Ophiostoma species, including *Ophiostoma borealis* sp. nov., infecting wounds of native broad-leaved trees in Norway

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Abstract

Ophiostoma spp. include important pathogens of trees and causal agents of sapstain. These fungi infect wounds on trees and are typically carried by insects, especially bark beetles. *Ophiostoma* spp. on coniferous hosts in the Northern Hemisphere are well known. However, other than for the serious pathogens *O. ulmi* and *O. novo-ulmi*, very little research has been done on the occurrence of this group of fungi on native broad-leaved trees, especially in the Nordic countries. In this study, surveys were conducted in several areas of Norway to isolate *Ophiostoma* spp. associated with wounds on native broad-leaved trees belonging to the genera *Betula*, *Fagus*, *Quercus*, *Sorbus* and *Tilia*. Morphological studies and comparisons of DNA sequences for the ITS, S.8S and part of the beta-tubulin gene regions were used to confirm the identity of the fungi collected. *Ophiostoma* spp., and especially their *Pesotum* anamorphs, were common on wounds on the trees sampled. In most cases, they were associated with wood stain. *Ophiostoma* spp. collected included predominantly *O. quercus*, *O. borealis* sp. nov., and *O. denticiliatum*. The results of this study emphasize that the diversity of *Ophiostoma* spp. on broad-leaved trees is still incompletely understood in Norway and other European countries.

Introduction

The Ophiostomatales (Ascomycetes) include many important pathogens and causal agents of sapstain in lumber. Amongst the pathogens, the Dutch elm disease fungi, *O. ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, are by far the best known (Wingfield et al., 1993; Brasier, 2000). Most species of *Ophiostoma* Sydow & P. Sydow, however, cause sapstain of freshly exposed wood and they can reduce the commercial value of wood products substantially (Wingfield *et al*., 1993).

The Ophiostomatales accommodate the genera *Ophiostoma* (with *Pesotum* J.L. Crane & Schokn. and *Sporothrix* Hektoen & C.F. Perkins anamorphs), *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr. (with *Hyalorhinocladiella* H.P. Upadhyay & W.B. Kendr. anamorphs), and *Grosmannia* Goid. (with *Leptographium* Lagerb. & Melin anamorphs) (Zipfel et al., 2006). In their sexual form, these fungi typically produce long-necked ascomata with sticky spores at their apices, which facilitate insect dispersal. Similarly, with the exception of *Sporothrix* spp., most of the asexual

structures are adapted for insect dispersal, producing their conidia in sticky droplets on erect conidiophores such as species of *Hyalorhinocladiella*, *Pesotum* and *Leptographium* (Wingfield *et al*., 1993).

Ophiostoma spp. infect wounds on trees, and are commonly vectored by insects, most often bark beetles (Coleoptera: Scolytinae), with which many of these fungi have specific associations (Six, 2003; Harrington, 2005). However, casual arthropod visitors of wounds such as mites, nitidulid beetles, and flies (Six, 2003; Harrington, 2005) are also important vectors of *Ophiostoma* spp. Gibbs (1980) and Juzwik & French (1983), showed that insects will, under certain circumstances, carry a mixed inoculum of both sapstain and pathogenic fungi. In some cases, the sapstain fungi can out-compete the pathogen and prevent further infection of wounds. This form of mutualism has been demonstrated between sapfeeding beetles, the oak wilt fungus *Ceratocystis fagacearum* (Bretz) J. Hunt and *O. quercus* (Georgev.) Nannf., a well-known sapstain fungus on hardwood trees (Gibbs, 1980; Juzwik *et al*., 1998).

Many *Ophiostoma* spp. have been reported from Scandinavia. In this regard, the type species of *Ophiostoma*, *O. piliferum* (Fr.) Sydow & P. Sydow, was described from pine and spruce in Sweden as long ago as 1823 (Fries, 1823). Early studies on these fungi in Scandinavia focused mainly on wood and/or pulp staining fungi in Sweden (Lagerberg *et al*., 1927; Melin & Nannfeldt, 1934) and Norway (Robak, 1932) and more recently in Norway by Roll-Hansen & Roll-Hansen (1980a, b). However, the majority of these studies have dealt with fungal associates of conifer-infesting bark beetles in Sweden (Mathiesen, 1950, 1951; Mathiesen-Käärik, 1953, 1960; Solheim & Långström, 1991), Norway (Solheim, 1986, 1992a, b, 1993) and Finland (Viiri, 1997). Until recently, the only records of *Ophiostoma* spp. on hardwood trees in Scandinavia were of the Dutch elm disease fungus, *O. ulmi*, reported from both Sweden (Mathiesen-Käärik, 1960) and Norway (Roll-Hansen, 1985; Venn, 1986), and of *O. piceae* (Münch) Syd. & P. Syd. from decayed *Betula pubescens* Ehrh. in Norway (Venn, 1972). In two recent studies of the fungal associates of the birch bark beetle, *Scolytus ratzeburgi* Janson, several *Ophiostoma* spp. were isolated from the beetles and their galleries in Finland (Linnakoski et al., 2008). These included the new species, *O. karelicum* Linnakoski, Z.W. de Beer & M.J. Wingf., *O. quercus* and an undescribed species closely related to *O. catonianum* (Goid.) Goid. (Linnakoski *et al*., 2008). In a subsequent study conducted in Norway, *O. karelicum* was also found to be the most common associate of *S. ratzeburgi* (Linnakoski *et al*., 2009). In addition, *O. quercus*, together with four unknown species, one of which was described as *O. denticiliatum* Linnakoski, Z.W de Beer & M.J. Wingf., were found. There were not enough isolates of the remaining species to support the description of new taxa, but they were shown to be closely related to *O. canum* (Munch) Syd. & P. Syd., *O. flexuosum* H. Solheim, and *O. multiannulatum* (Hedge. & R.W. Davidson) Hendr., respectively (Linnakoski *et al*., 2009),

The expanding global timber trade poses increasing risks regarding the introduction of alien bark beetles and fungal pathogens into countries worldwide (Skarpaas & Økland, 2009). Global warming represents another factor that is promoting the invasion of wood and tree-infesting insect pests such as bark beetles into areas previously unsuitable for them to survive (Logan *et al*., 2003; Raffa *et al*., 2008). Knowledge regarding the geographic origin and host range of fungal pathogens or fungi that might be commercially relevant is thus important for future pest risk assessments and for the development of effective quarantine and management strategies. Following this objective and as part of a collaborative project between research organizations in Norway and South Africa, funded by the Norwegian and South African Governments, a study was undertaken to survey for and to identify *Ophiostoma* spp. that infect wounds on native broadleaved tree species in Norway. Specifically the study aimed to address the paucity of detailed knowledge pertaining to this group of potentially important fungi in the country.

