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Characterisation of synnematous bark beetle-associated fungi from China, including *Graphium carbonarium* sp. nov.

D. Paciura · X. D. Zhou · Z. W. De Beer · K. Jacobs · H. Ye · M. J. Wingfield

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Abstract Ophiostomatoid fungi on trees are typically bark beetle associates that cause sapstain in timber and some are pathogens. Very little is known regarding the ophiostomatoid fungi associated with bark beetles in China and the aim of this study was to identify a collection of these fungi with synnematous anamorphs. Micromorphology and DNA sequences of the internal transcribed spacer regions (ITS) of the ribosomal DNA and the partial β-tubulin gene were used for identifications. The isolates could be divided in six morphological groups. DNA sequence comparisons with published data confirmed that these groups represented six species, four in the Ophiostomatales (Sordariomycetidae) and two in the Microascales (Hypocreomycetidae). The majority of these were isolated from conifer hosts. *Ophiostoma quercus, O. setosum*, *Pesotum fragrans* (Ophiostomatales) and *Graphium pseudormiticum* (Microascales) were found on *Tsuga dumosa* infested by a *Pissodes* sp. In addition, *O. quercus* and *P. fragrans* were found associated with *Tomicus yunnanensis* on *Pinus yunnanensis*, *P. fragrans* with a *Pissodes* sp. on *P. armandi*, and *O. piceae* with *Ips subelongatus* on *Larix olgensis*. Only two species, *O. quercus* and a new species in the *Graphium penicilliodes* complex, described here as *Graphium carbonarium* sp. nov., were isolated from *Pissodes* galleries on *Salix babylonica*. These results include several new fungus-host and fungus-insect associations, and *G. pseudormiticum* is reported here for the first time from China.

Keywords Conifers · *Graphium* · Hardwoods · *Ophiostoma* · *Pesotum* · Phylogeny · Taxonomy

D. Paciura · X. D. Zhou · Z. W. De Beer · M. J. Wingfield
Department of Microbiology and Plant Pathology,
Forestry and Agricultural Biotechnology Institute (FABI),
University of Pretoria,
Pretoria 0002, South Africa

X. D. Zhou (✉)
China Eucalypt Research Centre (CERC),
Chinese Academy of Forestry,
Wanshoushan, Beijing, China
e-mail: xu.zhou@fabi.up.ac.za

K. Jacobs
Department of Microbiology, University of Stellenbosch,
Stellenbosch, South Africa

H. Ye
Department of Biology, College of Life Sciences,
Yunnan University,
Kunming, China

Introduction

Ophiostomatoid fungi residing in genera such as *Ophiostoma*, *Grosmannia, Ceratocystiopsis* (Ophiostomatales), *Ceratocystis* and *Graphium* (Microascales) cause sapstain on freshly felled wood (Zipfel et al. 2006). These species can lead to wood degradation, sometimes causing significant economic losses (Seifert 1993). They are commonly associated with bark beetles (Coleoptera: Curculionidae, Scolytinae) infesting conifers and hardwoods (Wood and Bright 1992).

Over 170 million ha of natural forest, mainly consisting of conifers, is distributed throughout China, especially in the Northeastern and Southwestern regions of the country (Butterworth and Lei 2005). More than 160 species of conifer-infesting bark beetles have been reported from these forests (Yin et al. 1984), including several primary pests. Some of these species have killed millions of trees, and have been extensively studied (Yin et al. 1984; Ye 1991;

Yan et al. 2005). Although a significant body of knowledge has accumulated on bark beetles, very little is known regarding the fungal associates of the bark beetles of China. To date, only 13 ophiostomatoid species have been reported from this country in studies where identifications were confirmed with DNA sequences (Lin et al. 2003; Yamaoka et al. 2008; Lu et al. 2008, 2009a, b).

During the period of 2001–2004, extensive surveys were conducted in China as part of the China-South Africa Intergovernmental bark beetle-fungus project. Many fungal isolates with synnematous anamorphs were collected during the course of the China-South Africa project. The aim of this study was to characterize these isolates using both morphology and DNA sequence comparisons.

Materials and methods

Collection and isolation of fungi

Collections were made between 2001 and 2002 during extensive field excursions in two provinces in China: Jilin (Northeastern region) and Yunnan (Southwestern region). Hosts sampled included *Tsuga dumosa* (D.Don) Eichler, *Pinus yunnanensis* Franch., *P. armandii* Franch., *Salix babylonica* L. and *Larix olgensis* A. Henry. The techniques used to isolate the fungi from the beetles were those described by Zhou et al. (2004). From the purified cultures, only those producing synnematous anamorphs were selected for the present study. The following isolates collected from other areas and for which no sequence data existed, were also included for comparative purposes in the study: the ex-neotype isolate of *O. quercus* (CMW 2467 = CBS117913 = 0.80, Morelet 1992), an authentic isolate of *O. setosum* (CMW 16534 = CBS123602, Uzunovic et al. 2000), and the ex-type isolate of *G. basitruncatum* (Matsush.) Seifert & G. Okada (CMW29132 = CBS320.72 = JCM 9300).

All cultures used in this study are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. A complete set of subcultures of these isolates is maintained at Yunnan University, China. Representative specimens and cultures of the new taxon that was discovered have been deposited respectively in the National Collection of Fungi (PREM), Pretoria, South Africa, and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

Culture morphology

Isolates were grown on Oatmeal agar plates (Gams et al. 1998) at 25°C for 20 days. Fruiting structures were mounted in lactophenol on glass slides and examined using a light microscope. The isolates were grouped based on culture morphology and colour as defined in the colour charts of Rayner (1970). Scanning Electron Microscopy (SEM) was done for the new species description; the samples were prepared as follows: 10 mm diameter portions were cut of active fungal colonies (grown on PDA media for 10 days). Then the samples were fixed with 3% glutaraldehyde and 0.5% osmium tetraoxide on 0.1 M phosphate buffer and left overnight. After fixation, the samples were dehydrated in a graded ethanol series and let to dry overnight in a critical-point dryer; after that, the dried samples were mounted and covered with gold palladium alloy. The prepared samples were examined using a Joel JSM 840 scanning electron microscope. Four representative isolates of each group were selected for DNA sequencing and further morphological examination.

