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ORIGINAL PAPER

Two new *Leptographium* spp. reveal an emerging complex of hardwood-infecting species in the Ophiostomatales

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Abstract Species of *Leptographium* are generally characterized by mononematous conidiophores and are commonly associated with bark beetles and weevils. These species are responsible for sapstain and in some cases serious diseases on a range of primarily coniferous trees. In comparison with coniferous trees, the occurrence of *Leptographium* species on hardwood trees has been poorly studied in Europe. During a survey of ophiostomatoid fungi on various tree species in Norway and Poland, three unusual species, which fit the broader morphological description of *Leptographium* spp., were found in association with *Scolytus ratzeburgi*, *Dryocoetes alni* and *Trypodendron domesticum* on a variety of hardwoods, and from wounds on *Tilia cordata*. Phylogenetic analyses of sequence data for three gene regions (ITS2-LSU, β-tubulin, and TEF1-α) showed that these *Leptographium* species are phylogenetically closely related to each other and form a well-supported lineage that included *Grosmannia grandifoliae* and *Leptographium pruni*. The first species could be distinguished from the other *Leptographium* species based on conidiophores arising from spiral hyphae, chlamydospore-like structures and a hyalorhinocladiella-like synanamorph in culture. The second species differs from the previous one by having distinctly shorter conidiophores and smaller conidia. This species also produces a well-developed sporothrix-like synanamorph with denticulate conidiogenous cells. Based on these unusual morphological characteristics and distinct DNA sequences, these fungi were recognised as new taxa

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for which the names *Leptographium trypodendri* sp. nov. and *L. betulae* sp. nov. are provided. The third group of isolates belonged to *Grosmannia grandifoliae*, representing the first report of this species outside of the USA. The newly defined *G. grandifoliae* complex is the first species complex in *Leptographium s.l.* consisting of only hardwood-infecting species.

Keywords Bark beetle-associated fungi · Hardwoods · Ophiostomatoid fungi · *Leptographium trypodendri* · *Leptographium betulae*

Introduction

Species of *Leptographium* Lagerb. & Melin and *Grosmannia* Goid. (Ophiostomatales, Ascomycota) are well-known agents of sapstain and disease of respectively conifer wood and trees in North America, Europe and Asia (Harrington 1988; Jacobs and Wingfield 2001). These fungi are specifically adapted for dispersal by bark beetles and weevils (Coleoptera: Scolytinae) (Malloch and Blackwell 1993). The associations with these beetles vary from very specific to fairly unspecific (Kirisits 2004). Only a few of these fungi is considered primary pathogens, and the damage they cause are usually in association with their bark beetle vectors (Jacobs and Wingfield 2001).

Probably the best known of the conifer pathogens are the three host-specific varieties of *Grosmannia wageneri* (Goheen & F.W. Cobb) Zipfel et al., responsible for black stain root disease on native *Pinus* and *Pseudotsugae* spp. in disturbed stands in Western North America (Harrington and Cobb 1987; Cobb 1988). Another species, *Grosmannia clavigera* (R.C. Rob. and R.W. Davidson) Zipfel et al., is vectored by the mountain pine beetle (*Dendroctonus ponderosae* Hopkins) in an ongoing epidemic and range expansion of the beetle in Western Canada that is driven by climate change (Hicke et al. 2006; Alamouti et al. 2011). *Grosmannia clavigera* has been shown to be moderately virulent when inoculated in living pine trees (Solheim and Krokene 1998), but suggestions that the beetle depends on these fungi to kill trees have been challenged by Six and Wingfield (2011). In another scenario, *Leptographium procerum* (W.B. Kendr.) M.J. Wingf., a common associate of several bark beetle and weevil species, has been implicated as contributing to the death of millions of native pine trees in China in association with the red turpentine (*Dendroctonus valens* Le Conte) that was introduced into China from North America (Lu et al. 2010). The fact that *L. procerum* was originally described from Canada and the USA (Kendrick 1962), together with earlier population studies including only Chinese and North American isolates, led to assumptions that *L. procerum* was, like the beetle, introduced into China from the USA (Lu et al. 2009, 2011). However, several recent reports of *L. procerum* from various pine-infesting beetles in Europe (Jankowiak 2012; Jankowiak and Bilański 2013a, b, c), as well as a more comprehensive population study (Taerum et al. 2017), suggest that Europe is most likely the centre of origin of the fungus, implying that the *L. procerum*—*D. valens* is a most likely new host-vector association (Wingfield et al. 2016).

Of the more than 90 species currently treated in *Leptographium* sensu lato (De Beer et al. 2013), only twelve have been reported from hardwoods. One of these only, *L. calophylli* (Wiehe) J.F. Webber et al., is considered a significant pathogen, causing a serious wilting disease of *Calophyllum* trees in the Seychelles and Mauritius (Wiehe 1949; Webber et al. 1999). The fungus is vectored by the bark beetle *Cryphalus trypanus* Sampson (Wainhouse et al. 1998). These examples of pathogenic *Leptographium* spp. illustrate the risks that *Leptographium* spp. can pose in a changing climate and when introduced into new environments.

Among the above examples and in recent literature (e.g. Linnakoski et al. 2012; Huang and Chi-Y 2014; De Errasti et al. 2016), the use of the genus names *Leptographium* and *Grosmannia* might seem inconsistent. The reason for this is that for many years the taxonomy of the Ophiostomatales was defined based on morphological features of their sexual and asexual states. Under the dual nomenclature system, *Leptographium* accommodated asexual morphs, while species with known sexual states were treated in *Grosmannia* (Jacobs and Wingfield 2001; Zipfel et al. 2006). Applying the 'one fungus one name' principles adopted in the International Code of Nomenclature (ICN) for Algae, Fungi and Plants (Hawksworth 2011), De Beer and Wingfield (2013) re-evaluated the taxonomy of *Leptographium* and *Grosmannia* based on ribosomal DNA sequences. They recognized nine species complexes within

Leptographium sensu lato, showing that the type species for *Leptographium* and *Grosmannia* grouped in different complexes. They thus suggested that a more comprehensive study using sequences of more gene regions is needed to resolve the status of the two genera, and that for the interim, species names should not be changed and that new species should be treated in *Leptographium*.

During a recent survey of ophiostomatoid fungi on hardwood trees in Norway and Poland, an unidentified *Leptographium* species was isolated from *Betula verrucosa* Ehrh. infested with the bark beetle *Scolytus ratzeburgi* Jans. In addition, another unknown species of *Leptographium* was isolated from various tree species infested with the wood-boring beetle *Trypodendron domesticum* (L.) and the bark beetle *Dryocoetes alni* (Georg). Two isolates of a third species were also obtained from *Tilia cordata* Mill. trees. The aim of this study was to identify these fungi by comparing their morphology and DNA sequences to those of known species.

Materials and methods

Isolations, fungal isolates and herbarium specimens

Isolations were made from the bark beetle *S. ratzeburgi* and its galleries established in decaying trees of *B. verrucosa*, and from the wood-boring bark beetle *T. domesticum* and its galleries in *F. sylvatica* logs. Strains were collected at two localities in southern Poland during March–July 2011–2013 (Fig. 1). In Norway, isolations were done from the bark beetles *T. domesticum* and *D. alni* in *Alnus incana* (L.) Moench, *Betula pubescens* Ehrh., *Fraxinus excelsior* L. and *Quercus robur* L. In *A. incana* branches, *T. domesticum* coexisted with *D. alni*. The bark beetles were collected at three localities in southern Norway (Fig. 1). Isolates from *T. cordata* were made from stained wood from wounds in the trees during July 2016 in Poland (Fig. 1; Table 1).

Small pieces of the gallery tissue (4 × 4 mm^2) were removed with a sterile scalpel and placed on 2% malt extract agar (MEA) (20 g Biocorp malt extract, 20 g agar, 1000 ml distilled water), containing cycloheximide (200 mg, Aldrich-Sigma, St. Louis, Co. LLC.) and tetracycline sulfate (200 mg, Polfa, Tarchomin SA), incubated at 22 °C and later examined for fungal growth. Isolations directly from beetles were made by crushing them onto the surface of the same medium as above. In Norway, each bark beetle was divided in three parts, elytra, head and the rest, before placing the parts in three different Petri dishes with 2% MEA without any cycloheximide.

All isolates used in the study are listed in Table 1. These isolates were maintained in the culture collection of the Department of Forest Pathology, Mycology and Tree Physiology, University of Agriculture in Krakow, Poland. The Norwegian isolates are kept at the culture collection of Norwegian Institute of Bioeconomy. Ex-type isolates of new species described in this study were deposited in the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, the Netherlands, and in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Type specimens were deposited in the TUR Fungus collections of the Turku University, Finland and in the Mycological Herbarium, Natural History Museum, University of Oslo, Norway.