Materials and methods

Collection of isolates

Surveys of native broad-leaved trees in Norway were conducted in June and July 2004 and 2005. Sampling focused on wounds artificially induced on trees, freshly cut stumps of *Betula* spp. and *Populus tremula* L., logs and bark flaps of Betula spp. (*B. pendula* Roth and *B. pubescens*), *P. tremula*, *Quercus* spp. [*Q. petraea* (Matt.) Liebl. and *Q. robur* L.] and *Salix* spp. log ends at saw mills and loading depots. In 2004, sampling was restricted to forests in eastern Norway mainly around the town of Ås situated in the Boreonemoral vegetation zone, and a loading depot in South Western Sweden, near the town of Filipstad in the Southern Boreal Zone and not far from the Norwegian border. In 2005, sampling was done in both the southernmost part of Norway, in the Nemoral vegetation zone, and in many municipalities in Tromse county in northern Norway, situated in the Middle Boreal vegetation zone.

For the artificially induced wounds, an axe was used to remove sections of bark (10 x 20 cm^2) from living trees growing near lake Årungen in Ås, to expose the cambium. An uneven number of trees were wounded, depending on availability, including nine *Betula* spp., four *Quercus* spp., four *Sorbus aucuparia* L., four *Salix* spp. and one *P. tremula* tree. The wounds were inspected up to two months after they had been made. Pieces of bark and wood including cambial tissue were collected from all wounds that had signs of ophiostomatoid fungal infection (ascomata, synnemata, sap-staining) when examined with a 10X magnification hand lens. Sampling from freshly cut stumps and log ends at saw mills and loading depots was also done in a similar fashion by chopping pieces of infected tissues, especially where bark flaps protecting the wounds from desiccation were visible. Samples were stored in brown paper bags and transported to the laboratory. Dry samples were sprayed with water and sealed in plastic bags to induce sporulation of the fungi. Samples were examined daily for the development of fruiting bodies characteristic of *Ophiostoma* spp. and their anamorphs.

Isolations of the fungi were made by lifting spore masses directly from fruiting structures with a sterile needle onto 2% malt extract agar [MEA: 20 g malt extract, 15 g agar, Biolab, Midrand, South Africa and 1000 ml deionised water] containing 0.05 gfl of the antibiotic streptomycin (Sigma-Aldrich, Steinheim, Germany). Cultures were grown at 24°C for seven days to obtain pure colonies. Apart from isolates obtained during the survey, additional isolates of *O. quercus* from Austria, Uganda, and South Africa were included in the study for comparative purposes (Tab. 1). Replicates of each isolate were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and the culture collection of the Norwegian Forestry and Landscape Institute (NFU) in Ås. In addition, the ex-type cultures of the new species were deposited at the CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands).

Morphology of cultures

After purification, isolates were grouped into morphotypes based on culture morphology on MEA. From these morphotypes single drops of conidia or ascospores from selected isolates, or small pieces of mycelium, were transferred to Oatmeal agar media (OMA: 30 g Oats, 20 g Biolab agar and 1000 ml deionised water) to promote sporulation and for comparison with previously published characteristics. Cultures were incubated at 24°C until sporulation and then grouped into morphotypes based on colony colour (Rayner, 1970) and macromorphology.

Micromorphology

Fifty measurements of all characteristic morphological features were made for isolates chosen as the types for the new species and ten measurements were made for additional isolates. Measurements were recorded as (minimum -) mean minus st. dev. - mean plus st. dev. (- maximum). The means were then calculated for relevant morphological structures.

DNA isolation, PCR and DNA sequencing

Isolates, representing each of the different morphological groups were selected for DNA sequence comparisons. Single spore drops from pure cultures were transferred to 2% MEA and cultures were grown for 10 days. Mycelium was then scraped from the surface of cultures and transferred to 1.5 ml Eppendorf tubes using a sterile scalpel. DNA was extracted using the protocol described by Möller *et al*. (1992), except that 1O1l1of RNaseA (Roche Diagnostics) was added at the final step and incubated overnight at room temperature to digest RNA. The presence of DNA was verified by separating an aliquot of Sill on 1% agarose gels, containing ethidium bromide and visualized under Ultraviolet light. The internally transcribed spacer regions (ITS1 & 2) and 5.8S gene of the ribosomal RNA operon were amplified using an Eppendorf Mastercycler (Merck, Hamburg, Germany) and primers ITS1 and ITS4 (White *et al*., 1990). Parts of the beta-tubulin gene region were also amplified, using primers T10 (O'Donnell & Cigelnik, 1997) and Bt2b (Glass & Donaldson, 1995). The PCR and DNA sequencing protocols described by Kamgan Nkuekam *et al*. (2008a) were followed.

Compilation of sequence data sets

Contigs of both sequenced strands for each isolate were assembled using Sequence Navigator v. 1.01 (ABI PRISM, Perkin Elmer). Additional sequences of related *Ophiostoma* spp., including those from recent studies in Norway and Finland (Linnakoski *et al*., 2008, 2009), were obtained from GenBank (http://www.ncbi.nlm.nih.gov) to fortify the comparisons. Sequences were aligned using the E-INS-i option in the online version of MAFFT 6 (Katoh & Toh, 2008). To avoid the inclusion of large numbers of identical sequences in the phylogenetic analyses, ITS and beta-tubulin sequences of *O. quercus* were grouped based on haplotypes using MEGA 4.0.1 (Tamura *et al*., 2007). ITS haplotype groups were labeled A-H and the beta-tubulin haplotype groups 1-16. From each haplotype group, only one isolate was included in the analyses.

Phylogenetic analyses

Phylogenetic analyses of the ITS and beta-tubulin data sets were performed independently of each other, because reference sequences were not available for the same sets of isolates. For each data set, maximum parsimony (MP), Bayesian analyses (MB), and maximum likelihood (ML) analyses were done.

MP analyses were performed in similar manner for both data sets in PAUP 4.0b10 (Swofford, 1998). Only parsimony informative characters (196 for ITS and 122 for beta-tubulin) were included, using the following settings: 100 random sequence addition replicates, tree bisection-recognition (TBR) branch swapping, and 'multrees' option in effect. Confidence levels of the MP phylogenies were estimated with the bootstrap method (1000 replications).

Bayesian analyses based on Markov chain Monte Carlo (MCMC) were performed with MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). Ten million generations were run in four chains with sampling every 100 generations, which yielded 100 000 trees. Appropriate substitution models were determined using the Akaike Information Criterion (AIC) in MrModeltest 2.2

(http://www.abc.se/~riylander/). The model applied to the ITS region was GTR+I+G and for the beta-tubulin GTR+G was used. Burn-in values were determined using Tracer 1.4 (http://beast.bio.ed.ac.uk/Tracer). For ITS the first 500 trees were discarded as burnin and for beta-tubulin the first 1000 trees.

ML analyses were conducted on line using PhyML 3.0 (Guindon & Gascuel, 2003). The AIC was used in Modeltest 3.7 (Posada & Crandall, 1998) to select appropriate substitution models for the two data sets. For ITS, the selected model was GTR+I+G (proportion of invariable sites = 0.3779; gamma shape parameter = 0.6576) and for beta-tubulin it was GTR+G (gamma shape parameter = 0.2000).

Table 1. *Ophiostoma* isolates obtained in this study from native broad-leaved trees in Norway and Sweden. Also listed are isolates from Finland and Norway from the studies of Linnakoski *et at*. (2008, 2009), as well as isolates from elsewhere sequenced for reference purposes. Accession numbers of sequences obtained in the present study are printed in bold type.

