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Taxonomic re-evaluation of three related species of *Graphium*, based on morphology, ecology and phylogeny

Karin Jacobs[1]

Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-food Canada, Ottawa, Ontario, K1A 0C6 Canada

Thomas Kirisits

Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Universität für Bodenkultur Wien (BOKU), Hasenauerstrasse 38, A-1190 Vienna, Austria

Michael J. Wingfield

Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa

Abstract: Two fungi associated with bark beetles, *Graphium pseudormiticum* (described in 1994) and *Rhexographium fimbriisporum* (described in 1995), have two micromorphological characters in common. Both species produce conidia with conspicuous basal frills, and the conidia align in chains, despite being produced in slime. The association of *G. pseudormiticum* with the pine bark beetle, *Orthotomicus erosus*, and the association of *R. fimbriisporum* with the spruce bark beetle, *Ips typographus*, suggest ecological differences between the two fungal species. Analyses of micromorphology and phylogenetic analyses of aligned 18S and ITS sequences suggest that these two species are congeneric and should be classified in *Graphium* but that they represent distinct species. A collection of strains tentatively identified as *Graphium* spp., isolated from *Ips typographus* on *Picea abies*, *Ips cembrae* on *Larix decidua* and *Tomicus minor* on *Pinus sylvestris* in Austria share the same unusual basal conidial frills and conidial chains. Isolates from spruce were identified as *G. fimbriisporum* and those from pine as *G. pseudormiticum*. The strains from *Ips cembrae* on *Larix decidua*, distinguished by the reddish color of their colonies, microscopic structures and molecular characteristics, are described as the new species *Graphium laricis* sp. nov., and the close relationship of this species with the other two species is confirmed.

Key words: conidium development, *Graphium*, *Ips* spp., *Larix*, phylogeny, *Picea*, *Pinus*, *Rhexographium*

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[1] Corresponding author. E-mail: karin.jacobs@fabi.up.ac.za

INTRODUCTION

A diverse assemblage of fungi, belonging to a wide variety of systematic groups, is associated with bark beetles (Coleoptera: Scolytidae) on conifer and hardwood trees (Upadhyay 1981, Whitney 1982, Kirschner 1998, Jacobs and Wingfield 2001). The insects act as vectors of these fungi, and specific associations exist among individual bark beetle species and certain fungi. *Ophiostoma* Syd. & P. Syd species and related mitosporic fungi, in particular *Leptographium* Lagerb. & Melin and *Pesotum* J.L. Crane & Schokn., are the most common fungi associated with bark beetle species such as *Ips typographus* L. and *I. cembrae* Heer (Mathiesen-Kaärik 1953, Solheim 1986, van der Westhuizen et al 1995, Kirisits 1996, Krokene and Solheim 1996, Jacobs et al 1998, Yamaoka et al 1997, 1998, Stauffer et al 2001). Until recently, synnematous anamorphs of *Ophiostoma* were accommodated in *Graphium* Corda (Upadhyay 1981, Seifert and Okada 1993). However, recent studies have shown that the synnematous anamorphs of *Ophiostoma* phylogenetically are distant from other species in *Graphium* and should be accommodated in a distinct taxon (Okada et al 1998, 2000). Synnematous anamorphs of *Ophiostoma* thus were transferred to *Pesotum*, but taxa with annellidic conidiogenensis and microascaceous affinities related to *Graphium penicillioides* Corda are retained in *Graphium* (Okada et al 1998, 2000).

Graphium pseudormiticum M. Mouton & M.J. Wingf. was isolated and described in 1994 from galleries of the bark beetle *Orthotomicus erosus* Wollaston infesting pine in South Africa (Tribe 1990, 1992, Mouton et al 1994). *Orthotomicus erosus* is native to Europe and accidentally was introduced into South Africa in the 1970s (Kfir 1986, Tribe 1990, 1992). The fungus is characterized by darkly pigmented synnemata, percurrently proliferating conidiogenous cells and aseptate conidia that accumulate in mucilagenous masses, characteristic of the anamorph genus *Graphium*. Unlike other species in *Graphium*, strains of *G. pseudormiticum* produce conidia with conspicuous basal frills that accumulate in a chain-like formation (Mouton et al 1994). A detailed study of the conidium development in this species revealed that these are not true chains (Minter et al 1982, 1983) and are composed of conidia that adhere to

each other as a result of the attachment of the basal frills (Mouton et al 1994).

Morelet (1995) described *Rhexographium fimbriisporum* M. Morelet (as *R. fimbriasporum*) as a *Graphium*-like species almost identical to *G. pseudormiticum*, but he chose to separate it from *Graphium* in the monotypic genus *Rhexographium* M. Morelet based on apparent differences in conidiogenesis. *Rhexographium fimbriisporum* was isolated from the galleries of the bark beetle *Ips typographus* on Norway spruce (*Picea abies* [L.] Karst.) and is characterized by dark, synnematous conidiophores similar to those in *Graphium*. Morelet (1995) distinguished his strains from *Graphium* based on an apparently unique form of conidium development, although it was almost the same as that described for *G. pseudormiticum*. No reference was made to *G. pseudormiticum* because Morelet (1995) apparently was unaware of the study of Mouton et al (1994). This morphological similarity also was noted by Okada et al (1998), who speculated that *R. fimbriisporum* and *G. pseudormiticum* are synonomous.

In recent years, a number of intensive surveys of fungi associated with the bark beetle *Ips cembrae* on European larch (*Larix decidua* Mill.) have been conducted in Austria and Scotland (Kirisits et al 1998, 2000, Stauffer et al 2001). One of the aims of these surveys was to compare the ophiostomatoid fungi associated with *I. cembrae* with those of the closely related *I. typographus*, which have been well-studied (Mathiesen-Kaärik 1953, Solheim 1986, 1992, Kirisits 1996, Krokene and Solheim 1996, Viiri 1997). As part of these surveys, a fungus falling within the taxonomic circumscription of *Graphium*, and with conidium development similar to that of *G. pseudormiticum* and *R. fimbriisporum*, consistently was isolated from the galleries of *I. cembrae* in Scotland and Austria (Kirisits et al 1998, 2000, Stauffer et al 2001). Unlike *G. pseudormiticum* and *R. fimbriisporum*, this fungus is characterized by reddish-brown conidiophores and conidial masses.

The aim of this study was to re-evaluate the taxonomic placement of *R. fimbriisporum* and its relatedness to *G. pseudormiticum*. Furthermore, we have included a wide range of isolates, tentatively identified as *Graphium* spp., associated with bark beetles on spruce, larch and pine in Europe. Special consideration has been given to the identity of the commonly occurring red-colored fungus associated with *I. cembrae*.

MATERIALS AND METHODS

Isolation of fungal cultures.—The strains considered in this study (TABLE I) all originated from bark beetles, bark beetle galleries or sapwood associated with damage by these insects on conifers in South Africa, Austria, France and Scotland. Fungi were isolated from adult beetles or larvae by macerating the insects in a few drops of sterile water. The water with the macerated beetle or larva parts then was diluted and spread on the surface of malt-extract agar (MEA) (20 g Merck malt extract, 16 g Sigma agar, 1000 mL water) amended with 100 mg streptomycin sulphate after autoclaving. Fungi were isolated from stained sapwood by placing small pieces of wood on 2% MEA. Isolations also were made from stained sapwood of European larch logs, 6 wk after inoculation of adult *I. cembrae*, following similar methods to those described by Furniss et al (1990) and Krokene and Solheim (1996). Direct isolations were made from the conidial masses of synnemata occurring in the galleries of the insects. The plates were incubated at room temperature. Pure cultures were obtained by transferring mycelia or conidial masses from primary isolation dishes onto fresh MEA plates. All strains are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria (CMW), and the culture collection of the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Universität für Bodenkultur Wien (BOKU). Representative strains have been deposited in the Canadian Collection of Fungal Cultures and Herbarium (DAOM) and at Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS).

