used in morphological descriptions.

<sup>T</sup> Ex-type isolates; <sup>A</sup> Authentic isolates from the original collections.

## MATERIALS AND METHODS

### Isolation of fungi from bark beetles and galleries

Bark beetles and their galleries were collected from spruce and pine logs and naturally infested trees at four different sites in Finland and eight in Russia (Table 2). All sampling sites were located in the eastern parts of the Finland and in the Karelia region on the Russian side of the border. In July 2004, beetles were collected from the Ohtama region in Russia, at a location of extensive spruce bark beetle (*Ips typographus*) damage. In July–August 2005, more extensive isolations were done at the other research sites. Fungi were isolated from the bark beetles as well as their galleries (Fig. 1). Additional fungal isolates obtained in July 2007 from *Tomicus piniperda* infesting *Pinus sylvestris* in Russia were included in the study. Samples were collected from natural wounds but also using a trap-tree method. Methods of fungal isolation were the same as those described by Linnakoski et al. (2008).

Purified cultures were grouped according to culture morphology. From each morphological group, isolates representing different sites, beetle and host tree species were selected for DNA sequencing. They were deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands and the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Isolates of the new taxa are also maintained in the VTT Culture collection, VTT Technical Research Centre of Finland, Espoo, herbarium specimens were deposited in Kuopio Museum of Natural History (KUO), Kuopio, Finland, and taxonomic novelties in MycoBank (Crous et al. 2004). Several isolates of known species for which DNA sequences were not available, were also included in the study for comparative purposes (Table 2).

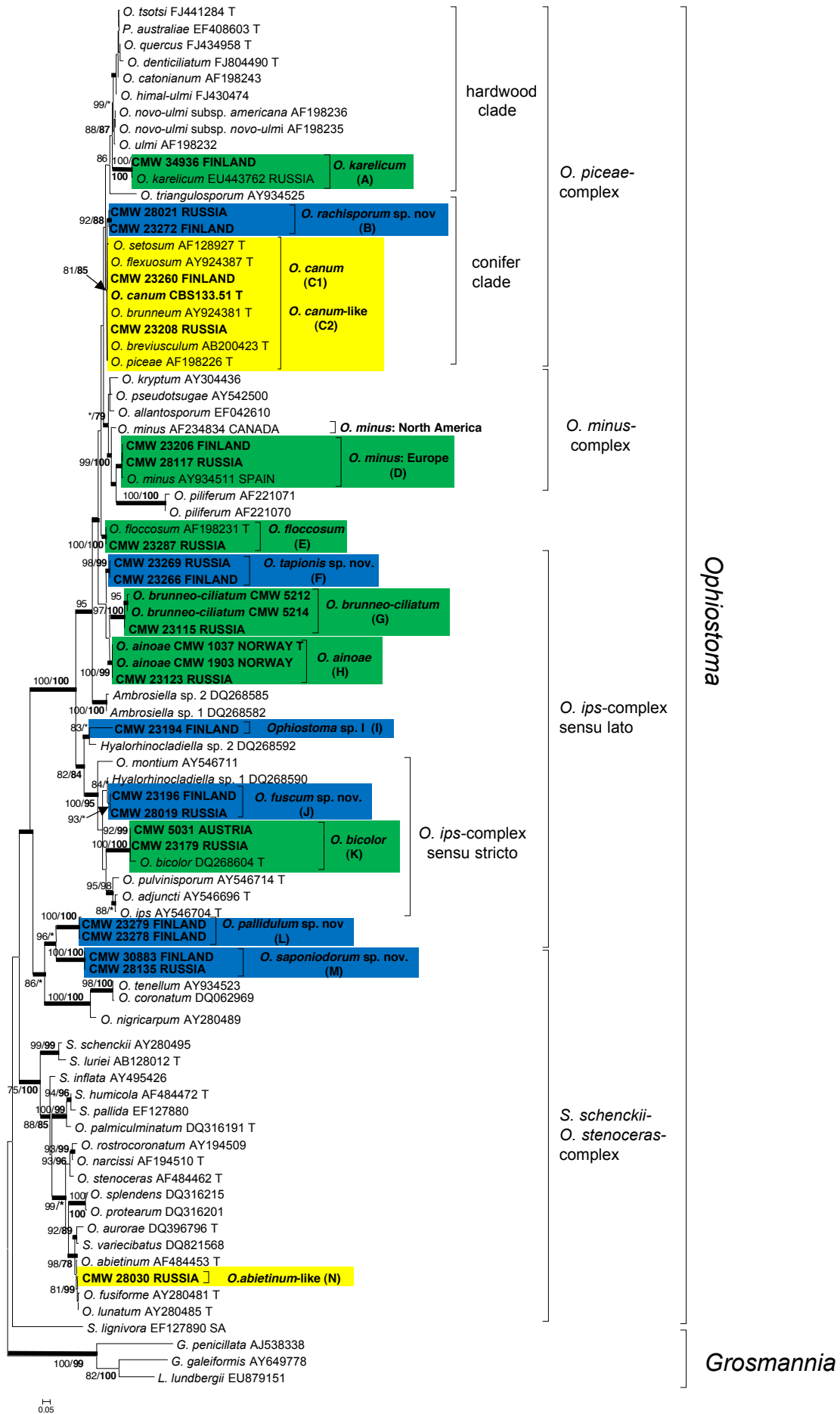
### DNA extraction and PCR

Fungal isolates were grown on malt extract agar (MEA; 20 g Difco Bacto™ malt extract from Becton, Dickinson & Company, Sparks, USA, 20 g Difco Bacto™ agar, and 1 L Milli-Q water) plates. DNA was extracted using PrepMan Ultra Sample preparation reagent (Applied Biosystems, Foster City, CA, USA) using a slight modification on the manufacturer's protocol (Linnakoski et al. 2008). The ITS 1 and 2 regions, including the 5.8S gene, were amplified using primers ITS1-F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). Part of the  $\beta$ -tubulin gene region was amplified using primers Bt2a and Bt2b (Glass & Donaldson 1995). Primer Bt2b was replaced in some cases with primer T10 (O'Donnell & Cigelnik 1997).

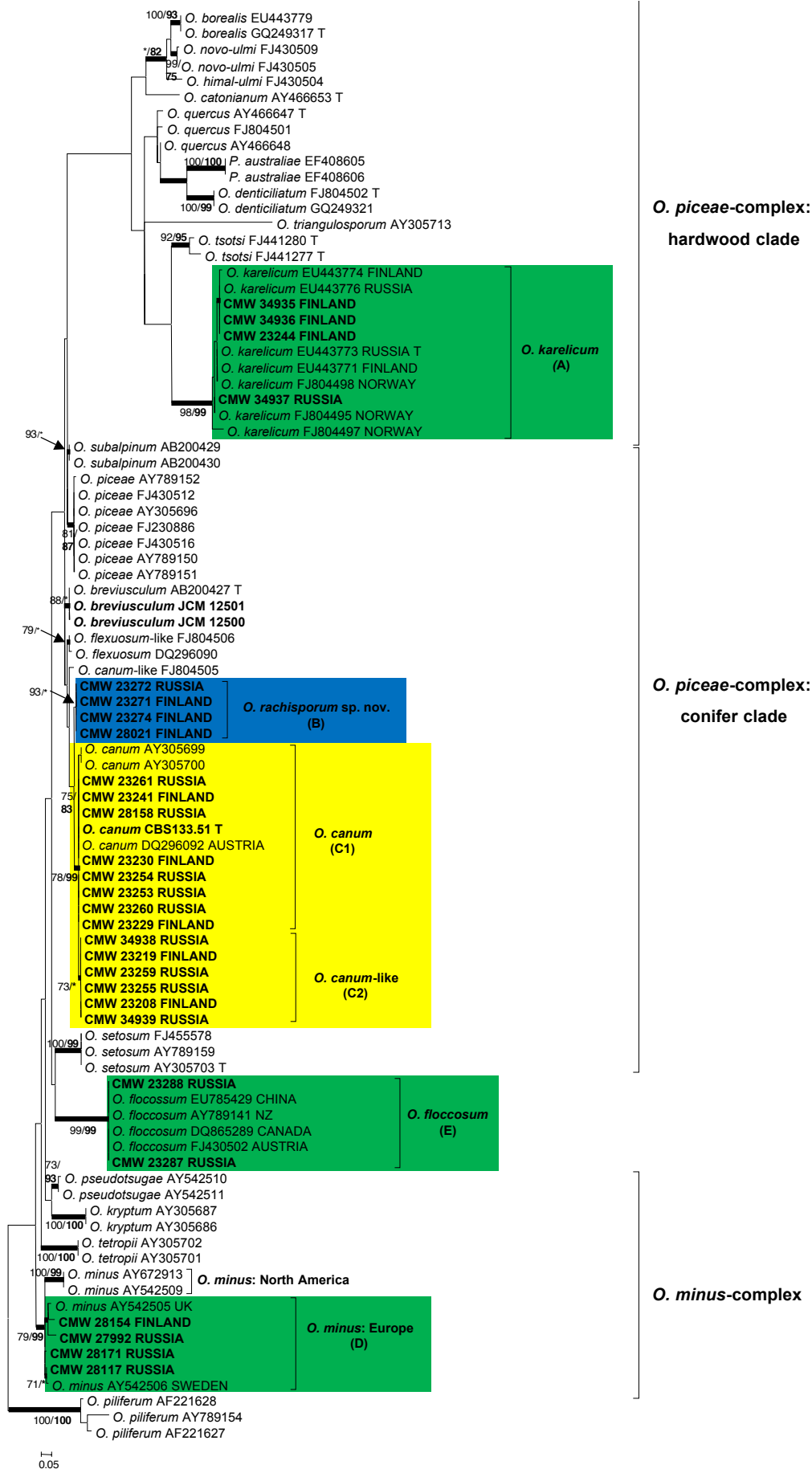
Gene fragments were amplified in 25  $\mu$ L reaction mixture containing 0.25  $\mu$ L of Super-Therm DNA polymerase mixture (250 U) (Hoffman-La Roche, Nutley, USA), 2.5  $\mu$ L of reaction buffer (10 $\times$ ) and 2.5  $\mu$ L of MgCl<sub>2</sub> (25 mM) (supplied with the enzyme), 2.5  $\mu$ L of dNTPs (2 mM each) and 0.25  $\mu$ L of each primer (10  $\mu$ M). Amplification reactions were performed using an Eppendorf Mastercycler® Personal (Perkin-Elmer, Hamburg, Germany). The PCR conditions for ITS gene region were: an initial denaturation step at 95 °C for 2 min, followed by 35 cycles of 30 s at 95 °C, 30 s at 54 °C and 1 min at 72 °C, and a final chain elongation at 72 °C for 8 min. The  $\beta$ -tubulin gene was amplified using a denaturation step at 95 °C for 2 min, followed by 35 cycles of 30 s at 95 °C, 30 s at 56 °C and 1 min at 72 °C, and a final chain elongation at 72 °C for 8 min. Amplified products were purified using the High Pure PCR Product Purification Kit (Roche Molecular Biochemicals, Indianapolis, USA) and sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on the ABI Prism 377 Autosequencer (Applied Biosystems, Foster City, CA, USA), using the same primers used for the PCR.