Species	[a]CMW no.	ITS		β-tubulin		Hosts	Origin	Collectors
		HT[b]	Acc. no.	HT[b]	Acc. no.			
O. quercus	5928	E	**EF408598**			*Acacia mearnsii*	Uganda	Roux
	15528	G	**EF408579**	10	**GQ249305**	*Betula* sp.	Norway, Årungen	Roux, Solheim
	15544	E	**EF408581**			*Sorbus* sp.	Norway, Årungen	Roux
	15592	B	**GQ249322**			*Populus* sp.	Norway, Årungen	Roux, Solheim
	15608	G	**=EF408579**	10	**=GQ249305**	*Betula* sp.	Sweden, Filipstad	Roux, Solheim
	15704	H	**GQ249324**			*Quercus* sp.	Norway, Årungen	Roux
	17828	E	**=EF408586**	8	**GQ249303**	*Quercus robur*	Austria	Kirisits
	17877	E	**EF408588**			*Tilia cordata*	Austria	Kirisits
	17892	E	**EF408586**			*Q. robur*	Austria	Kirisits
	17893	H	**EF408590**	8	**=GQ249303**	*Fagus sylvatica*	Austria	Kirisits
	17894	E	**EF408589**			*T. cordata*	Austria	Kirisits
	19182	G	**=EF408579**	1	**GQ249299**	*Populus* sp.	Norway, Tresnes	Kamgan, Solheim
	19187	F	**GQ249323**			*Quercus* sp.	Norway, Øydna	Kamgan, Solheim
	19193	H	**=EF408590**	16	**GQ249310**	*Populus* sp.	Norway, Øydna	Kamgan, Solheim
	19198			8	**GQ249302**	*Quercus* sp.	Norway, Øydna	Kamgan, Solheim
	19201			11	**GQ249306**	*Quercus* sp.	Norway, Øydna	Kamgan, Solheim
	19214			15	**GQ249309**	*Quercus* sp.	Norway, Øydna	Kamgan, Solheim
	19231			7	**GQ249301**	*Quercus* sp.	Norway, Salthaug	Kamgan, Solheim
	19240			5	**GQ249300**	*Quercus* sp.	Norway, Salthaug	Kamgan, Solheim
	19241			14	**GQ249308**	*Quercus* sp.	Norway, Salthaug	Kamgan, Solheim
	19243			9	**GQ249304**	*Quercus* sp.	Norway, Salthaug	Kamgan, Solheim
	19257			12	**GQ249307**	*Quercus* sp.	Norway, Lyngdal	Kamgan, Solheim
	20452	D	**EF408563**			*Rapanea melanophloeos*	South Africa	Roux
O. quercus	29491	A	**FJ804488**	10	FJ804501	*S. ratzeburgi* ex *Betula* sp.	Norway, Hobøl	Linnakoski
O. denti-ciliatum	15748		**GQ249331**		**GQ249321**	*Betula* sp.	Sweden, Filipstad	Roux, Solheim
	19185				**GQ249320**	*Betula* sp.	Norway, Tresnes	Kamgan, Solheim
	29493[T]		FJ804490		FJ804502	*S. ratzeburgi* ex *Betula* sp.	Norway, Hobøl	Linnakoski
	29494[T]		FJ804491		FJ804503	*S. ratzeburgi* ex *Betula* sp.	Norway, Hobøl	Linnakoski
	D091318		FJ804492		FJ804504	*S. ratzeburgi* ex *Betula* sp.	Norway, Hobøl	Linnakoski
***O. borealis* sp. nov.**	17860		**EF408594**		**GQ249311**	*T. cordata*	Austria	Kirisits
	18893		**GQ249325**		**GQ249312**	*Betula* sp.	Norway, Salangen	Kamgan, Solheim
	18919		**EF408592**		**GQ249313**	*B. pubescens*	Norway, Sørreisa	Kamgan, Solheim
	18941		**GQ249326**		**GQ249314**	*Betula* logs	Norway, Målselv	Kamgan, Solheim
	18953		**GQ249327**		**GQ249315**	*Betula* logs	Norway, Målselv	Kamgan, Solheim
	18956		**GQ249328**		**GQ249316**	*Betula* logs	Norway, Målselv	Kamgan, Solheim
	18966[T]		**EF408593**		**GQ249317**	*B. pubescens*	Norway, Målselv	Kamgan, Solheim
	19263		**GQ249329**		**GQ249318**	*Betula* logs	Norway, Målselv	Kamgan, Solheim
	19297		**GQ249330**		**GQ249319**	*Populus* sp.	Norway, Sørreisa	Kamgan, Solheim
	19320		**EF408591**			*B. pubescens*	Norway, Målselv	Kamgan, Solheim
	23112[c]		EU443765		EU443778	*S. ratzeburgi* gallery on *Betula* sp.	Finland	De Beer
	23113[c]		EU443765		EU443779	*S. ratzeburgi* gallery on *Betula* sp.	Finland	De Beer

[a] CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
[b] Published sequences of *O. quercus* were assigned to haplotype (HT) groups (numbered alphabetically for ITS and numerically for ß-tubulin), each group consisting of identical sequences. Only one sequence per group was deposited in Genbank.
[c] Isolates identified as *O. catonianum*-like by Linnakoski *et al*. (2008).
[T] Ex-type isolates.

Results

Collection of isolates

In total, 230 cultures were obtained from bark and cambial samples collected from more than 200 logs, stumps and stems of broad-leaved tree species from five different native tree genera in Norway and Sweden. Most collections were obtained from *Betula* spp. (121) and *Quercus* spp. (80), while ophiostomatoid fungi was also obtained from *Populus tremula*, *Sorbus aucuparia* and *Salix* spp.

Morphology of cultures

Most isolates obtained produced only *Pesotum* anamorphs, while only a few produced both ascomata and synnemata in culture. Based on the cultural variation, a total of 73 isolates including representative strains from different tree genera, different geographical locations sampled and different morpho-groups were selected for DNA sequencing (Table 2) to obtain an indication of the species diversity within the samples collected in Norway and Sweden.

DNA sequencing and phylogenetic analyses

All isolates selected for DNA sequencing produced fragments of approximately 650 bp using the primers ITS1 and ITS4, while the beta-tubulin fragments were about 500 bp in size. Alignment of sequences for isolates from Norway (Table 1) with related sequences from GenBank resulted in a total of 683 characters including gaps, with 403 constant characters, 84 parsimony uninformative characters and 196 parsimony informative characters for the ITS data set. For the beta-tubulin data set 343 characters including gaps, with 204 constant characters, 17 parsimony-uninformative characters and 122 parsimony informative characters were obtained. MP analyses resulted in 64 trees of 475 steps for ITS, and 1512 trees of 446 steps for beta-tubulin data sets. For the respective data sets the consistency indexes (Cl) were 0.747 and 0.608, while the retention indexes (RI) values were 0.864 and 0.901. For each data set a 50% majority rule tree obtained from Bayesian analyses is presented (Figs 1 and 2). Bayesian posterior probabilities $\geq$ 90%, and MP and ML bootstrap values $\geq$ 75%, are indicated at the relevant nodes.