Morphological comparisons.—All measurements and microscopic observations were made on fungal structures grown 7–10 d on 2% MEA and oatmeal agar (OA) (Gams et al 1998, Jacobs et al 2003), incubated in the dark or in incidental light at 25 C. Measurements are reported as the maximum and minimum values of 50 measurements as well as the mean ± standard deviation. Fungal structures were mounted on slides in 85% lactic acid and examined with phase or differential interference contrast microscopy. Color codes were determined with the Methuen handbook of color (Kornerup and Wanscher 1978). Growth rates were determined on both MEA and OA at 25 C with incident light. Cycloheximide tolerance was determined on MEA plates amended with 100 mg/L cycloheximide at 25 C with incident light.

For scanning electron microscopy (SEM), small blocks of agar about 5 mm wide were cut from sporulating colonies and fixed in 4% glutaraldehyde and 0.5% osmium tetroxide in a 0.1 M phosphate buffer, dehydrated in a graded ethanol series, then critical-point dried (Tousimis SAMDRI PVT-3). Specimens were mounted and coated with gold-palladium alloy (Technics Sputter Coater) and examined with a Phillips XL30 Environmental Scanning Electron microscope.

DNA sequencing and phylogenetic analyses.—Single conidial strains used for phylogenetic analysis were grown on commercial potato-dextrose agar (PDA) (Difco) for 10 d in the dark at 25 C. DNA extractions from pure cultures were prepared with the UltraClean™ Microbial DNA kit (MoBio Laboratories, California, U.S.A.). Successful isolation of DNA was confirmed on 1% agarose gels. Amplifications of the 18S gene and ITS1–5.8S-ITS2 (internal transcribed spacer regions of the ribosomal DNA) were made using

TABLE I. Strains of *Graphium pseudormiticum*, *Rhexographium fimbriisporum* (= *Graphium fimbriisporum* comb. nov.) and *Graphium laricis* sp. nov. used in this study

Species	Isolate no.	Genbank no.	Origin and year of isolation	Host	Associated insect	Collector
Graphium pseudormiticum	CMW 503 (ex-type strain)	AY148166[a] AY148186[b]	South Africa, 1984	*Pinus* sp.	*Orthotomicus erosus*	G. Tribe
	PREM 51539 (type)		South Africa	*Pinus* sp.	*Orthotomicus erosus*	M.J. Wingfield
	CMW 5611 (IFFF I/2/2/5)	AY148167[a] AY148185[b]	Horn, Austria, 1998	*Pinus sylvestris*	*Tomicus minor*	T. Kirisits
Rhexographium fimbriisporum (= *Graphium fimbriisporum* comb. nov.)	CMW 5605 (MPFN 281) (ex-type strain)	AY148171[a] AY148177[b]	France	*Picea abies*	*Ips typographus*	M. Morelet
	MPFN 1494 (type)		France	*Picea abies*	*Ips typographus*	M. Morelet
	CMW 3352 (CBS 422.94, IFFF PR/1/2/3, CMW 5606)	AY148169[a] AY148172[a] AY148178[b] AY148180[b]	Prinzersdorf, Austria, 1992	*Picea abies*	*Ips typographus*	T. Kirisits
	CMW 3353 (CBS 421.94, IFFF R/4/1/2, CMW 5607)	AY148170[a] AY148173[a] AY148179[b]	Old growth forest Rothwald, Austria, 1992	*Picea abies*	*Ips typographus*	T. Kirisits
	CMW 5608 (IFFF R/8/3/2)	AY148174[a]	Old growth forest Rothwald, Austria, 1992	*Picea abies*	*Ips typographus*	T. Kirisits
	CMW 5609 (IFFF HIE/15)	AY148175[a]	Hiesberg, Austria, 1998	*Picea abies*	*Ips typographus*	T. Kirisits
	CMW 5610 (IFFF HIE/19)	AY148168[a] AY148176[b]	Hiesberg, Austria, 1998	*Picea abies*	*Ips typographus*	T. Kirisits
Graphium laricis sp. nov.	CMW 5598 (DAOM 229754, IFFF Scotland/4)	AY148160[a] AY148181[b]	Atholl, Scotland, 1997	*Larix decidua*	*Ips cembrae*	T. Kirisits, M.J. Wingfield and D.B. Redfern
	CMW 5599 (DAOM 229755, IFFF Scotland/8)	AY148160[a]	Elgin, Scotland, 1997	*Larix decidua*	*Ips cembrae*	T. Kirisits, M.J. Wingfield and D.B. Redfern
	CMW 5600 (DAOM 229756, IFFF Scotland/11)		Peebles, Scotland, 1997	*Larix decidua*	*Ips cembrae*	T. Kirisits, M.J. Wingfield and D.B. Redfern
	CMW 5601 (DAOM 229757, IFFF IC/L/MEA/13) (ex-type strain)	AY148162[a] AY148183[b]	Kindberg, Austria, 1995	*Larix decidua*	*Ips cembrae*	T. Kirisits and P. Baier

TABLE I. Continued

Species	Isolate no.	Genbank no.	Origin and year of isolation	Host	Associated insect	Collector
	CMW 5602 (DAOM 229758, IFFF IC/38)	AY148163[a] AY148184[b]	Glein, Austria, 1997	*Larix decidua*	*Ips cembrae*	T. Kirisits
	CMW 5603 (DAOM 229759, IFFF IC/GL/26/4)	AY148164[a] AY148182[b]	Glein, Austria, 1997	*Larix decidua*	*Ips cembrae*	T. Kirisits
	CMW 5604 (DAOM 229760, IFFF Conidia/1)	AY148165[a]	Ehrwald, Austria, 1997	*Larix decidua*	*Ips cembrae*	T. Kirisits