DNA isolation, PCR and sequencing

After the isolates were grouped based on culture morphology, DNA sequence comparisons were used to confirm the separation and preliminary identification of the groups. DNA was extracted from single spore cultures obtained for each of the representative isolates. Fungal spores from the selected isolates were suspended in sterile deionised water; spore suspensions spread onto the surface of 2% MEA (20 g Biolab malt extract, 20 g Biolab agar, and 1,000 mL deionised water) plates, and then incubated overnight at room temperature in the dark. Germinating single spores were transferred individually to fresh PDA plates. Actively growing mycelia from 8-day-old PDA plates were scraped into 2 mL Eppendorf tubes, freeze-dried and ground to a powder in liquid nitrogen. DNA extraction was done following the method described by Jacobs et al. (2004). DNA concentrations were estimated using the NanoDrop-1000 Spectrophotometer v. 3.2 (NanoDrop Technologies Inc. Wilmington, DE 19810, United States) and visualized on a 1% agarose gel stained with ethidium bromide.

Primers ITS1 and ITS4 (White et al. 1990) were used to amplify the internal transcribed spacer regions (ITS) of the ribosomal DNA operon for all isolates. Primers Bt2a and Bt2b (Glass and Donaldson 1995) were used to amplify the partial β-tubulin gene region for species where the ITS-region is known not to distinguish between closely related species (Table 1). The PCR reaction mix contained 1X of PCR buffer, 2.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.2 mM of each primer, and 2.5 U/μL of Taq-polymerase enzyme (Roche Diagnostics, Mannheim, Germany). The PCR reactions were carried out on a thermal cycler (Mastercycler® Perkin Elmer Corporation, Massachusetts, USA) under the following conditions: initial denaturation at 94°C for 3 min, followed by 35 amplification cycles each consisting

Table 1 Isolates of synnematous ophiostomatoid fungi from Yunnan and Jilin provinces in China, for which DNA sequences were determined. Authentic isolates representing known species were included for comparative purposes

Species	Isolate number	Host/insect	Origin	GenBank acc.	
				ITS	β-tubulin
Ophiostoma quercus	CMW2467[a]	*Quercus* sp.	Nogent, France	FJ434958	FJ455576
	CMW11772	*Pinus yunnanensis* / *Tomicus yunnanensis*	Lufeng, Yunnan	FJ434957	FJ455575
	CMW12086	*Tsuga dumosa* / *Pissodes* sp.	Dali, Yunnan	FJ434955	FJ455573
	CMW12147	"	"	FJ434956	FJ455574
	CMW12287	"	"	FJ434947	FJ455563
	CMW12295	*Salix babylonica* / *Pissodes* sp.	Lijiang, Yunnan	FJ434944	FJ455560
	CMW12296	"	"	FJ434945	FJ455561
	CMW12298	"	"	FJ434946	FJ455562
	CMW12332	*T. dumosa* / *Pissodes* sp.	Dali, Yunnan	FJ434948	FJ455565
	CMW12338	"	"	FJ440715	FJ455568
	CMW12348	"	"	FJ440716	FJ455566
	CMW12363	"	"	FJ434949	FJ455564
	CMW12371	"	"	FJ434952	FJ455570
	CMW12380	"	"	FJ434950	FJ455567
	CMW12512	"	"	FJ434954	FJ455572
O. piceae	CMW11935	*Larix olgensis* / *Ips subelongatus*	Wangqing, Jilin	FJ434973	FJ455597
	CMW11936	"	"	FJ434974	FJ455598
	CMW11937	"	"	FJ434975	FJ455599
O. setosum	CMW11914	*Abies* sp.	Chuxiong, Yunnan	FJ440717	FJ455594
	CMW12057	*T. dumosa* / *Pissodes* sp.	Dali, Yunnan	FJ440719	FJ455592
	CMW12058	"	"	FJ440718	FJ455593
	CMW12289	"	"	FJ434959	FJ455577
	CMW12290	"	"	FJ434960	FJ455578
	CMW12334	"	"	FJ434961	FJ455579
	CMW12336	"	"	FJ434963	FJ455581
	CMW12339	"	"	FJ434964	FJ455582
	CMW12344	"	"	FJ434966	FJ455584
	CMW12374	"	"	FJ434967	FJ455585
	CMW12377	"	"	FJ434968	FJ455586
	CMW12383	"	"	FJ434969	FJ455587
	CMW12384	"	"	FJ434970	FJ455588
	CMW12387	"	"	FJ434972	FJ455590
	CMW16534[b]	*Tsuga heterophylla*	Canada	FJ440720	FJ455595
Pesotum fragrans	CMW12376	*T. dumosa* / *Pissodes* sp.	Dali, Yunnan	FJ434976	FJ455600
	CMW12388	*P. armandii* / *Pissodes* sp.	"	FJ434977	FJ455601
	CMW24416	*P. yunnanensis* / *T. yunnanensis*	Kunming, Yunnan	FJ434978	FJ455602
Graphium pseudormiticum	CMW12285	*T. dumosa* / *Pissodes* sp.	Dali, Yunnan	FJ434981	NA
G. carbonarium sp. nov.	CMW12418	*S. babylonica* / *Pissodes* sp.	Lijiang, Yunnan	FJ434980	FJ455603
	CMW12420	"	"	FJ434979	FJ455604

[a] Ex-neotype isolate of *O. quercus* (Morelet 1992)

[b] Authentic isolate of *O. setosum* (Uzunovic et al. 2000)

of 1 min denaturation at 92°C, annealing for 45 sec at 54–58°C, depending on the primers used, and elongation of 1 min at 72°C. The PCR was concluded by a final elongation cycle of 4 min at 72°C.