Cultures of *Leptographium pruni* Masuya and M.J. Wingf. were sourced from the culture collection of the Tohoku Research Center of Forestry and Forest Products Research Institute, Nabeyashiki, Shimi-Kuriyagawa, Morioka, Japan (Hayato Masuya), and the ex-type isolate of *L. pruni* from the Japan Collection of Microorganisms (JCM). Some isolates of *L. pruni* were also deposited in the CBS collection. Two isolates of *G. grandifoliae* (R.W. Davidson) Zipfel et al., which is closely related to *L. pruni* were also included for the morphological and sequence analysis (Table 1). These isolates were also deposited in the CBS collection. Taxonomic descriptions and nomenclatural data were registered in MycoBank (www.MycoBank.org) (Robert et al. 2013).

DNA extraction, PCR and sequencing

Fungal isolates were grown on 2% malt extract agar [MEA: 20 g malt extract L^{-1} (Biocorp™, Warszawa, Poland), 20 g Biocorp™ $agar^{-1}$and 1 L distilled water] in 60 mm plastic Petri dishes for 1–2 weeks prior to DNA extraction. DNA was extracted using the Genomic Mini AX Plant Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol.

Three gene regions were amplified for sequencing and phylogenetic analyses, including ITS2-LSU, βT and TEF1-α. The following primers were used: ITS3

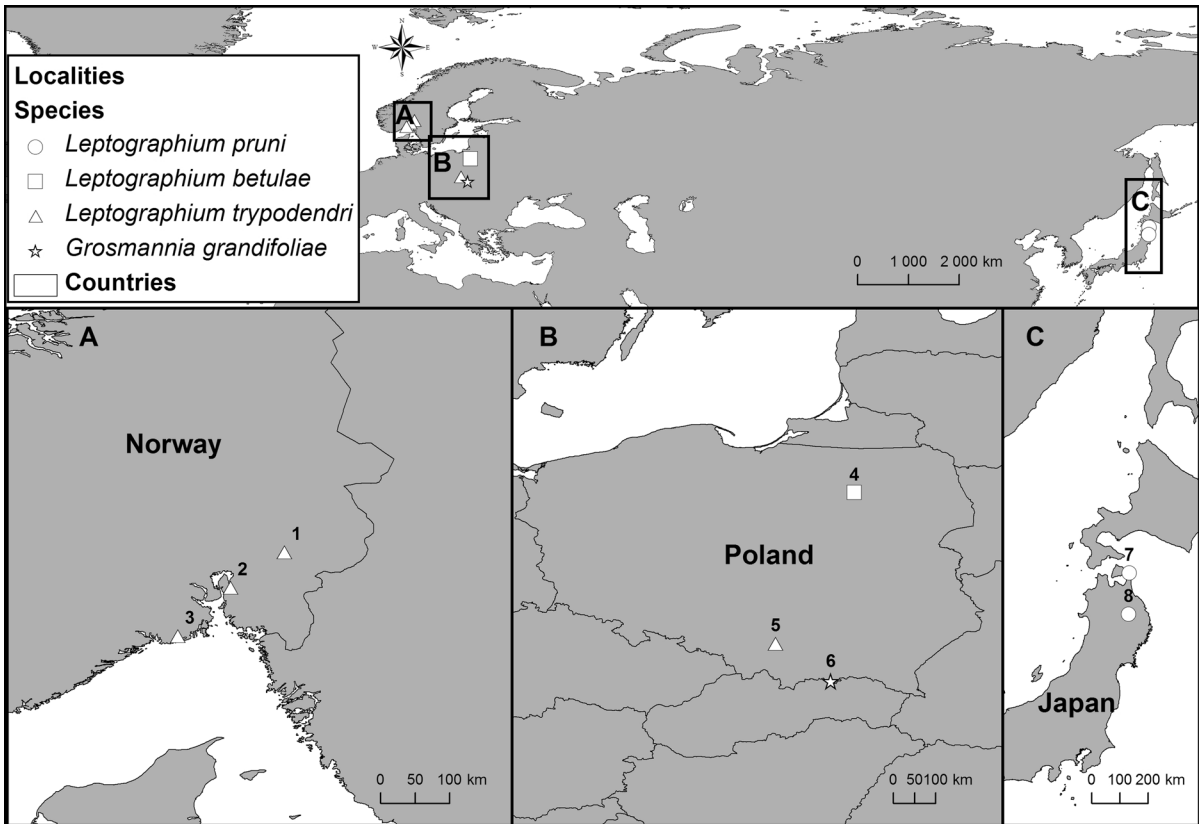


Fig. 1 Origin of isolates used in this study: *1*—Nes, Ullershov, Norway (59°41′20.79″N, 10°45′10.18″E); *2*—Ås, Syverud, Norway (59°41′20.79″N, 10°45′10.18″E); *3*—Larvik, Norway (59° 3′37.00″N, 10° 4′6.68″E); *4*—Myszyniec, Poland (53°23′ 5.89″N, 21°22′58.08″E); *5*—Krzeszowice, Poland (50°9′20.46″ N, 19°42′8.06″E; *6*—Muszyna, Poland (49°21′5.97″N, 20°52′ 47.83″E); *7*—Higashidori, Aomori, Japan (41°16′3.05″N, 141° 20′24.07″E); *8*—Iwate, Morioka, Japan (39°56′58.81″N, 141° 18′49.38″E)

and LR3 (White et al. 1990) for ITS2-LSU, T10 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) for βT, and EF1-F and EF2-R (Jacobs et al. 2004) for TEF1-α.

Amplification of the gene regions was performed under the following conditions: a denaturation step at 98 °C for 30 s followed by 35 cycles of 5 s at 98 °C, 10 s at 52–64 °C (depending on the type of primer and fungal species) and 30 s at 72 °C, and a final chain elongation at 72 °C for 8 min. Gene fragments were amplified in a 25 µL reaction mixture containing 0.25 µL of Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland), 5 µL Phusion HF buffer (5×), 0.5 µL of dNTPs (10 mM), 0.75 µL DMSO (100%) and 0.5 µL of each primer (25 µM). Amplification reactions were performed in the LabCycler Gradient (Sensoquest Biomedical Electronics GmbH, Germany). The PCR products were visualized under UV light on a 2% agarose gel stained with Midori Green DNA Stain (Nippon Genetic, Europe).

Amplified products were sequenced with the BigDye® Terminator v 3.1 Cycle Sequencing Kit (AB Applied Biosystems, Foster City, CA, USA) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA), at the DNA Research Centre (Poznań, Poland) using the same primers that were used for the PCR. The sequences (Table 1) were deposited in NCBI GenBank and compared with those in GenBank using the BLASTn algorithm.

Sequence analyses

BLAST searches using the BLASTn algorithm were performed to retrieve similar sequences from