Table 2. Isolates of *Ophiostoma* identified as part of the present study from broad-leaved hosts in different parts of Norway and Sweden (underlined) during surveys in 2004 and 2005.

Host Tree	*O. quercus*	*O. borealis*	*O. denticiliatum*
Betula	[a]15608, 15528, 18878, 18883, 18939, 19265, 19266, 19267, 19270, 19272, 19276, 19277, 19285, 19287, 19296, 19314, 19317, 22055, 22066, 22070, 22072	18893, 18919, 18941, 18953, 18956, 18966, 19263, 19320	15748, 19185
Populus	15592, 18917, 19175, 19178, 19182, 19183, 19184, 19192, 19193, 19254, 19258	19297	
Quercus	15704, 15746, 15750, 19187, 19198, 19201, 19212, 19214, 19222, 19223, 19240, 19241, 19242, 19243, 19244, 19247, 19248, 19250, 19251, 19252, 19253, 19255, 19256, 19257, 19261		
Salix	19260, 19264		
Sorbus	15544, 15560, 15566		

[a]All numbers represent CMW numbers if cultures in the Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

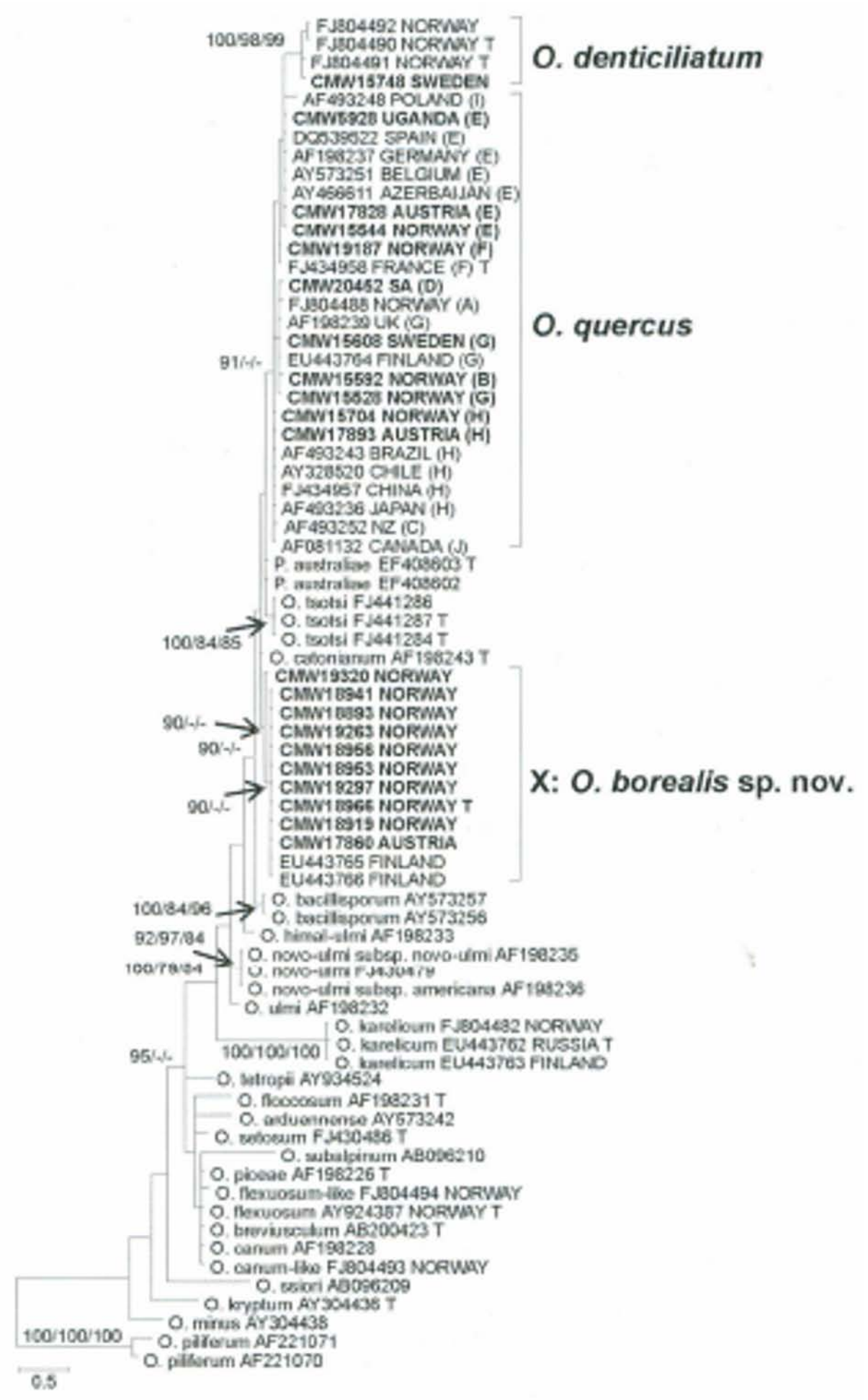

100/98/99
FJ804492 NORWAY
FJ804490 NORWAY T
FJ804491 NORWAY T
CMW15748 SWEDEN
O. denticiliatum
AF493248 POLAND (I)
CMW5928 UGANDA (E)
DQ539622 SPAIN (E)
AF198237 GERMANY (E)
AY573251 BELGIUM (E)
AY456611 AZERBAIJAN (E)
CMW17828 AUSTRIA (E)
CMW15544 NORWAY (E)
CMW19187 NORWAY (F)
FJ434958 FRANCE (F) T
CMW20452 SA (D)
FJ804488 NORWAY (A)
AF198239 UK (G)
O. quercus
CMW15608 SWEDEN (G)
91/-/-
EU443764 FINLAND (G)
CMW15592 NORWAY (B)
CMW15628 NORWAY (G)
CMW15704 NORWAY (H)
CMW17893 AUSTRIA (H)
AF493243 BRAZIL (H)
AY328520 CHILE (H)
FJ434957 CHINA (H)
AF493236 JAPAN (H)
AF493252 NZ (C)
AF081132 CANADA (J)
P. australiae EF408603 T
P. australiae EF408602
O. tsotsi FJ441286
O. tsotsi FJ441287 T
100/84/85
O. tsotsi FJ441284 T
O. catonianum AF198243 T
CMW19320 NORWAY
CMW18941 NORWAY
CMW18893 NORWAY
90/-/-
CMW19263 NORWAY
90/-/-
CMW18958 NORWAY
CMW18953 NORWAY
X: O. borealis sp. nov.
90/-/-
CMW19297 NORWAY
CMW18966 NORWAY T
CMW18919 NORWAY
CMW17860 AUSTRIA
EU443765 FINLAND
EU443766 FINLAND
100/84/96
O. bacillisporum AY573257
O. bacillisporum AY573256
92/97/84
O. himal-ulmi AF198233
O. novo-ulmi subsp. novo-ulmi AF198235
100/78/84
O. novo-ulmi FJ430479
O. novo-ulmi subsp. americana AF198236
O. ulmi AF198232
O. karelicum FJ804482 NORWAY
100/100/100
O. karelicum EU443762 RUSSIA T
95/-/-
O. karelicum EU443763 FINLAND
O. tetropii AY934524
O. floccosum AF198231 T
O. arduennense AY573242
O. setosum FJ430486 T
O. subalpinum AB096210
O. piceae AF198226 T
O. flexuosum-like FJ804494 NORWAY
O. flexuosum AY924387 NORWAY T
O. breviusculum AB200423 T
O. canum AF198228
O. canum-like FJ804493 NORWAY
O. sslori AB096209
O. kryptum AY304436 T
O. minus AY304438
100/100/100
O. pilleriana AF221071
O. pilleriana AF221070
0.5