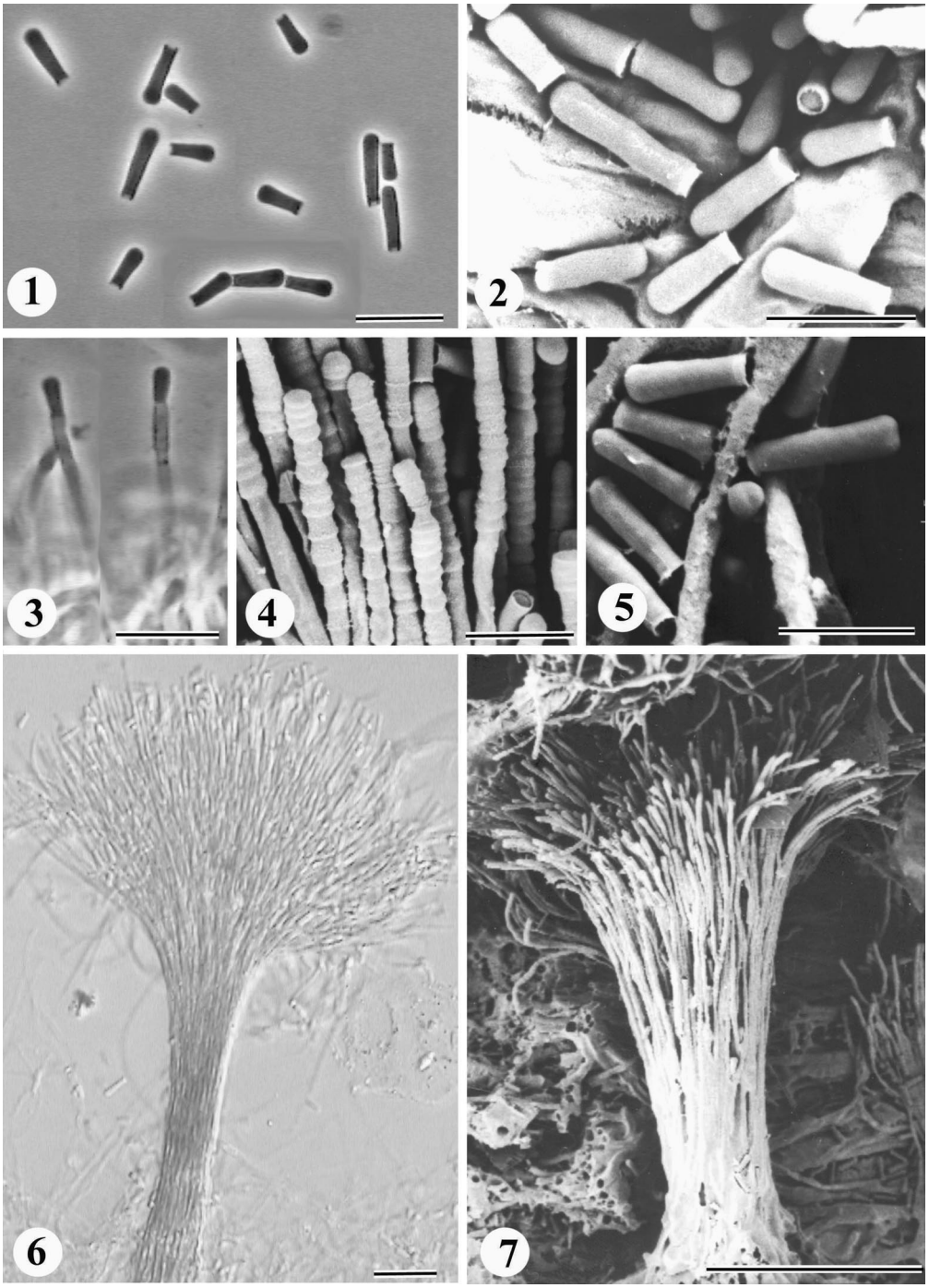
CMW represents the culture collection at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa. MPFN represents the culture collection at the Laboratoire de Pathologie Forestière, INRA, Centre de Recherches de Nancy, 54280 Champenoux, France. CBS represents the culture collection at the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. DAOM represents the Canadian Collection of Fungal Cultures and Herbarium, at ECORC, Agriculture and Agri-food Canada, Ottawa, Canada. IFFF represents the culture collection at the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Universität für Bodenkultur Wien (BOKU). Sequences coding for the ITS1-5.8S-ITS2 and the 18S regions are designated a and b, respectively.

standard protocols for PCR reactions. PCR reactions were conducted with Ready-To-Go (RTG) PCR-beads (Amersham Pharmacia Biotech, Piscataway, New Jersey, U.S.A.) and the primers NS1 and ITS4 (White et al 1990). PCR products were purified with the UltraClean™ PCR-cleanup kit (Mo-Bio Laboratories, California, U.S.A.) and sequenced with the Big-dye terminator cycle sequencing premix kit (Perkin Elmer Applied Biosystems California, U.S.A.) on an ABI PRISM 310 automatic sequencer (Perkin Elmer Applied Biosystems California, U.S.A.). Primers NS1, NS2, NS3, NS4, NS5, NS6, NS7, NS8, ITS1, ITS2, ITS3, and ITS4 (White et al 1990) were used to sequence both strands of the 18S and ITS regions. Sequence contigs were assembled with Sequencher version 4.0.5. (Gene Codes Corporation, Michigan, U.S.A.); a computer-generated alignment (Wisconsin Package Version 10.1, Genetics Computer Group (GCG), Madison, Wisconsin; Canadian Bioinformatics Resource (http://www.cbr.nrc.ca/) of the resulting sequences together with imported reference sequences from GenBank then was adjusted manually in PAUP* version 4.0b8 (Phylogenetic Analysis Using Parsimony) (Swofford 1999). Bases from the 3′ end and 5′ end of the 18S region and 5′ ends of the ITS1 as well as the 5′ end of large ribosomal subunit were excluded from the analysis of datasets to align them with sequences deposited in GenBank.

For analysis, the 18S and ITS-datasets were constructed and compared with sequences of closely related species and genera deposited in GenBank. Phylogenetic relationships were inferred using a heuristic search in PAUP* version 4.0b8. The aligned 18S dataset consisted of 931 characters. In all sequences, characters were treated in the analysis as unweighted and gaps were treated as a fifth base. One area with only single-strand sequences was excluded from the 18S analysis (characters 451–564 in the aligned dataset). Five hundred fifty-one characters were constant, 105 parsimony uninformative and 275 parsimony informative. The aligned ITS dataset consisted of 549 characters. Three hundred six characters were constant, 69 parsimony uninformative and 174 parsimony informative. A heuristic search was performed on both datasets with tree-bisection-reconnection (TBR) branch swapping. Starting trees were obtained through stepwise addition. The resulting trees were used to obtain a consensus tree. Confidence levels were estimated using a Bootstrap analysis (1000 replicates) with the "fast" stepwise addition option.

RESULTS

Morphology.—Comparison of the type specimens of *Graphium pseudormiticum* (PREM 51539) and *Rhexographium fimbriisporum* (MPFN 1494), as well as the ex-type strains, confirmed that *Rhexographium fimbriisporum* (MPFN 281 = CMW 6505 = CBS 870.95) has similar morphological features to *G. pseudormiticum* (CMW 503) (FIGS. 1–7). Both have conidia characterized by conspicuous basal frills and are aligned in chains, as described in detail for *G. pseudormiticum* by Mouton et al (1994) (FIGS. 1–2). In



FIGS. 1–7. Light and scanning micrographs of the ex-type strain of *Graphium fimbriisporum* (MPFN281 = CBS 870.95). FIG. 1. Light micrograph of the conidia with conspicuous basal frills forming short chains (Bar = 10 μm). FIG. 2. Scanning electron micrograph of conidia showing conspicuous basal frills. (Bar = 5 μm). FIG. 3. Conidiogenous cells showing annellidic conidium development (Bar = 10 μm). FIG. 4. Conidiogenous cells with pronounced annellations (Bar = 5 μm) . FIG. 5. Conidiogenous cell showing delayed secession of conidia (Bar = 5 μm). FIG. 6. Light micrograph of a synnematous conidioma with parallel stipe hyphae (Bar = 20 μm). FIG. 7. Scanning electron micrograph of a synnematous conidioma with parallel stipe hyphae (Bar = 5 μm).

TABLE II. Comparison between *Graphium pseudormiticum*, *Rhexographium fimbriisporum* (= *Graphium fimbriisporum* comb. nov.) and *Graphium laricis* sp. nov.

