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Filamentous Fungi and Yeasts Associated with Mites Phoretic on *Ips typographus* in Eastern Finland

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Abstract: The European spruce bark beetle (*Ips typographus*) has become a major forest pest in Finland in recent years. The beetle is a well-known vector of mainly ophiostomatoid fungi causing blue-stain of timber and pathogens that have the ability to amplify the insect damage. It also vectors other associated organisms, such as phoretic mites. The ecology of these mites remains poorly understood, including their associations with fungi. In this study, we considered filamentous fungi and yeasts associated with mites phoretic on *I. typographus*. Fungal identifications were based on DNA sequences and phylogenetic analyses of the ITS and/or partial β-tubulin gene regions. Fifteen fungal species were detected, including eight yeasts and seven filamentous fungi. Eleven percent of the beetles carried mites and of these 74% carried at least one fungal species. An average of two fungal species were carried per mite. The most commonly found filamentous fungi were *Grosmannia penicillata* (25%), *Ophiostoma bicolor* (19%), *O. ainoae* (12%) and *O. brunneolum* (12%). Of the yeast species, the most commonly found was *Wickerhamomyces bisporus* (47%). This study is the first to report yeasts associated with *I. typographus* and its phoretic mites in Finland. Majority of the filamentous fungal species found are those previously reported in association with *I. typographus*. The results also confirmed that many of the fungal species commonly found on *I. typographus* are also associated with its phoretic mites. However, the nature of the symbiosis between the mites, beetles and fungal associates remains to be understood.

Keywords: boreal forests; fungal diversity; insect-fungus symbiosis; ophiostomatoid fungi; spruce bark beetle; yeasts

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1. Introduction

The European spruce bark beetle (*Ips typographus* L.) is a major forest pest in Europe. In recent years, its importance has increased also in the Northern Europe due to changing climate [1,2]. The beetle is a vector of fungi and other microorganisms, such as phoretic mites and nematodes [3–5]. Particularly common fungal associates are ophiostomatoid fungi (Ophiostomatales and Microascales), a polyphyletic group of fungi having morphological and chemical co-adaptations to arthropod dispersal [6,7]. The majority of these fungi are saprophytic species, but some are tree-killing pathogens having the ability to amplify the insect damage.

Fungi associated with *I. typographus* have been relatively well studied in Europe. Certain species such as *Endoconidiophora polonica* (Siemaszko) Z.W. de Beer, T.A. Duong & M.J. Wingfield, *Grosmannia penicillata* (Grosmann) Goid., *Grosmannia europhioides* (E.F. Wright & Cain)

Zipfel, Z.W. de Beer & M.J. Wingf. and *Ophiostoma bicolor* R.W. Davidson & D.E. Wells are amongst the most frequently found fungi associated with the beetle [8–11]. Although *I. typographus* and its fungal associates represents one of the most extensively studied examples of bark beetle-fungi interactions, new fungal species discoveries and descriptions are not uncommon [12–14]. Recent studies have reported occurrence of spatial and temporal differences in the fungal species assemblages [3,10] and showing volatile organic compounds influencing the beetle-fungi interactions [6]. Despite these advances, many aspects like multitrophic interactions involving beetles, mites and fungi and factors maintaining these complex interactions remain poorly known [15].

Increasing evidence suggests that bark beetle-associated mites are important vectors of fungi present in beetle galleries [10,16–19]. The mites can carry fungal spores on their bodies or in specialized structures (sporothecae) to new host trees [19]. Some mite species are mycetophagous feeding on fungi; mites can promote mutualistic fungi and increase fungal survival by reducing antagonistic fungi in the bark beetle [20,21].

The vast majority of studies have focused on the diversity of ophiostomatoid fungi associated with *I. typographus* and to some extent also with its phoretic mites. In contrast, little is known regarding the other fungi involved in these interactions. In addition to ophiostomatoid fungi, yeasts have also been recognized as constant components in bark and ambrosia beetle galleries [22,23] and in the guts of the beetles [24]. Yeasts have most likely been overlooked in majority of the previous collections. Particularly common amongst these yeasts are those in the Ascomycotes that rely on vectors to move to new hosts trees [25].

The aim of the study was to provide baseline knowledge on fungal diversity associated with mites phoretic on *I. typographus* infesting *Picea abies* L. in Eastern Finland.

2. Materials and Methods

2.1. Study Area and Collection of Samples

Wind-felled *P. abies* trees infested by *I. typographus* were sampled from June to September 2017 in two spruce-dominated forests in North Karelia province; Kuhasalo (N 62°34′46″; E29°44′13″) and Rasimäki (N 62°30′62″; E 29°58′27″). Adult living beetles were collected either directly from the galleries (each beetle from a separate gallery, maximum ten beetles from the same tree individual) in Kuhasalo or using Ipsowit® Standard (Witasek Pflanzen-Schutz GmbH, Feldkirchen in Kärnten, Austria) in Rasimäki pheromone funnel traps that were emptied weekly during the sampling period and stored individually in Eppendorf tubes. To prevent living mite contamination of the mycology laboratory, the beetles were stored at −20 °C at least for 24 h prior to fungal isolations. Beetles were morphologically identified using a dissecting microscope. At the same time, phoretic mites on the beetles were collected individually, crushed with sterilized tools and plated directly onto 2% malt extract agar (MEA; 2% malt extract from Biokar Diagnostics, Beauvais, France and 2% agar from Fisher Scientific, Mexico) in Petri dishes containing 0.05 g/L of streptomycin sulphate (Sigma-Aldrich, St. Louis, MO, USA). The plates were incubated in the dark at 25 °C for 2–4 weeks and inspected regularly for fungal growth. Emerging mycelium and spore masses (including yeasts) were transferred to fresh MEA plates (without streptomycin) and subcultured until pure cultures were obtained. Occasionally, 2% water agar was necessary to use to obtain pure cultures. Purified cultures were grouped based on morphological and culture characteristics and at least one isolate from each group was subjected to DNA-based identification. The isolates obtained in this study were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1). The isolates were also stored in the culture collection of the Natural Resources Institute Finland (Luke), Helsinki, Finland.

Table 1. Fungal isolates obtained from mites phoretic on *Ips typographus* in Eastern Finland.