Fig. 1. Phylogenetic tree produced by a Bayesian analysis of the ITS sequence data. *Ophiostoma piliferum* was used as outgroup taxon. Posterior probabilities above 90% are presented at nodes, followed by bootstrap values above 75% for respectively MP and ML analyses. Isolates sequenced in this study are printed in bold type. Sequences of *O. quercus* isolates were assigned to haplotype groups, indicated as (A-J). T = ex-type isolates.

Based on ITS analyses (Fig. 1), most of the Norwegian isolates selected for sequencing grouped with *O. quercus* isolates from previous studies. The Norwegian *O. quercus* isolates belonged to haplotypes A, B, E, F, G, H and the Swedish isolate to haplotype G (Table 1). A single isolate from Sweden (CMW 15748) grouped with *O. denticiliatum* isolates from *Scolytus ratzeburgii* on birch in Norway (Linnakoski *et al*. 2009). Nine Norwegian and one of the reference isolates from Austria, that were considered to be *O. quercus*, formed a lineage (X in Fig. 1) distinct from *O. quercus*. Lineage X also included two isolates from *S. ratzeburgii* galleries on birch in Finland, referred to as *O. catonianum*-like by Linnakoski *et al*. (2008).

Analyses of the beta-tubulin data (Fig. 2) gave results similar to those obtained with ITS. The Norwegian *O. quercus* isolates belonged to haplotypes 1, 5, 7, 8, 9, 10, 11, 12, 14, 15 and 16, and the Swedish isolate to haplotype 10 (Tab. 1). The *O. quercus* lineage showed substantial variability within the group, but with very high statistical support delineating the species clearly from its closest relative, *O. tsotsi* Grobbelaar, Z.W. de Beer & M.J. Wingf. (Grobbelaar *et al*. 2010). Beta-tubulin sequence data also showed that in addition to the Swedish isolate (CMW 15748) grouping with *O. denticiliatum* in the ITS analysis, a Norwegian isolate from birch (CMW 19185) also belonged to this species. The same group of isolates constituting lineage X in the ITS analysis, formed a strongly supported group closest to, but clearly distinct from the Dutch elm disease fungi and *O. catonianum*.

Taxonomy

Phylogenetic analyses of the ITS (Fig. 1) and partial beta-tubulin (Fig. 2) gene regions, revealed that several isolates from Norway and Austria represented an undescribed taxon (lineage X). This was further supported by morphological comparisons and a new species is, therefore, described as follows:

Ophiostoma borealis sp. novo G. Kamgan Nkuekam, H. Solheim & Z.W. de Beer-MB 518853 Figs 3-9

Etymology: The species name refers to the boreal ecosystem where the fungus was collected.

Coloniae in OMA olivaceo-griseae vel fumeae, in MEA bubalinae vel melleae, in MEA ad 25°C ad 41 mm in 10 diebus crescentes. Optime crescunt ad 20°C. *Bases* ascomatae nigrae globosae (74-) 103-175 (-274) µm longae, (72-)103-170 (-233) µm latae, cum trichomatibus hypharum; *colla* nigra (295-) 341-1041 (-1828) µm longa. *Hyphae* ostiolorum divergentes (17-) 26-48 (-58) µm longae. *Ascosporae* reniformes, non septatae (3.4-) 3.8-4.3 (-4.6) µm longae, (1.4-) 1.5-1.9 (-2.1) µm latae. Anamorphae biformes. Anamorpha *Pesotum* conidiophoris erectis atrobrunneis apicem versus pallescentibus (82-) 129-212 (-246) µm longis. *Capitula conidiogena* maxime (33-) 48-77 (-100) µm lata, laete brunnea, apicem versus hyalinescentia. *Cellulae conidiogenae* hyalinae acerosae (5-) 14-30 (-44) µm longae, (0.5-) 1-1.7 (-2.2) µm latae. *Conidia* non septata, hyalina, oblonga vel obovoidea (3.8-) 4-5.7 (-6.9) µm × (1-) 1.8-2 (-2.8) µm. Conidiophorae *Sporothrix* hyalinae (15-) 19-45 (-77) × (1-) 1.8-2.4 (-2.7) µm cum denticulis prominentis.