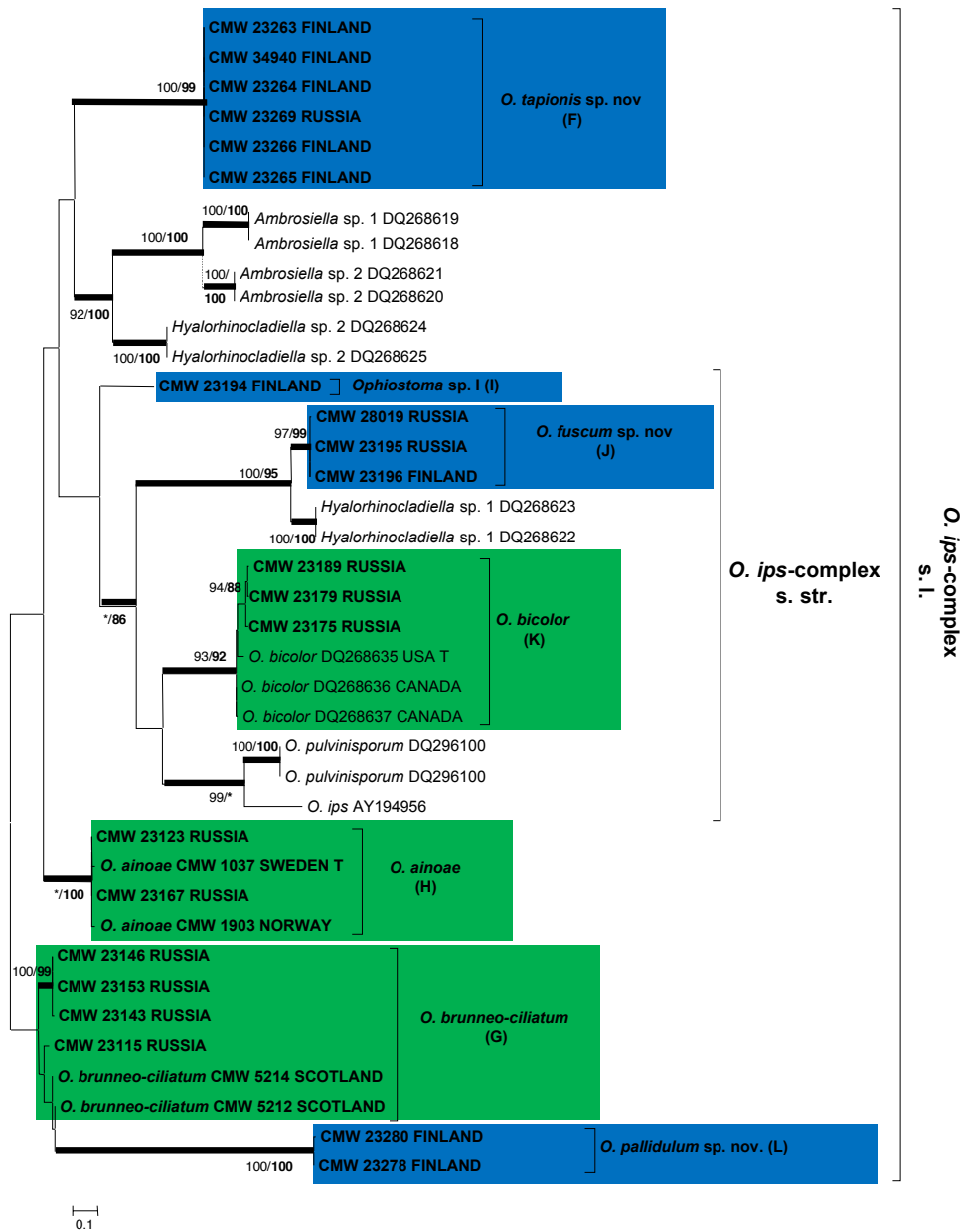
**Fig. 1** a. Part of a typical boreal forest in Finland; b. exposed galleries of *Hylurgops palliatus* on *Pinus sylvestris*; c. trapping logs in Ilomantsi, Finland; d. galleries of *Trypodendron lineatum* on *Picea abies*; e. galleries of *Tomicus minor* on *P. sylvestris*.



**Fig. 2** Phylogram obtained from ML analyses of the ITS region. Novel sequences obtained in this study are printed in **bold** type. ML bootstrap support values (1 000 replicates) (normal type) and MP Jackknife values (10 000 replicates) (**bold** type) above 75 % are indicated at the nodes. Posterior probabilities (above 90 %) obtained from BI are indicated by bold lines at the relevant branching points. \* = bootstrap values lower than 75 %. T = ex-type isolates. Scale bar = total nucleotide difference between taxa. Green = known species; blue = new species; yellow = species of uncertain identity.



**Fig. 3** Phylogram obtained from ML analyses of the  $\beta$ -tubulin gene of the *O. piceae*- and *O. minus* complexes. Novel sequences obtained in this study are printed in **bold** type. ML bootstrap support values (1 000 replicates) (normal type) and MP Jackknife values (10 000 replicates) (**bold** type) above 75 % are indicated at the nodes. Posterior probabilities (above 90 %) obtained from BI are indicated by bold lines at the relevant branching points. \* = bootstrap values lower than 75 %. T = ex-type isolates. Scale bar = total nucleotide difference between taxa. Green = known species; blue = new species; yellow = species of uncertain identity.



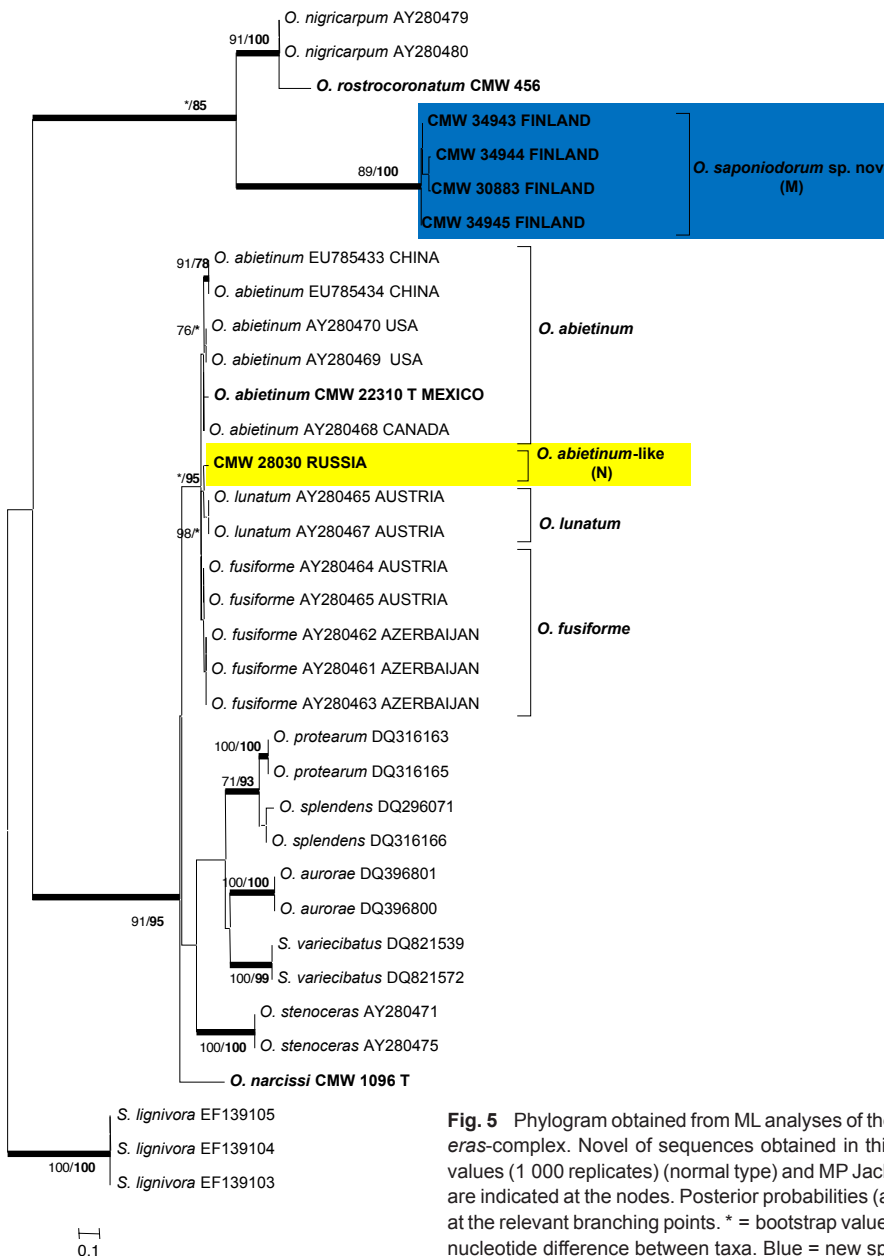
**Fig. 4** Phylogram obtained from ML analyses of the  $\beta$ -tubulin gene of species in the *O. ips*-complex s.l. Novel sequences obtained in this study are printed in **bold** type. ML bootstrap support values (1 000 replicates) (normal type) and MP Jackknife values (10 000 replicates) (**bold** type) above 75 % are indicated at the nodes. Posterior probabilities (above 90 %) obtained from BI are indicated by bold lines at the relevant branching points. \* = bootstrap values lower than 75 %. T = ex-type isolates. Scale bar = total nucleotide difference between taxa. Green = known species; blue = new species.

### Sequence analyses

For each isolate, sequences obtained using the forward and reverse primers were aligned and consensus sequences determined using Geneious Pro v4.0.4 for MacIntosh (Biomatters, Auckland, New Zealand). BLAST searches were conducted for preliminary identifications, after which datasets that included published GenBank sequences were compiled in Molecular Evolutionary Genetic Analyses (MEGA) v3.1 (Kumar et al. 2004). Sequences were aligned online with MAFFT v6 (Kato et al. 2002), using the FFT-NS-i option with a gap opening penalty of 1.53 and an offset value of 0.00. All sequences generated in this study were deposited in GenBank (Table 2). Accession numbers for sequences from reference isolates obtained from GenBank are presented in the phylogenetic trees (Fig. 2–5). Datasets were analysed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). MP analyses were conducted using TNT v1.1 (Goloboff et al. 2008) run on the computer clusters of the CSC, IT Centre for Science, Espoo,

Finland. Heuristic searches with 10 000 replicates of random addition sequences (RAS) and tree bisection and reconnection (TBR) branch swapping were carried out. Gaps were treated as a fifth character for all datasets (Odgen & Rosenberg 2007). A Jackknife test (JK) with 10 000 replicates was used for counting the support values. ML analyses were performed using RAxML v7.0.4 (Stamakis et al. 2008) assuming the GTR+G substitution model, run on the CIPRES cluster at the San Diego Supercomputing Center. Support for the nodes was estimated from 1 000 bootstrap replicates (Felsenstein 1985). BI analyses based on a Markov Chain Monte Carlo (MCMC) were carried out with MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003). The MCMC chains were run for five million generations using the best fitting model selected by the AIC in MrModeltest v2.3 (<http://www.abc.se/~nylander/>). Trees were sampled every 100 generations resulting in 50 000 trees from both runs. From these, the first 200, 250, 100 and 100 trees were discarded as burn-in, as calculated for the respective datasets. The remaining trees were used to construct majority rule consensus trees.





**Fig. 5** Phylogram obtained from ML analyses of the  $\beta$ -tubulin gene of species in the *S. schenckii*-*O. stenoceras*-complex. Novel of sequences obtained in this study are printed in **bold** type. ML bootstrap support values (1 000 replicates) (normal type) and MP Jackknife values (10 000 replicates) (**bold** type) above 75 % are indicated at the nodes. Posterior probabilities (above 90 %) obtained from BI are indicated by **bold** lines at the relevant branching points. \* = bootstrap values lower than 75 %. T = ex-type isolates. Scale bar = total nucleotide difference between taxa. Blue = new species; yellow = species of uncertain identity.