Species	Isolate no		Mite No.	GenBank Acc. No.	
	Personal [1]	CMW [2]		ITS	β-Tubulin
Ceratocystiopsis minuta (Siemaszko) H.P. Upadhyay & W.B. Kendr.	A10-1aba	51,536	A7-1d		MW345787
	A7-1d	51,426	A7-1		MW345786
	B3-1aab		B3-1		
	B3-6balla	51,448	B3-6		MW345784
	B6-1ebb	51,350	B6-1		MW345785
Chionosphaera cuniculicola R. Kirschner, Begerow & Oberw.	B1-2aaaa	51,590	B1-1	MW256646	
Endoconidiophora polonica	B3-7ab	51,333	B3-7	MW256648	
	B5-7ab		B5-7	MW256650	
	B6-2aba	51,327	B6-2	MW256649	
	B6-2ac	51,463	B6-2		
	B6-3ca		B6-3	MW256647	
Fontanospora fusiramosa Marvanová, Peter J. Fisher & Descals	B1-1b2	51,453	B1-1		
Graphium fimbriisporum (M. Morelet) K. Jacobs, Kirisits & M.J. Wingf.	A4-2aab		A4-2		
	A6-1ad	51,382	A6-1	MW256653	
	A7-2aca	51,389	A7-2	MW256652	
	B6-2ad	51,329	B6-2	MW256651	
	B6-3cb		B6-3		
Grosmannia penicillata	A6-2b	51,417	A6-2		
	A6-2cbb	51,432	A6-2		MW345794
	A6-3adb		A6-3		
	A6-3c	51,353	A6-3		MW345789
	A9-1cba	51,412	A9-1		MW345788
	B5-1b	51,337	B5-1		MW345793
	B6-1c	51,338	B6-1		MW345790
	B6-1ca		B6-1		
	B6-2b	51,435	B6-2		
	B6-2c		B6-2		
	B6-2eca	51,446	B6-2		MW345791
	B5-1dbb	51,423	B5-1		MW345792
	A10-1b	51,376	A10-1		MW345795
	A10-3ada		A10-1		MW345796
	A6-3da	51,470	A6-3		
Kuraishia capsulata (Wick.) Y. Yamada, K. Maeda & Mikata	A4-2aaaa	51,570	A4-2	MW256635	
	G83-1aa	51,578	G83	MW256634	
Kuraishia molischiana Dlauchy, G. Péter, Tornai-Leh. & Kurtzman	B2-3aa	51,574	B2-3	MW256633	
Nakazawaea sp.	B8-1aaa		B8-1		
	B8-1abb	51,543	B8-1	MW256638	
	B2-3b	51,542	B2-3	MW256639	
Ogataea glucozyma (Wick.) Y. Yamada, K. Maeda & Mikata	F30-1caa	51,585	F30	MW256636	
Ogataea ramenticola (Kurtzman) Kurtzman & Robnett	B3-1aaaa	51,591	B3-1	MW256637	
Ophiostoma ainoae H. Solheim	A4-2aab	51,342	A4-2		MW345797
	A4-2b-1	51,464	A4-2		
	A7-2db	51,361	A7-2		MW345800
	A9-1cbbb	51,354	A9-1		MW345798
	B6-2ea	51,459	B6-2		
	B6-2ebb		B6-2		
	B6-2ecb	51,341	B6-2		MW345799

Table 1. *Cont.*

Species	Isolate no		Mite No.	GenBank Acc. No.	
	Personal [1]	CMW [2]		ITS	β-Tubulin
Ophiostoma bicolor	A10-2b	51,355	A10-1		MW345809
	A7-2ab	51,367	A7-2		MW345806
	A7-2b	51,364	A7-2		
	A7-2da	51,462	A7-2		
	B1-1d	51,442	B1-1		MW345805
	B3-1aba		B3-1		
	B3-1b	51,352	B3-1		MW345804
	B3-1d	51,425	B3-1		
	B3-7ad	51,343	B3-7		MW345807
	A10-1dac	51,456	A10-1		
	A10-1db	51,427	A10-1		
	B3-7ac		B3-7		
	A7-1b		A7-1		MW345808
Ophiostoma brunneolum Linnak., Z.W. de Beer & M.J. Wingf.	B1-3ab		B1-3		
	B1-3gab	51,328	B1-3		
	B1-3gbb	51,371	B1-3		MW345801
	B3-7cb		B3-7		
	B8-1ac	51,454	B8-1		MW345802
	B8-2ba	51,422	B8-2		MW345803
	B8-2bb	51,346	B8-2		
	B8-cb	51,375	B8-2		
Wickerhamomyces bisporus (O. Beck) Kurtzman, Robnett & Basehoar-Powers	A6-1aaba	51,547	A6-1	MW256640	
	A6-3aaa	51,544	A6-3	MW256641	
	A7-2aaa	51,575	A7-2	MW256644	
	B1-1a		B1-1		
	B1-3aaa	51,584	B1-1	MW256642	
	B2-1aaa	51,581	B2-1	MW256645	
	B2-2aaa	51,589	B2-2	MW256643	

[1] Personal collection stored at the culture collection of the Natural Resources Institute Finland (Luke), Helsinki, Finland; [2] CMW: Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

2.2. DNA Extraction, PCR and Sequencing

Prior to DNA extraction, the fungi were grown in 5 mL of liquid culture medium (2% malt extract and 0.5% yeast extract from Biolab, Midrand, South Africa) in 20 mL glass vials, for 24 h with shaking (120 rpm). The fungal tissues were then transferred into 1.5 mL Eppendorf tubes and freeze-dried. The tubes were snap-frozen in liquid nitrogen and the mycelium was ground into fine powder using micro pestles. DNA was extracted from grounded mycelium powder following the method as described by [10].

The internal transcribed spacer gene (ITS) regions including the 5.8S gene and in some cases the partial beta-tubulin (BT) gene were amplified and sequenced. The primers used for PCR amplification and sequencing were ITS1-F [26] and ITS4 [27] for the ITS region and T10 [28] for the BT region. The reaction mixture contained 0.2 µL of KAPA Taq DNA Polymerase (5 U/µL) (Kapa Biosystems, Cape Town, South Africa), 2.5 µL of MyTaq™ Reaction Buffer (5×), 2.5 µL of dNTPs (10 mM), 0.5 µL of 50 mM $MgCl_2$ and 0.50 µL of each primer (10 mM stock concentration), 2 µL fungal genomic DNA and PCR graded water to the final volume of 25 µL. PCR amplifications were performed using the following conditions: denaturation at 95 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C and a final extension at 72 °C for 10 min. An aliquot of 4 µL of each PCR product was stained with 1 µL of 6 × Orange DNA Loading Dye (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania), run on 2% agarose gel at 90 V along with a O'GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania), stained with GelRed (Biotium, Hayward, USA) and visualised with a Gel Doc EZ Imager (Bio-Rad Laboratories, Hercules, CA, USA)). Amplified PCR products were

purified using the EXO-SAP (Exonuclease I—Shrimp Alkaline Phosphatase, Thermo Fisher Scientific, Waltham, MA, USA) protocol.

The same primers used for PCR amplification were also used for sequencing PCR except that Bt2a primer [29] was used instead of T10 primer for sequencing of BT products. The sequencing reactions consisted of 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 mM stock concentration), 1 µL of the purified PCR product and PCR grade water to the final volume of 12 µL. The thermal cycling conditions were: 25 cycles of 10 s at 96 °C, 5 s at 52 °C and 4 min at 60 °C. Sequencing products were then cleaned using ethanol/sodium acetate precipitation. Sequencing fragment analysis was conducted on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the DNA Sequencing Facility of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

2.3. Sequence Analyses and Fungal Identification

Consensus sequences were assembled with the Geneious R6 (Biomatters Ltd., Auckland, New Zealand), after which preliminary identification of the isolates was performed using the BLAST searches in GenBank (http://www.ncbi.nlm.nih.gov (accessed on March 2019)) applying a megablast algorithm. ITS and BT data sets for the different genera or species complexes including type sequences (when available) of closely related species and sequences from different geographical origins to show relationships with other geographic isolates of the same species, were compiled with MEGA v.7 [30]. The data sets were aligned using the online version of MAFFT v.7 [31] with the automatic option of selecting the most suitable multiple alignment strategy for each data set.