Table 1 Isolates used in the present study

Species	Isolate no[a]				Host	Insect	Origin	GenBank accession no		
	CMW	CBS	Herbarium	Other				ITS2-LSU	βT	TEF1-α
L. trypodendri sp. nov.	44156[b]	142722	http://mus.utu.fi/TFU.206894[P]	KFL20FTDRJ	*Fagus sylvatica*	*Trypodendron domesticum*	Krzeszowice, Poland	KY801826	KY801779	KY801803
L. trypodendri	43188[b]	142723	http://mus.utu.fi/TFU.206895	KFL23FbTDRJ	*F. sylvatica*	*T. domesticum*	Krzeszowice, PL	KY801827	KY801780	KY801804
L. trypodendri	43182[b]	142724	http://mus.utu.fi/TFU.206896[H]	KFL66FTDRJ	*F. sylvatica*	*T. domesticum*	Krzeszowice, PL	KY801828	KY801781	KY801805
L. trypodendri	43185[b]	142725	http://mus.utu.fi/TFU.206897[P]	KFL3514FTDRJ	*F. sylvatica*	*T. domesticum*	Krzeszowice, PL	KY801829	KY801782	KY801806
L. trypodendri	43187	142726	http://mus.utu.fi/TFU.206898	KFL72FTDRJ	*F. sylvatica*	*T. domesticum*	Krzeszowice, PL	KY801830	KY801783	KY801807
L. trypodendri	43184	142727	http://mus.utu.fi/TFU.206899	KFL68FTDRJ	*F. sylvatica*	*T. domesticum*	Krzeszowice, PL	KY801831	KY801784	KY801808
L. trypodendri	43183	142728	http://mus.utu.fi/TFU.206900	KFL6114bFTDRJ	*F. sylvatica*	*T. domesticum*	Krzeszowice, PL	KY801832	KY801785	KY801809
L. trypodendri				N2016-0574/2/6	*Alnus incana*	*Dryocoetes alni*	Nes, Ullershov Norway	KY801833	KY801786	KY801810
L. trypodendri		142729	OF-304919[P]	N2016-0661/2/1	*Alnus incana*	*D. alni*	Ås, Syverud, N	KY801834	KY801787	KY801811
L. trypodendri		142730	OF-304920[P]	N2016-0681/2/5	*Alnus incana*	*T. domesticum*	Larvik, N	KY801835	KY801788	KY801812
L. betulae sp. nov.	43190[b]	142731	http://mus.utu.fi/TFU.206901[P]	KFL20BSRRJ	*Betula verrucosa*	*Scolytus ratzeburgi*	Myszyniec, Poland	KY801836	KY801789	KY801813
L. betulae	43194[b]	142732	http://mus.utu.fi/TFU.206902	KFL170BSRRJ	*B. verrucosa*	*S. ratzeburgi*	Myszyniec, PL	KY801837	KY801790	KY801814
L. betulae	44157[b]	142733	http://mus.utu.fi/TFU.206903[P]	KFL378BSRRJ	*B. verrucosa*	*S. ratzeburgi*	Myszyniec, PL	KY801838	KY801791	KY801815
L. betulae	43193	142739	http://mus.utu.fi/TFU.206904	KFL419BSRRJ	*B. verrucosa*	*S. ratzeburgi*	Myszyniec, PL	KY801839	KY801792	KY801816
L. betulae	43191[b]	142734	http://mus.utu.fi/TFU.206905[H]	KFL382BSRRJ	*B. verrucosa*	*S. ratzeburgi*	Myszyniec, PL	KY801840	KY801793	KY801817
L. betulae	43192			KFL341BSRRJ	*B. verrucosa*	*S. ratzeburgi*	Myszyniec, PL	KY801841	KY801794	KY801818
L. betulae				KFL242BSRRJ	*B. verrucosa*	*S. ratzeburgi*	Myszyniec, PL		KY801795	KY801819
L. betulae				KFL212BSRRJ	*B. verrucosa*	*S. ratzeburgi*	Myszyniec, PL		KY801796	KY801820
Leptographium pruni	10417	120197		JCM11708[H]	*Prunus jamasakura*	*Polygraphus ssiori*	Iwate, Morioka, Japan	KY801842	KY801797	KY801821
L. pruni		142735		HM 35.1	*P. jamasakura*	*P. ssiori*	Higashidori, Aomori, J	KY801843	KY801798	KY801822
L. pruni		142736		HM 96.1	*P. jamasakura*	*P. ssiori*	Higashidori, Aomori, J	KY801844	KY801799	KY801823
Grosmannia grandifoliae		142737		KFL36316NLRJ	*Tilia cordata*	wound	Muszyna, Poland	KY801845	KY801800	KY801824
G. grandifoliae		142738		KFL36516NLRJ	*Tilia cordata*	wound	Muszyna, PL	KY801846	KY801801	KY801825

[a] CMW Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; CBS Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; TFU the TUR Fungus collections of the Turku University, Finland; OF the Mycological Herbarium, Natural History Museum, University of Oslo, Norway; KFL Culture collection of the Department of Forest Pathology, Mycology and Tree Physiology; University of Agriculture in Krakow, Poland; JCM Japan Collection of Microorganisms, Riken BioResource Center, Koyadai, Tsukuba, Ibaraki, Japan; HM Culture collection of the Tohoku Research Center of Forestry & Forest Products Research Institute, Nabeyashiki, Shimi-Kuriyagawa, Morioka, Japan (Hayato Masuya), N Culture Collection at Norwegian Institute of Bioeconomy, Norway

[b] Isolates used in growth and morphological studies; [P]ex-paratype; [H]ex-holotype

GenBank (http://www.ncbi.nlm.nih.gov). Accession numbers of these sequences are presented in the corresponding phylogenetic trees (Figs. 2, 3, 4). The DNA sequences obtained in this study were deposited in GenBank and their accession numbers are presented in the Table 1.

Individual data sets for the ITS2-LSU, the βT and the TEF1-α gene regions were used for phylogenetic analyses. Data sets were compiled and edited in Molecular Evolutionary Genetic Analysis (MEGA) v6.06 (Tamura et al. 2013). The ITS2-LSU sequences of the isolates of each new species and *L. pruni* that were most closely related to them (Table 1), were compared with those of 72 other species in *Leptographium s. l.* obtained from GenBank to show the placement of the species within the genus. Two protein coding gene regions (βT and TEF1-α) for 23 isolates (Table 1) were sequenced for the delineation of closely related species.

Sequence alignments were performed using the online version of MAFFT v7 (Katoh and Standley 2013). The ITS, βT and TEF1-α data sets were aligned using the E-INS-i strategy with a 200PAM/ $\kappa = 2$ scoring matrix, a gap opening penalty of 1.53 and an offset value of 0.00. Aligned data sets of the protein-coding genes were compared to gene maps constructed by Yin et al. (2015) to determine the presence or absence of introns and confirm that introns and exons were appropriately aligned (Table 2). For ML and Bayesian analyses, the best-fit substitution models for each data set were established using the corrected Akaike Information Criterion (AICc) in jModelTest 2.1.10 (Guindon and Gascuel 2003; Darriba et al. 2012).

Phylogenetic analyses were performed for each of the data sets using two different methods: maximum likelihood (ML) and Bayesian inference (BI). Maximum likelihood (ML) searches were conducted in PhyML 3.0 (Guindon et al. 2010), via the Montpelier online server (http://www.atgc-montpellier.fr/phyml/) with 1000 bootstrap replicates. BI analyses based on a Markov Chain Monte Carlo (MCMC) were carried out with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). The MCMC chains were run for 10 million generations using the best-fit model. Trees were sampled every 100 generations, resulting in 100,000 trees from both runs. The burn-in value for each dataset was determined in Tracer v1.4.1 (Rambaut and Drummond 2007).

Fig. 2 Phylogram obtained from ML analyses of the ITS2-LSU region showing the placement of isolates obtained from Poland and Norway in *Leptographium s. l.* Sequences obtained during this study are presented in *bold* type. The phylogram was obtained from Maximum Likelihood (ML) analyses. Bootstrap values >75% for ML and posterior probabilities >75% obtained from Bayesian (BI) analyses are presented at nodes as follows: ML/BI. *Bootstrap values <75%. Ⓗ species from hardwood hosts

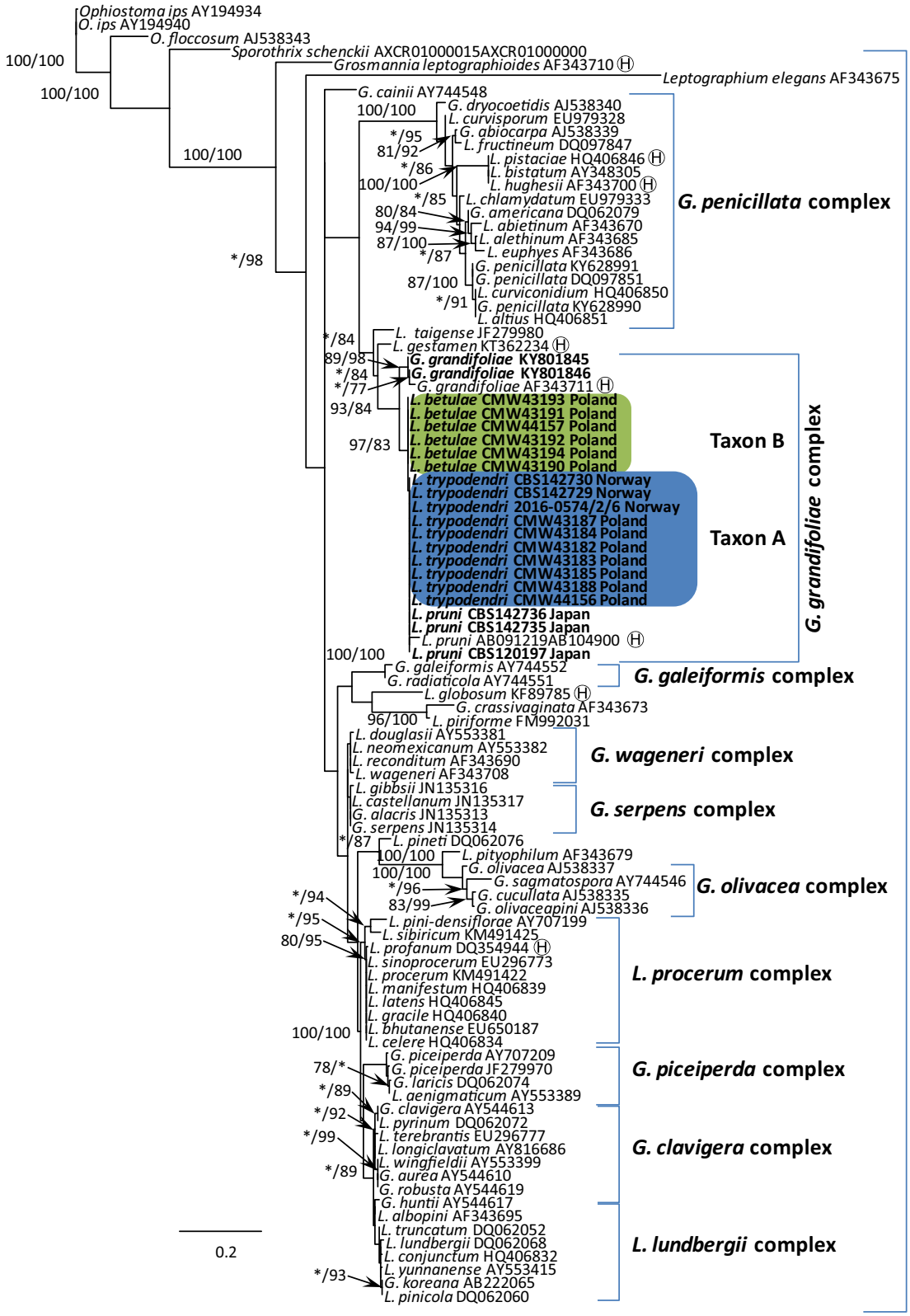
Morphological characterization

Morphological characteristics for selected isolates and herbarium specimens chosen to represent the type specimens were examined. Cultures were grown on 2% MEA with and without host tree twigs to induce potential ascocarp formation. The autoclaved twigs with bark were placed in the middle of the agar plates. Fungi were grown as single isolate cultures, and crossings were made following the technique described by Grobbelaar et al. (2010). Cultures were incubated at 25 °C and inspected regularly for fruiting structures.