PCR products were detected under UV light on a 2% agarose gel stained with ethidium bromide. The PCR products were purified using Sephadex® G-50 (SIGMA-ALDRICH, Amersham, Biosciences Limited, Sweden),

according to the manufacturer's instructions. The PCR fragments were sequenced using 10 μL sequencing reactions with the primers mentioned for each region and Big Dye™ Terminator v. 3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, CA, United States). The sequencing products were cleaned with Sephadex® G-50 and analyzed using an ABI Prism™ 3100 Genetic Analyzer (Applied Biosystems). Contigs of forward and reverse sequences were assembled in MEGA (Molecular Evolutionary Genetics Analysis) v. 4.0 (Tamura et al. 2007) for each isolate.

Phylogenetic analyses

BLAST searches were conducted with each consensus sequence in GenBank to obtain a preliminary identification of the fungi and to place them in groups. Related sequences from reliable sources were downloaded from GenBank and aligned with sequences from this study using the online version of MAFFT v. 5.3 (Katoh et al. 2002). Closely related taxa were used as outgroups for the different DNA sequence analyses. For ITS data sets of the *Ophiostoma* and *Graphium* groups, *Leptographium lundbergii* Lagerb. & Melin and *Pseudallescheria boydii* (Shear) McGinnis, A.A. Padhye & Ajello were respectively used, while *O. floccosum* Math.-Käärik was the outgroup for the β-tubulin analyses.

Maximum parsimony (MP) and distance analyses (using the neighbor-joining tree (NJ) building algorithm) were done for each gene region in PAUP (Phylogenetic Analysis Using Parsimony) v. 4.01b (Sinauer Associates Inc., Sunderland, Massachusetts, USA). For distance analyses, the most suitable substitution models for the respective data sets were selected using Modeltest v. 3.7 (Posada and Crandall 1998). For MP and NJ, confidence levels were determined by doing 1,000 bootstrap replicates.

Bayesian analyses were conducted on each data set in MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003). Four incrementally heated simultaneous Monte Carlo Markov chains were run over 5 million generations, applying the respective substitution models as were determined for each data set in MrModeltest v. 2.2 (http://www.abc.se/~nylander/). Trees were sampled every 100 generations. Burn-in was determined and the necessary number of trees was discarded before a 50% majority rule consensus tree was calculated from the remaining trees and reconstructed in MEGA v. 4.0.

Morphology

Representative cultures from each of the species determined by DNA sequence comparisons were studied microscopically to confirm their identification based on published morphological descriptions of the species. A total of 30 measurements were made for each structure, and the ranges and averages were computed.

Growth studies

Growth studies in culture were conducted for each species using three isolates per species. Plates containing 2% MEA were inoculated with 5 mm diameter disks taken from actively growing colonies that had been grown for 10 days in the dark. Five replicates per isolate were incubated at each 5°C interval for temperatures ranging from 5 and 35° C. Diameters of all colonies were measured after both 4 and 8 days. An additional set was prepared to incubate the strains at 32°C as suggested by Harrington et al. (2001) to differentiate the species in the *O. piceae* complex. Growth comparisons of the new taxon were made with the ex-type culture of *G. basitruncatum* (CBS 320.72 = CMW 29132), using 2% MEA and OA at the same range of temperatures mentioned above.

Results

Collection of samples and culture morphology

In total, 81 isolates with synnematous anamorphs and they were collected during the survey in China and included in the present study. The 81 cultures could be separated into six morphological groups based on culture morphology.

PCR, sequencing and phylogenetic analyses

The amplification of the ITS region of the rRNA operon resulted in fragments of approximately 650 bp. Amplicons for the β-tubulin gene region were approximately 450 bp in length.

BLAST results showed that isolates residing in four of the morphological groups were related to species with *Pesotum* anamorphs in the Ophiostomatales. The remaining two groups were related to *Graphium* species in the Microascales. Due to the phylogenetic distance between these two orders, two different datasets were compiled with isolates belonging to the respective orders. After alignment, the ITS and β-tubulin datasets containing *Ophiostoma* species consisted of 783 and 286 characters, respectively. The ITS data set for *Graphium* species consisted of 545 characters after alignment.

MP, NJ and Bayesian analyses resulted in similar topologies for each of the three data sets. NJ trees of the respective datasets are presented as Figs. 1, 2 and 3, with bootstrap results from the NJ and MP trees, and posterior probabilities obtained from the Bayesian analyses, presented as indicated in the legends.