Three phylogenetic methods were applied: maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI). ML was performed with the online version of PhyML 3.0 [32], using automatic model selection by SMS [33] and Akaike information criterion (AIC) [34]. Branch support was estimated with Approximate Likelihood-ratio Test (aLRT) [35]. MP analyses were conducted using PAUP v.4.0a164 [36]. Gaps and missing data were excluded in the MP analyses. BI analyses based on a Markov Chain Monte Carlo (MCMC) simulation were carried out with MrBayes v.3.2.2 [37] with best-fitting evolutionary model determined using MrModeltest 2.3 [38] based on the Akaike Information Criterion (AIC). The MCMC chains were run for five million generations using a sample frequency of 100 (resulting in 50,000 trees). Burn-in values were determined for the respective data sets and all trees sampled during the burn-in phase having were discarded. The resulting majority trees were viewed with MEGA v.7 [30] or FigTree v.1.4.3 [39] and post-edited with Adobe Illustrator CC 2018 (Adobe Inc., San Jose, CA, USA).

3. Results

3.1. Collection of Beetles and Mites

In total, 180 (60 from the galleries and 120 from the pheromone traps) living adults *I. typographus* beetles were collected and inspected for the presence of phoretic mites. Thirty-nine phoretic mites were found, of which seven were nymphs. Eleven percent of the beetles carried mites. When phoretic mites were present, the number per individual beetle ranged between 1–7 and an average of two mites were carried per beetle. The majority of the phoretic mites were found attached on the elytral declivity surface (49%) or ventral surface between the first pair of legs (33%). Based on morphological identification the mites were species of *Uropodina* (Acari: Mesostigmata).

3.2. Isolation and Identification of Fungi

At least one fungal species was isolated from 87% of the collected mites. The number of fungal species per individual mite ranged between 1–4, with an average of two fungal species carried per mite. In total, 145 fungal isolates were obtained in this study. Of these 75 isolates, representing the different morphological groups, were subjected for

DNA sequencing (Table 1). The amplified DNA fragments were approximately 600 and 500 bp long for the ITS region and for the partial BT, respectively. The preliminary BLAST analyses identified the filamentous fungi as members of Ophiostomatales and Microascales (Ascomycota). Most of the yeasts were Ascomycetes, only one Basidiomycete yeast was isolated.

The identities of the fungal species were further confirmed by phylogenetic analysis (Figures 1–8). The filamentous fungi included in total seven species, which included three *Ophiostoma sensu lato*, one *Leptographium s. lat.* species (Ophiostomatales) (Figures 1–3) and three species of Microascales (Figures 4–6). The ascomycetous yeast species included six species of Saccharomycetales (Figure 7) and a single yeast-like species of uncertain taxonomic placement (not included in phylogenetic analysis). The single basidiomycetous yeast-like fungus resided in the Agaricostilbales (Figure 8).

3.3. Ophiostomatales

The *Ophiostoma* spp. resided in two species complexes, *Ophiostoma ips* (Figure 1) and *Ophiostoma clavatum* (Figure 2). Analysis of BT data set confirmed that isolates residing in the *O. ips* species complex represented *O. bicolor* (Figure 1). A total of 16 isolates belonged in the *O. clavatum* complex (Figure 2). These included *Ophiostoma brunneolum* and *Ophiostoma ainoae* isolates (Table 1). The *O. ainoae* isolates grouped with the ex-type isolate of *O. ainoae* based on the BT data. There was variation amongst the *O. brunneolum* isolates sequences, but also they formed a distinct clade together with the ex-type isolate of this species.

The isolates in the *Leptographium s. lat.* resided in the *G. penicillata* species complex (Figure 3). All the isolates (in total 14) formed a clade with *G. penicillata* based on BT data. The sequences were most similar to other previously originating from Finland, as well as from China and Austria. The ex-type isolate of *G. penicillata* had 3–4 bp differences with the isolates obtained in this study. It also groups with the ex-type sequence of *Grosmannia fenglinhense* R. Chang, Z.W. de Beer & M.J. Wingf, but this seems to be a typo in the original publication by [3], where the GenBank number MH124324 is indicated to be the BT sequence for *G. fenglinhense*, but in the GenBank the identification is provided as *G. penicillata*.

3.4. Microascales

The isolates belonging to Microascales were identified as species of *Ceratocystiopsis*, *Endoconidiophora* and *Graphium* (Figures 4–6). Based on the BT sequences analysis, the *Ceratocystiopsis* isolates included two cryptic species that grouped within *Cop. minuta* complex containing sequences originating from Europe and Japan (Figure 4). The ITS sequence analysis confirmed that the *Endoconidiophora* isolates represented *E. polonica* (Figure 5). The sequences obtained in this study were identical to those of the ex-type isolate of that species originating from Poland. The remaining isolates were identified as *Graphium fimbriisporum* (Figure 6).