### Morphological studies

DNA sequence analyses suggested that some of the isolates considered in this study represented undescribed species of *Ophiostoma*. In an attempt to obtain sexual structures and to determine the thallism of the purportedly unknown taxa discovered, mating experiments were conducted. For each species, a single conidial culture was prepared from each of the available isolates, that was then crossed in all possible combinations with each other. The method described by Grobbelaar et al. (2010) was used where sterilised pine and spruce twigs (depending on the origin of isolate) were placed on the agar plates to encourage sporulation. For controls, isolates were crossed against themselves. Mating experiments were conducted on three different media including water agar (WA; 15 g Difco Bacto™ agar and 1 L Milli-Q water), MEA and oatmeal agar (OA; 15 g oatmeal, 15 g Difco Bacto™ agar and 1 L Milli-Q water). Cultures were grown in an incubator at 20 °C and inspected regularly for fruiting structures. The viability of ascospores produced from the crosses was confirmed by transferring a drop of ascospores from an ascoma from each positive cross to fresh MEA. These plates were then incubated and monitored. Culture morphology was used to confirm their identity.

For the species descriptions, anamorph and teleomorph (where present) fruiting structures were mounted in 85 % lactic acid on glass slides and observed using a Nikon Eclipse 50i phase contrast microscope (Nikon Corporation Tokyo, Japan). A Nikon DS-Fi1 camera system (Nikon Corporation, Tokyo, Japan) was used to capture photographic images. For the description of new species, measurements were made of 50 each of the relevant anamorph and teleomorph structures. Micronematal anamorph structures were studied using slide cultures (Riddell 1950). Averages, ranges and standard deviation of the measurements were computed. The measurements are presented in the format (minimum–) mean minus standard deviation – mean plus standard deviation (–maximum).

For scanning electron microscopy (SEM), agar disks were cut from the sporulating colonies and fixed in 2.5 % glutaraldehyde in a 0.15 M phosphate buffer overnight and 1 % osmium tetroxide, and dehydrated in an ethanol series. The samples were critical-point dried in liquid carbon dioxide with a Balzers Union CPD 010 (Balzers Union AG, Balzers, Liechtenstein) and sputter-coated with copper or gold at minimum 20 nm thickness in an Emitech K675X sputter coater (Emitech, Ashford, UK). A LEO 1550 scanning electron microscope (Beamtech Nordiska

**Table 3** Number of *Ophiostoma* isolates obtained from 11 bark beetle species and their galleries during the course of this study.

Beetle species* → Fungus species ↓	<i>Picea abies</i> (spruce) (species 1–8)												<i>Pinus sylvestris</i> (pine) (species 3–11)								Total			
	1		2		3		4		5		6		7		8		9		10			11		
	b	g	b	g	b	g	b	g	b	g	b	g	b	g	b	g	b	g	b	g		b	g	
<b>Finland</b>																								
<i>O. abietinum</i> -like	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
<i>O. ainoae</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
<i>O. bicolor</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
<i>O. brunneo-ciliatum</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
<i>O. canum</i>	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	10	0	13		
<i>O. canum</i> -like	17	0	0	0	15	0	1	1	15	0	9	0	0	7	0	0	0	8	0	27	0	100		
<i>O. floccosum</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
<i>O. fuscum</i> sp. nov.	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1		
<i>O. karelicum</i>	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	3		
<i>O. minus</i> European	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	2		
<i>O. pallidulum</i> sp. nov.	0	0	0	0	1	0	5	0	1	0	0	0	0	1	0	0	0	0	0	1	0	9		
<i>O. rachisporum</i> sp. nov.	0	0	0	0	0	0	0	0	1	0	0	0	0	10	0	0	0	0	0	0	0	11		
<i>O. saponiodorum</i> sp. nov.	2	1	0	0	0	0	0	0	0	9	1	0	0	0	0	0	0	0	0	0	0	13		
<i>O. tapionis</i> sp. nov.	7	0	0	0	0	0	1	0	10	0	1	0	0	0	0	0	0	0	0	2	0	21		
<i>Ophiostoma</i> sp. I	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1		
Total isolates	28	1	0	0	17	0	8	1	29	0	19	2	0	0	19	0	0	9	0	41	0	174		
<b>Russia</b>																								
<i>O. abietinum</i> -like	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1		
<i>O. ainoae</i>	26	25	0	0	0	2	0	0	6	1	0	0	0	0	0	0	0	0	0	3	0	63		
<i>O. bicolor</i>	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	25		
<i>O. brunneo-ciliatum</i>	50	5	0	0	0	0	0	0	0	9	2	0	0	0	0	0	0	3	0	2	71			
<i>O. canum</i>	3	4	0	0	0	0	0	10	4	3	1	0	0	0	0	0	3	2	14	21	65			
<i>O. canum</i> -like	5	11	1	0	25	23	0	0	27	14	18	15	0	4	0	6	7	24	30	7	3	220		
<i>O. floccosum</i>	0	3	4	1	0	0	0	13	0	2	2	1	1	0	0	3	2	1	0	0	0	33		
<i>O. fuscum</i> sp. nov.	0	2	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	4		
<i>O. karelicum</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3		
<i>O. minus</i> European	0	4	0	0	1	4	0	0	2	1	3	0	0	1	0	1	0	2	1	15	5	51		
<i>O. pallidulum</i> sp. nov.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
<i>O. rachisporum</i> sp. nov.	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1		
<i>O. saponiodorum</i> sp. nov.	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1		
<i>O. tapionis</i> sp. nov.	0	0	0	0	0	0	0	0	2	3	0	0	0	0	0	0	0	0	0	0	0	5		
<i>Ophiostoma</i> sp. I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
Total isolates	109	54	5	1	26	29	0	0	60	23	38	20	1	6	1	1	9	11	29	50	30	40	543	

\* Bark beetle species: 1 = *Ips typographus*; 2 = *Ips* sp.; 3 = *Dryocoetes autographus*; 4 = *Hylastes brunneus*; 5 = *Hylurgops palliatus*; 6 = *Pityogenes chalcographus*; 7 = *Pityogenes* sp.; 8 = *Trypodendron lineatum*; 9 = *Ips sexdentatus*; 10 = *Tomicus piniperda*; 11 = *Tomicus minor*.  
b = beetles; g = galleries.

AB, Gnesta, Sweden) operating at 5 kV was used to examine the specimens.

Three representative isolates (Table 2) of each unknown *Ophiostoma* species were chosen to determine the optimal temperature for growth in culture. Agar plugs 8 mm diam bearing mycelium of isolates were cut from actively growing margins of colonies, and placed at the centres of 9 cm Petri dishes containing 2 % MEA. Five replicate plates were prepared for each isolate at each temperature considered. The growth rates of isolates were determined at temperatures ranging from 5 to 35 °C (± 0.5 °C) at 5 °C intervals in the dark. For one species, the growth rate was determined also at 40 and 45 °C. Colony diameter (three colony diameter measurements per plate) on each plate was measured after 4 and 8 d of incubation in each temperature. Mean radial growth rates (mm/day) at 25 °C were calculated as an average of readings.

## RESULTS

### Collections of bark beetles and fungi

Altogether 11 bark beetle species were collected from Finland and Russia during the course of this study (Table 3). The majority of bark beetle species occurred on both pine and spruce. *Ips sexdentatus*, *Tomicus minor* and *T. piniperda* were found only on pine, while *Ips typographus* and *Ips* sp. were encountered only on spruce. The isolations from bark beetles and their galleries yielded a total of 717 isolates resembling *Ophiostoma* spp. (Table 3), producing typical *Pesotum*, *Hyalorhinochlaediella* and/or *Sporothrix*-like anamorph structures in culture. Isolates representing other genera of the *Ophiostomatales* were excluded from the present study.

### DNA sequence analyses

The preliminary analyses indicated that all isolates selected for this study represented *Ophiostoma* spp., even though sexual

structures were not observed for all groups. Due to the large number of isolates collected and sequenced, representative isolates of different beetles, hosts and countries were selected for each taxon for the final sequence datasets. In several cases, the ITS data did not distinguish clearly between closely related species, but they were useful to assign isolates to species complexes within *Ophiostoma* (Fig. 2). The  $\beta$ -tubulin gene region was used to identify isolates to the species level. Due to differences in the presence and absence of  $\beta$ -tubulin introns between species complexes in *Ophiostoma* (Zipfel et al. 2006),  $\beta$ -tubulin datasets for the different complexes were analyzed separately from each other (Fig. 3–5).

Aligned DNA sequences for the ITS gene region yielded 839 characters, including gaps (Fig. 2). Alignments of the three  $\beta$ -tubulin data subsets consisted respectively of 305, 690 and 276 characters, including gaps (Fig. 3–5). The Bayesian analyses for ITS and  $\beta$ -tubulin gene regions produced trees with topologies similar to those of the ML and MP analyses. The best fitting substitution models selected for Bayesian analyses were GTR+I+G (Fig. 2, 4, 5) and GTR+G (Fig. 3).

Comparisons of ITS sequences obtained for isolates in this study, with sequences from GenBank and the reference isolates (Fig. 2), showed that our isolates could be assigned to 14 taxa that are designated A to N. These resided in four species complexes (Harrington et al. 2001, de Beer et al. 2003a, Zipfel et al. 2006) within the genus *Ophiostoma*. These were the *O. piceae*-, *O. minus*-, *O. ips*- and *S. schenckii*-*O. stenoceras*-complexes (Fig. 2). In the ITS-tree (Fig. 2), the lineages representing *O. karelicum* (A), the European clade of *O. minus* (D), *O. floccosum* (E), *O. brunneo-ciliatum* (G), *O. ainoae* (H), *O. bicolor* (K) and five apparently undescribed taxa (B, F, J, L, M) were well-resolved. Isolates in lineages C and N could not be distinguished from closely related species in the ITS tree (Fig. 2).

The  $\beta$ -tubulin gene region amplified for all species in the *O. piceae*- and *O. minus*-complexes (Fig. 3) included exons 4, 5, and 6, as well as intron 4, but intron 5 was absent. Four of the groups of isolates in the study formed well-supported lineages, three of which included reference sequences of known species, confirming their identities as *O. karelicum* (A), the European clade of *O. minus* (D), and *O. floccosum* (E). The fourth lineage (B) did not include any isolates of known species, but was clearly distinct from its closest relative, *O. canum*. The isolates residing in phylogenetic lineage C could be separated in two sublineages (C1 and C2) based on morphology and these are further discussed in the morphology section. However, lineages C1 and C2 had ITS sequences identical to each other and an authentic isolate of *O. canum* (CBS 133.51). The  $\beta$ -tubulin sequences of lineage C1 were identical to that of the *O. canum* isolate, but differed only in a single base pair at the 30th position of exon 5, from the sequences of lineage C2.

The  $\beta$ -tubulin sequences of species in the *O. ips*-complex (Fig. 4) had an intron/exon composition similar to those of the *O. piceae*- and *O. minus*-complexes. However, they varied greatly from these complexes and were more appropriately aligned and analyzed as a separate dataset. Analyses of the  $\beta$ -tubulin data for isolates in the *O. ips*-complex confirmed the identities of three known species from amongst the isolates obtained in this study. These were *O. brunneo-ciliatum* (G), *O. ainoae* (H) and *O. bicolor* (K). The remaining isolates grouped in four lineages (F, I, J, L) with good statistical support, corresponding to the lineages revealed in the ITS tree and represented undescribed taxa.