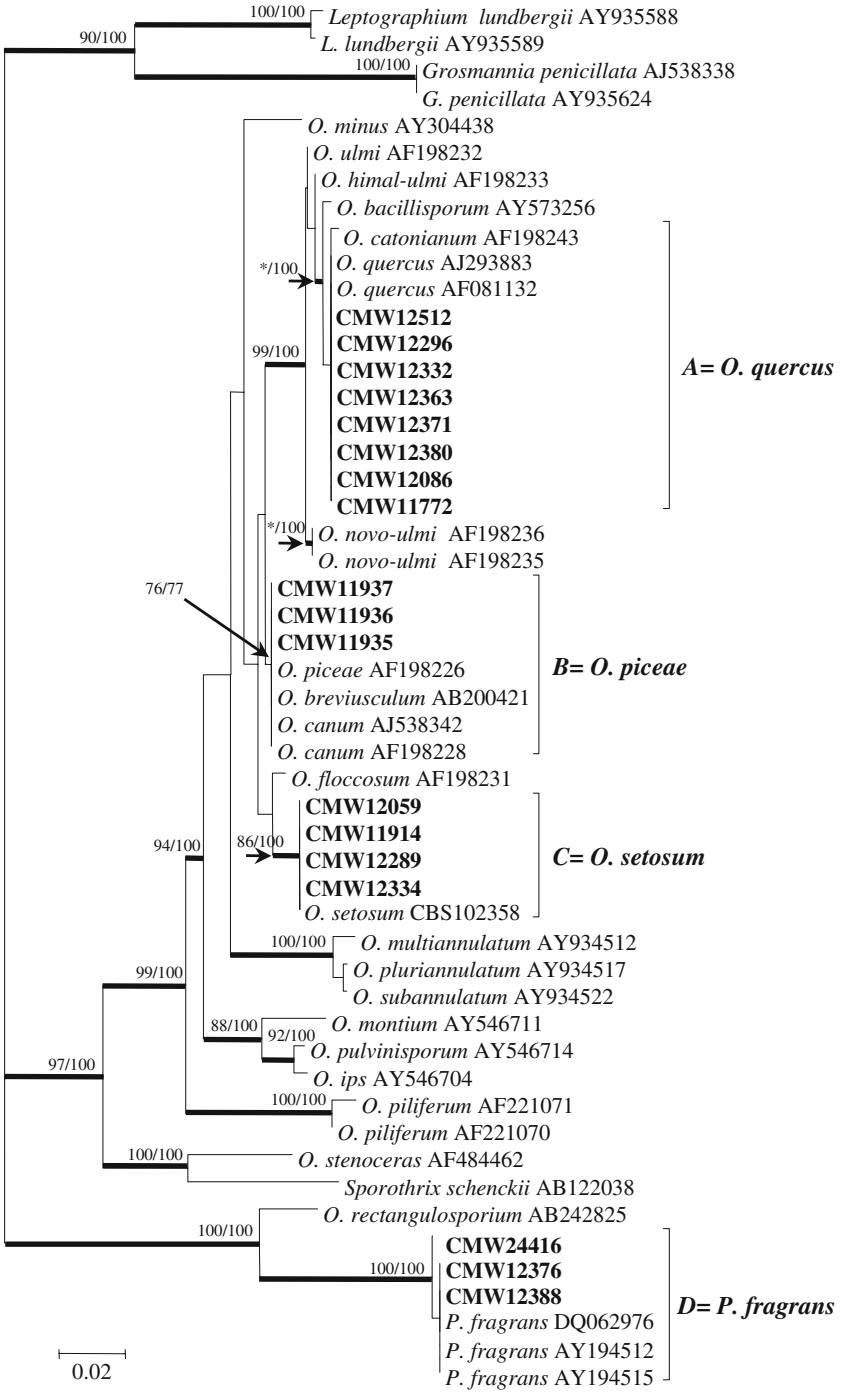


Fig. 1 NJ tree obtained from ITS sequence data of *Pesotum* isolates (Ophiostomatales) from China (*bold type*). *Dark branches* indicate posterior probabilities >0.95. Bootstrap values at nodes are for 1,000 replicates (Neighbor-Joining/Maximum Parsimony). * are bootstrap values <75%

Comparison of ITS sequences (Fig. 1) showed that four groups from this study respectively grouped with isolates of *Ophiostoma quercus* and *O. catonianum* (Goid.) Goid. (group A), *O. piceae, O. canum* (Münch) Syd. and *O. breviusculum* W.Hsin Chung, Yamaoka, Uzunovic, J.J. Kim (group B), *O. setosum* Uzunovic, Seifert, S.H. Kim & C. Breuil (group C) and *P. fragrans* (Math.-Käärik) G. Okada & Seifert (group D). ITS sequences, however, could not distinguish satisfactorily between the species in groups A and B. β-tubulin sequences (Fig. 2) distinguished between the species within these two groups. The Chinese isolates in groups A and B respectively grouped with *O. quercus* and *O. piceae*, with considerable variability amongst the *O. quercus* isolates.