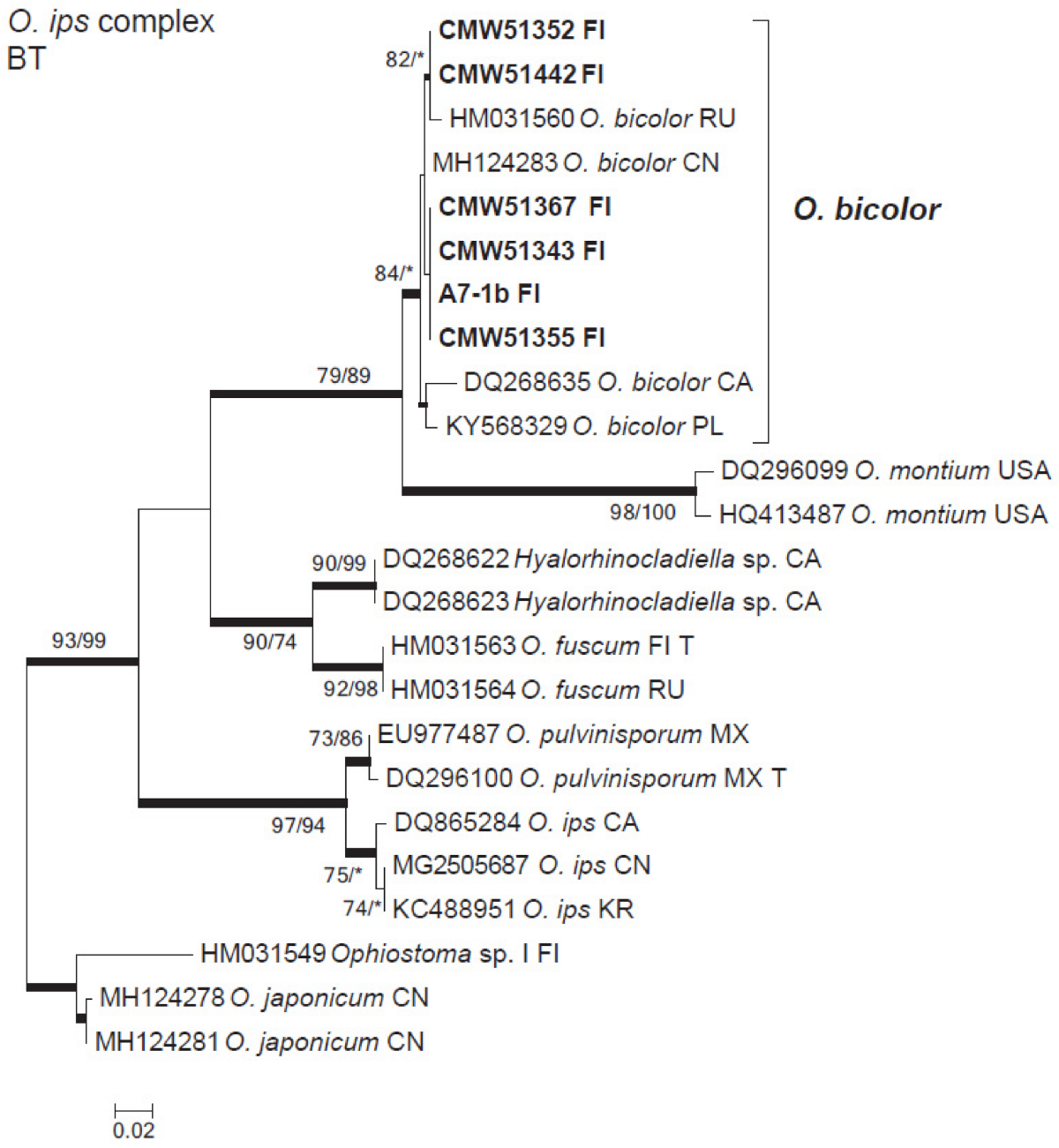

Figure 1. Phylogenetic tree of *Ophiostoma ips* species complex obtained from maximum likelihood (ML) analyses of the β-tubulin data set. Sequences generated in this study are printed in bold type. Bootstrap values above 75% for ML/maximum parsimony (MP) are presented at the nodes. Posterior probabilities (above 90%) obtained from Bayesian (BI) are indicated by bold lines at the relevant branching points. * = bootstrap values < 75%. T = ex-type isolates. Country abbreviations follow ISO 3166 Country Codes. Scale bar = total nucleotide difference between taxa.

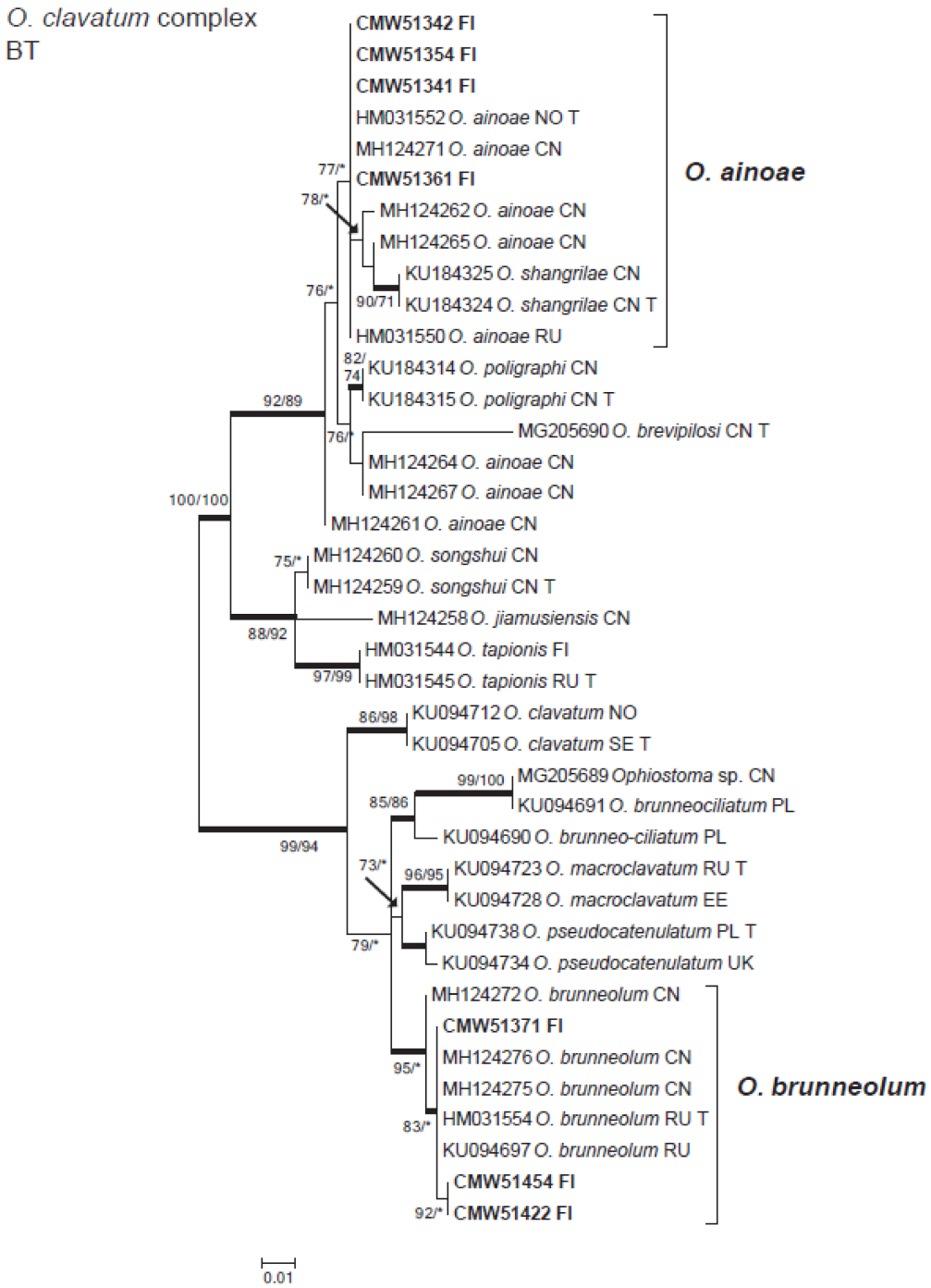

Figure 2. Phylogenetic tree of *Ophiostoma clavatum* species complex obtained from maximum likelihood (ML) analyses of the β-tubulin data set. Sequences generated in this study are printed in bold type. Bootstrap values above 75% for ML/maximum parsimony (MP) are presented at the nodes. Posterior probabilities (above 90%) obtained from Bayesian (BI) are indicated by bold lines at the relevant branching points. * = bootstrap values < 75%. T = ex-type isolates. Country abbreviations follow ISO 3166 Country Codes. Scale bar = total nucleotide difference between taxa.