The intron/exon arrangement of the  $\beta$ -tubulin data for the *S. schenckii*-*O. stenoceras*-complex (Fig. 5) differed from the other complexes, lacking intron 4 but with intron 5 present. Analyses of these data revealed that group M formed a well-

supported lineage peripheral to the majority of species in the complex but most closely related to *O. nigricarpum* and *O. rostro-coronatum*. The single isolate representing group N, resided in a well-supported lineage that included *O. abietinum*, *O. fusiforme* and *O. lunatum*. However, within the larger group, this isolate formed a distinct lineage between the other three species and thus could not be assigned to any of the three known species with certainty.

### Morphology

Morphological characters of the isolates identified and new species in the phylogenetic studies are described in detail with the species descriptions in the Taxonomy section. However, the morphological differences between the C1 and C2 groups of isolates that formed a monophyletic lineage (Fig. 2, 3) containing an authentic isolate of *O. canum* (CBS 133.51) are worth noting here. The morphology of lineage C1 isolates corresponded with that of *O. canum* (CBS 133.51) of which the *Pesotum* anamorph produces characteristic globose conidia (Mathiesen 1950, 1951, Harrington et al. 2001). The lineage C2 isolates were different from those of C1, producing obovoid conidia.

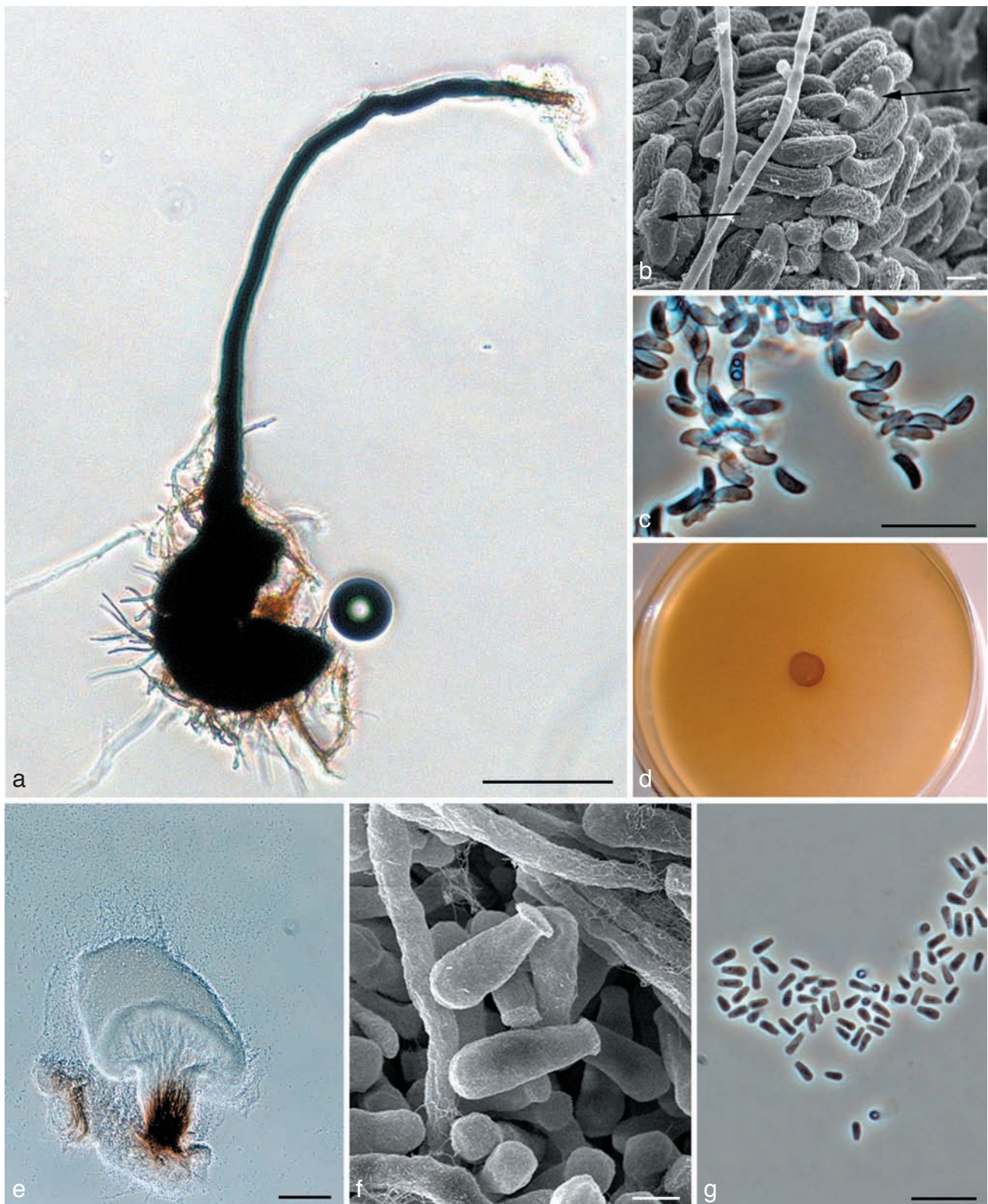
The two single isolates representing lineages I and N, both produced only anamorphs in culture. The isolate in lineage I (CMW 23194) primarily formed a *Hyalarhinochloidiella*-like anamorph but also had a *Pesotum*-like synanamorph, while CMW 28030 (lineage N) produced a well-defined *Sporothrix* anamorph with denticulate conidiogenous cells.

### Mating experiments

Matings between the single conidial cultures resulted in successful crosses, producing ascomata for isolates in lineages B and M, as well as the isolates identified as the European clade of *O. minus* (lineage D). All crosses, including those of the controls for lineages B and D produced ascomata, confirming that these isolates are homothallic. However, lineage M isolates only produced ascomata when different isolates were crossed, and in none of the control crosses, confirming heterothallism in this species. The ascomata resulting from all the crosses produced viable ascospores that gave rise to colonies typical of the parent isolates on MEA. Isolates representing lineages F, J and L did not form ascomata in culture.

### Taxonomy

Based on phylogenetic analyses and morphological comparisons, the isolates collected in this study could be assigned to 15 different taxa, all in the genus *Ophiostoma*. Seven of these are of known species and these include *O. karelicum*, *O. canum*, representatives of the European clade of *O. minus*, *O. floccosum*, *O. brunneo-ciliatum*, *O. ainoae* and *O. bicolor*. One species (lineage C2) could not be distinguished from *O. canum* based on DNA sequences, but had a distinct morphology, and this is consequently treated as '*O. canum*-like'. Another species (lineage N), represented by a single isolate, could not be distinguished from *O. abietinum*, *O. fusiforme* and *O. lunatum* with confidence and we refer to it as '*O. abietinum*-like'. Lineage I, a distinct and undescribed taxon, was represented only by a single isolate, and we have chosen not to describe this species until additional isolates are available for study. This species is thus treated as *Ophiostoma* sp. 'I'. Based on the results of this study, the remaining five lineages (B, F, J, L, M) clearly represent undescribed taxa that are described here as new species of *Ophiostoma*. Two of the novel taxa (lineages B and M) produced ascomata in culture, while the remaining three species (lineages F, J, L) are known only by their anamorph states.



**Fig. 6** Morphological characters of teleomorph and anamorph structures of *Ophiostoma rachisporum* sp. nov. a. Ascoma; b. scanning electron micrograph (SEM) of ascospores; c. ascospores; d. fourteen day old culture on malt extract agar (MEA); e. *Pesotum* anamorph; f. SEM of conidia; g. conidia. — Scale bars: a, e = 100  $\mu$ m; b, f = 1  $\mu$ m; c, g = 10  $\mu$ m.

## Taxon B

***Ophiostoma rachisporum*** Linnakoski, Z.W. de Beer & M.J. Wingf., sp. nov. — MycoBank MB518881; Fig. 6

Anamorph. *Pesotum*-like  
Synanamorph. *Sporothrix*-like

Ascomata post 30 d in agaro cum farina avenae remulisque laricis sterilisatis evoluta, superficialia vel in medio subimmersa. Bases atrae globosae (97–)111–182(–228)  $\mu$ m diametro. Colla ascomatum nigra, recta vel subfalcata, (230–)282–530(–784)  $\mu$ m longa. Hyphae ostiolares absunt. Asco-

sporaе hyalinae, unicellulares, lateraliter visae allantoidea medio cristata (3–)3.5–4(–4.5)  $\times$  1–1.5(–2)  $\mu$ m, a fronte visae cylindricae. Anamorphae *Pesotum* macronematalis et *Sporothriciformis* micronematalis adsunt. Mycelium hyalinum, in agaro superficiale. Crescit optime in 20  $^{\circ}$ C.

*Etymology.* The epithet *rachisporum* refers to the ridged ascospores in this species.

*Ascomata* developing after 30 d on oatmeal agar with sterilised spruce twigs: superficial or partly embedded on media (Fig. 6a). *Bases* dark, globose, (97–)111–182(–228)  $\mu$ m diam; ornamented with brown hyphal hairs (17–)31–70(–103)  $\mu$ m

long, 1–1.5(–2)  $\mu\text{m}$  wide at apex, (1–)1.5–2(–2.5)  $\mu\text{m}$  wide at base. *Ascomatal necks* black, straight or slightly curved, (230–)282–530(–784)  $\mu\text{m}$  long, (22–)30–43(–50)  $\mu\text{m}$  wide at base, (10–)13–20(–25)  $\mu\text{m}$  wide at the apex. *Ostiolar hyphae* absent. *Asci* not observed. *Ascospores* hyaline, 1-celled, allantoid in side view, with a ridge in the middle (3–)3.5–4(–4.5)  $\times$  1–1.5(–2)  $\mu\text{m}$ , cylindrical in frontal view, (2.5–)3–4(–4.5)  $\times$  1–1.5(–2)  $\mu\text{m}$ , globose in end view (Fig. 6b–c). *Pesotum* macronematal anamorph predominant (Fig. 6e). *Synnemata* hyaline, pigmented at the base, (135–)200–365(–547)  $\mu\text{m}$  long including capitulum, (22–)35–75(–110)  $\mu\text{m}$  wide at base; conidiogenous cells (10–)12–21(–30)  $\times$  1  $\mu\text{m}$ ; *conidia* hyaline, 1-celled, smooth, oblong, (2–)3–4(–4.5)  $\times$  1–1.5  $\mu\text{m}$ , (Fig. 6f–g) aggregating into a cream-white mucilaginous spore drop. Synnematal anamorph usually abundant on oatmeal agar, malt extract agar and water agar. Attached to substratum by brown rhizoid-like hyphae. *Sporothrix*-like micronematal anamorph present. *Conidiogenous cells* arising directly from hyphae, (9–)15–27(–35)  $\times$  1–1.5  $\mu\text{m}$ ; *conidia* hyaline, smooth, obovoid to bacilliform, (3–)3.5–5(–7)  $\times$  1–1.5(–2)  $\mu\text{m}$ .

**Culture characteristics** — Mycelium superficial on the agar, hyaline, with white to greyish aerial mycelium (Fig. 6d). Colonies slow growing, reaching 31 mm diam in 8 d at 25 °C, mean radial growth rate 3 ( $\pm$  0.3) mm/d. Growth reduced at 5 and 30 °C. No growth observed at 35 °C. Optimal temperature for growth 20 °C.

**Sexuality** — Homothallic.

**Host range** — Associated with *Trypodendron lineatum* and their galleries on *Picea abies* and *Pinus sylvestris*, and *Hylurgops palliatus* on *P. sylvestris*.