	G. pseudormiticum (Mouton et al 1994)	*R. fimbriisporum* (= *G. fimbriisporum*) (Morelet 1995)	*G. laricis*
Conidiophore length	113–263 μm	118–230 μm	103–218 μm
Conidial dimensions	4–7.5 × 1.5–2.5 μm	4.4–12 × 2.0 μm	4–6 × 1–2
Conspicuous basal frill	Present	Present	Present
Conidia in chains	Present	Present	Present
Rhizoids	Present	Absent	Absent
Host	*Pinus* spp.	*Picea abies*	*Larix decidua*
Associated insects (Mouton et al 1994, Morelet 1995, Kirschner 1998, Kirisits 1996, Kirisits et al 1998, 2000)	*Ips sexdentatus* *Orthotomicus erosus* *Orthotomicus laricis* *Tomicus minor*	*Crypturgus cinereus* *Crypturgus pusillus* *Dryocoetes autographus* *Ips amitinus* *Ips typographus* *Hylurgops glabratus* *Hylurgops palliatus* *Pityogenes chalcographus* *Polygraphus poligraphus* *Trypodendron lineatum*	*Ips cembrae*
Known distribution (Mouton et al 1994, Morelet 1995, Kirschner 1998, Kirisits 1996, Kirisits et al 1998, 2000)	South Africa, Austria, Germany	France, Austria, Germany	Scotland, Austria

both these fungi, conidia develop after percurrent proliferation of the conidiogenous cells and pronounced annellations are clearly visible (FIGS. 3–4). Conidia in *R. fimbriisporum* display delayed secession (FIG. 5), similar to that observed in *G. pseudormiticum* (Mouton et al 1994) (not considered by Morelet 1995) (TABLE II). *Rhexographium fimbriisporum*, however, has larger conidia than those observed and documented for *G. pseudormiticum* (Mouton et al 1994, Morelet 1995). While *G. pseudormiticum* is characterized by dark olive-green colonies (K&W-3D7), *R. fimbriisporum* has camel brown (K&W-6D4) colonies. Thus, based on morphology, *R. fimbriisporum* and *G. pseudormiticum* are similar but probably represent distinct species within *Graphium*.

Phylogeny.—The morphological similarity but taxonomic distinctiveness of *G. pseudormiticum* and *R. fimbriisporum* was confirmed by analyses of DNA sequence data. Heuristic analysis of the 18S sequence data resulted in 12 most-parsimonious trees (FIG. 8). Strains of *G. pseudormiticum* and *R. fimbriisporum* clustered together with other species of *Graphium*, including the type species, *G. penicillioides,* in a clade within the Microascales. These data confirm that *R. fimbriisporum* should be classified in *Graphium* and that it does not represent a distinct genus. Analysis of the ITS dataset resulted in 16 most-parsimonious trees (FIG. 9). *Rhexographium fimbriisporum* and *G. pseudormiticum* clustered with other species of *Graphium*, including the type species *G. penicillioides*. The ex-type strain of *R. fimbriisporum*, together with other strains from *Ips typographus* on *Picea abies*, formed a clade separate from *G. pseudormiticum*, confirming the unique nature of the former fungus. One strain (CMW 5611 = IFFF I/2/2/5) from pine infested by *Tomicus minor* clustered with the ex-type strain of *G. pseudormiticum* (CMW 503) and thus represents this fungus. Based on both morphological characterization and analyses of DNA sequence data, we propose this new combination for *R. fimbriisporum*.

Graphium fimbriisporum (Morelet) K. Jacobs, T. Kirisits & M.J. Wingf. comb. nov.
≡ *Rhexographium fimbriisporum* M. Morelet, Ann. Soc. Sci. Nat. Aschéol. ToulonVar. 47, 90. 1995 (basionym).

Strains from Larix decidua.—Strains from *Larix decidua* infested by *Ips cembrae* are characterized by a pattern of conidium development similar to that observed in *G. pseudormiticum* and *G. fimbriisporum*. The conidia of these strains are characterized by conspicuous basal frills that are produced in chains (FIGS. 10, 16). Unlike *G. pseudormiticum* and *G. fimbriisporum*, strains from *Larix decidua* have reddish (K&W-7D7) conidiophores and conidial masses. Furthermore, sequences of the 18S region showed that the strains from *Larix* phylogenetically are related