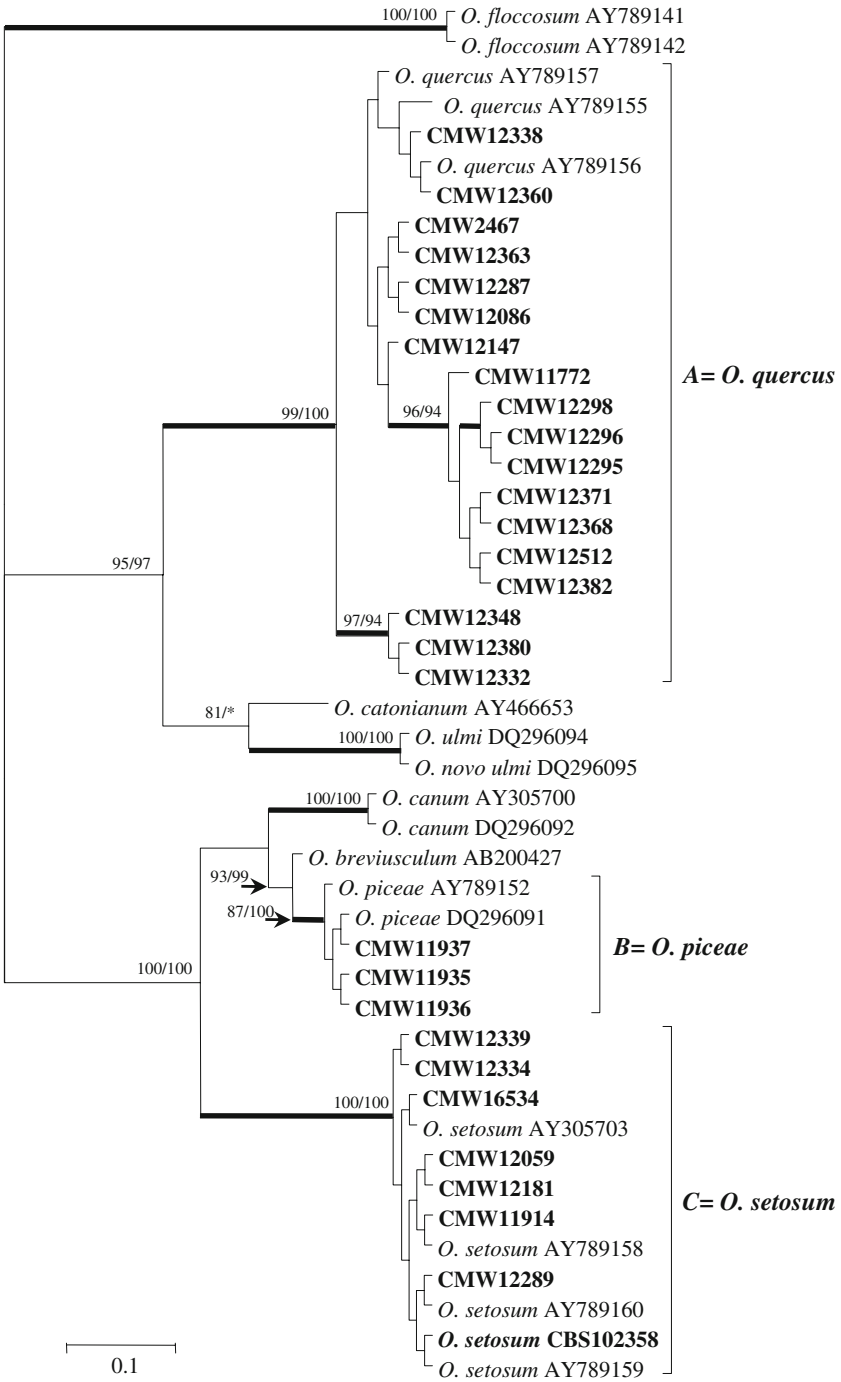


Fig. 2 NJ tree obtained from β-tubulin sequence data of *Pesotum* isolates (Ophiostomatales) from China (*bold type*). *Dark branches* indicate posterior probabilities >0.95. Bootstrap values at nodes are for 1,000 replicates (Neighbor-Joining/Maximum Parsimony). * are bootstrap values <75%

Analyses of the ITS sequences for isolates belonging to the *Microascales* (Fig. 3), showed that these isolates separated into two lineages corresponding with the two morphological groups based on culture morphology. The ITS sequence of one isolate (CMW12285) was identical to that of the ex-type isolate (CMW503) of *G. pseudormiticum* M. Mouton & M.J. Wingf. (group E); while the other two isolates resided in a group distinct from any known *Graphium* species (group F), with significant statistical support in all the analyses.

Morphology

All isolates had synnematous anamorphs in culture (Figs. 4–7, 19–22) and sexual structures were never observed.

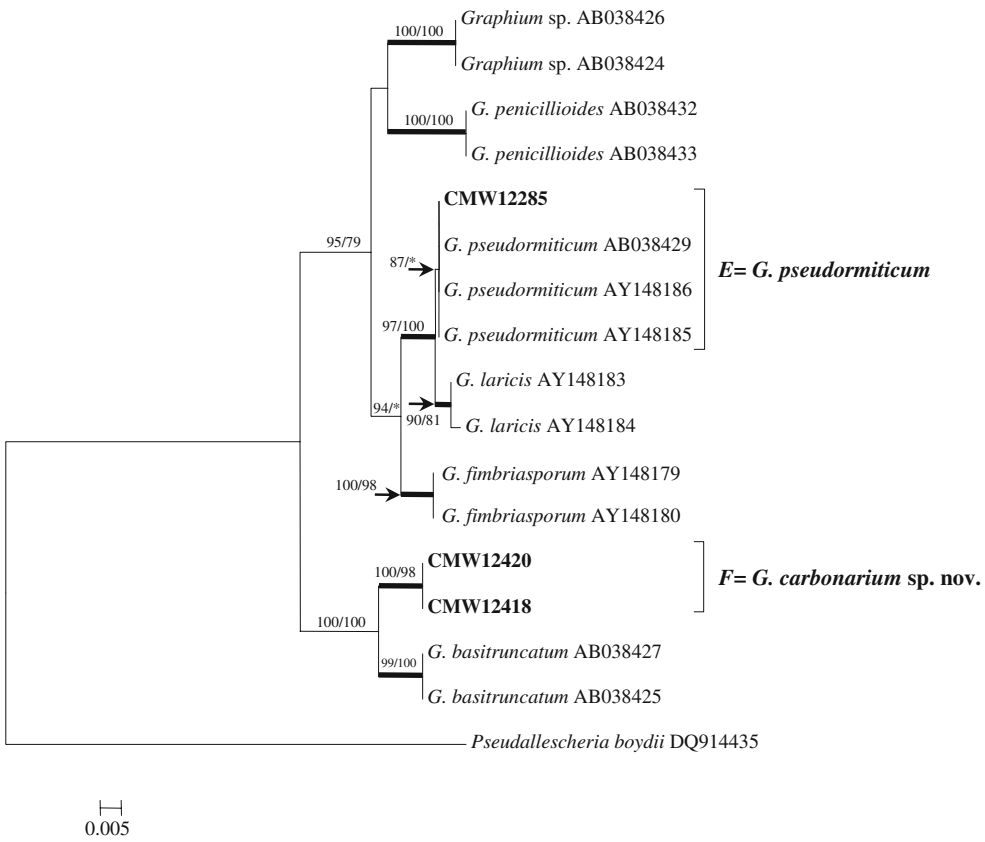


Fig. 3 NJ tree obtained from ITS sequence data of *Graphium* isolates (Microascales) from China (*bold type*). *Dark branches* indicate posterior probabilities >0.95. Bootstrap values at nodes are for 1,000 replicates (Neighbor-Joining/ Maximum Parsimony). * are bootstrap values <75%

Cultures from groups A to C had *Sporothrix* synanamorphs (Figs. 15–17), while those of group F had a *Scedosporium*-like anamorph (Figs. 31–33).Group F isolates and the species most closely related to it, *G. basitruncatum* (Figs. 20, 24, 28, 31), differed mainly in the presence of larger synnemata and conidia of the Chinese isolates, and their ability to grow at 35°C.

Taxonomy

Morphology, growth and phylogenetic analyses confirmed the identities of six synnematous Ophiostomatoid fungi from China. Five of these are known taxa and their morphology is treated only briefly. One group of isolates was clearly distinct from all other species and it is therefore described here as a new species of *Graphium*.