**Distribution** — Presently known from Ilomantsi and Punkaharju, Finland; Kivennapa, Russia.

**Specimens examined.** FINLAND, Southern Savonia, Punkaharju, on *Trypodendron lineatum* infesting *Pinus sylvestris*, Feb. 2006, R. Linnakoski, holotype KUO 021868 (dried culture obtained from cross between CMW 23272  $\times$  CMW 23274, Herbarium of Kuopio Museum of Natural History, Finland), cultures ex-type CBS 128119 = CMW 23272, CBS 128123 = CMW 23274; Southern Savonia, Punkaharju, on *Hylurgops palliatus* infesting *Pinus sylvestris*, Feb. 2006, R. Linnakoski, paratype KUO 021871 (dried culture obtained from CMW 23273).

## Taxon F

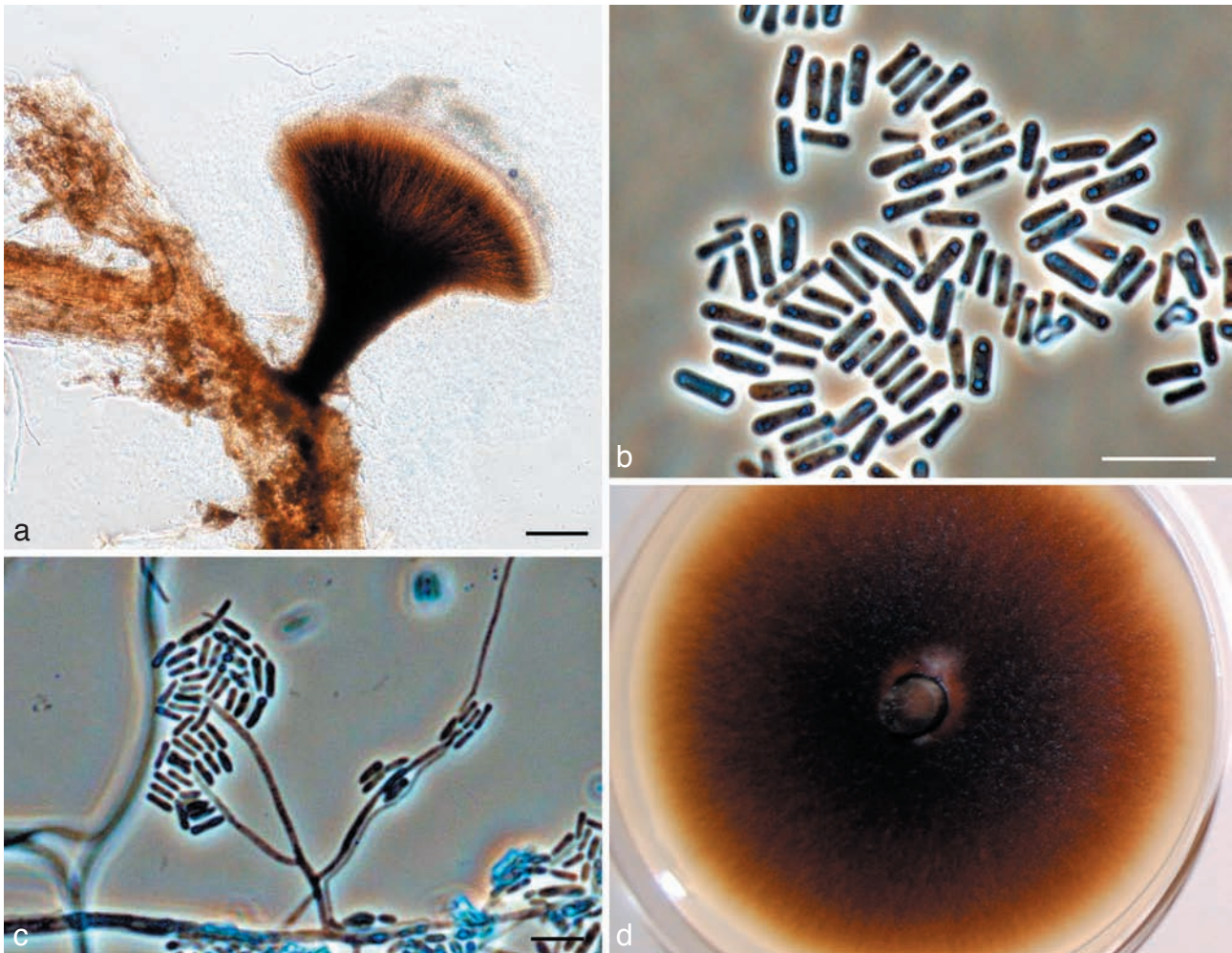
***Ophiostoma tapionis*** Linnakoski, Z.W. de Beer & M.J. Wingf., sp. nov. — MycoBank MB518882; Fig. 7

*Anamorph.* *Hyalorhinocladiella*-like

Teleomorpha ignota. Anamorpha micronematalis *Hyalorhinocladiella* adest. Cellulae conidiogenae (11–)15–25(–31)  $\times$  1–2  $\mu\text{m}$ ; conidia obovoidea vel bacilliformia (4–)4.5–6(–8)  $\times$  (1–)1.5–2.5(–3)  $\mu\text{m}$ . Mycelium primo hyalinum, deinde medio brunnescens, in agaro superficiale. Crescit optime in 20 °C.



**Fig. 7** Morphological characters of anamorph structures of *Ophiostoma tapionis* sp. nov. a. *Hyalorhinocladiella* anamorph; b. conidia; c. SEM of conidia; d. fourteen day old culture on MEA. — Scale bars: a = 100  $\mu\text{m}$ ; b = 10  $\mu\text{m}$ ; c = 3  $\mu\text{m}$ .



**Fig. 8** Morphological characters of anamorph structures of *Ophiostoma fuscum* sp. nov. a. *Pesotum*-like anamorph; b. conidia; c. *Hyalorhinocladiella* anamorph; d. fourteen day old culture on MEA. — Scale bars: a = 100  $\mu$ m; b, c = 10  $\mu$ m.

**Etymology.** The epithet *tapionis* refers to Tapio, a forest god of Finnish mythology. He figures prominently in the Kalevala, the national epic of Finland.

**Teleomorph** unknown. *Hyalorhinocladiella* micronematal anamorph present (Fig. 7a). *Conidiogenous cells* arising directly from hyphae, (11–)15–25(–31)  $\times$  1–2  $\mu$ m; *conidia* hyaline, smooth, obovoid to bacilliform, (4–)4.5–6(–8)  $\times$  (1–)1.5–2.5(–3)  $\mu$ m (Fig. 7b–c).

**Culture characteristics** — *Colonies* at first hyaline, later becoming moderately brown at the centre (Fig. 7d). Mycelium superficial on the agar. Cultures slow growing, reaching 44 mm diam in 8 d at 25  $^{\circ}$ C, mean radial growth rate 4.5 mm ( $\pm$  1) mm/d. Growth reduced at 5 and above 30  $^{\circ}$ C. No growth observed at 35  $^{\circ}$ C. Optimal temperature for growth 20  $^{\circ}$ C.

**Sexuality** — Unknown.

**Host range** — Associated with *Hylurgops palliatus* on *Picea abies* and *Pinus sylvestris*, *Hylastes brunneus* and *Tomicus minor* on *P. sylvestris*, *Ips typographus* and *Pityogenes chalcographus* on *P. abies*.

**Distribution** — Presently known from Punkaharju, Finland; Lisino-Corpus and Kivennapa, Russia.

**Specimens examined.** FINLAND, Southern Savonia, Punkaharju, on *Hylurgops palliatus* infesting *Picea abies*, Feb. 2006, R. Linnakoski, holotype KUO 021872 (dried culture obtained from CBS 128120 = CMW 23265), Herbarium of Kuopio Museum of Natural History, Finland), cultures ex-type CBS 128120 = CMW 23265; Southern Savonia, Punkaharju, on *Hylastes brunneus* infesting *Pinus sylvestris*, Feb. 2006, R. Linnakoski, paratype KUO 021873 (dried culture obtained from CBS 128122 = CMW 23266). — RUSSIA, Lisino-Corpus, on *Hylurgops palliatus* infesting *Pinus sylvestris*, Feb. 2006, R. Linnakoski, paratype KUO 021875 (dried culture obtained from CBS 128121 = CMW 23269).

## Taxon J

***Ophiostoma fuscum*** Linnakoski, Z.W. de Beer & M.J. Wingf., sp. nov. — MycoBank MB518883; Fig. 8

*Anamorph.* *Hyalorhinocladiella*-like  
*Synanamorph.* *Pesotum*-like

*Teleomorpha ignota.* Anamorpha micronematalis *Hyalorhinocladiella* dominans. Cellulae conidiogenae (5–)15–23(–25)  $\times$  1–1.5  $\mu$ m; conidia obovoidea vel bacilliformia (3.5–)4–5(–6)  $\times$  1–1.5  $\mu$ m. Anamorpha macronematalis *Pesotiformis* adest. Synnemata atrobrunnea (240–)284–539(–954)  $\mu$ m longa capitulo incluso. Coloniae aequaliter brunneae, in agaris superficiale. Crescit optime in 25  $^{\circ}$ C.

**Etymology.** The epithet *fuscum* refers to the dark brown colour of the macronematal anamorph and colonies.

**Teleomorph** unknown. *Hyalorhinocladiella* micronematal anamorph predominant. *Conidiogenous cells* (5–)15–23(–25)  $\times$  1–1.5  $\mu$ m; *conidia* hyaline, smooth, obovoid to bacilliform, (3.5–)4–5(–6)  $\times$  1–1.5  $\mu$ m (Fig. 8c). *Pesotum*-like macronematal anamorph present (Fig. 8a–b). *Synnemata* dark brown, (26–)30–66(–109)  $\mu$ m long including capitulum, (26–)30–66(–109)  $\mu$ m wide at base; *conidiogenous cells* (9–)15–24(–30)  $\times$  1–1.5  $\mu$ m (Fig. 8a); *conidia* hyaline, 1-celled, smooth, oblong, (4–)5–6(–7)  $\times$  1–1.5  $\mu$ m, aggregating into a cream-white mucilaginous spore drop (Fig. 8b). Attached to substratum by brown rhizoid-like hyphae.

**Culture characteristics** — Colonies evenly brown (Fig. 8d). Mycelium superficial on the agar, greyish aerial mycelium present. Cultures rather slow growing, reaching 53 mm diam in 8 d at 25  $^{\circ}$ C, mean radial growth rate 6.1 mm ( $\pm$  0.3) mm/d.

Growth reduced at 5 and above 30 °C. Optimal temperature for growth 25 °C.

Sexuality — Unknown.

Host range — Associated with *Ips typographus* and *Pityogenes chalcographus* on *Picea abies*.

Distribution — Presently known from Pyhäselkä, Finland; Kivennapa and Lisino-Corpus, Russia.

*Specimens examined.* FINLAND, Pyhäselkä, on *Pityogenes chalcographus* infesting *Picea abies*, Aug. 2005, Z. W. de Beer, holotype KUO 021877 (dried culture obtained from CMW 23196, Herbarium of Kuopio Museum of Natural History, Finland), cultures ex-type CMW 23196. — RUSSIA, Lisino-Corpus, on *Ips typographus* infesting *Picea abies*, Feb. 2006, R. Linnakoski, paratype KUO 021876 (dried culture obtained from CBS 128124 = CMW 23195); Kivennapa, on *Pityogenes chalcographus* infesting *Picea abies*, Feb. 2006, R. Linnakoski, paratype KUO 021878 (dried culture obtained from CMW 28019).