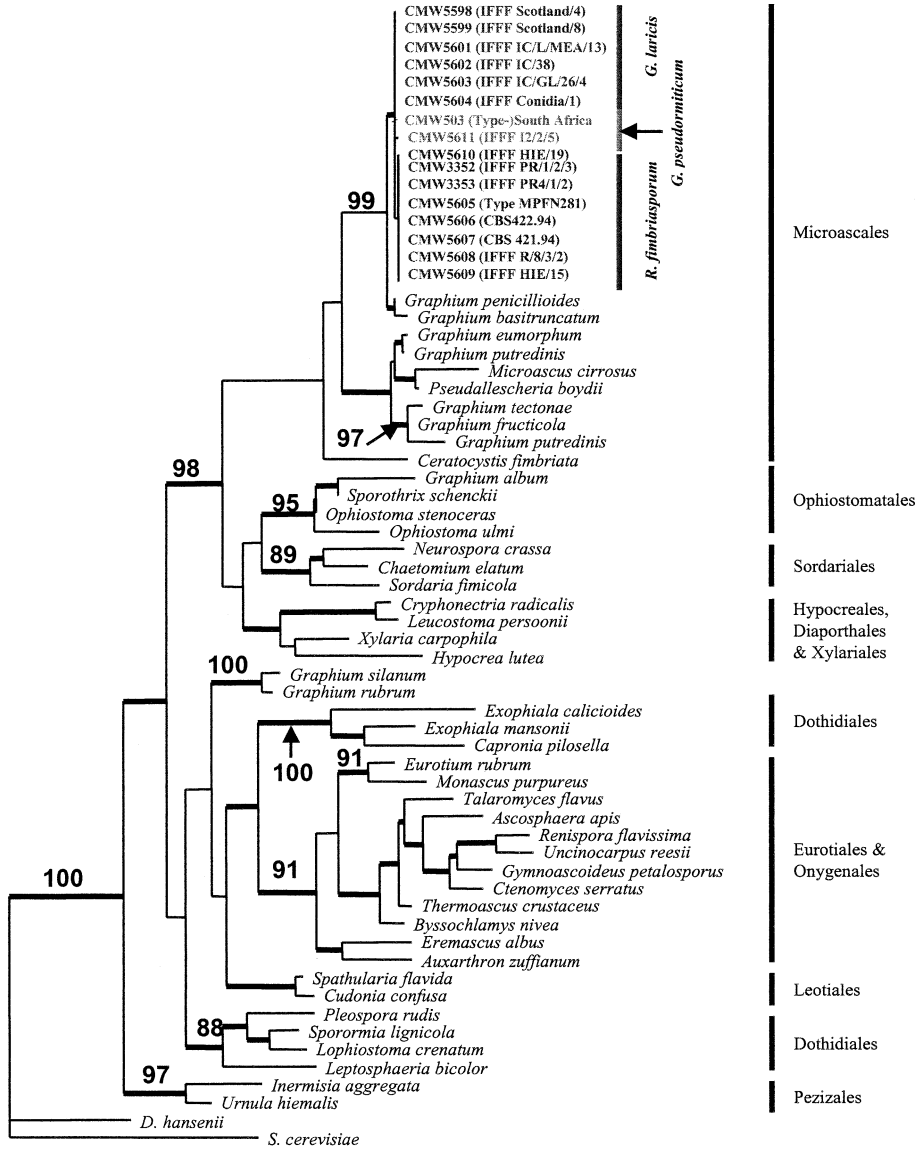


FIG. 8. One of the 12 most-parsimonious trees generated by heuristic analysis of the 18S dataset (1071 steps, RI = 0.811, CI = 0.511, HI = 0.489). Branches with 100% support in a consensus tree are indicated in bold. Bootstrap values are indicated above the branches. These strains from GenBank were included: *Graphium penicillioides* (AB038423); *Graphium basitruncatum* (AB038421); *Graphium eumorphum* (AB007684); *Graphium putredinis* (AB007683; AB007673); *Microascus cirrosus* (M89994); *Pseudoallescheria boydii* (M89782); *Graphium tectonae* (AB007662); *Graphium fructicola* (AB007659); *Ceratocystis fimbriata* (CFU43777); *Graphium album* (AB007657); *Sporothrix schenckii* (M85053); *Ophiostoma stenoceras* (M85054); *Ophiostoma ulmi* (M83261); *Neurospora crassa* (X04971); *Chaetomium elatum* (M83257); *Sordaria fimicola* (X69851); *Cryphonectria radicalis* (L42442); *Leucostoma persoonii* (M83259); *Xylaria carpophila* (Z49785); *Hypocrea lutea* (D14407); *Graphium silanum* (AB007661); *Graphium rubrum* (AB007660); *Exophia calicioides* (AB007655); *Exophiala mansonii* (X78480); *Capronia pilosella* (U42473); *Monoascus purpureus* (M83260); *Eurotium rubrum* (U00970); *Talaromyces flavus* (M83262); *Ascosphaera apis* (M83264); *Renispora flavissima* (U29393); *Uncinocarpus reesii* (URU29394); *Gymnoascoideus petalosporus* (U29392); *Ctenomyces serratus* (U29391); *Thermoascus crustaceus* (M83263); *Byssochlamus nivea* (M83256); *Eremascus albus* (M83258); *Auxarthron zuffianum* (U29395); *Spathularia flavida* (Z30239); *Cudonia confusa* (Z30240); *Sporormia lignicola* (LU42478); *Lophiostoma crenatum* (U42485); *Pleospora rudis* (U00975); *Leptosphaeria bicolor* (U04202); *Innermisia aggregata* (Z30241); *Urnula hiemalis* (Z49754); *Debaryomyces hansenii* (X58053); *Saccharomyces cerevisiae* (J01353).

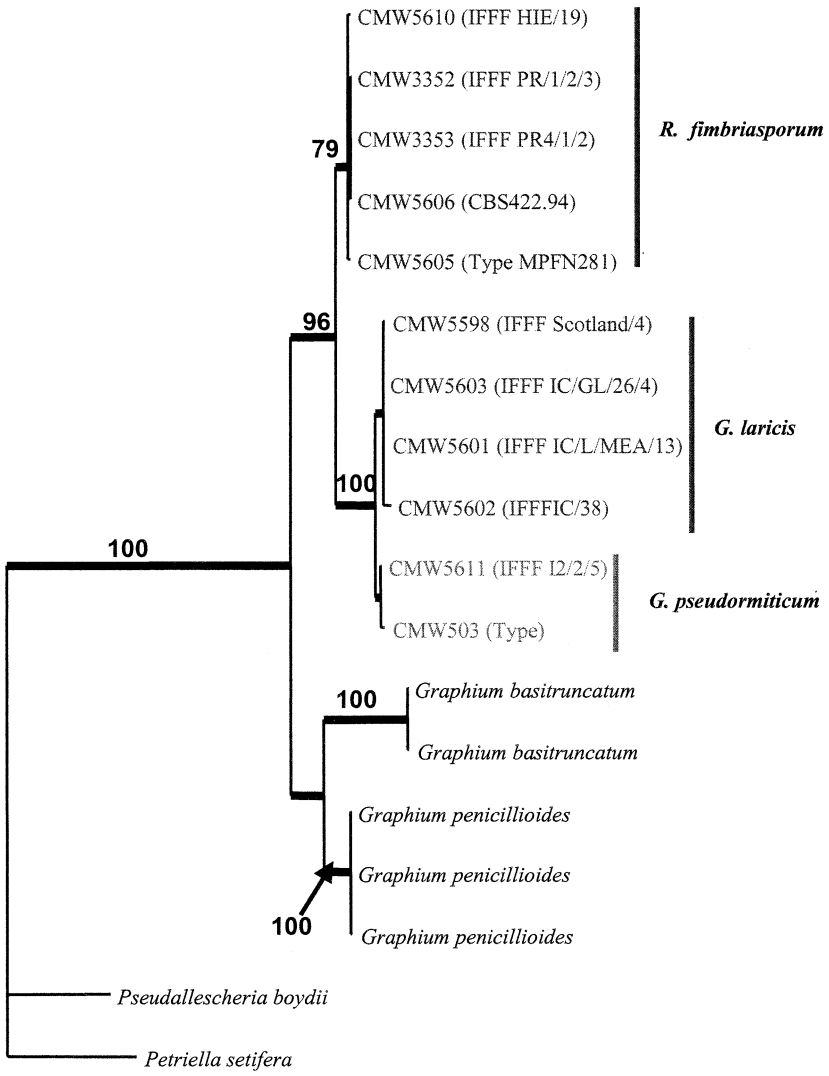


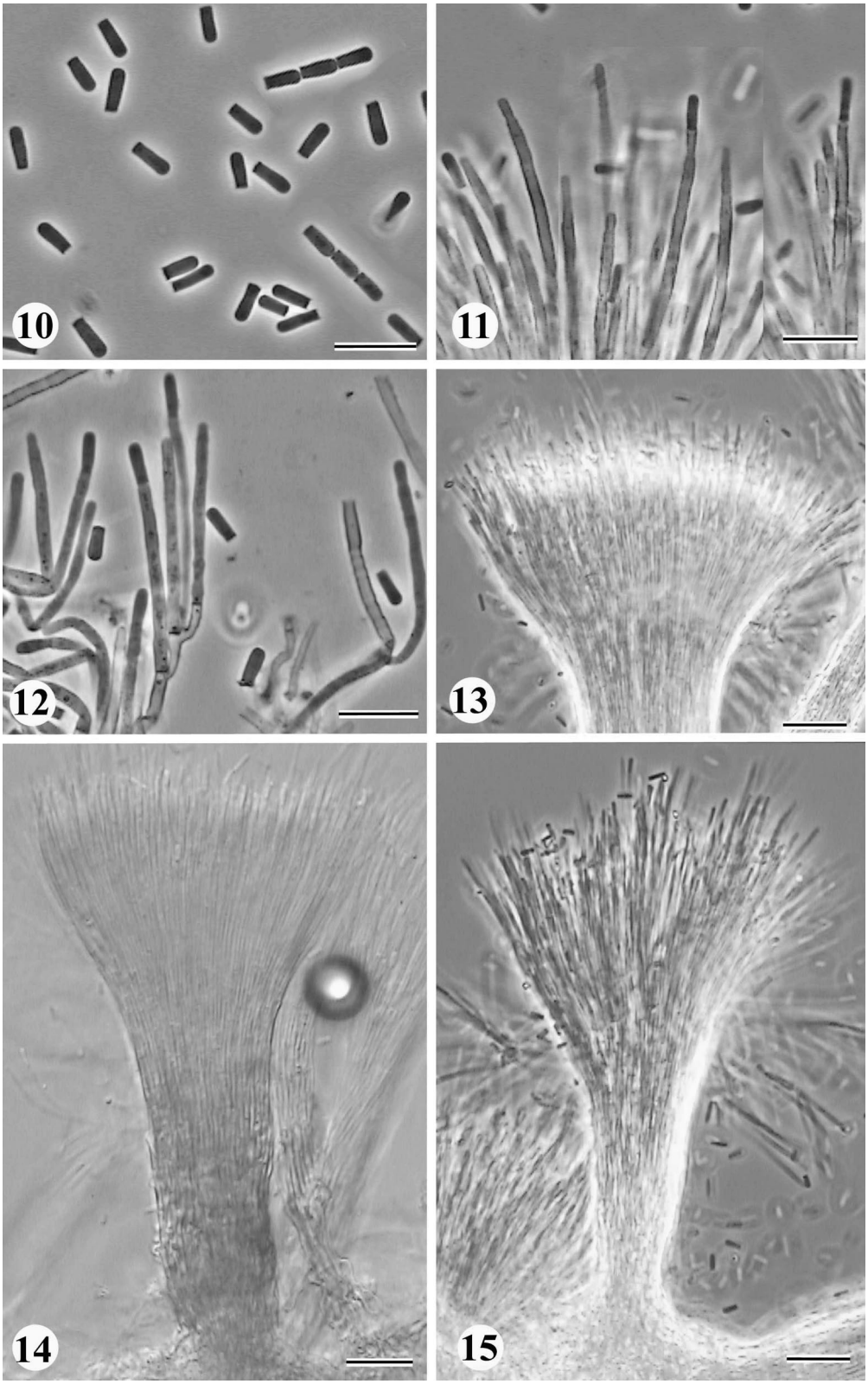
FIG. 9. One of the eight most-parsimonious trees generated by heuristic analysis of the ITS dataset (549 steps, RI = 0.932, CI = 0.915, HI = 0.085). Branches with 100% support in a consensus tree are indicated in bold. Bootstrap values are indicated above the branches. These strains from GenBank were included: *Graphium basitruncatum* (AB038425; AB038427); *Graphium penicillioides* (AB038430; AB038432; AB038431); *Pseudoallescheria boydi* (AF022486); *Petriella setifera.*