Morphological characteristics were examined by mounting the sexual and asexual fruiting structures in 80% lactic acid on glass slides, and these were observed using Nikon Eclipse 50*i* microscope (Nikon® Corporation, Tokyo, Japan) and Invenio 5S digital camera (DeltaPix®, Maalov, Denmark) to capture photographic images. Measurements were made of 50 each of the taxonomically relevant structures where this was possible with Coolview 1.6.0 software (Precoptic®, Warsaw, Poland). Averages, ranges and standard deviations were computed for the measurements. The measurements are presented in the format '(min–max) (mean–SD)'.

Culture characteristics

Growth characteristics of isolates in pure culture were considered for four representative isolates of each of the studied species (Table 1). Four replicate plates per isolate were used for each temperature (5, 10, 15, 20, 25, 30 and 35 °C). Agar disks 5 mm diam. were cut from actively growing margins of colonies of each isolate to be tested, and placed at the center of plates containing 2% MEA. Colony diameters (three measurements per plate) were determined 7 and 14 days after inoculation and growth rates were calculated as mm/d.


Ophiostoma ips AY194934
O. ips AY194940
O. floccosum AJ538343
Sporothrix schenckii AXCR01000015AXCR01000000
Grosmannia leptographioides AF343710 Ⓗ
Leptographium elegans AF343675
G. cainii AY744548
G. dryocoetidis AJ538340
L. curvisporum EU979328
G. abiocarpa AJ538339
L. fructineum DQ097847
L. pistaciae HQ406846 Ⓗ
L. bistatum AY348305
L. hughesii AF343700 Ⓗ
L. chlamydatum EU979333
G. americana DQ062079
L. abietinum AF343670
L. alethinum AF343685
L. euphyes AF343686
G. penicillata KY628991
G. penicillata DQ097851
L. curviconidium HQ406850
G. penicillata KY628990
L. altius HQ406851
G. penicillata complex
L. taigense JF279980
L. gestamen KT362234 Ⓗ
G. grandifoliae KY801845
G. grandifoliae KY801846
G. grandifoliae AF343711 Ⓗ
L. betulae CMW43193 Poland
L. betulae CMW43191 Poland
L. betulae CMW44157 Poland
L. betulae CMW43192 Poland
L. betulae CMW43194 Poland
L. betulae CMW43190 Poland
Taxon B
L. trypodendri CBS142730 Norway
L. trypodendri CBS142729 Norway
L. trypodendri 2016-0574/2/6 Norway
L. trypodendri CMW43187 Poland
L. trypodendri CMW43184 Poland
L. trypodendri CMW43182 Poland
L. trypodendri CMW43183 Poland
L. trypodendri CMW43185 Poland
L. trypodendri CMW43188 Poland
L. trypodendri CMW44156 Poland
Taxon A
L. pruni CBS142736 Japan
L. pruni CBS142735 Japan
L. pruni AB091219AB104900 Ⓗ
L. pruni CBS120197 Japan
G. grandifoliae complex
G. galeiformis AY744552
G. radiaticola AY744551
G. galeiformis complex
L. globosum KF89785 Ⓗ
G. crassivaginata AF343673
L. piriforme FM992031
L. douglasii AY553381
L. neomexicanum AY553382
L. reconditum AF343690
L. wageneri AF343708
G. wageneri complex
L. gibbsii JN135316
L. castellanum JN135317
G. alacris JN135313
G. serpens JN135314
G. serpens complex
L. pineti DQ062076
L. pityophilum AF343679
G. olivacea AJ538337
G. sagmatospora AY744546
G. cucullata AJ538335
G. olivaceapini AJ538336
G. olivacea complex
L. pini-densiflorae AY707199
L. sibiricum KM491425
L. profanum DQ354944 Ⓗ
L. sinoprocerum EU296773
L. procerum KM491422
L. manifestum HQ406839
L. latens HQ406845
L. gracile HQ406840
L. bhutanense EU650187
L. celere HQ406834
L. procerum complex
G. piceiperda AY707209
G. piceiperda JF279970
G. laricis DQ062074
L. aenigmaticum AY553389
G. piceiperda complex
G. clavigera AY544613
L. pyrinum DQ062072
L. terebrantis EU296777
L. longiclavatum AY816686
L. wingfieldii AY553399
G. aurea AY544610
G. robusta AY544619
G. clavigera complex
G. huntii AY544617
L. albopini AF343695
L. truncatum DQ062052
L. lundbergii DQ062068
L. conjunctum HQ406832
L. yunnanense AY553415
G. koreana AB222065
L. pinicola DQ062060
L. lundbergii complex
Leptographium s. l.
0.2

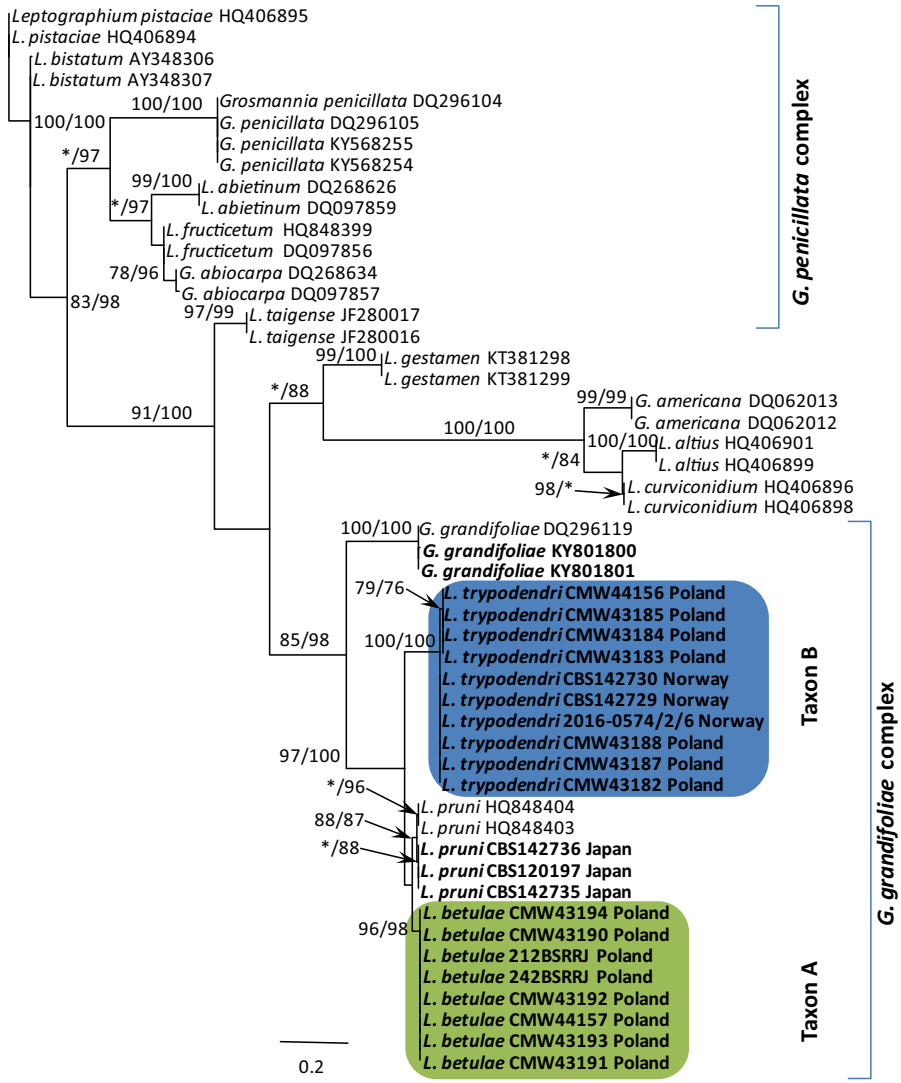


Fig. 3 Phylogram obtained from the βT sequences of *Leptographium* spp. and *Grosmannia* spp. related to the Polish and Norwegian isolates. Sequences obtained during this study are presented in *bold* type. The phylogram was obtained from Maximum Likelihood (ML) analyses. Bootstrap values >75% for ML and posterior probabilities >75% obtained from Bayesian (BI) analyses are presented at nodes as follows: ML/BI. *Bootstrap values < 75%

Results

Morphological characteristics

Despite their close phylogenetic relationship, the two unknown *Leptographium* spp. were morphologically distinct from each other as well as from the closely related *L. pruni* and *G. grandifoliae*. They formed olivaceous leptographium-like asexual states and sporothrix- or hyalorhinocladiella-like synanamorphs. Morphological differences between the two new taxa and the other species are discussed in the *Notes* provided for the new species descriptions in the "Taxonomy" section. The optimal growth temperature for all the isolates was 25 °C.