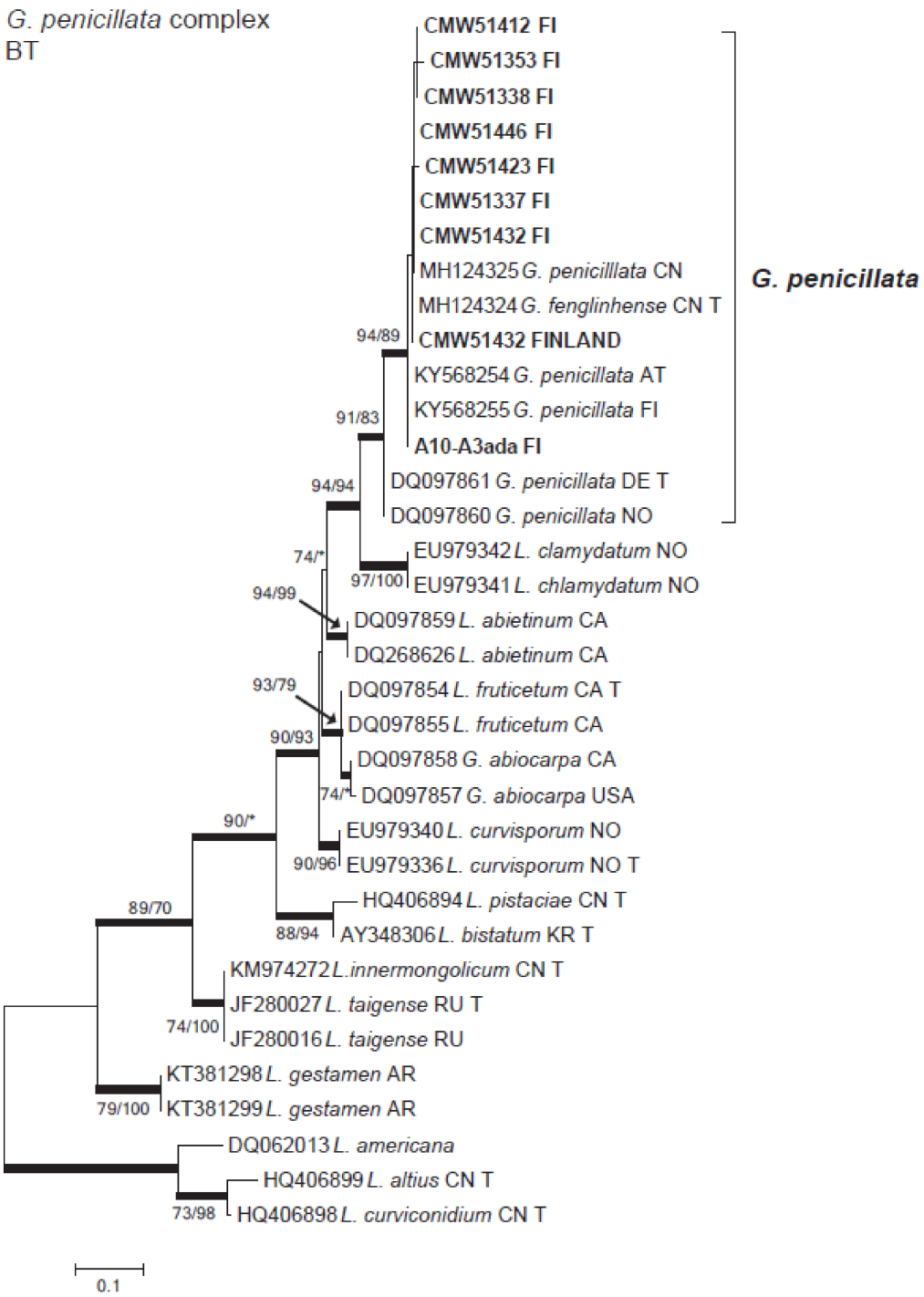

Figure 3. Phylogenetic tree of *Grosmannia penicillata* species complex obtained from maximum likelihood (ML) analyses of the β-tubulin (BT) data set. Sequences generated in this study are printed in bold type. Bootstrap values above 75% for ML/maximum parsimony (MP) are presented at the nodes. Posterior probabilities (above 90%) obtained from Bayesian (BI) are indicated by bold lines at the relevant branching points. * = bootstrap values < 75%. T = ex-type isolates. Country abbreviations follow ISO 3166 Country Codes. Scale bar = total nucleotide difference between taxa.