#### Taxon L

***Ophiostoma pallidulum*** Linnakoski, Z.W. de Beer & M.J. Wingf., *sp. nov.* — MycoBank MB518884; Fig. 9

*Anamorph.* *Hyalorhinocladiella*-like

Teleomorpha ignota. Anamorpha micronematalis *Hyalorhinocladiella* adest. Cellulae conidiogenae (9–)11–19(–26) × 1–1.5 µm; conidia obovoidea vel bacilliformia (2–)2.5–3.5(–4) × 1–1.5 µm. Coloniae hyalinae, in agar superficiale. Crescit optime in 30 °C.

*Etymology.* The epithet *pallidulum* refers to the pale colour of the colonies.

*Teleomorph* unknown. *Hyalorhinocladiella* micronematal anamorph present (Fig. 9a–c). *Conidiogenous cells* (9–)11–19(–26) × 1–1.5 µm (Fig. 9a–b); *conidia* hyaline, smooth, obovoid to bacilliform, (2–)2.5–3.5(–4) × 1–1.5 µm (Fig. 9c).

*Culture characteristics* — Colonies hyaline, white aerial mycelium present (Fig. 9d). Mycelium superficial on the agar. Rather slow growing, reaching 55 mm diam in 8 d at 25 °C,

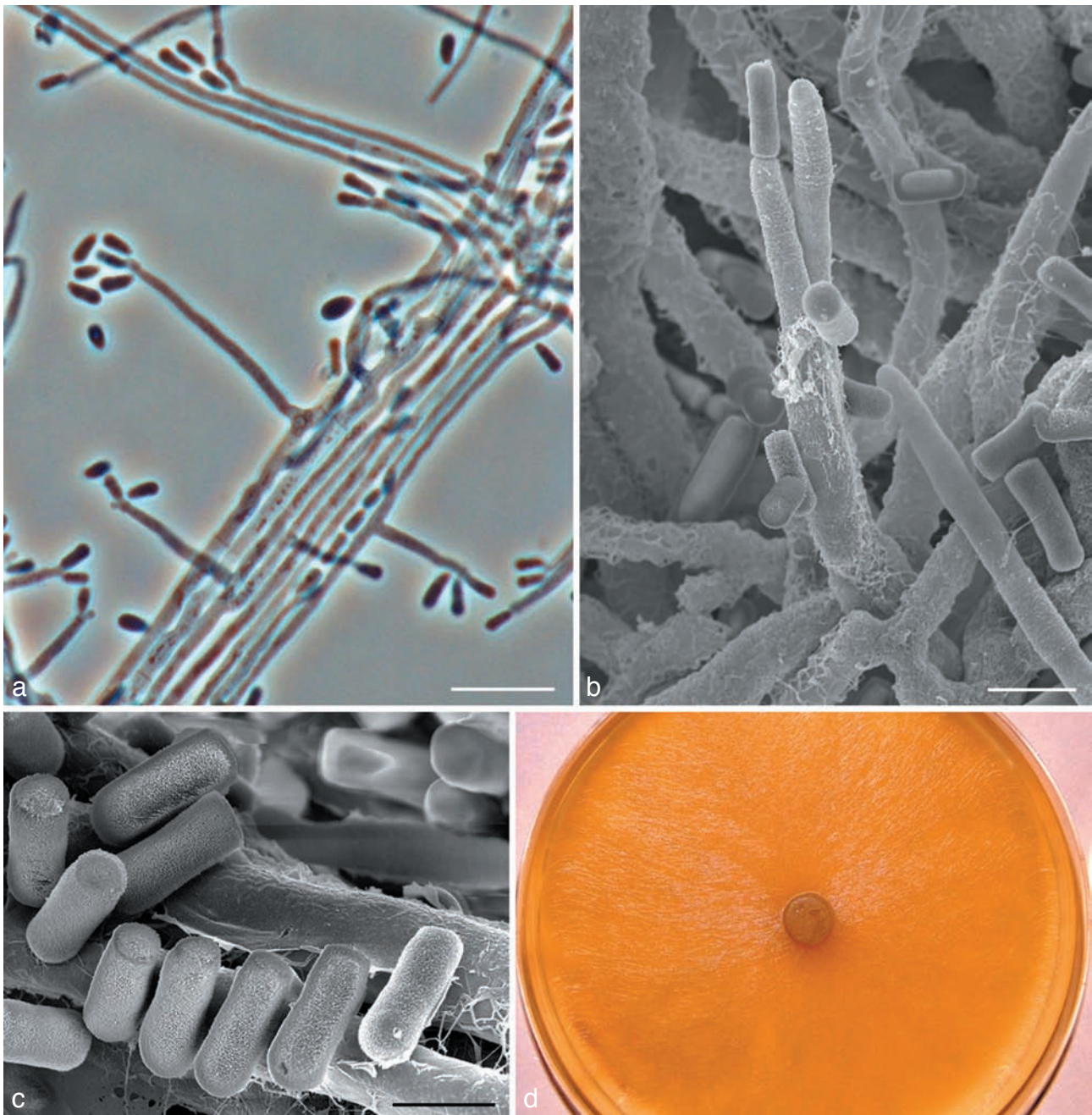
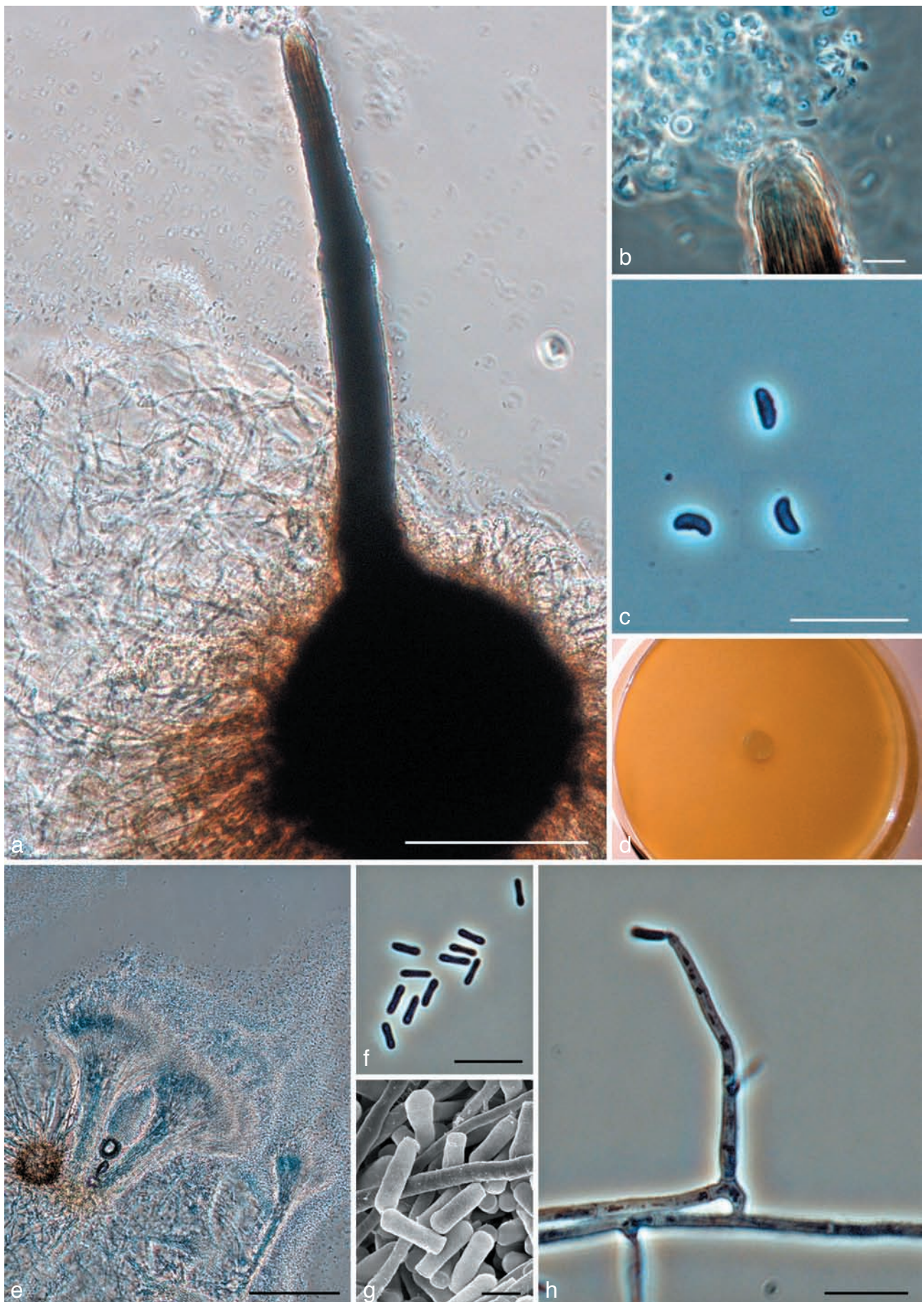


Fig. 9 Morphological characters of *Ophiostoma pallidulum* sp. nov. anamorph structures. a. *Hyalorhinocladiella* anamorph; b. SEM of conidiogenous cells of *Hyalorhinocladiella* anamorph; c. SEM of conidia; d. fourteen day old culture on MEA. — Scale bars: a = 10 µm; b = 3 µm, c = 2 µm.



**Fig. 10** Morphological characters of *Ophiostoma saponiodorum* sp. nov. anamorph and teleomorph structures. a. Ascoma; b. tip of neck with ascospores; c. ascospores; d. fourteen day old culture on MEA; e. *Pesotum*-like anamorph; f. conidia; g. SEM of conidia; h. conidiogenous cell and conidia of *Hyalorhino-cladiella*-like anamorph. — Scale bars: a, e = 100  $\mu$ m; b, c, f, h = 10  $\mu$ m; g = 2  $\mu$ m.



mean radial growth rate 6 mm ( $\pm$  1) mm/d. Growth reduced at 5 and above 30 °C. No growth observed at 35 °C. Optimal temperature for growth 30 °C.

Sexuality — Unknown.

Host range — Associated with *Hylastes brunneus*, *Hylurgops palliatus*, *Tomicus minor*, *Trypodendron lineatum* and *Dryocoetes autographus* on *Pinus sylvestris*.

Distribution — Presently known from Punkaharju, Finland.

*Specimens examined*: FINLAND, Punkaharju, on *Hylastes brunneus* infesting *Pinus sylvestris*, Feb. 2006, R. Linnakoski, holotype KUO 021879 (dried culture obtained from CBS 128118 = CMW 23278, Herbarium of Kuopio Museum of Natural History, Finland), cultures ex-type CBS 128118 = CMW 23278; on *Hylastes brunneus* infesting *Pinus sylvestris*, Feb. 2006, R. Linnakoski, paratype KUO 021880 (dried culture obtained from CMW 23279); on *Hylurgops palliatus* infesting *Pinus sylvestris*, Feb. 2005, R. Linnakoski, paratype KUO 021881, (dried culture CBS 127076 = CMW 23280).

## Taxon M

***Ophiostoma saponiodorum*** Linnakoski, Z.W. de Beer & M.J.

Wingf., sp. nov. — MycoBank MB518885; Fig. 10

*Anamorph*. *Pesotum*-like

*Synanamorph*. *Hyalorhinocladiella*-like

Ascomata post 30 d in agarum cum farina avenae remulisque laricis sterilisatis evoluta, superficialia vel in medio subimmersa. Bases atrae globosae (110–)155–211(–263)  $\mu$ m diametro. Colla ascomatum nigra, recta vel subfalcata, (171–)315–449(–527)  $\mu$ m longa, interdum duo vel tres in quoque ascomate in cultura. Hyphae ostiolaris absunt. Ascospores hyalinae, unicellulares, lateraliter visae allantoidae (2.5–)3–4(–4.5)  $\times$  1–1.5  $\mu$ m, a fronte visae cylindricae. Anamorphae *Pesotum* macronematalis et *Hyalorhinocladiella* micronematalis adsunt. Mycelium odore saponis, hyalinum, in agarum superficiale. Crescit optime in 30–35 °C. Crescere potest in 40 °C.