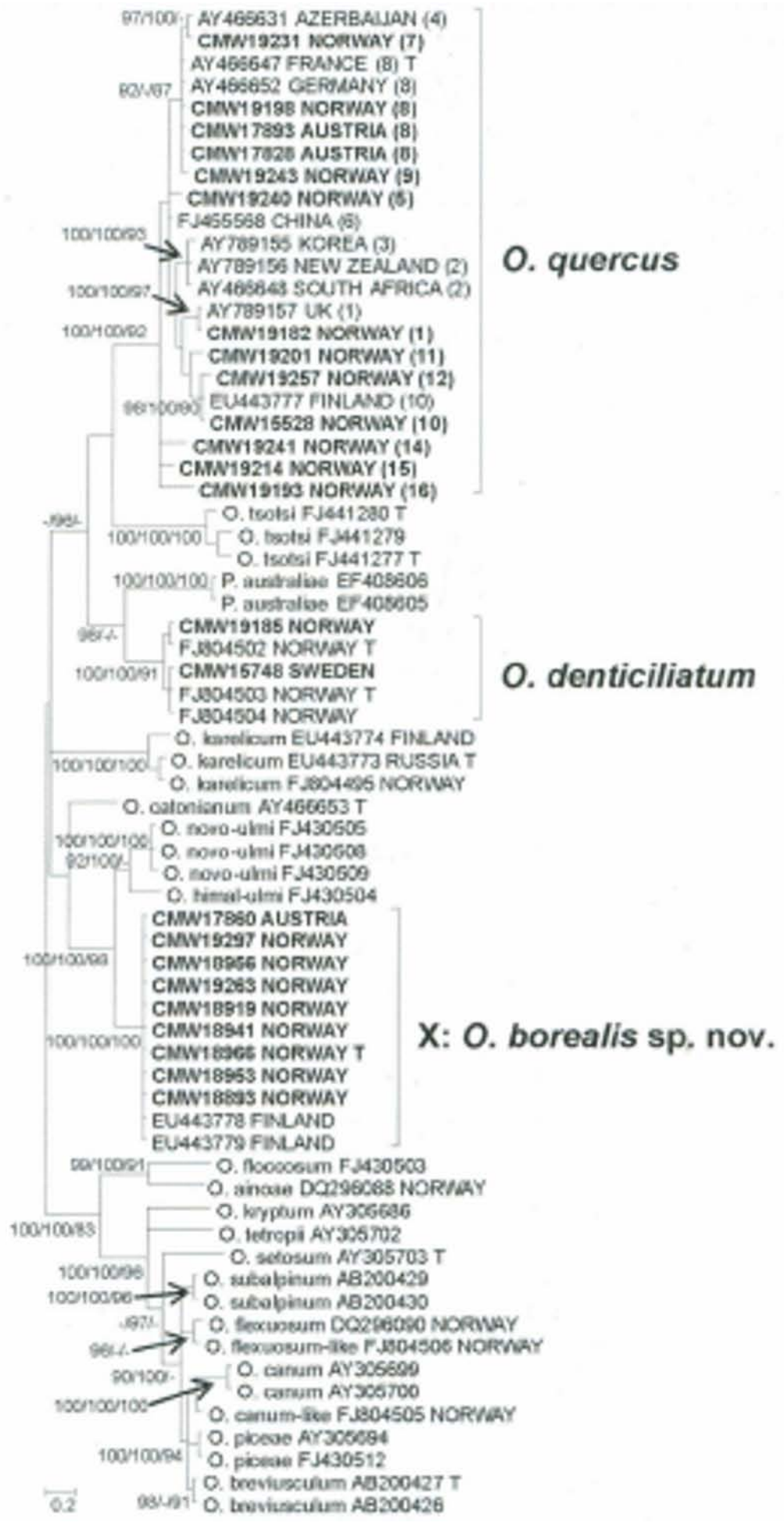
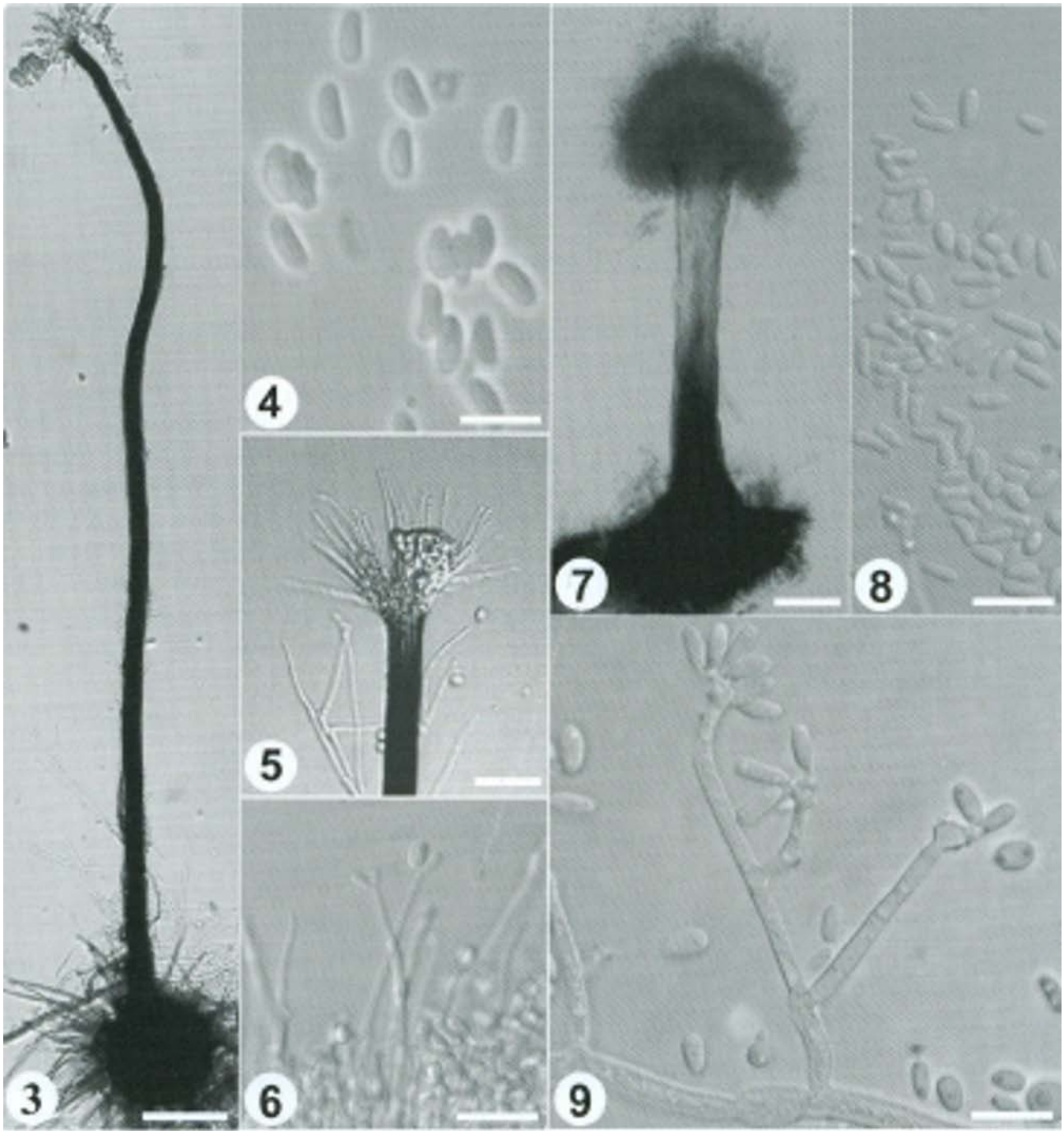

97/100/-
AY466631 AZERBAIJAN (4)
CMW19231 NORWAY (7)
AY466647 FRANCE (8) T
82/-/87
AY466652 GERMANY (8)
CMW19198 NORWAY (8)
CMW17893 AUSTRIA (8)
CMW17828 AUSTRIA (8)
CMW19243 NORWAY (9)
CMW19240 NORWAY (6)
FJ455568 CHINA (6)
100/100/93
AY789155 KOREA (3)
AY789156 NEW ZEALAND (2)
100/100/97
AY466648 SOUTH AFRICA (2)
AY789157 UK (1)
100/100/92
CMW19182 NORWAY (1)
CMW19201 NORWAY (11)
CMW19257 NORWAY (12)
96/100/90
EU443777 FINLAND (10)
CMW16528 NORWAY (10)
CMW19241 NORWAY (14)
CMW19214 NORWAY (15)
CMW19193 NORWAY (16)
O. quercus
-/99/-
O. tsotsi FJ441280 T
100/100/100
O. tsotsi FJ441279
O. tsotsi FJ441277 T
100/100/100
P. australiae EF408606
P. australiae EF408605
96/-/-
CMW19185 NORWAY
FJ804502 NORWAY T
100/100/91
CMW15748 SWEDEN
FJ804503 NORWAY T
FJ804504 NORWAY
O. denticiliatum
O. karelicum EU443774 FINLAND
100/100/100
O. karelicum EU443773 RUSSIA T
O. karelicum FJ804496 NORWAY
O. catonianum AY466653 T
100/100/100
O. novo-ulmi FJ430505
O. novo-ulmi FJ430508
92/100/-
O. novo-ulmi FJ430509
O. himal-ulmi FJ430504
CMW17860 AUSTRIA
CMW19297 NORWAY
100/100/99
CMW18956 NORWAY
CMW19263 NORWAY
CMW18919 NORWAY
CMW18941 NORWAY
100/100/100
CMW18966 NORWAY T
CMW18953 NORWAY
CMW18893 NORWAY
EU443778 FINLAND
EU443779 FINLAND
X: O. borealis sp. nov.
99/100/91
O. flocoosum FJ430503
O. ainoae DQ296088 NORWAY
O. kryptum AY305686
100/100/83
O. tetropii AY305702
O. setosum AY305703 T
100/100/96
O. subalpinum AB200429
100/100/96
O. subalpinum AB200430
-/97/-
O. flexuosum DQ296090 NORWAY
96/-/-
O. flexuosum-like FJ804506 NORWAY
90/100/-
O. canum AY305699
O. canum AY305700
100/100/100
O. canum-like FJ804505 NORWAY
O. piceae AY305694
100/100/94
O. piceae FJ430512
O. brevisuculum AB200427 T
0.2
98/-/91
O. brevisuculum AB200426