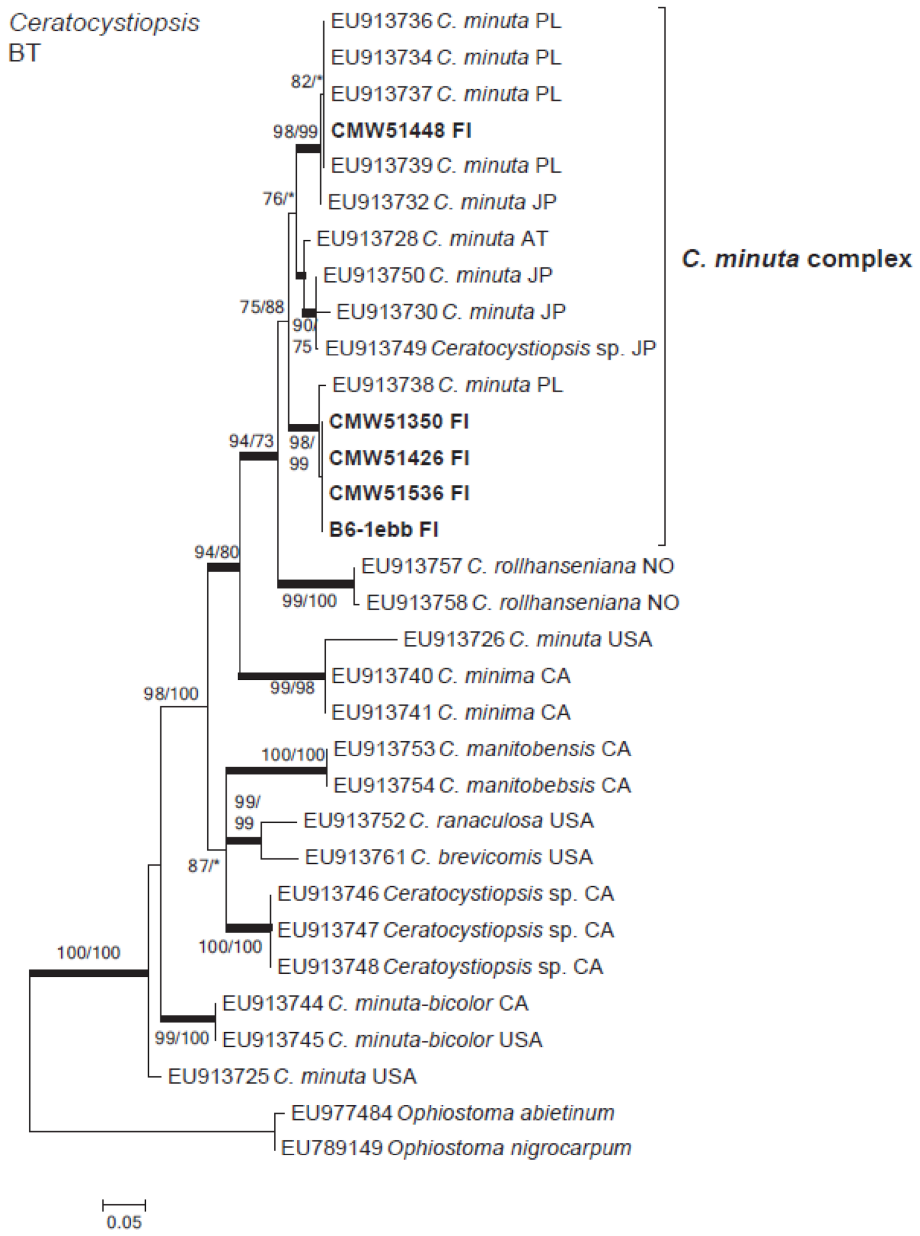

Figure 4. Phylogenetic tree of *Ceratocystiopsis* obtained from maximum likelihood (ML) analyses of the β-tubulin (BT) data set. Sequences generated in this study are printed in bold type. Bootstrap values above 75% for ML/maximum parsimony (MP) are presented at the nodes. Posterior probabilities (above 90%) obtained from Bayesian (BI) are indicated by bold lines at the relevant branching points. * = bootstrap values < 75%. T = ex-type isolates. Country abbreviations follow ISO 3166 Country Codes. Scale bar = total nucleotide difference between taxa.

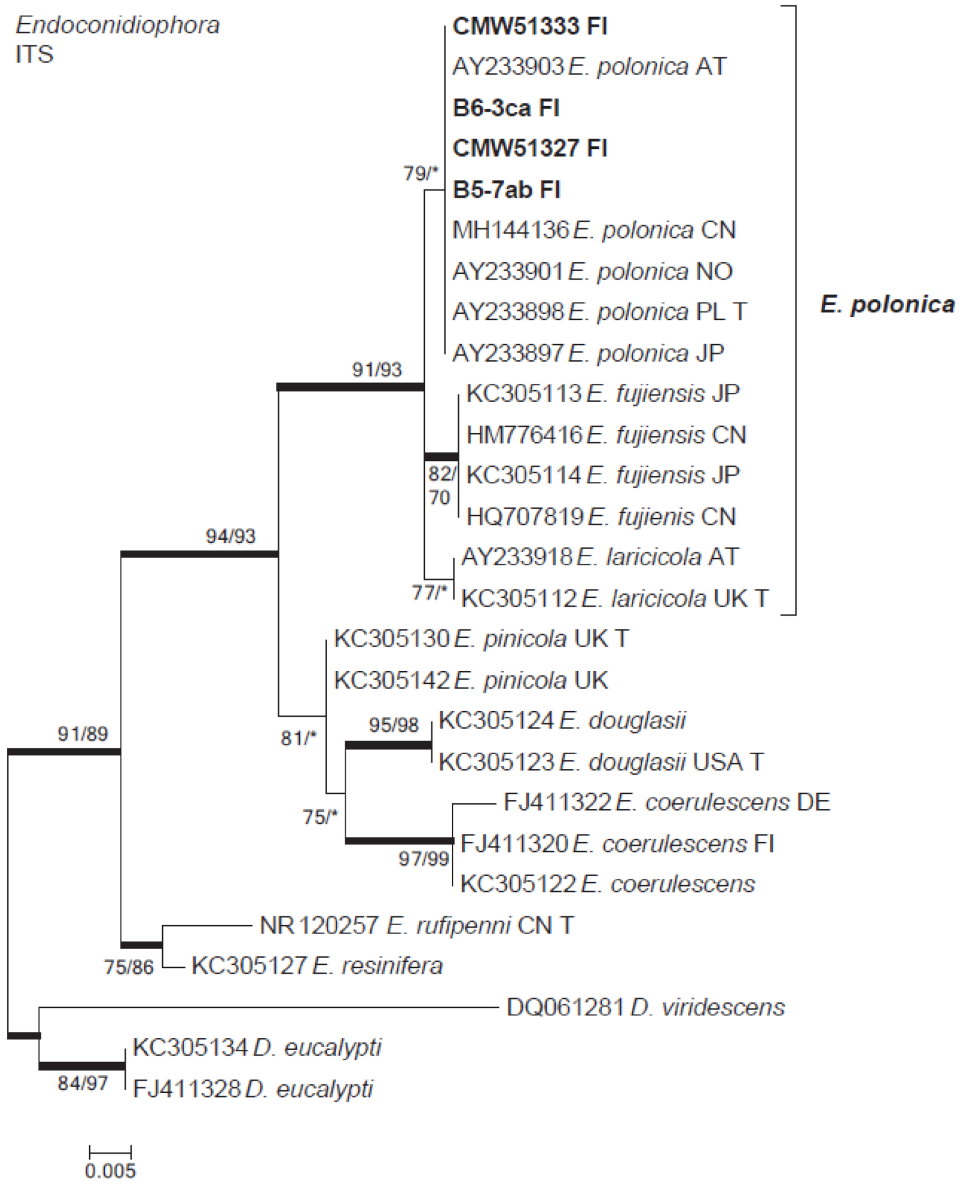

Figure 5. Phylogenetic tree of *Endoconidiophora* obtained from maximum likelihood (ML) analyses of the internal transcribed spacer region (ITS) data set. Sequences generated in this study are printed in bold type. Bootstrap values above 75% for ML/maximum parsimony (MP) are presented at the nodes. Posterior probabilities (above 90%) obtained from Bayesian (BI) are indicated by bold lines at the relevant branching points. * = bootstrap values < 75%. T = ex-type isolates. Country abbreviations follow ISO 3166 Country Codes. Scale bar = total nucleotide difference between taxa.