DNA sequence analyses

The amplified DNA fragments were approximately 860–918 bp long for the 5.8S-ITS2-LSU region, 372–545 bp long for the partial βT, and 805–834 bp long for the TEF1-α genes. The aligned data set for

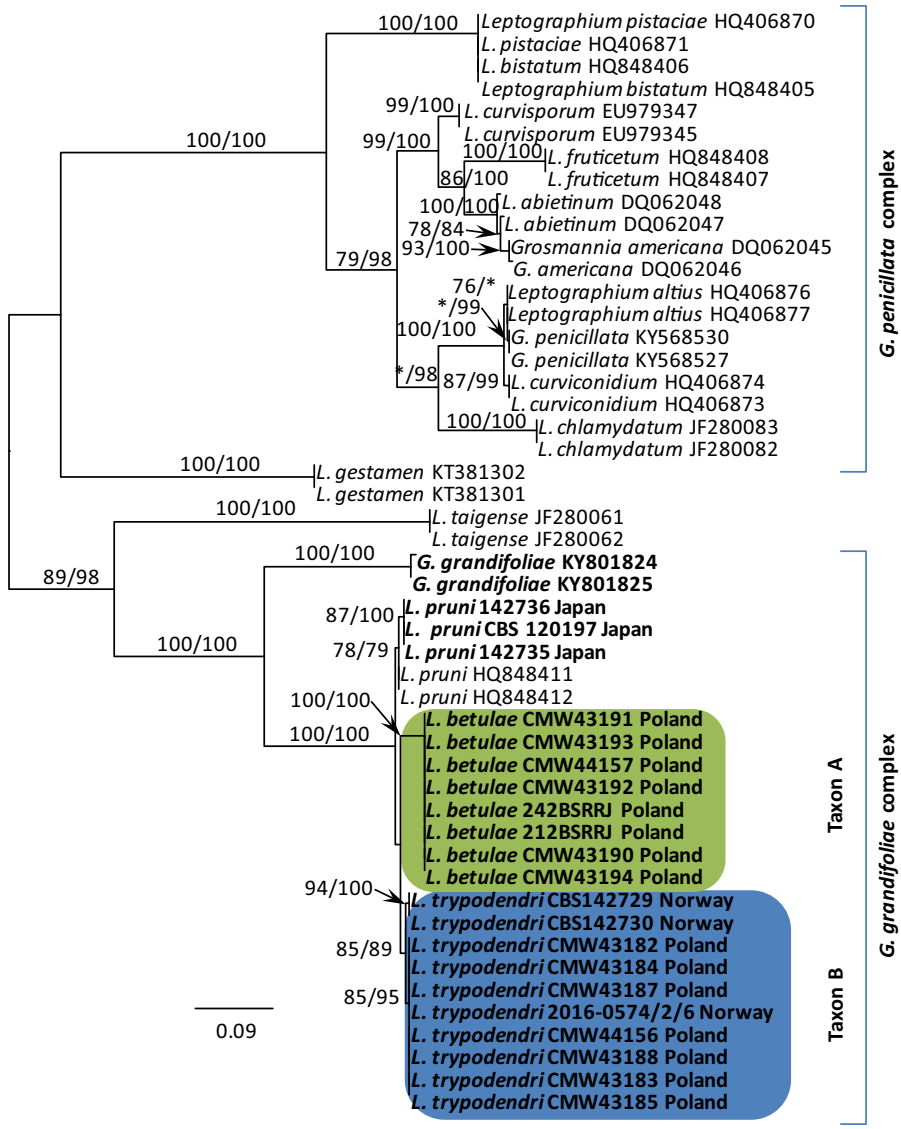


Fig. 4 Phylogram obtained from the TEF1-α sequences of *Leptographium* spp. and *Grosmannia* spp. related to the Polish and Norwegian isolates. Sequences obtained during this study are presented in *bold* type. The phylogram was obtained from Maximum Likelihood (ML) analyses. Bootstrap values >75% for ML and posterior probabilities >75% obtained from Bayesian (BI) analyses are presented at nodes as follows: ML/BI. *Bootstrap values <75%

the ITS2-28S region gene region included 97 taxa and 629 characters (with gaps). The βT data set consisted of 50 taxa and 484 characters (with gaps), and included the partial exon 3, intron 3, exon 4, intron 4, exon 5, intron 5 and partial exon 6. The TEF1-α data set consisted of 49 taxa and 708 characters (with gaps), including partial intron 4, exon 5, intron 5, and partial exon 6. The BI and ML analyses for each data set produced trees with similar topologies (Figs. 2–4). The best-fitting substitution model selected for ML/BI analyses was HKY+I+G for all data sets.

In the ITS2-LSU tree (Fig. 2), the two unidentified species from Poland grouped with *L. pruni* and *G. grandifoliae* in a well-supported monophyletic lineage adjacent to the *G. penicillata* complex. In

addition, the two isolates from *Tilia* grouped with the ex-type isolate of *G. grandifoliae*. However, the ITS2-LSU data did not distinguish clearly between *L. pruni* and the Polish isolates.

Analyses of the partial βT gene distinguished clearly between *G. grandifoliae,* including the two isolates from *Tilia,* and *L. pruni,* and separated the other isolates from Poland in two well-supported clades, Taxa A and B. Taxon A includes three isolates from Norway (Fig. 3). Together, the four taxa again formed a monophyletic clade with good support. In the βT data set, 35/384 (9.1%) positions were variable. Little or no intraspecific sequence variation was found within each of the four species in the complex. Intraspecific variability of the βT and TEF1-α genes was only detected for *L. trypodendri* in two positions i.e. 70, 175 and six positions i.e. 94, 124, 131, 158, 451 and 502, respectively (Table 2).

The phylogram based on the TEF1-α data resolved *G. grandifoliae, L. pruni* and Taxa A and B, with the latter three grouping closely together (Fig. 4). Together, the four species again formed a well-supported monophyletic lineage.

Fig. 5 Morphological characteristics of *Leptographium trypodendri* sp. nov. (CBS 142,724). **a** Conidiophore. **b** Conidiophores arising in loosely arranged groups on spiral hyphae. **c** Conidiogenous apparatus with two primary branches. **d** Conidia. **e, f** Hyalorhinocladiella-like synanamorph. **g** Chlamydospore-like structures. **h** Culture on MEA

Taxonomy

Based on DNA sequences and morphological differences, Taxa A and B from Poland and Norway could be distinguished from *L. pruni* and *G. grandifoliae*, and are thus described below as novel species.

Taxon A

Leptographium trypodendri R. Jankowiak, B. Strzałka and R. Linnakoski, sp. nov. Fig. 5 MB 821669.

Etymology: *trypodendri* refers to the fungus being collected mainly from *Trypodendron*.