closely to *G. pseudormiticum* and *G. fimbriisporum* (FIG. 8). However, the ex-type strain of *G. pseudormiticum* (CMW 506) is characterized by a 433 bp Group I intron in the 18S gene. The intron is located at nt1430 position of the model organism *Saccharomyces cerevisiae.* No introns were observed in strains from *Larix decidua* or *G. fimbriisporum.* In the analysis of the ITS dataset, the strains from *Larix decidua* formed a clade close to *G. pseudormiticum* and *G. fimbriisporum* but constituted a discrete group within the clade (FIG. 9). Thus, based on morphology, ecological characteristics and analyses of DNA sequences, we conclude that the red-colored *Graphium*, commonly occurring in the galleries of *I. cembrae* on *L. decidua* in Europe, represents a previously undescribed species for which we provide this description

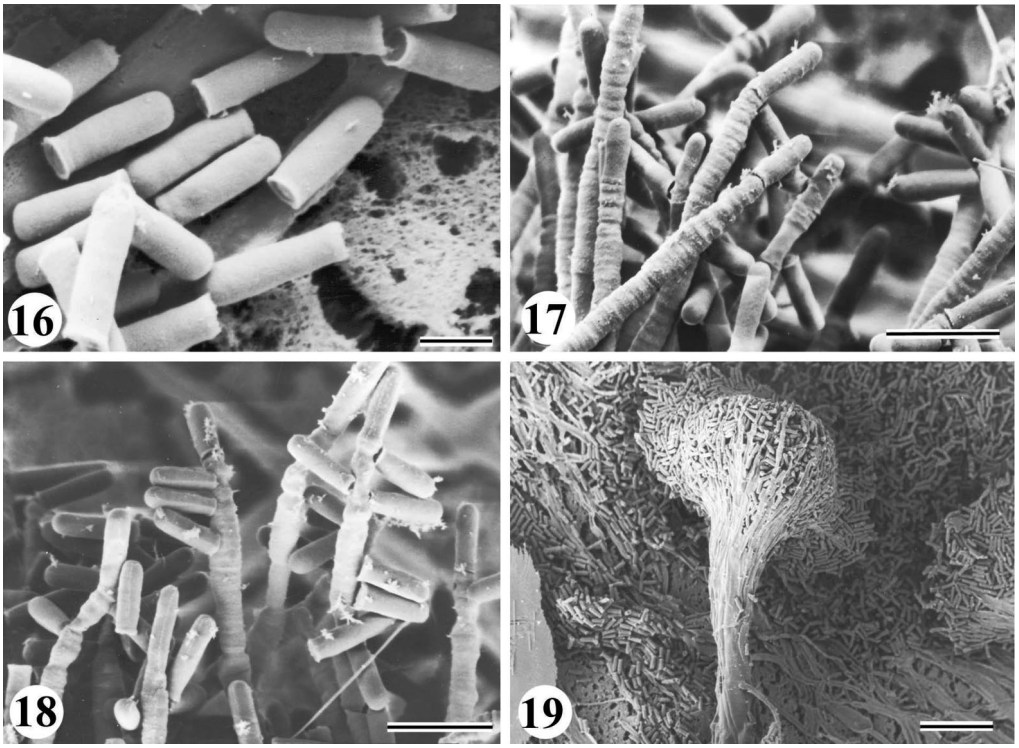
Graphium laricis K. Jacobs, T. Kirisits et M.J. Wingf. sp. nov. FIGS. 10–21.

Etymology; from the host *Larix.*

Coloniae in MEA 25 C pervenientes diametrum 15 mm post 12 dies, in OA ad 25 C pervenientes diametrum 25 mm post 12 dies. Cycloheximidum non tollitur. Coloniae perinum incoloratae in MEA et OA, poster rubescentes. Conidiophora macronematos, synnematos, stipes rubellus ad basim, hyalinum versus apicem. Synnemata (103–) 157 ± 32 (–218) μm longus, (10–) 30 ± 2 (–55) latus in medio, (18–) 44 ± 17 (–88) μm ad apicem, rhizoidea ad basim parce evoluta. Cellulae conidiogenae annellatae, hyalinae, (19–) 21 ± 8 (–52) μm longae. Conidia unicellularia, hyalina, cylindrica, subrotumda, fimbriis basilaribus prominentibus 4.0–6.0 μm × 1.0–2.0 μm. Holotypus: DAOM 229757.