*Etymology*. The epithet *saponiodorum* refers to the soapy aroma of the colonies.

*Ascomata* developing after 30 d on oatmeal agar with sterilised spruce twigs when two mating types are paired: superficial or partly embedded on media (Fig. 10a). *Bases* dark, globose, (110–)155–211(–263)  $\mu$ m diam; ornamented with brown hyphal hairs (27–)45–94(–119)  $\mu$ m long, 1–1.5(–2)  $\mu$ m wide at apex, 1.5–2(–3)  $\mu$ m wide at base. *Ascomatal necks* black, straight or slightly curved, (171–)315–449(–527)  $\mu$ m long, (28–)32–40(–46)  $\mu$ m wide at base, (9–)11–16(–20)  $\mu$ m wide at the apex. In culture, necks sometimes 2 or 3 per ascoma. *Ostiolar hyphae* absent (Fig. 10b). *Asci* not observed. *Ascospores* hyaline, 1-celled, allantoid in side view, (2.5–)3–4(–4.5)  $\times$  1–1.5  $\mu$ m, cylindrical in frontal view, (2.5–)3–4(–4.5)  $\times$  1–1.5  $\mu$ m, globose in end view (Fig. 10b–c).

*Pesotum*-like macronematal anamorph predominant (Fig. 10e–g). *Synnemata* hyaline, (118–)188–297(–370)  $\mu$ m long including capitulum, (15–)20–43(–78)  $\mu$ m wide at base; *conidiogenous cells* (8–)17–24(–28)  $\times$  1–1.5(–2)  $\mu$ m (Fig. 10e); conidia hyaline, 1-celled, smooth, oblong, (3–)3.5–4(–5)  $\times$  (0.5–)1–1.5  $\mu$ m, aggregating into a cream-white mucilaginous spore drop (Fig. 10f–g). *Synnematal anamorph* usually plentiful on oat meal agar. *Hyalorhinocladiella*-like micronematal anamorph present (Fig. 10h). *Conidiogenous cells* arising directly from hyphae, (9–)14–24(–35)  $\times$  1–2(–2.5)  $\mu$ m; *conidia* hyaline, smooth, obovoid to bacilliform, (3–)4–6(–7)  $\times$  1–1.5(–2)  $\mu$ m.

*Culture characteristics* — Mycelium hyaline, white aerial mycelium present (Fig. 10d). Colonies rather fast growing, reaching 72 mm diam in 8 d at 25 °C, mean radial growth rate 8 mm ( $\pm$  0.5) mm/d. Capable growing at high temperatures (40 °C). No growth observed at 5 °C and 45 °C. Optimal temperature for growth 30–35 °C.

Sexuality — Heterothallic.

Host range — Associated with *Ips typographus* and *Pityogenes chalcographus* on *Picea abies*.

Distribution — Presently known from Pyhäselkä and Jouhterinen, Finland; Roikonkoski, Russia.

*Specimens examined*. FINLAND, North Karelia, Pyhäselkä, on *Ips typographus* infesting *Picea abies*, Aug. 2005, Z.W. de Beer, holotype KUO 021882 (dried culture obtained from cross between CBS 127078 = CMW 34945  $\times$  CBS 128125 = CMW 30883, Herbarium of Kuopio Museum of Natural History, Finland), cultures ex-type CBS 127078 = CMW 34945; on *Ips typographus* infesting *Picea abies*, Aug. 2005, Z.W. de Beer, paratype KUO 021886 (dried culture obtained from CBS 128125 = CMW 30883); on *Pityogenes chalcographus* infesting *Picea abies*, Aug. 2005, Z.W. de Beer, paratype KUO 021887 (dried culture obtained from CBS 127079 = CMW 34944).

## DISCUSSION

In this study, 717 fungal isolates resembling *Ophiostoma* were found in association with 11 different bark beetle species and their galleries, infesting *Picea abies* and *Pinus sylvestris* in the Karelia region of Finland and Russia. These fungi included seven known species: *Ophiostoma ainoae*, *O. bicolor*, *O. brunneo-ciliatum*, *O. canum*, *O. floccosum*, *O. karelicum*, and the European lineage of *O. minus*. Six new *Ophiostoma* spp. were discovered, five of which were described here as *O. fuscum*, *O. pallidulum*, *O. rachisporum*, *O. saponiodorum* and *O. tapionis*. The remaining novel taxon emerging from the analyses was not described formally because it was represented by only a single isolate. The identity of two species, one closely related to *O. canum* and the other close to *O. abietinum* could not be resolved with certainty and these require further investigation. *Ophiostoma canum* is reported for the first time from Finland, and *O. brunneo-ciliatum*, *O. canum* and *O. floccosum* for the first time from Russia. The presence of all the other species previously known to be present in these countries is confirmed for the first time based on DNA sequence comparisons.

### The *O. piceae*-complex and similar species

Phylogenetic inference based on DNA sequence comparisons as well as morphological features made it possible to assign isolates obtained in this study to one of four species complexes in *Ophiostoma*. These included *O. piceae*-, *O. minus*-*O. ips*- and *S. schenckii*-*O. stenoceras*-complexes. The majority of the isolates resided in the *O. piceae*-complex. This complex was defined by Harrington et al. (2001) as a monophyletic lineage accommodating species having unsheathed, allantoid ascospores and both synnematos *Pesotum* and mononematos *Sporothrix* anamorphs. DNA sequence comparisons for additional species and gene regions have revealed that the *O. piceae*-complex includes at least two major lineages, respectively representing species isolated from hardwoods (Linnakoski et al. 2008, 2009, Nkuekam et al. 2008, Grobbelaar et al. 2009, 2010) and coniferous hosts (Uzunovic et al. 2000, Chung et al. 2006). In all species of the *O. piceae*-complex for which  $\beta$ -tubulin sequences are available, this gene region includes intron 4 and lacks intron 5 (Zipfel et al. 2006, Grobbelaar et al. 2009).

In the present study, the most commonly encountered species were *O. canum* and a closely related putatively new species, which we have referred to as *O. canum*-like. These species were isolated from all locations in association with several different bark beetle species infesting both pine and spruce. The two species could not be distinguished based on ITS and  $\beta$ -tubulin sequences, but they are morphologically distinct. *Ophiostoma canum* produces globose conidia, while the *O. canum*-like species produces obovoid conidia, which are more similar to *O. piceae* and other species in the *O. piceae*-complex (Harrington et al. 2001). We were not able to amplify the EF 1- $\alpha$  gene region for these species, but additional gene regions should be explored further to distinguish between these taxa.

*Ophiostoma canum* was originally described from sapstained pine in Germany (Münch 1907). It has since been reported from *Tomicus piniperda* and *T. minor* in Sweden (Mathiesen 1950, Rennerfelt 1950) and Japan (Masuya et al. 1999), although identification of isolates in the latter study was based only on morphology. Results of the present study are first to suggest that the species is vectored by other beetle species such as *Ips typographus*, *Hylastes brunneus*, *Hylurgops palliatus*, *Pityogenes chalcographus* and *Trypodendron lineatum*.

The unidentified *O. canum*-like species that was frequently isolated in this study is morphologically similar to *O. piceae*. Surprisingly, despite the relatively large number of different bark beetle species and locations sampled in this study, no isolates of *O. piceae* (sensu Morelet 1992, Brasier & Kirk 1993, Harrington et al. 2001) were obtained. *Ophiostoma piceae* had been reported from Finland and Russia in several earlier studies (Potlajczuk & Schekunova 1985, Viiri 1997, Pashenova et al. 2007), as well as from similar beetles and hosts in neighbouring Scandinavian countries (Lagerberg et al. 1927, Mathiesen 1950, Rennerfelt 1950, Roll-Hansen & Roll-Hansen 1980, Solheim 1986). The *O. canum*-like species emerging from this study might be the same as isolates from Scandinavia previously identified as *O. piceae* based on morphology only. Beta-tubulin sequences of these *O. canum*-like isolates differ substantially (Fig. 2, 3) from those of authentic *O. piceae*-isolates from other studies (Jacobs & Kirisits 2003, Kim et al. 2005, Bommer et al. 2009, Grobbelaar et al. 2009). DNA sequence comparisons must thus be used to confirm the identification of the so-called *O. piceae* isolates from previous studies in Scandinavia and Russia.

The third species belonging to *O. piceae*-complex found emerging from the present study is also related to *O. canum* and the *O. canum*-like species, but could be distinguished from these taxa in ITS and  $\beta$ -tubulin sequences. This species was described as *O. rachisporum*, which produces both *Pesotum* and *Sporothrix* anamorphs and allantoid ascospores. The species was mainly isolated from *Trypodendron lineatum* on both pine and spruce. It is consequently well placed in the conifer lineage of the *O. piceae*-complex based on phylogeny, morphology and ecology.

It was surprising to find *O. karelicum*, a representative of the hardwood clade in *O. piceae*-complex, in the present study. This fungus has consistently been isolated in association with the birch bark beetle, *Scolytus ratzeburgi* and has been found in Finland, Russia and Norway (Linnakoski et al. 2008, 2009). In the present study, six isolates were found in association with *Ips typographus* and *Tomicus minor* from pine and spruce. These results suggest that *O. karelicum* can be vectored by bark beetle species other than *S. ratzeburgi*, possibly together with predators and mites associated with these insects.

Another species previously associated with the *O. piceae*-complex, *O. floccosum* (Harrington et al. 2001), was found in association with several bark beetle species infesting both pine and spruce in Russia. This species groups only peripherally to the hardwood and conifer clades of the *O. piceae*-complex (Zipfel et al. 2006, Grobbelaar et al. 2009), but based on morphology, resides within the complex (Harrington et al. 2001). *Ophiostoma floccosum* is widely distributed, but usually reported in low numbers associated with bark beetles primarily on pine trees (Mathiesen 1951, Harrington et al. 2001, de Beer et al. 2003b, Jacobs et al. 2003, Kim et al. 2007, Lu et al. 2009, Yamaoka et al. 2009).

### The *O. minus*-complex

The *O. minus*-complex presently includes *O. minus*, *O. pseudo-tsugae*, and *O. kryptum* (Jacobs & Kirisits 2003, Jacobs et al. 2003, Gorton et al. 2004). These species are distinguished

from those in the *O. piceae*-complex by ascospores with short necks, elongated ascospores and *Hyalorhinocladiella* anamorphs (Hedgcock 1906, Upadhyay 1981, Jacobs & Kirisits 2003, Jacobs et al. 2003). The  $\beta$ -tubulin intron composition corresponds with that of the *O. piceae*-complex, which includes intron 4 and lacks intron 5 (Gorton et al. 2004). Although *O. tetropii* groups among species of the *O. minus* complex based on our  $\beta$ -tubulin analysis and that of Jacobs & Kirisits (2003), it groups closer to the hardwood clade of the *O. piceae* complex based on ITS (Jacobs & Kirisits 2003, Jacobs et al. 2003). Its ascospores can also be distinguished from those of the other three species in the complex by its much longer necks (Jacobs et al. 2003). We thus do not treat *O. tetropii* as part of the *O. minus* complex until more data proves otherwise.