Fig. 2. A midpoint-rooted phylogenetic tree produced by a Bayesian analysis of the beta-tubulin sequence data. Posterior probabilities above 90% are presented at nodes, followed by bootstrap values above 75% for respectively MP and ML analyses. Isolates sequenced in this study are printed in bold type. Sequences of *O. quercus* isolates were assigned to haplotype groups, indicated as (1-16). T = ex-type isolates.



Figs 3-9. Morphological characteristics of *Ophiostoma borealis* sp. novo (CMW 18966). Teleomorph: **3.** globose ascomatal base (scale bar = 50 µm), **4.** reniform ascospores (scale bar = 10 µm), **5.** divergent ostiolar hyphae (scale bar = 20 µm). *Pesotum* anamorph: **6.** conidiogenous cells with conidia at the tips of sympodially proliferating conidiogenous cells (scale bar == 10 µm), **7.** synnemata (scale bar = 20 µm) showing hyphal hairs at base, **8.** oblong to cylindrical conidia (scale bar = 10 µm). *Sporothrix* anamorph: **9.** conidiogenous cell with emerging conidia (scale bar = 10 µm).

Conidia non septata, hyalina, obovoidea, interdum manifeste acerosa (7.6-) 8.9-11.3 (-13.8) x (2.9-) 3.2-4 (-4.8) µm.

Teleomorph state produced on *Betula pubescens* chips after random crossing of strains after approximately two months. *Ascomatal* bases black, globose (74-) 103-175 (-274) µm long and (72-) 103-170 (-233) µm wide with hyphal hairs. *Ascomatal* necks black (295-) 341-1041 (-1828) µm long, base of necks smooth (22-) 32-53 (-69) µm wide, middle of necks (15-) 18-26 (-35) µm wide, tips of necks (9-) 13-18 (-25) µm wide. *Ostiolar hyphae* present, divergent (17-) 26-48 (-58) µm long. *Asci* evanescent. *Ascospores* reniform in side view, aseptate (3.4-) 3.8-4.3 (-4.6) µm long and (1.4-) 1.5-1.9 (-2.1) µm wide, accumulating in round, creamy spore drops.

Colonies Olivaceous grey (21""i) or smoke grey (21""d) on OMA with conidiophores forming light-colored slimy heads, becoming creamy toward the centers and arranged in mostly circular rings, reverse mouse grey (15""d) to almost black. On MEA colonies buff (19"d) with conidiophores forming cream-colored slimy heads scattered over the colony mostly surrounded by mat of mycelia, reverse colonies buff (19"d) becoming honey (19"b) toward the middle of the plate. Colony diameter reaching 41 mm in 10 days on MEA at 25 ºC. Optimal growth temperature 20 ºC, no growth at 5 ºC or at 35 ºC.

Pesotum anamorph: *Conidiophores* synnematal, erect, dark brown at the bases, becoming lighter towards the apex, (82-) 129-212 (-246) µm long, (9-) 13-21 (-30) µm wide in the middle, (16-) 20-31 (-38) µm wide at the base. *Conidiogenous heads* (33-) 48-77 (-100) µm across the widest part, light brown becoming hyaline towards the apex. *Conidiogenous cells*, hyaline, acerose (5-) 14-30 (-44) µm long, (0.5-) 1-1.7 (-2.2) µm wide, tapering towards the apex. *Conidia* produced through holoblastic, sympodial development. Conidia aseptate, hyaline, oblong to obovoid, accumulating in slimy heads on the apices of the synnemata, (3.8-) 4-5.7 (-6.9) µm × (1-) 1.8-2 (-2.8) µm.

Sporothrix anamorph: conidiophores hyaline, cylindrical and branched, tapering towards the apex, (15-) 19-45 (-77) x (1-) 1.8-2.4 (-2.7) µm, prominent denticles present. *Conidia*, aseptate, hyaline, obovoid, distinctly acerose in some cases (7.6-) 9-11.3 (-13.8) × (3-) 3.2-4 (-4.8) µm.

Specimens examined: Norway, Troms, Målselv, Rostadalen, Dalhaug, isolated from wounds on logs of newly felled *Betula pubescens*. June 2005, G. Kamgan Nkuekam & H. Solheim, holotype PREM 59745, living culture CMW 18966, CBS 123222, NFLI 05-703/2.

Additional specimens: Norway, Troms, Målselv, but from three different sites (or localities); Dalhaug in Rostadalen, Moeng near Øverbygd and Sandbakken near Olsborg, isolated from wounds on logs of newly felled *B. pubescens*. June 2005, G. Kamgan Nkuekam & H. Solheim, paratypes, PREM 59742 (Living cultures CMW 18953, CBS 123220, NFLI 05-677/2); PREM 59747 (Living cultures CMW 18919, CBS 123221, NFLI 05-605/2); PREM 59744 (Living cultures CMW 18956, NFLI 05-689); PREM 59746 (Living cultures CMW 18941, NFLI 05-659/2).

Discussion

Results of this study have shown that there is a relatively low diversity of *Ophiostoma* spp. on hardwoods in Norway. More than 200 isolates were obtained in a fairly limited study period, showing for the first time that *Ophiostoma* spp. are common on native hardwood trees in Norway. Because of the large number of isolates collected, and because it is notoriously difficult to identify *Ophiostoma* spp., such as those in the *O. piceae* complex (Harrington *et al*., 2001), isolates were subjected to a preliminary screening based primarily on culture morphology. Representative isolates were selected for each morphological group and the resulting 73 isolates were further characterized based on DNA sequence comparisons. This resulted in the identification of three distinct

Ophiostoma spp.: *O. borealis* sp. nov., *O. denticiliatum* and *O. quercus*.