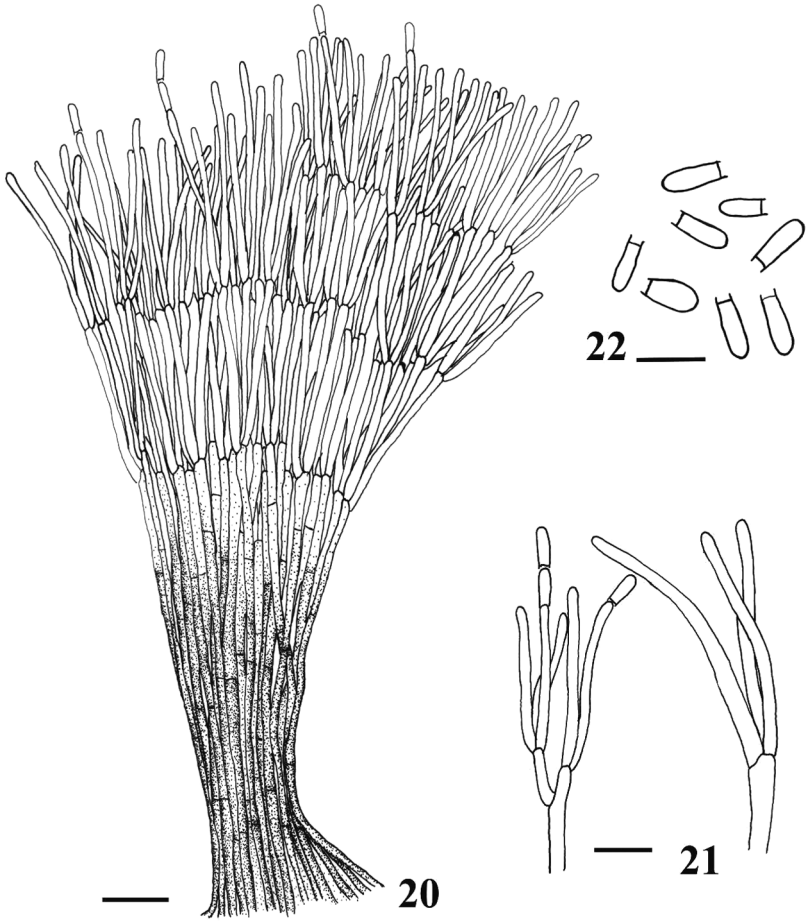
FIGS. 10–15. Light micrographs of the ex-type strain of *Graphium laricis* (CMW 5601 = IFFF IC/L/MEA/13). FIG. 10. Cylindrical conidia with conspicuous basal frill, occurring in short chains (Bar = 10 μm). FIG. 11. Conidiogenous cell showing annellidic conidium development (Bar = 10 μm). FIG. 12. Three-level branching pattern of the conidiophore (Bar = 10 μm). FIG. 13. Divergent capitulum of conidiogenous cells (Bar = 20 μm). FIGS. 14, 15. Synnematous conidiomata with parallel hyphae in the stipes (Bar = 20 μm).



FIGS. 16–19. Scanning electron micrographs of *Graphium laricis*. FIG. 16. Conidia showing conspicuous basal frills (Bar = 2 μm), FIG. 17. Conidiogenous cells with pronounced annellations (Bar = 5 μm). FIG. 18. Conidiogenous cells showing delayed secession of conidia (Bar = 5 μm). FIG. 19. Synnema of *G. laricis* (Bar = 20 μm).

Colonies on MEA reaching 15 mm diam., on OA reaching 25 mm diam after 12 d at 25 C. Not tolerant to low concentrations of cycloheximide (100 mg/L). Colony color on MEA white, becoming reddish-brown (K&W-7D7) toward the center, sporulation on MEA sparse, colony margin smooth, reverse white, becoming reddish-brown toward the center. Colony colorless on OA, becoming cinnamon brown (K&W-6D6) with age. Sporulation and formation of synnemata abundant on OA, sparse aerial mycelia developing with age. Mucilaginous conidial masses reddish-brown, giving the colony a reddish-brown appearance, reverse reddish-brown, colony margin smooth. Hyphae mostly submerged in agar with little aerial mycelium, hyaline, smooth-walled, not constricted at the septa, 2–5 μm wide. *Synnemata* arising singly or in groups, consisting of a stipe of parallel hyphae, a divergent capitulum of conidiogenous cells and a mucilaginous mass of conidia. Synnematal stipe, reddish at the base, becoming hyaline toward the apex, (103–) 157 ± 32 (–218) μm long, (10–) 30 ± 2 (–55) μm wide at the center, (18–) 44 ± 17 (–88) μm wide at the apex. Rhizoids at the base scanty. Conidiophores produced in the divergent capitulum with three series of branches, with 3–5 metulae or conidiogenous cells per branch point, metulae and conidiogenous cells (4–) 11 ± 7 (–21) long and 1.0–2.0 μm wide, conidium development annellidic, inconspicuous, conidial dehiscence sometimes delayed and thus giving the impression of sympodial proliferation. *Conidia* aseptate, hyaline, cylindrical rounded with conspicuous basal frill 4.0–6.0 μm × 1.0–2.0 μm, accumulating in hyaline mucilaginous masses forming chain-like formations at the apices of conidiophores, turning reddish-brown (K&W-7D7) with age.

HOLOTYPE: DAOM 229757, Austria, Styria, Kindberg, isolated from a larvae of *Ips cembrae* on *Larix decidua*, 1 Jul 1995, collected and isolated: T. Kirisits and P. Baier, (living cultures: CMW 5601 = IFFF IC/L/MEA/13). Additional specimens: DAOM 229754, United Kingdom, Scotland, Atholl, isolated from conidiophores in the galleries of *Ips cembrae* on *Larix decidua*, 28 Aug 1997, collected and isolated: T. Kirisits, M.J. Wingfield and D.B. Redfern, (living cultures: CMW 5598 = IFFF Scotland/4); DAOM559755, United Kingdom, Scotland, Elgin, isolated from conidiophores in the galleries of *Ips cembrae* on *Larix decidua*, 29 Aug 1997, collected and isolated: T. Kirisits, M.J. Wingfield and D.B. Redfern, (living cultures: CMW 5599 = IFFF Scotland/8); DAOM 229756, United Kingdom, Scotland, Peebles, isolated from conidiophores in the galleries of *Ips cembrae* on *Larix decidua*, 26 Aug 1997, collected and isolated: T. Kirisits, M.J. Wingfield and D.B. Redfern, (living cultures: CMW 5600 = IFFF Scotland/11); DAOM 229758, Austria, Styria, Glein, isolated from an adult *Ips cembrae* on *Larix decidua*, 25 Jun 1997, collected and isolated: T. Kirisits, (living cultures: CMW 5602 = IFFF IC/38); DAOM 229759,



FIGS. 20–22. Line drawings of *G. laricis*, FIG. 20. Synnema with parallel hyphae in the stipe and diverging conidiophores in the capitulum (Bar = 20 μm). FIG. 21. Branching patterns of the metulae (Bar = 10 μm). FIG. 22. Conidia with conspicuous basal frills (Bar = 10 μm).

Austria, Styria, Glein, isolated from the sapwood of *Larix decidua* inoculated with *Ips cembrae* 6 wk earlier, 14 Aug 1997, collected and isolated: T. Kirisits, (living cultures: CMW 5603 = IFFF IC/GL/26/4); DAOM 229760, Austria, Tyrol, Ehrwald, isolated from conidiophores in the galleries of *Ips cembrae* on *Larix decidua*, Jul 1997, collected and isolated: T. Kirisits, (living cultures: CMW 5604 = IFFF Conidia/1).

DISCUSSION

In this study we distinguish three species of *Graphium*, which commonly occur in the galleries of bark beetles that infest conifers in Europe. Until recently, the occurrence of *Graphium* spp. in bark beetle galleries would not have been considered unusual. This is because the dominant fungi in this niche are *Ophiostoma* spp. (Mathiesen 1951, Mathiesen-Kaärik 1953, Solheim 1986, 1992, Wingfield et al 1993, Krokene and Solheim 1996, Viiri 1997), and the synnematous anamorphs of these fungi generally have been classified in *Graphium* (Upadhyay 1981, Seifert and Okada 1993). However, the taxonomy of *Graphium* long has been in disarray and it has taken a recent series of studies based on DNA sequence data to resolve this problem. In the first of these studies, Okada et al (1998) re-established *Pesotum* for synnematous anamorphs of *Ophiostoma* and reserved *Graphium* for fungi related to the Microascales (Crane and Schocknecht 1973, Okada et al 1998). The next necessary step was the typification of the type species of *Graphium*, *G. penicillioides* [epitype designated by Okada et al (2000)], and this study confirmed that *Graphium* is unrelated to species of *Ophiostoma*. The results of this study provide further evidence that two phylogenetically unrelated groups of fungi produce synnematous anamorphs, present in the galleries of bark beetles.