Several phylogenetic studies have shown a distinction between European isolates of *O. minus* and North American isolates that represent the type of *O. minus* (Hedgcock 1906, Gorton & Webber 2000, Gorton et al. 2004, Lu et al. 2009). The isolates collected in this study from Russia and Finland grouped with the European clade of *O. minus*, which probably corresponds with *O. pini*. *Ophiostoma pini* was described from pine by Münch (1907), but since 1956 has been considered a synonym of *O. minus* (Hunt 1956, Olchowecki & Reid 1974, Upadhyay 1981). Further studies including an inspection of the type material, additional cultures of both species, and comparisons of sequences for gene regions additional to those previously considered, will be needed to clarify the taxonomy of this group.

*Ophiostoma minus* is one of the most important agents of blue stain in Europe and North America (Solheim & Långström 1991, Seifert 1993). The species is frequently isolated from coniferous trees, mainly from pine species attacked by *Tomicus piniperda* in Europe (Mathiesen 1950, Mathiesen-Käärik 1953, Lieutier et al. 1989, Solheim & Långström 1991), but it is also occasionally found in association with other beetle species in the region (Käärik 1980, Upadhyay 1981, Kirisits 2004). A single isolate with sequence similarity to the European lineage was recently reported from China (Lu et al. 2009). In the present study, *O. minus* was most commonly isolated from pine attacked by *T. minor*, but also in association with several other beetle species.

### The *O. ips*-complex

Species in *Ophiostoma ips*-complex (s.str.) are characterised by cylindrical or allantoid ascospores with pillow-shaped sheaths, and they produce a continuum of anamorph forms ranging from primarily *Hyalorhinocladiella*-type structures to more rarely *Pesotum*-like synnematous structures (Zipfel et al. 2006). Five species have been shown to reside in the *O. ips*-complex s.str. based on phylogenetic inference. These include *O. adjuncti*, *O. bicolor*, *O. ips*, *O. montium* and *O. pulvinisporum* (Kim et al. 2003, Zhou et al. 2004, 2006, Massoumi Alamouti et al. 2007). Kim et al. (2003) showed that the  $\beta$ -tubulin gene of *O. montium* lacked introns 4 and 5 and consequently, this species was excluded from our analyses of the  $\beta$ -tubulin dataset, in which all the other species had intron 4, and no intron 5. However, based on ITS sequences and morphology, *O. montium* remains best placed in the *O. ips*-complex s.str., at least until sequence data for other gene regions can resolve its status.

Massoumi Alamouti et al. (2007) included an undescribed *Hyalorhinocladiella* sp. (1) in the *O. ips*-complex. Analyses in the present study and the results of Massoumi Alamouti et al. (2007) showed that several species group close to, but not as part of the monophyletic *O. ips*-complex s.str. (Fig. 2, 4). For the purpose of this discussion, we treat this larger lineage as the *O. ips*-complex s.l. The larger lineage includes an undescribed *Hyalorhinocladiella* sp. (2) and two undescribed *Ambrosiella* spp. from the study of Massoumi Alamouti et al. (2007). Data

from the present study also suggest a similar placement for *O. ainoae* and *O. brunneo-ciliatum*, somewhere between the *O. ips*- and *O. piceae*-complexes, but closer to the *O. ips*-complex s.str. The sheathed, pillow-shaped ascospores and *Pesotum*-like anamorphs of *O. ainoae* (Solheim 1986) and *O. brunneo-ciliatum* (Mathiesen-Käärik 1953) correspond well with those of species in the *O. ips*-complex s.str.

From the bark beetle-associated isolates collected in Finland and Russia, seven species could be placed in the *O. ips*-complex s.l. as defined above. The first of these was *O. brunneo-ciliatum*, previously isolated from pines and larch infested by *Ips* spp. in Europe and Japan (Mathiesen-Käärik 1953, Lieutier et al. 1989, Yamaoka et al. 1998). Isolates in the present study came from Russia and mainly from *I. typographus* on spruce, but also from *Pityogenes chalcographus*, *Tomicus minor* and *T. piniperda* on pine. The fungus seems to be a constant associate on larch of *I. cembrae* in Europe and *I. subelongatus* in Japan (Yamaoka et al. 2009) and was shown to be only mildly pathogenic to its host trees (Lieutier et al. 1989, Yamaoka et al. 1998).

Another species found in the present study was *O. ainoae*, normally associated with *Ips typographus* infesting spruce in Europe (Solheim 1986, Jankowiak 2005). All our *O. ainoae* isolates were collected from Russian sites, mainly in association with *I. typographus*, but also with *Dryocoetes autographus* and *Hylurgops palliatus* infesting spruce, and *T. minor* infesting pine. This species has also been reported from *I. typographus* f. *japonicus* from Japan (Yamaoka et al. 1997), but an isolate from the original Japanese collection groups in *Grosmania*, close to *G. cucullata*, based on ribosomal DNA sequences (Okada et al. 1998, Gebhardt et al. 2005). The appropriate placement of the Japanese species thus needs to be verified using DNA sequence comparisons and including additional cultures.

The third known species from the present study belonging to the *O. ips*-complex was *O. bicolor*, originally described by Davidson (1955). It is a common species in the Northern Hemisphere, occurring on spruce infested by *Ips* spp. (Davidson et al. 1967, Käärik 1975, Solheim 1986, Yamaoka et al. 1997, Jankowiak 2005, Massoumi Alamouti et al. 2007). In our study, isolates of *O. bicolor* were collected from Russia in an area with extensive *I. typographus* damage on spruce. These ecological characteristics are consistent with the previously reported fungus-vector relationships for the species.

DNA sequence comparisons for two gene regions of the new taxa described here as *O. tapionis*, *O. fuscum* and *O. pallidulum* confirmed that these species form discrete, well-supported lineages within the broader *O. ips*-complex. None of the new species produced teleomorphs in culture, but all three produced a *Hyalorhinocladiella* anamorph, typical of species in the complex. One species, *O. fuscum*, also had a *Pesotum*-like synnematomous anamorph, similar to those produced by *O. ips* (Benade et al. 1995, Okada et al. 1998) and *O. pulvinisporum* (Zhou et al. 2004). In the ITS tree (Fig. 2), *O. pallidulum* grouped most closely to *O. nigricarpum* and *O. saponiodorum*, which are outliers of the *S. schenckii*-*O. stenoceras* complex. However, the *Hyalorhinocladiella* anamorph and  $\beta$ -tubulin intron 4 of *O. pallidulum* resemble those of species in the larger *O. ips*-complex (s.l.), in which we have treated it (Fig. 2, 4). *Ophiostoma fuscum*, *O. pallidulum* and *O. tapionis* were isolated from various beetle species on pine and spruce. *Ophiostoma pallidulum* was found only in Finland.

The species that remains undescribed, *Ophiostoma* sp. I, was isolated only once from *Dryocoetes autographus* on spruce in Finland. This isolate also produces a *Hyalorhinocladiella* anamorph and a *Pesotum*-like synanamorph, which supports its phylogenetic placement in the *O. ips*-complex close to other undescribed *Hyalorhinocladiella* species (Fig. 2, 4) from Canada

(Massoumi Alamouti et al. 2007). Additional isolates are needed to justify the description of *Ophiostoma* sp. I as new.

### The *S. schenckii*-*O. stenoceras*-complex

The *S. schenckii*-*O. stenoceras*-complex is characterized by reniform ascospores without a sheath, a *Sporothrix* anamorph (de Beer et al. 2003a), and the absence of intron 4 and presence of intron 5 in the  $\beta$ -tubulin gene (Zipfel et al. 2006). The complex accommodates several *Sporothrix* species associated with human disease (Marimon et al. 2007), soil, wood (Aghayeva et al. 2004, de Meyer et al. 2008), or *Protea infructescences* with their suite of insects and mites (Roets et al. 2008). It is interesting that most species in the *S. schenckii*-*O. stenoceras*-complex does not have bark beetle associates, which are typical for the all other genera and species complexes in the *Ophiostomatales*.

Isolates obtained in the present study included one new taxon, *O. saponiodorum*, that grouped in a lineage with *O. coronatum*, *O. nigricarpum*, *O. rostricornatum* and *O. tenellum*, peripheral to the major lineage of the *S. schenckii*-*O. stenoceras*-complex (Fig. 2, 5). These species are all of North American origin and most have been collected from stained conifer wood (Davidson 1958, 1966, Olchowecki & Reid 1974). The exception is *O. rostricornatum* that was isolated from birch pulpwood (Eslyn & Davidson 1976). Like these species, *O. saponiodorum* also produced unshathed, allantoid ascospores. However, *O. saponiodorum* differs from other species in the complex with which it groups that have *Sporothrix* anamorphs. The micronematous anamorph of *O. saponiodorum* lacks clear denticles characteristic of *Sporothrix* species (de Hoog 1974), and thus looks more *Hyalorhinocladiella*-like (Upadhyay & Kendrick 1975). In addition, it has a hyaline *Pesotum*-like synanamorph. Olchowecki & Reid (1974) described the anamorph of *O. coronatum* as 'micronematous or semimicronematous', which might suggest a similar type of structure, although this cannot be seen in their illustrations. Hyaline synnemata, referred to by some authors as so-called *Hyalopesotum* anamorphs (Upadhyay & Kendrick 1975), are not a unique character and have been reported for several other species from different lineages in the *Ophiostomatales*. These are now considered to be a reduced form of the more common *Pesotum*-like synnemata (Wingfield et al. 1991, Okada et al. 1998), which often occur together with *Sporothrix* or *Hyalorhinocladiella* anamorphs.

The identity of lineage N, consisting only of a single isolate from pine in Russia, could not be resolved. The isolate is closely related to *O. abietinum*, *O. fusiforme* and *O. lunatum*. *Ophiostoma abietinum* was originally described from galleries of a *Pseudohylesinus* sp. on *Abies vejari* in Mexico (Marmolejo & Butin 1990). It has subsequently often been reported erroneously as *O. nigricarpum*-like and occurs in low numbers associated with pine bark beetles in various countries (de Beer et al. 2003a, Aghayeva et al. 2004, Thwaites et al. 2005, Zhao et al. 2005, Zhou et al. 2006, Lu et al. 2009). *Ophiostoma fusiforme* and *O. lunatum* have been found only on hardwoods in Europe (Aghayeva et al. 2004). Considering its host, it seems possible that the Russian isolate represents a new haplotype of *O. abietinum*, but additional isolates and sequences for an additional number of gene regions will be necessary to resolve its identity.

### Conclusions

The aim of this study was to characterize *Ophiostoma* species associated with pine- and spruce-infesting bark beetles in Finland and neighbouring Russia. From a superficial assessment of our results, the diversity of *Ophiostoma* species is higher in Russia than in Finland, with 10 species found in Russia, and only seven from Finland.

Results of the present study identified some of the fungal associates of *Hylastes brunneus*. These include *Ophiostoma canum*, an *O. canum*-like species, *O. pallidulum* and *O. tapionis*. To the best of our knowledge, there have been no previous reports regarding the fungal associates of *H. brunneus*.

A surprising outcome of the present study is that there are many more *Ophiostoma* spp. occurring on pine and spruce in the boreal forests of Finland and Russia, than has previously been recognized. The number of new taxa encountered, covering a relatively small geographical area considered in this survey, emphasizes the importance of developing a clear understanding of the possible threats of moving timber and wood products, without knowledge of the micro-organisms that might also be moved. It will, therefore, be important to expand these surveys to wider geographical areas of Finland and Russia, and also to consider the pathogenicity of the species involved.

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