This study resulted in the discovery of a new *Ophiostoma* sp., *O. borealis* based on sequence analyses. The fungus groups in the hardwood inhabiting clade of the *O. piceae* complex and is closely related to *O. catonianum* and *O. novo-ulmi*. The fact that *O. borealis* was collected from Northern Norway and also identified from cultures from Austria and sequences from Finland (Linnakoski *et al*., 2008), suggests that this species has a wide distribution in Europe. Although the majority of isolates originated from *Betula*, its presence on *Populus* in Norway and *Tilia cordata* Mill. in Austria suggests that it has a broad host range. Given the difficulty in distinguishing between the synnematal anamorphs of *O. borealis* and other *Ophiostoma* spp., it is possible that *O. borealis* has been recorded in Europe before, but under the names *O. piceae* or *O. quercus*.

Morphological differences between *O. borealis* and its closest relatives, *O. quercus*, *O. novo-ulmi* and *O. catonianum*, are difficult to define due to variation in the dimensions of structures often present within a single culture. However, when ranges of dimensions are compared (Table 3), some differences between the species are evident. The most notable of these is that *O. borealis* produces substantially shorter synnemata than the other species, although length ranges overlap with *O. catonianum*. *Ophiostoma quercus* can be distinguished from *O. borealis* and the other two species by the presence of very long conidia produced by its *Sporothrix* anamorph. Where *O. borealis* and *O. quercus* occur on a variety of host tree species, *O. novo-ulmi* and *O. catonianum* have been recorded only from *Ulmus* and *Pyrus* spp, respectively. *Ophiostoma borealis*, *O. quercus* and *O. novo-ulmi* are heterothallic, while *O. catonianum* is homothallic. Furthermore, in the illustrations of Goidánich (1935), *O. catonianum* produces allantoid ascospores similar to the other species, but with ends slightly more pointed.

Table 3. Comparison of the most relevant morphological features distinguishing *O. borealis* sp. nov. from *O. quercus*, *O. novo-ulmi* and *O. catonianum*. All measurements in µm.

	O. borealis sp. nov. (present study)	*O. quercus* (Grobbelaar *et al.* 2009; Halmschlager *et al.* 1994)	*O. novo-ulmi* (Brasier 1991)	*O. catonianum* (Goidánich 1935)
Perithecia:				
neck length	295-1827	306-2036	230-1070	475-630
base width	72-232	110-217	75-140	110-165
Ascospore: length	3.4-4.6	2.6-4.8	4.5-6	3-5
width	1.4-2.1	0.9-2.4	1-1.5	1.5-1.8
***Pesotum* stage:**				
synnemata	**82-260**	227-1097	< 2000	26-550
conidia	**3.8-6.9 × 1-2.8**	2.5-4.8 × 1-2.4	2-6 × 1-3	3-5 × 1.6-2
***Sporothrix* stage:**				
conidia	7.6-13.8	14-233	4.5-14	10-16
Thallism	heterothallic	heterothallic	heterothallic	homothallic
Host range	hardwoods	hardwoods	restricted to *Ulmus* spp.	only known from *Pyrus* sp.

Of the *Ophiostoma* spp. identified, *O. quercus* was by far the most common. This fungus has previously been reported only from the birch bark beetle at a single site in Norway. However, the report of *O. piceae* as common in decayed *Betula* logs in Norway (Venn, 1972) probably more appropriately reflects *O. quercus*. Results of this study considerably extend the host range and distribution of *O. quercus* to five genera of hardwood hosts spread over all the major vegetation zones in Norway.

It was not surprising to find *O. quercus* on hardwood trees in this study. The fungus is well-known to have a wide distribution in the northern hemisphere on deciduous timber, although many reports have been obscured by taxonomic confusion. Many early reports of the fungus on hardwoods were of *O. piceae* and it is only relatively recently that *O. piceae* isolates from hardwoods have been recognized as most probably representing the distinct fungus, *O. quercus* (Morelet, 1992; Brasier & Kirk, 1993; Halmschlager *et al*., 1994; Kim *et al*., 1999). Some authors have also referred to the fungus as *O. querci* which might have confused the literature pertaining to it even further (De Beer *et al*., 2003a). Interestingly, the fungus has a cosmopolitan distribution on hardwoods and is less commonly found on conifers. It has been reported from native and non-native trees in North America (Kim *et al*., 1999; Juzwik *et al*., 1998), South America (Harrington *et al*., 2001; De Beer *et al*., 2003b; Geldenhuis *et al*., 2004; Zhou *et al*., 2004), Europe (Morelet, 1992; Brasier & Kirk, 1993; Halmschlager *et al*., 1994; Carlier *et al*., 2006; Romon *et al*., 2007; Grobbelaar *et al*., 2009), Africa (De Beer *et al*., 2003b; Zhou *et al*., 2006; Kamgan Nkuekam *et al*., 2008a, b), East Asia (Kim *et al*., 2005; Masuya *et al*., 2009; Paciura *et al*., 2010) and Australasia (Harrington *et al*., 2001; Thwaites *et al*., 2005). It is thus not unusual to find the fungus commonly associated with wounds on broad-leaved trees in Norway, and also in Austria and Sweden.

Ophiostoma denticiliatum was isolated only twice in this study, on both occasions from birch. The species was first isolated and described from the birch bark beetle, *Scolytus ratzeburgi*, in Norway (Linnakoski *et al*., 2009). Interestingly, it was not isolated from the galleries of the beetle in that study, and neither was it found in a previous study of the beetle and its associated fungi in Finland and Russia (Linnakoski *et al*., 2008). It is thus unlikely to be an obligate associate of the beetle, but might be associated with hyperphoretic mites as has been found for many other *Ophiostoma* spp. (Bridges & Moser, 1983; Moser *et al*., 1989). Linnakoski *et al*. (2009), reported that *O. denticiliatum* cultures reach only 35 mm in 10 days and this is substantially slower than *O. quercus* cultures that reach 41-57 mm in the same period (Grobbelaar *et al*., 2009). This is in contrast to *O. borealis* cultures that reach 41 mm in ten days and thus only the lower end of the growth range for *O. quercus*. Because of its aggressive growth, *O. quercus* might easily out-compete *O. borealis* and more so, *O. denticiliatum*, on exposed sapwood surfaces. This could explain why fewer isolates of the latter species were collected in this study.

Linnakoski *et al*. (2008, 2009), recorded seven *Ophiostoma* spp. from the birch bark beetle and its galleries and these resulted from relatively limited collections. In contrast, the present study included relatively extensive sampling but resulted in only three *Ophiostoma* spp. being found. This, surprisingly limited diversity might reflect the sampling strategy that would have favoured the rapidly growing and apparently aggressive *O. quercus*. In contrast, if isolations from wound-visiting arthropods had been included, a greater diversity of these fungi might have emerged.

This study has improved our knowledge about the occurrence, host range and geographic distribution of *Ophiostoma* spp. in Norway. *Ophiostoma quercus* is without doubt a widespread species occurring on a wide range of substrates as confirmed again in this study. *Ophiostoma borealis*, although only representing nine of the sequenced isolates, could be more widespread than indicated, due to the rough screening used to select isolates for DNA sequence comparisons. However, this study has clearly shown that there is much to be learned regarding the occurrence of *Ophiostoma* spp. in Norway, and this is despite the fact that this is one of the best studied fungal genera in the world.

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