Ophiostomatales

Ophiostoma quercus (Georgev.) Nannf., Svenska Skogsvårdsföreningens Tidskrift 32: 408 (1934)

(Figs. 4, 8, 12, 15)

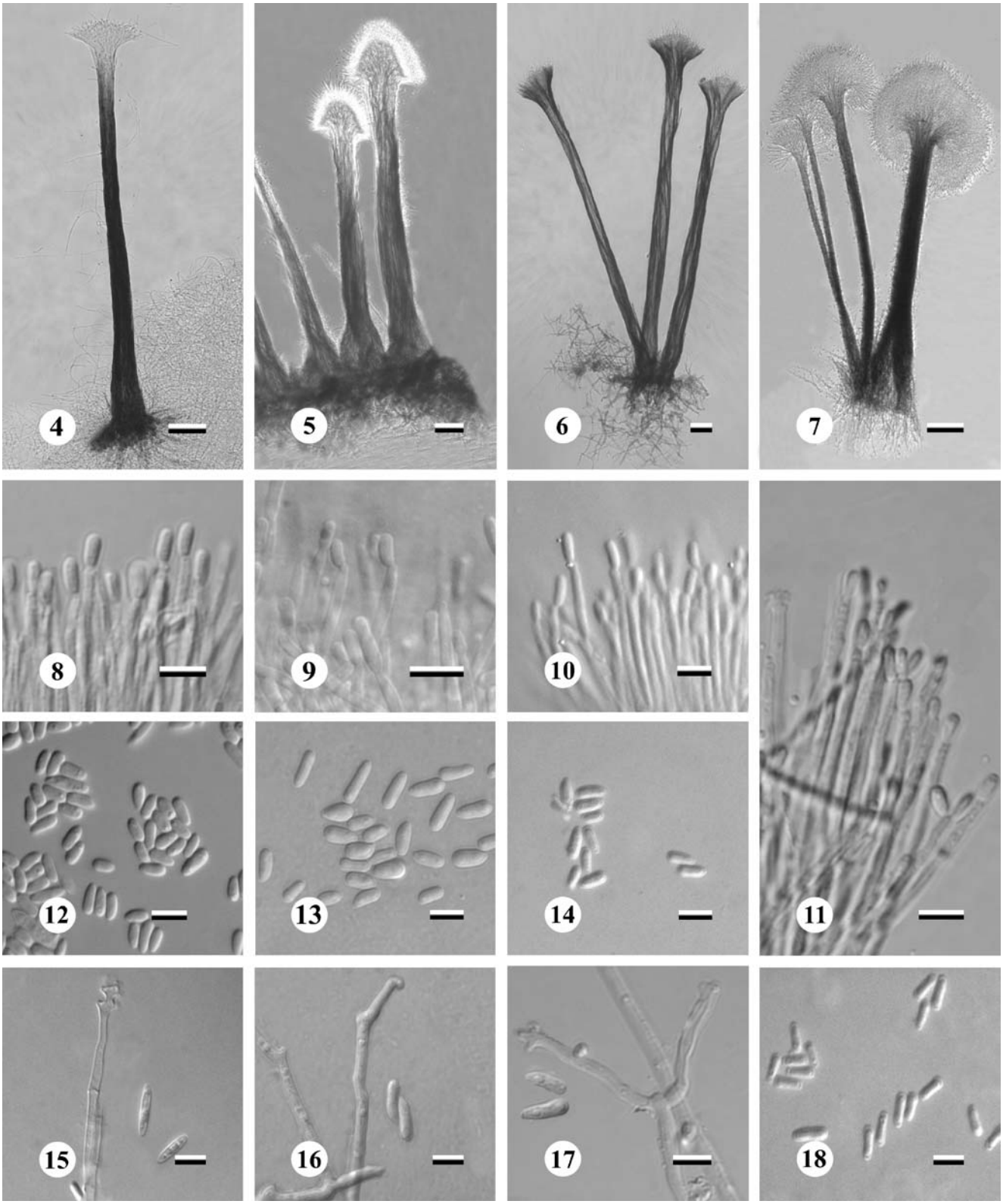
Teleomorph: not observed.

Anamorph: *Pesotum roboris* (Georgescu, Teodoru & Badea) Grobbelaar, Z.W. de Beer & M.J. Wingf., Mycol Prog 8(3): 223 (2009)