Taxonomic confusion relating to *Graphium* has resulted in considerable difficulty in providing names for synnematous fungi in the galleries of bark beetles (Mathiesen-Kaärik 1953, Wingfield and Gibbs 1991, Viiri 1997, Zhou et al 2001, Jacobs et al 2003). These

fungi tend to be similar morphologically, and identifications based on micromorphology and/or colony characters are likely to continue to be difficult. However, *Graphium* and *Pesotum* now are clearly separated and a reasonably robust set of sequence data is available for most species of *Graphium*. This study represents an example of the use of these sequence data, together with morphology and ecological information, to characterise fungi of previously confused taxonomic status.

Graphium pseudormiticum, *G. fimbriisporum* and *G. laricis* appear to represent a species complex within *Graphium*, characterized by apparently unique conidium ontogeny and morphology (Mouton et al 1994, Morelet 1995). *Graphium pseudormiticum*, *G. fimbriisporum* and *G. laricis* not only are similar in their patterns of conidium development, but all three species have synnemata ranging in length from 100 to 260 μm, although those of *G. laricis*, on average, are slightly shorter. *Graphium fimbriisporum* can be distinguished from the other two species based on its slightly longer conidia. Colony color is also a useful character to distinguish among these species. *Graphium pseudormiticum* is characterized by olivaceous-green (K&W-3D7) colonies, and *G. fimbriisporum* has colonies that are almost dark brown (K&W-6D4). This is in contrast to the colonies of *G. laricis*, which have a very obvious reddish (K&W-7D7) color.

The three species of *Graphium* treated in this study occupy three discrete ecological niches. *Graphium pseudormiticum* initially has been isolated from galleries of *O. erosus* on *Pinus* spp. in South Africa (Mouton et al 1994), and we suspect it to be associated with a wide variety of pine bark beetles in Europe. *Graphium fimbriisporum* appears to be commonly associated with *Ips typographus* infesting spruce in Europe (Morelet 1995, Kirisits 1996, Kirisits et al 2000). In surveys in Austria it also has been isolated from various other bark beetle species on Norway spruce, including *Pityogenes chalcographus* L., *Ips amitinus* Eichh., *Hylurgops glabratus* Zett., *Hylurgops palliatus* Gyll. and *Dryocoetus autographus* Ratz. (Kirisits 1996, Kirisits et al 2000). In contrast, *G. laricis* is one of the dominant fungi found on *I. cembrae* and in its galleries on *Larix decidua* in Austria and Scotland (Kirisits et al 1998, 2000, Stauffer et al 2001).

When *G. pseudormiticum* first was described, it was assumed that the fungus was of European origin. This is because of its association with *Orthotomicus erosus*, which is well known and native to Europe, from where it accidentally was introduced to South Africa (Tribe 1990, 1992). Kirschner (1998) investigated the fungi associated with bark beetles in Germany and reported *Graphium pseudormiticum* as a common associate of various bark beetles on spruce (*Crypturgus cinereus*, *Crypturgus pusillus*, *Dryocoetes autographus*, *Hylurgops palliatus*, *Polygraphus poligraphus*, *Trypodendron lineatum*, *Pityogenes chalcographus* and *Ips typographus*) and pine (*Ips sexdentatus* and *Orthotomicus laricis*). He did not distinguish between isolates from spruce (= *G. fimbriisporum*) and those from pine (= *G. pseudormiticum*) as separate species and might not have been aware of the description of *G. fimbriisporum* by Morelet (1995). In a recent survey of ophiostomatoid fungi associated with bark beetles in South Africa, Zhou et al (2001) failed to detect *G. pseudormiticum*. They thus considered this species to be an infrequent or occasional associate of *O. erosus*. A possible explanation for this could relate to a lack of ability to compete in its non-native environment or the fact that it naturally occurs at a low frequency.

Detailed surveys of the fungi associated with *Ips typographus* and *I. cembrae* have been conducted in Japan (van der Westhuizen et al 1995, Yamaoka et al 1997, 1998, Jacobs et al 1998), where both insects are common on Yezo spruce (*Picea jezoensis* Carr.) and Japanese larch (*Larix kaempferi* [Lamb.] Carr.), respectively. Neither *G. fimbriisporum* nor *G. laricis* were found in these studies. Likewise, *G. fimbriisporum* and *G. pseudormiticum* have not been mentioned in most previous investigations of the mycobiota of bark beetles in Europe (e.g., Mathiesen-Käärik 1953, Solheim 1986, 1992, Krokene and Solheim 1996, Viiri 1997). We think that these fungi might have been overlooked in these surveys. After clarification of their taxonomic placement, further studies now are necessary to elucidate the biogeography and vectors of *G. fimbriisporum*, *G. pseudormiticum* and *G. laricis*.

While no observations are available for *G. pseudormiticum*, circumstantial evidence suggests that *G. fimbriisporum* and *G. laricis* are only very weak pathogens or saprophytes on their host species. They also are thought to display limited, if any, abilities to stain the sapwood of their hosts. In investigations in Austria, *G. fimbriisporum* and *G. laricis* commonly were isolated directly from *Ips typographus* and *Ips cembrae*, respectively, but less frequently were isolated from the sapwood of insect-infested spruce and larch (Kirisits 1996, Kirisits et al 2000, Kirisits, unpubl data). Isolation results indicate that *G. fimbrisporum* and *G. laricis* are secondary colonizers of the sapwood of infested trees, following primary invaders such as *Ceratocystis polonica* (Siemaszko) C. Moreau and *Ceratocystis laricicola* Redfern & Minter (Solheim 1992, Kirisits et al 2000, Kirisits, unpubl data). One isolate of *G. laricis* also was included in an inoculation experiment on European larch, in which it caused only small necrotic lesions in the phloem, no blue stain

and only very little desiccation in the sapwood of test trees (Kirisits et al 2000, Kirisits, unpubl data).

Ips typographus and *I. cembrae* are well-known and important insect pests in Europe and Asia (Schmitschek 1931, Crooke and Bevan 1957, Nobuchi 1974, Postner 1974, Redfern et al 1987, Christiansen and Bakke 1988, Stauffer et al 2001) and both have high quarantine status in North America. Many studies have been conducted on fungi associated with the former species (Mathiesen-Kaärik 1953, Solheim 1986, 1992, Krokene and Solheim 1996, Kirisits 1996, Kirisits et al 2000, Jacobs et al 1998, Viiri 1997, Yamaoka et al 1997) but this is less so for the latter (van der Westhuizen et al 1995, Yamaoka et al 1998, Kirisits et al 1998, 2000, Stauffer et al 2001). One of the objectives of this study was to compare fungi associated with native and introduced *I. cembrae* in Austria and Scotland, respectively. The discovery of *G. laricis* as a common inhabitant in this niche in both locations perhaps is not surprising. It does, however, illustrate the fact that still relatively little is known about the fungi associated with important insect pests of conifers, many of which are greatly feared in terms of quarantine and international trade.

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