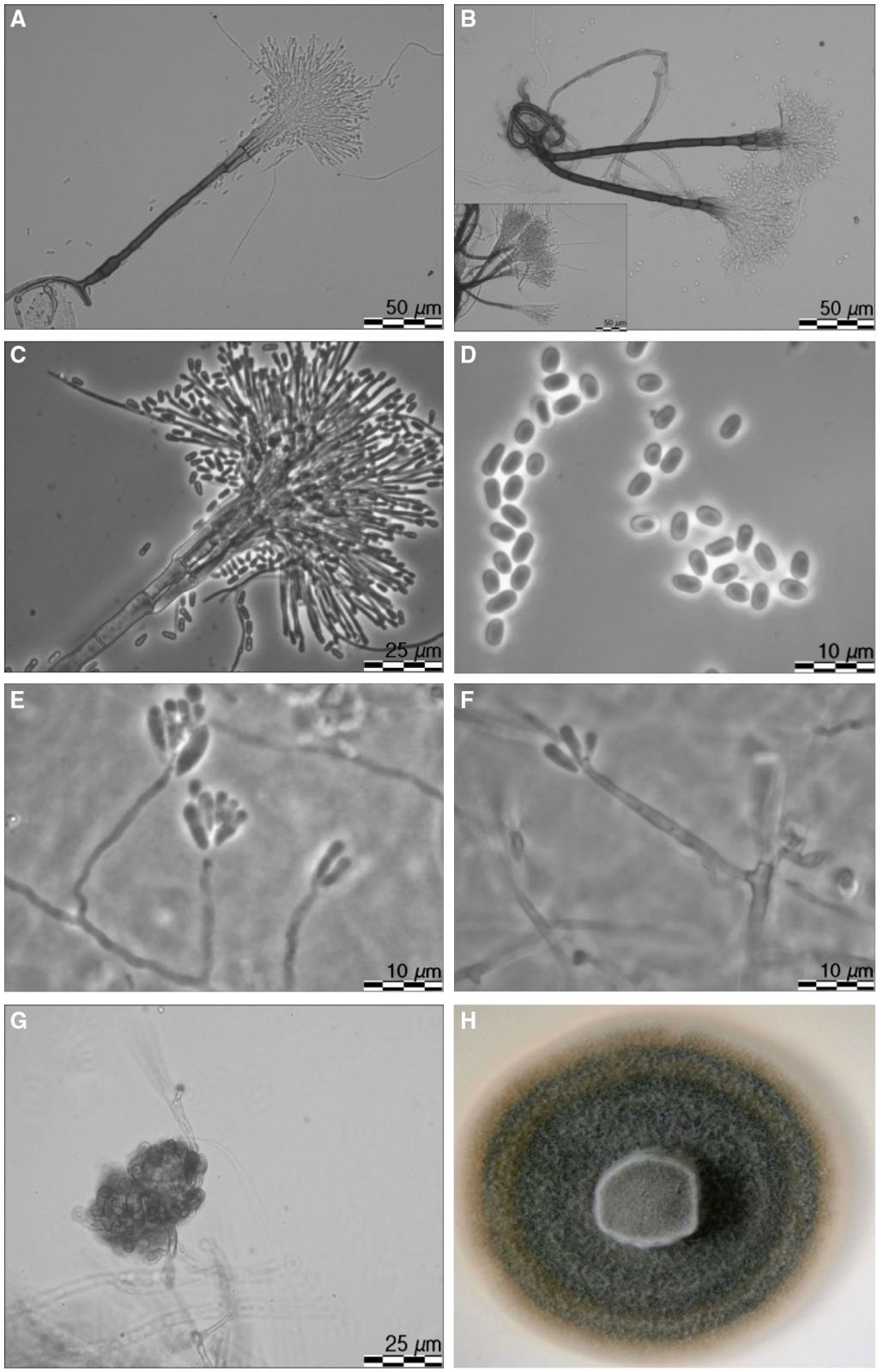
Sexual state not observed. *Conidiophores* macronematous, arising laterally from hyphae, single solitary or often also in loosely arranged groups on

Table 2 Comparison of polymorphic sites of protein-coding genes of *Leptographium pruni* and both new taxa

Species	Strains	Genes*: BT	Genes*: TEF1-α
		iiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiieeee	iiiiiiiiiiiiiiiiiiieiiee
		11111111122233	111111345567
		12222222244455666677788367777788902604	24445589123555050276
		70234567835814067902902930134589223270	56760120204441278812040
L. trypodendri sp. nov	CMW44156	AACGCAGTAAGTCAA-TACGTCGCGGGTGAACCTACAC	ATCTAACCTT-GGCC--CTGCCT
	CMW43188	AACGCAGTAAGTCAA-TATGTCGCGGGTGTACCTACAC	ATCTAACCTT-GGCC--CTGCCT
	CMW43182	AACGCAGTAAGTCAA-TATGTCGCGGGTGTACCTACAC	ATCTAACCTT-GGCC--CTGCCT
	CMW43185	AACGCAGTAAGTCAA-TACGTCGCGGGTGAACCTACAC	ATCTAACCTT-GGCC--CTGCCT
	CMW43187	AACGCAGTAAGTCAA-TATGTCGCGGGTGTACCTACAC	ATCTAACCTT-GGCC--CTGCCT
	CMW43184	AACGCAGTAAGTCAA-TACGTCGCGGGTGAACCTACAC	ATCTAACCTT-GGCC--CTGCCT
	CMW43183	AACGCAGTAAGTCAA-TACGTCGCGGGTGAACCTACAC	ATCTAACCTT-GGCC--CTGCCT
	N2016-0574/2/6	AACGCAGTAAGTCAA-TATGTCGCGGGTGTACCTACAC	ATCTAACCTTCGGCC--CTGCCT
	CBS142729	AACGCAGTAAGTCAA-TATGTCGCGGGTGTACCTACAC	ATCTAACCTTCGATC-TCCTCCT
	CBS142730	AACGCAGTAAGTCAA-TATGTCGCGGGTGTACCTACAC	ATCTAACCTTCGATC-TCCTCCT
L. betulae sp. nov.	CMW43190	T--------GTGAGGGCGCAT-A-A---GAATTCGGGC	C-TT--CG-T-AGC-TTCCTCTA
	CMW43194	T--------GTGAGGGCGCAT-A-A---GAATTCGGGC	C-TT--CG-T-AGC-TTCCTCTA
	CMW44157	T--------GTGAGGGCGCAT-A-A---GAATTCGGGC	C-TT--CG-T-AGC-TTCCTCTA
	CMW43193	T--------GTGAGGGCGCAT-A-A---GAATTCGGGC	C-TT--CG-T-AGC-TTCCTCTA
	CMW43191	T--------GTGAGGGCGCAT-A-A---GAATTCGGGC	C-TT--CG-T-AGC-TTCCTCTA
	CMW43192	T--------GTGAGGGCGCAT-A-A---GAATTCGGGC	C-TT--CG-T-AGC-TTCCTCTA
	KFL242BSRRJ	T--------GTGAGGGCGCAT-A-A---GAATTCGGGC	C-TT--CG-T-AGC-TTCCTCTA
	KFL212BSRRJ	T--------GTGAGGGCGCAT-A-A---GAATTCGGGC	C-TT--CG-T-AGC-TTCCTCTA
L. pruni	JCM11708	T--------ATGAGGGCGCAA-A-G---TTGTTCGCGT	CTC-ACTTTGTGGCC-TTCTTCA
	CBS142735	T--------ATGAGGGCGCAA-A-G---TTGTTCGCGT	CTC-ACTTTGTGGCC-TTCTTCA
	CBS142736	T--------ATGAGGGCGCAA-A-G---TTGTTCGCGT	CTC-ACTTTGTGGCC-TTCTTCA

* "i" indicates introns, while "e" represents exons in the various genes

Numbers written vertically above columns indicate the relative positions in the alignments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spiral hyphae, without rhizoidal hyphae at the bases (Fig. 5a, b). *Stipes* erect, light to dark brown, 1–7 septa, 31–130 (mean 74.9 ± 19.8) µm long and 2.5–5 (mean 3.6 ± 0.6) µm wide at base. *Conidiogenous apparatus* 39.5–86 (mean 62.7 ± 11) µm long (excluding conidial mass) consisting of (1–) 2 (–3) series of branches-type B (more than two branches) (Jacobs and Wingfield 2001) (Fig. 5c). Primary branches dark olivaceous, cylindrical, smooth, 8.5–26 × 2.5–6 µm. *Conidiogenous cells* hyaline, tapering from base to apex, 3.5–31 (mean 23 ± 6) × 1–2 (mean 1.6 ± 0.2) µm. *Conidia* hyaline, oblong to elliptical, sometimes allantoid or obovoid, 2.5–4.5 (mean 3.4 ± 0.43) × 1–2 (mean 1.6 ± 0.2) µm, accumulating around the conidiogenous apparatus in a hyaline mucilaginous mass (Fig. 5 d).

Hyalorhinocladiella-like micronematal asexual state present (Fig. 5e, f). *Conidiogenous cells* arising directly from hyphae, 5–53 (mean 23.3 ± 10.7) × 0.5–2.0 (mean 1.2 ± 0.4) µm; *conidia* hyaline, cylindrical, obovate or pyriform, 3–7.5 (mean 4.7 ± 0.9) × 0.5–3.5 (mean 2 ± 0.7) µm. *Chlamydospores* present in young and older cultures, 16–71.5 (mean 37.5 ± 10.6) µm in diameter (Fig. 5g).

Culture characteristics colonies on 2% MEA at first light gray, becoming darker (Fig. 5h). Aerial mycelium abundant, laniferous, often forming subvisible "annular zones" and later "cloud-like structures". Aerial mycelium hyaline to pale brown, sometimes brown, 1.5–3 (mean 2.3 ± 0,4) µm thick, occurring singly, rarely aggregated in strands of 2–10 hyphae. Optimal growth temperature at 25 °C. Culture growth rates 2.2 mm/day (±0.1) at 20 °C and 2.4 mm/day (±0.1) at 25 °C. No growth observed at 5 °C and at 35 °C.