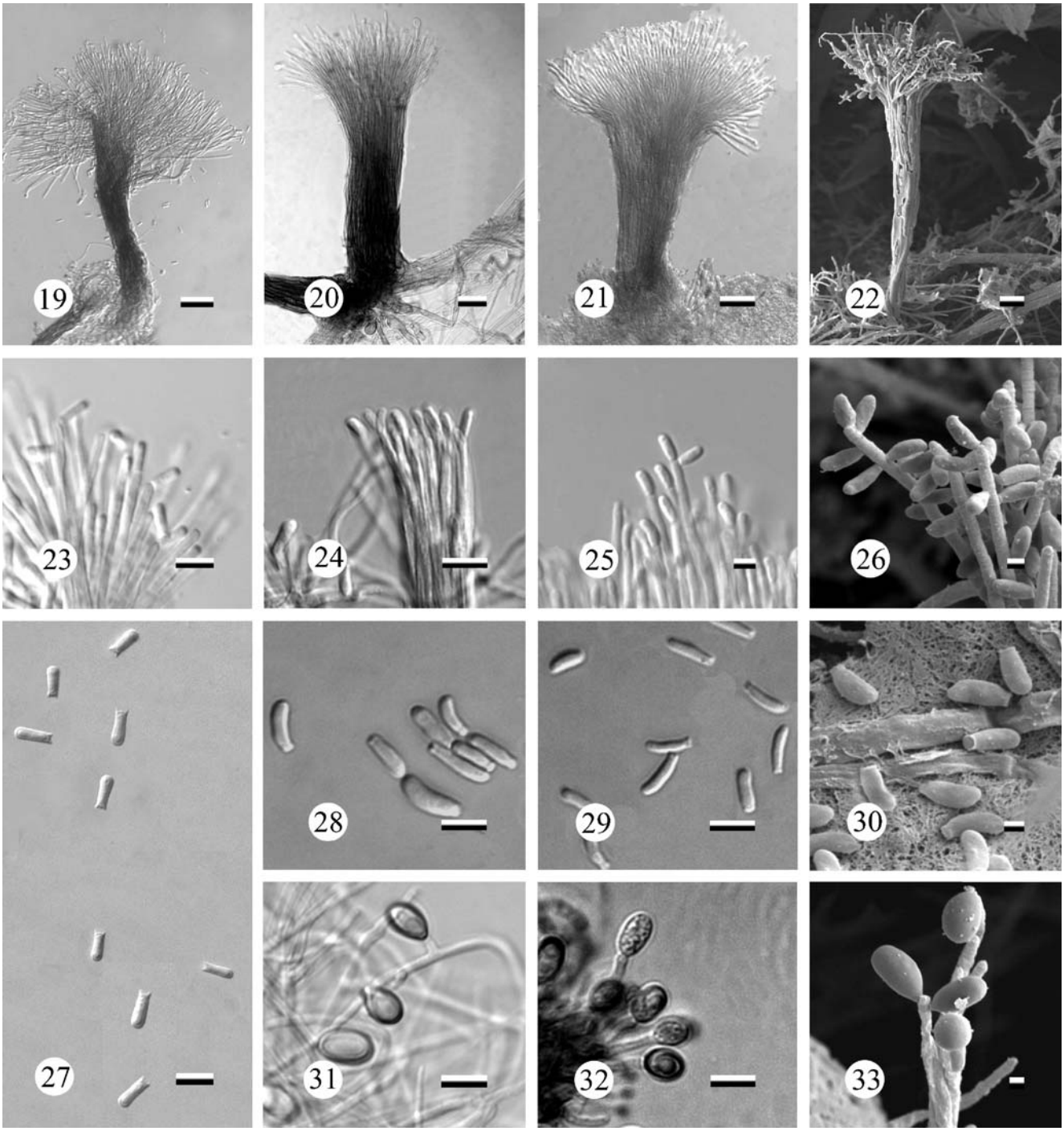
Synanamorph: *Sporothrix roboris* (Georgescu & Teodoru) Grobbelaar, Z.W. de Beer & M.J. Wingf., Mycol Prog 8(3): 223 (2009)

The *O. quercus* isolates in this study showed a wide morphological variation, with conidiophore lengths between (256-)464–713(-1004) µm; and conidia between 3–5(-7) µm long and 1–2µm wide. Conidial shapes varied from oblong and ellipsoid to elongated. The colony colour varied between isabelline (19″i), hazel (11′k), olivaceous buff (21‴d), umber (9 m) and light buff (17′f) in PDA media, while in OA media the colonies were light buff (17′ f) and later became dark olivaceous (21‴m). A *Sporothrix* synanamorph was present in 85% of the isolates and rhizoids were present at synnematal bases in all isolates. The optimal growth temperature was 25°C, poor growth was observed at 5 and 30°C, but these isolates started to grow slowly after 5 days at 32°C. These observations are consistent with descriptions of *O. quercus* previously presented (Georgevitch 1927; Morelet 1992; Halmschlager et al. 1994; Harrington et al. 2001; Grobbelaar et al. 2009).

Habitat: Associated with bark beetles of the genera *Crypturgus*, *Brachyderes, Hylastes*, *Hylurgops*, *Hypothenemus*, *Ips*, *Leperisinus*, *Pissodes*, *Scolytus*, *Taphrorychus*,



Figs. 4–18 Anamorph stages of *Ophiostoma* species with *Pesotum* anamorphs isolated from conifers and hardwoods in China. **Figs 4, 8, 12, 15**. *O. quercus* CMW12363 **4**. Synnema. **8**. Conidiogenous cell. **12**. Conidia. **15**. *Sporothrix* synanamorph. **Figs 5, 9, 13, 16**. *O. piceae* CMW11936. **5**. Synnema. **9**. Conidiogenous cell. **13**. Conidia. **16**. *Sporothrix* synanamorph. **Figs 6, 10, 14, 17**. *O. setosum* CMW12339. **6**. Synnema. **10**. Conidiogenous cell. **14**. Conidia. **17**. *Sporothrix* synanamorph. **Figs 7, 11, 18**. *Pesotum fragrans* CMW12376. **7**. Synnema. **11**. Conidiogenous cell. **18**. Conidia. (*Bars* 4–7=50 μm. *Bars* others:=5 μm)



Figs. 19–33 *Graphium* species. **Figs 19, 23, 27**. *G. pseudormiticum* CMW12285. **19**. Synnema. **23**. Conidiogenous cell. **27**. Conidia. **Figs 20, 24, 28, 31**. *G. basitruncatum* CBS320.71 (Ex-type isolate). **20**. Synnema. **24**. Conidiogenous cell. **28**. *Graphium* conidia. **31**. *Scedosporium* synanamorph. **Figs 21, 25, 29, 32**. *G. carbonarium* sp. nov. CMW12420 **21**. Synnema. **25**. Conidiogenous cell. **29**. *Graphium* conidia. **32**. *Scedosporium* synanamorph. **Figs 22, 26, 30, 33**. SEM photos of *G. carbonarium* sp. nov. CMW12420 **22**. Synnema. **26**. Conidiogenous cell. **30**. *Graphium* conidia. **33**. *Scedosporium* synanamorph. (*Bars* 19–21=10 µm. Bars 22, 26, 30, 33=1 µm. *Bars* others=5 µm)

and *Xyloterus*, on species of *Abies, Betula, Cunninghamia*, *Cupressocyparis, Dacrydium, Eucalyptus*, *Fagus*, *Grevillea, Larix, Nothofagus*, *Olinia, Pinus*, *Podocarpus, Prumnopitys, Pseudotsuga, Quercus* and *Ulmus*.

Known distribution: Europe, Asia, Africa, Australia, New Zealand, North and South America.