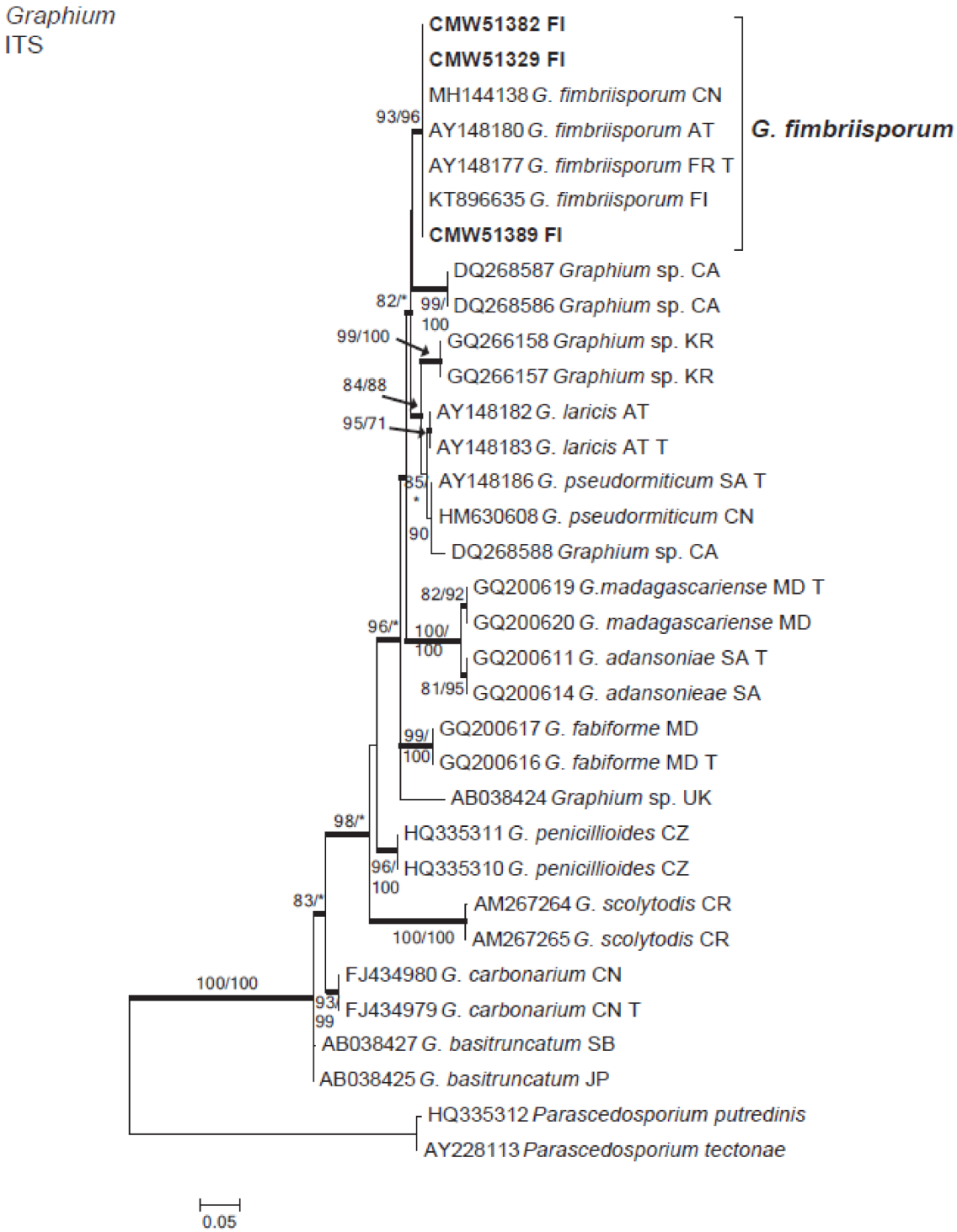

Figure 6. Phylogenetic tree of *Graphium* obtained from maximum likelihood (ML) analyses of the internal transcribed spacer region (ITS) data set. Sequences generated in this study are printed in bold type. Bootstrap values above 75% for ML/maximum parsimony (MP) are presented at the nodes. Posterior probabilities (above 90%) obtained from Bayesian (BI) are indicated by bold lines at the relevant branching points. * = bootstrap values < 75%. T = ex-type isolates. Country abbreviations follow ISO 3166 Country Codes. Scale bar = total nucleotide difference between taxa.

Figure 7. Phylogenetic tree of Saccharomycetales yeasts obtained from maximum likelihood (ML) analyses of the internal transcribed spacer region (ITS) data set. Sequences generated in this study are printed in bold type. Bootstrap values above 75% for ML/maximum parsimony (MP) are presented at the nodes. Posterior probabilities (above 90%) obtained from Bayesian (BI) are indicated by bold lines at the relevant branching points. * = bootstrap values < 75%. T = ex-type isolates. Country abbreviations follow ISO 3166 Country Codes. Scale bar = total nucleotide difference between taxa.

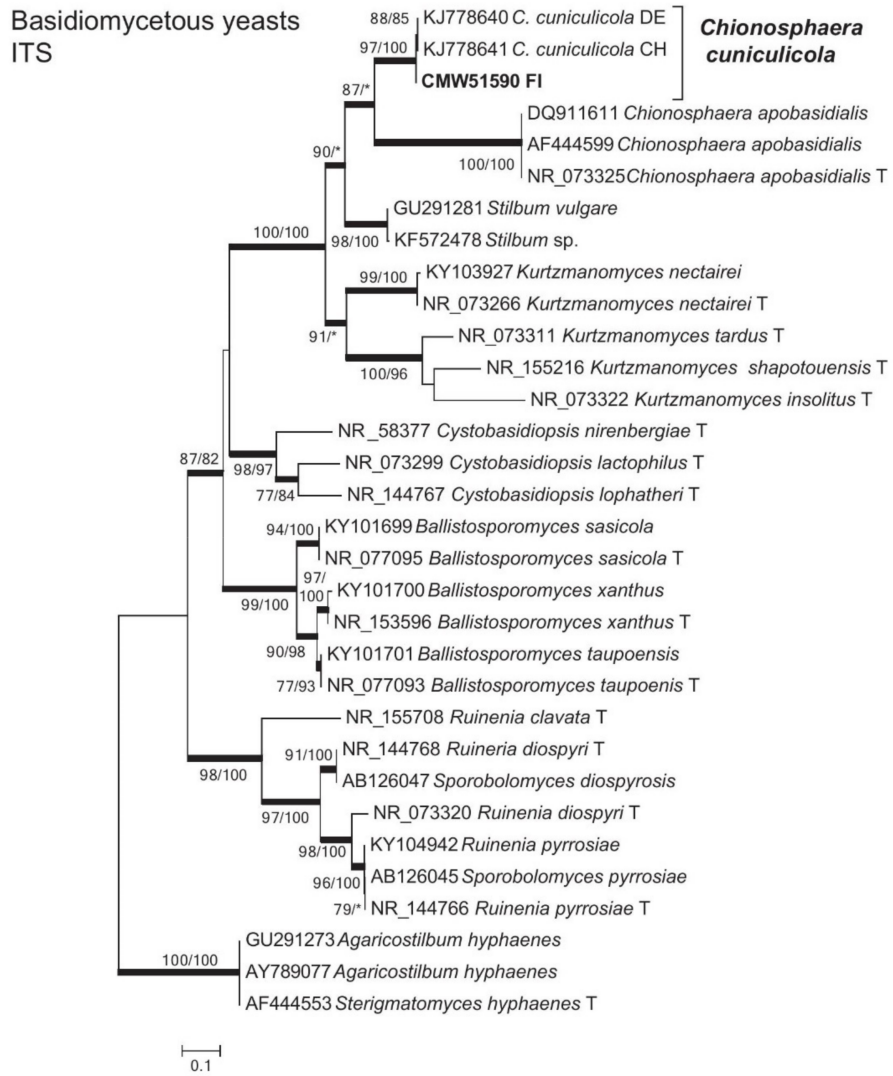

Figure 8. Phylogenetic tree of Basidiomycota yeasts obtained from maximum likelihood (ML) analyses of the internal transcribed spacer region (ITS) data set. Sequences generated in this study are printed in bold type. Bootstrap values above 75% for ML/maximum parsimony (MP) are presented at the nodes. Posterior probabilities (above 90%) obtained from Bayesian (BI) are indicated by bold lines at the relevant branching points. * = bootstrap values < 75%. T = ex-type isolates. Country abbreviations follow ISO 3166 Country Codes. Scale bar = total nucleotide difference between taxa.