Type material POLAND, Krzeszowice, from *Trypodendron domesticum* beetle infesting *Fagus sylvatica*, 12 April 2013, *R Jankowiak*, holotype TUR http://mus.utu.fi/TFU.206896, culture ex-holotype CBS 142724 = CMW 43182; POLAND, Krzeszowice, from *Trypodendron domesticum* beetle infesting *Fagus sylvatica*, 12 April 2013, *R Jankowiak*, paratype TUR http://mus.utu.fi/TFU.206894, culture ex-paratype CBS 142722 = CMW44156; POLAND, Krzeszowice, from *Trypodendron domesticum* beetle infesting *Fagus sylvatica*, 12 April 2013, *R Jankowiak*, paratype http://mus.utu.fi/TFU.206897, culture ex-paratype CBS14725 = CMW43185; POLAND, Krzeszowice, from *Trypodendron domesticum* beetle infesting *Fagus sylvatica*, 12 April 2013, *R. Jankowiak*, paratype OF-304919, culture ex-paratype CBS 142729; NORWAY, Ås, from *Dryocoetes alni* beetle infesting *Alnus incana*, 7 February 2016, *A Truls*, paratype OF-304920, culture ex-paratype CBS 142730; NORWAY, Larvik, from *Trypodendron domesticum* beetle infesting *Alnus incana*, 4 April 2016, *A Truls*.

Fig. 6 Morphological characteristics of *Leptographium betulae* sp. nov. (CBS 142,734). **a** Conidiophore. **b** Conidiophores arising in loosely arranged groups on hyphae. **c** Conidiogenous apparatus with two primary branches. **d** Conidia. **e, f** Sporothrix-like synanamorph, **g** Culture on MEA ▶

Host trees: *Alnus incana, Betula pubescens, Fagus sylvatica, Fraxinus excelsior* and *Quercus robur*

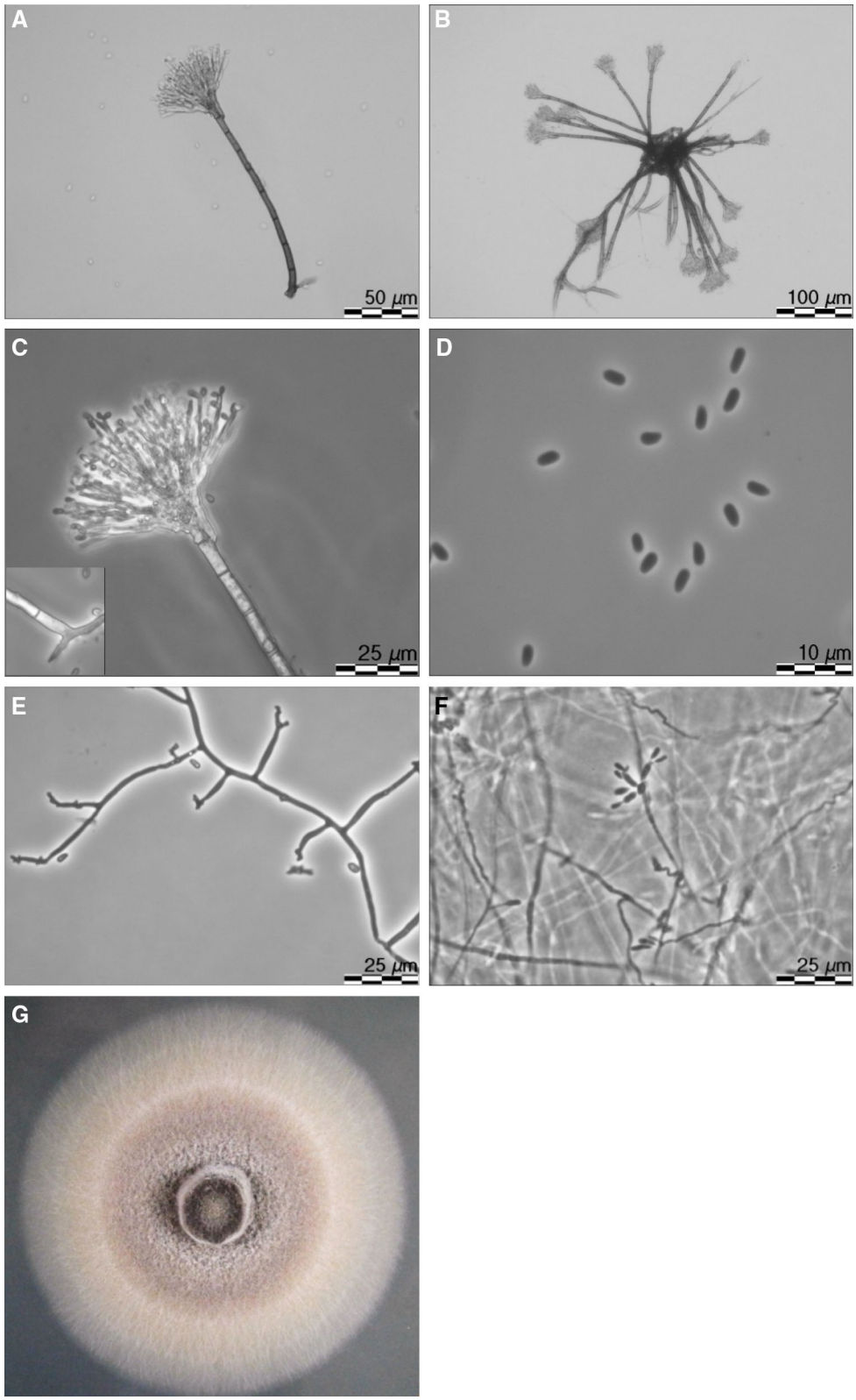
Insect vectors: *Dryocoetes alni*, *Trypodendron domesticum*

Known distribution: Norway, Poland

Notes Isolates of *L. trypodendri* grouped close to *L. pruni* in all phylogenetic analyses (Figs. 2–4), and can clearly be separated from the latter species based on sequences of two protein-coding genes (Figs. 3, 4). These two species differ in 35 bp in β-tubulin and 15 bp in TEF1-α (Table 2).

Morphologically, *L. trypodendri* differs from *L. pruni* in having shorter conidiophores, longer conidiogenous apparatus, chlamydospore-like structures, and with no growth observed at 35 °C on MEA. The only other *Leptographium* spp. known to produce chlamydospores are *L. piriforme* Greif et al. (Greif et al. 2006) and *L. chlamydatum* (Jacobs et al. 2010), both unrelated to the species treated in this study. Furthermore, *L. trypodendri* has a hyalorhinocladiella-like synanamorph versus a sporothrix-like form of *L. pruni* with more pronounced denticles. In turn, the closely related *G. grandifoliae* forms perithecia and has larger conidiophores having distinct rhizoids. *Leptographium trypodendri* differs from *L. betulae* in having shorter stipes, longer conidiogenous apparatuses and larger conidia. However, the most obvious distinguishing characteristic of these taxa is the presence of different synanamorphs: *L. trypodendri* is associated with a hyalorhinocladiella-like synanamorph, while *L. betulae* has a sporothrix-like synanamorph.

Leptographium trypodendri was found respectively on 43, 63 and 77% of *T. domesticum* beetles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from *A. incana* on the three sample plots in south-eastern Norway. It was isolated more rarely (25%) on *F. excelsior* and *Q. robur*, but fewer bark beetles were collected on these tree species. Only one *D. alni* beetle carried *L. trypodendri* on each of the three sample plots, and on those places *D. alni* was collected from the same branches as *T. domesticum*. Similarly, to the Norwegian results, *L. trypodendri* was often recorded in association with *T. domesticum* in Poland. It was isolated from 52% of the beetles collected from *F. sylvatica* logs.

Taxon B

Leptographium betulae R. Jankowiak, B. Strzałka & R. Linnakoski, sp. nov. Fig. 6 MB 821670.

Etymology: *betulae* refers to the fungus being collected only from *Betula verrucosa*.

Sexual state not observed. *Conidiophores* macronematous, arising laterally from hyphae, solitary or often also in loosely arranged groups on hyphae, without rhizoidal hyphae at the bases (Fig. 6a, b). *Stipes* erect, pale brown to dark brown, 1–6-septate, 54.5–423 (mean 166.4 ± 70.9) µm long and 2–7 (mean 3.9 ± 1) µm wide at base. *Conidiogenous apparatus* 27–70 µm (mean 49.4 ± 9.7) long (excluding conidial mass) consisting of 1–3 branches (branch type B) (Jacobs and Wingfield 2001) (Fig. 6c). Primary branches olivaceous, cylindrical, smooth, 11–29.5 × 3.5–5 µm. *Conidiogenous cells* hyaline, tapering from base to apex, 7.5–13.5 (mean 10 ± 2) × 1.5–3 (mean 2.2 ± 0,4) µm. *Conidia* hyaline, obovate, elliptical, sometimes allantoid, 2–4 (mean 2.9 ± 0,3) × 1.0–1.5 (mean 1.3 ± 0.2) µm, accumulating around the conidiogenous apparatus in a hyaline mucilaginous mass (Fig. 6d).

Sporothrix-like micronematal asexual state present. *Conidiophores* micronematous sporothrix-like, arising individually from undifferentiated hyphae (Fig. 6e, f). *Conidiogenous cells* cylindrical, variable in shape and size, usually widest at the basal part and slightly tapering, having distinct appendages at their tip, 21–74 (mean 43 ± 13.5) µm long, 1–3 µm wide at the base. *Conidia* hyaline, 1-celled, oblong to oval, 3–7 (mean 5 ± 1) × 1.5–3.5 (mean 2.5 ± 0.6) µm, sometimes developing into larger ramoconidia, producing distinct denticles and secondary conidia. Ramoconidia hyaline, 1-celled, clavate, 5.0–12.0 (mean 7.5 ± 1.9) × 1–3 (mean 2 ± 0.5) µm. The secondary conidia hyaline, 1-celled, ellipsoidal, 2–4 (mean 3 ± 0.6) × 1–2.5 (mean 1.6 ± 0.4) µm.

Culture characteristics colonies on 2% MEA initially hyaline, whitish, later light gray, with abundant aerial mycelium (Fig. 6g). Mycelium laniferous, without "annular zones". Hyphae 2–3 (mean 2.4 ± 0.4) µm thick, hyaline to light gray, sometimes pale brown, occurring singly or aggregated in strands of 2–10 hyphae. Macronematous and micronematous conidiophores present. Optimal growth temperature at 25 °C. Culture growth rates 2.4 mm/day (±0.1) at 20 °C, 2.6 mm/day (±0.1) at 25 °C, and 0.9 mm/day (±0.1) at 35 °C. No growth observed at 5 °C.

Type material POLAND, Myszyniec, from galleries of *Scolytus ratzeburgi* infesting *Betula verrucosa*, 24 July 2011, *R Jankowiak*, holotype TUR http://mus.utu.fi/TFU.206905, culture ex-holotype CBS 142734 = CMW 43191; POLAND, Myszyniec, from galleries of *Scolytus ratzeburgi* infesting *Betula verrucosa*, 24 July 2011, *R Jankowiak*, paratype TUR http://mus.utu.fi/TFU.206901, culture ex-paratype CBS 142731 = CMW43190; POLAND, Myszyniec, from galleries of *Scolytus ratzeburgi* infesting *Betula verrucosa*, 24 July 2011, *R Jankowiak*, paratype TUR http://mus.utu.fi/TFU.206903, culture ex-paratype CBS 142733 = CMW44157; POLAND, Myszyniec, from galleries of *Scolytus ratzeburgi* infesting *Betula verrucosa*, 24 July 2011, *R Jankowiak*.

Host tree: *Betula verrucosa*
Insect vectors: *Scolytus ratzeburgi*
Known distribution: Poland

Notes Isolates of *L. betulae* grouped close to but distinct from *L. pruni* in phylogenetic analyses (Fig. 2), and can clearly be separated from the latter based on sequences of the two protein-coding gene regions (Figs. 3, 4). *Leptographium betulae* differs from *L. pruni* in 8 bp in β-tubulin, and 16 bp in TEF1-α (Table 2). Morphologically, it differs from *L. pruni* in having smaller conidia and lower optimal growth temperature on MEA. *Leptographium betulae* appears to be *S. ratzeburgi*-specific on *B. verrucosa* in Poland, occurring only in galleries of this beetle species (7%), always together with another *S. ratzeburgi*-associated fungus, *O. karelicum* Linnakoski, Z.W. de Beer & M.J. Wingf. (Jankowiak et al., unpublished data).

Discussion

Two new species of *Leptographium,* as well as two Polish isolates of *G. grandifoliae,* were discovered from European hardwoods in this study. These three species, together with *L. pruni*, formed the first well-supported lineage in *Leptographium s.l.* consisting only of hardwood-infecting species. The species in this complex are characterised by relatively small conidiophores arranged in loosely groups and synanamorphs of the hyalorhinocladiella- to sporothrix-like forms. The oldest known species in the complex, *G. grandifoliae*, is only known by a sexual stage. The two new species are potential symbiotic associates of the European hardwood ambrosia beetle, *T. domesticum,* and the European birch bark beetle, *S. ratzeburgi*. These results are unusual as by far, the majority of *Leptographium* spp. are known as associates of conifer-infesting bark beetles, especially root-feeding species.

In comparison with conifer-infesting bark beetles, the interactions between ophiostomatoid fungi and bark beetle species occurring on hardwood trees have been poorly studied. For this reason, the nine well-defined *Leptographium s.l.* species complexes recognized to date all include only conifer-infecting species. The *G. penicillata* complex includes among its more than 20 species three species from hardwoods: *L. hughesii* K. Jacobs et al. from *Parashorea* in Borneo and Vietnam (Jacobs et al. 1998), *L. eucalyptophylum* K. Jacobs et al. from *Eucalyptus* spp. in West Africa (Jacobs et al. 1999), and *L. pistaciae* Paciura et al. from *Pistacia* trees in China (Paciura et al. 2010). Of the nine species in the *L. procerum* complex (Yin et al. 2015), only *L. profanum* K. Jacobs et al. comes from the roots of various hardwoods in the USA (Jacobs et al. 2006). The phylogenetic placement of three hardwood-infesting species remains uncertain (De Beer et al. 2013; De Beer and Wingfield 2013): *L. calophylli*, *G. francke-grosmanniae* (R.W. Davidson) Zipfel et al. in association with *Elateroides dermestoides* (L.) beetles (Lymexylidae) from oak in Europe (Davidson 1971), and *L. brevicolle* K. Jacobs and M.J. Wingf. from *Trypodendron retusus* (Le Conte) galleries on aspen in the USA (Davidson 1958; Jacobs and Wingfield 2001). Three more species from hardwoods are placed peripheral to or between other species complexes in *Leptographium s.l.*: *G. leptographioides* (R. W. Davidson) Zipfel et al. from *Quercus* in the USA (Davidson 1942; Linnakoski et al. 2012), *L. verrucosum* (Gebhardt, R. Kirschner and Oberw.) Z.W. de Beer & M.J. Wingf. from *Xyleborus dryographus* (Ratzeburg) (Scolytinae) from *Quercus* in Germany (Gebhardt et al. 2002; Musvuugwa et al. 2015), and *L. globosum* Y.T. Huang and Chi Y. Chen from fallen hardwood in Taiwan (Huang and Chi-Y 2014).

The two remaining species from hardwoods, *G. grandifoliae* from stained *Fagus grandifoliae* Ehrh. wood in the USA (Davidson 1976), and *L. pruni* from *Polygraphus ssiori* Niijima attacking *Prunus* in Japan (Masuya et al. 2004), grouped close to each other, but not in a supported lineage, in *Leptographium s.l.* in the LSU phylogeny of De Beer and Wingfield (2013). The two species also formed an unsupported clade, sister to a new species, *L. gestamen* De Errasti & Z.W. de Beer from galleries of *Gnathotrupes* ambrosia beetles on *Nothofagus* in Argentina (De Errasti et al. 2016). Although *L. gestamen* also grouped with *G. grandifoliae*, *L. pruni* and the two newly described species from the present study in a lineage with some support in the ITS2-LSU region (Fig. 1), it did not form part of a monophyletic lineage with these species in the β-tubulin (Fig. 2) and TEF1-α (Fig. 3) trees. We thus restrict the newly defined species complex to include *G. grandifoliae*, *L. pruni, L. betulae* and *L. trypodendri,* and name it after the species that was first described, *G. grandifoliae* (Davidson 1976). Our study also represents the first report of *G. grandifoliae* from outside the USA and from another host than *Fagus*.

Apart from morphological differences between *L. betulae* and *L. trypodendri* described in the taxonomy section, the two species also differ with regards to their host range and beetle vectors. *Leptographium trypodendri* seems to be very common on *T. domesticum* attacking *A. incana* in south-eastern Norway, and *F. sylvatica* in Poland, and less often from *F. excelsior* and *Q. robur* in Norway. It was found on rare occasions also on *D. alni*. Our results suggest that *L. betulae* is only associated with *S. ratzeburgi* attacking *B. verrucosa*.

While the majority of previous studies have focused on conifer-associated beetle species and their fungal associates, the diversity of hardwood-associated ophiostomatoid fungi has yet to be explored and understood in more detail in Europe and elsewhere. The results of the present study clearly demonstrate that the species diversity of hardwood-infesting

Leptographium species occurring in Europe might be underestimated. Therefore, it will be important to expand these surveys to cover larger geographic areas including more host tree and insect vector species in Europe.

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Conflict of interest The authors declare that have no conflict of interest.

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