3.5. Yeasts

The Ascomycete yeasts included six species of Saccharomycetales (Figure 7) and a single yeast-like species (*Fontanospora fusirasimosa*) of uncertain taxonomic placement (Table 1). The most commonly found were isolates identified as *Wickerhamomyces bisporus*. They formed a well-supported clade with the ex-type isolate of *W. bisporus* and sequences from isolates and uncultured environmental sample sequences originating from bark beetles and their galleries, including *I. typographus*.

The other ascomycetous yeasts included two species of *Ogataea* and *Kuraishia* (Figure 7). The isolate representing *Ogataea ramenticola* grouped together with the ex-type isolate of the species. The other *Ogataea* species was closely related to *Ogataea glucozyma*. However, its identity requires further confirmation as several other *Ogataea* species grouped within the same phylogenetic clade. The *Kuraishia* species found in this study were identified as *Kuraishia capsulata* and *Kuraishia molischiana*. One putatively novel ascomycetous yeast species in the genus *Nakazawaea* was detected (Figure 7). The isolates obtained in this study grouped in

a well-supported clade with the other bark beetle-derived sequence data from isolates or uncultured environmental samples originating from Finland, Russia and North America.

The single basidiomycetous yeast species was identified as *Chionosphaera cuniculicola* (Figure 8). It formed a well-supported phylogenetic clade with the other *C. cuniculicola* isolates originating from conifer-inhabiting beetles.

4. Discussion

This study contributes to limited research on fungi associated with mites phoretic on *I. typographus*. It is also the first to report yeasts associated with mites phoretic on *I. typographus* in Finland. In total, 145 fungal cultures representing 15 taxa were isolated, including seven filamentous fungi and eight yeasts or yeast-like species. The most common were the ophiostomatoid species residing in the *Ophiostoma* s. lat. and *Leptographium* s. lat. (Seifert et al. 2013). The most commonly detected ophiostomatoid species included *G. penicillata*, *O. bicolor* and species residing in the recently defined *O. clavatum* complex [14]. Of the yeast species, an ascomycetous yeast *W. bisporus* was the most frequently found.

Over thirty mite species, of which 15 phoretic, have been reported in association with *I. typographus* in Finland [5]. Based on Penttinen et al. (2013) study, the most abundant mites associated with the beetle are members of Mesostigmata, Oribata and Prostigmata. In the present study, the mites were morphologically identified as *Uropodina* (Mesostigmata). Mesostigmatic mites have also been reported as common *I. typographus*-associates in studies conducted in other European countries [40,41]. Unlike the case for *I. typographus*, very little is known regarding the relationships between mites and fungi. In this regard, [42] suggested that phoretic mites of Cerambycidae could be implicated in the transmission of fungi found in their galleries. Our results support the relatively limited previous studies where mites have commonly been found in association with ophiostomatoid fungi [10,16–18,43]. This is not particularly surprising, as these organisms (beetles, mites and fungi) share the same habitat and likely form complex, multi-partite interactions in the host tree galleries. However, certain fungal species have been more consistently reported in association with mites phoretic on *I. typographus* rather than with the beetles. Most notable of these is the presence of *O. bicolor* in Finland, Sweden and Japan [10,43,44]. Chang et al. [3] also hypothesized that this commonly found species could be predominantly a mite-associated fungus. Some of the mite species are mycetophagous [20,21] and it is possible that *O. bicolor* is of nutritional importance to certain mites.

All the other ophiostomatoid species found in this study (*O. ainoae*, *O. brunneolum*, *G. penicillata*, *Cop. minuta*, *E. polonica* and *Gr. fimbriisporum*) have previously been reported in association with *I. typographus* in Europe [8–11,13,45]. A previous study has shown that the pathogenic fungus *E. polonica* is capable for the degradation of phenolic defense compounds of Norway spruce and thus may have an important role in the bark beetle ability to colonize trees [6]. Species in the *O. clavatum* complex are well-known associates of *Ips* species [14]. Two species, *O. ainoae* and *O. brunneolum* residing in this complex were detected in the present study. This is the first report of *O. brunneolum* in Finland. *Cop. minuta* found in this study is a fungus that has rarely been reported in Finland [46,47] and its identity confirmed for the first time using DNA-based identification. The species remains a taxonomic challenge and appears to represent a cryptic species complex rather than a single species [48]. *Cop. minuta* isolates originating from the present study likely include two species for which the taxonomic boundaries remain to be resolved.

An interesting outcome of this study was the dominant presence of a number of yeast species existing in association with the phoretic mites. The yeast diversity in this habitat has received little attention, but recent studies have reported that yeasts are common bark beetle-associates that have only emerged as relevant after DNA sequencing techniques have been applied to taxonomic studies [49,50]. Consistent with the previous studies recently summarized by Davis [23], yeasts in the Saccharomycetaceae were the most frequently found also in this study. *Wickerhamomyces bisporus* was the most frequently isolated yeast and it has also been also previously found in association with *I. typographus* in Europe [50].

Species of *Wickerhamomyces* have been reported from galleries and guts of wood-boring insects [51,52], indicating their common association with beetles.

The other Saccharomycetaceae isolates included members of *Ogataea*, *Kuraishia* and *Nakazawaea*, which is consistent with the study of [53]. The common occurrence of these yeast genera as part of *I. typographus* mycobiome is also supported by the recent high-throughput sequencing study [24]. One of the species, *K. capsulata*, has been amongst the most commonly reported yeast species in surveys of *I. typographus* and other *Ips* species [23,50,53,54]. The *Nakazawaea* species detected in this study represents a putatively novel species, apparently common associate of *I. typographus* and other bark beetles, that remains to be formally described. The only basiodiomycetous yeast detected was *C. cuniculicola*. It was originally described as commonly associated with various bark beetle species, including *I. typographus*, in several locations in Europe [55]. It has also been found associated with the invasive pine-infesting beetle, *Dendroctonus valens* LeConte in China [56].

The results of this study highlight the fact that there remains much to learn regarding the intricate interactions of fungi, mites and other organisms associated with *I. typographus*. This despite the fact that it is an extensively studied example of bark beetle-microbial associations and as highlighted in the recent review of Biederman et al. (2020). Furthermore, mites phoretic on *I. typographus* are associated with rich fungal diversity, including yeasts that probably have functional roles in mite and bark beetle ecology.

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Data Availability Statement: The sequence data generated in this study (accession numbers in Table 1) are openly available in GenBank (http://www.ncbi.nlm.nih.gov).

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