Material examined: CHINA, Dali and Lijiang, Yunnan Province, from *Pissodes* sp. on *Pinus yunnanensis* and

Tsuga dumosa, January 2005, D. Paciura (CMW12295, CMW12332, CMW12363, CMW12371, CMW12380).

Ophiostoma piceae (Münch) Syd. in Sydow & Sydow, Annales Mycologici 17(1): 43 (1919)

(Figs. 5, 9, 13, 16)

Teleomorph: not observed.

Anamorph: *Pesotum piceae* J.L. Crane & Schokn. Am. J. Bot. 60: 348 (1973).

Synanamorph: *Sporothrix*.

Pesotum anamorph of isolates examined in this study had short to medium size synnemata (243-)358–487(-518) µm and conidia oblong to obovoid 4–6 µm length and 2–3 µm wide, rhizoids present. All the isolates had a *Sporothrix* synanamorph. The colony colour in PDA was umber (9 m). Optimal growth was obtained at 25°C; there was no growth at 5 and 30°C. These observations are consistent with those for *O. piceae* by Münch (1907), Upadhyay (1981) and Harrington et al. (2001).

Habitat: Associated with bark beetles of the genera *Brachyderes, Hylastes, Hylurgops, Hylurgus, Ips, Orthotomicus, Tetropium, Tomicus* and *Xyleborus*; on several conifer and hardwood hosts of the genera *Abies, Acer, Betula, Chamaecyparis, Crataegus, Fagus, Larix, Laurelia, Magnolia, Nothofagus, Picea, Pinus, Populus, Pseudotsuga, Quercus, Thuja, Tsuga* and *Ulmus*.

Known distribution: Europe, Asia, Africa, Australia, New Zealand, North and South America.

Material examined: CHINA, Wangqing, Jilin Province, from *Ips subelongatus* on *Larix olgensis*, January 2005, X. D. Zhou (CMW11935, CMW11936, CMW11937, CMW11938).

Ophiostoma setosum Uzunovic, Seifert, S.H. Kim & C. Breuil, Myc. Res. 104(4): 490 (2000)

(Figs. 6, 10, 14, 17)

Teleomorph: not observed.

Anamorph: *Pesotum cupulatum* McNew & T.C. Harr., Mycologia 93(1): 121 (2001)

Synanamorph: *Sporothrix*.

Chinese isolates had more variation in conidiophore length of synnemata than *O. quercus* strains, ranging from (378-)491–1257(-3341) µm, and conidia from 3–5(-7) µm length and 1–2 µm wide. Conidia were elongated, ellipsoid and oblong. Rhizoids produced by 38% of the isolates. Most of the strains showed the typical crown of marginal hyphae at the upper part of the synnematal stipes. A *Sporothrix* synanamorph was present in 38% of the isolates. The colony colour varied between umber (9 m) and isabelline (19″i) in PDA media and olivaceous (21″m), isabelline (19″i) and umber (9 m) in OA media. These isolates grew optimally at 25°C and the growth did not occur at 5 and 30°C. Results corresponded with descriptions of *O. setosum* by Uzunovic et al. (2000) and Harrington et al. (2001).

Habitat: Associated with *Hylastes ater* on *Pinus radiata*. Other hosts include *Tsuga heterophylla, Pseudotsuga menziesii, Pinus contorta* and *Picea glauca*.

Known distribution: Canada, USA, New Zealand and Korea.

Material examined: CHINA, Dali and Chuxiong, Yunnan Province, from *Pissodes* sp. on *Tsuga dumosa* and *Abies* sp., January 2005, D. Paciura, X.D. Zhou (CMW12334, CMW12337, CMW12339, CMW11914, CMW12057).

Pesotum fragrans (Math. Käärik) G. Okada & Seifert, Can.J.Bot. 76 (9):1503 (1998)

(Figs. 7, 11, 18)

Teleomorph: not observed.

Pesotum fragrans isolates were readily distinguishable from the other *Pesotum* species based on morphology. Length of conidiophores varied from (378-)438–619(-708) µm. Conidia were oblong to obovoid with truncated bases, 3–6(-7) µm long and 1–2 µm wide. No rhizoids were present. The typical lemon yellow (23) pigmentation of the media was observed in PDA and OA media, the colony colour was isabelline (19″i) in PDA and light buff (17′f) in OA medium. The optimal temperature for growth was 25°C, with no growth at 5 and 30°C. Morphology of the Chinese isolates corresponded with descriptions of *P. fragrans* by Mathiesen-Käärik (1954), Okada et al. (1998) and Jacobs and Seifert (2004).

Habitat: Associated with *Ips sexdentatus* on *Pinus sylvestris, Tetropium fuscum* on *Picea glauca* and *P. rubens, Trypodendron lineatum* on *Abies balsamea, Hylurgops palliatus, Hylastes attenuatus, Orthotomicus erosus* and *Hypothenemus eruditus* on *P. radiata, Hylastes angustatus* on *Pinus patula*, and *Cryphalus piceae* on *Pseudotsuga* sp.

Known distribution: Sweden, Spain, Poland, Korea, Canada, USA, New Zealand, Australia and South Africa.

Material examined: CHINA, Dali, Midu and Kunming, Yunnan province, from *Pissodes* sp. on *T. dumosa* and *P. armandii; Tomicus yunnanensis* on *P. yunnanensis*, January 2005, D. Paciura and X.D. Zhou (CMW12376, CMW12388, CMW24416).

Microascales

Graphium pseudormiticum M. Mouton & M.J. Wingf., Mycol. Res. 98(11): 1273 (1994)

(Figs. 19, 23, 27)

Teleomorph: not observed.

The single isolate from China was characterized by relatively short synnemata, (118-)135–166(-195) µm, light brown olivaceous (19″k) with rhizoids. Conidiogenous cells were annellated and conidia cylindrical, (5-)6–7(-8) µm long and 2–3 µm wide, with a conspicuous basal frill. Colony hazel (11′k) on OA and dark olivaceous (21‴m) on

PDA media. Optimal growth temperature 30°C. The morphology compared well with the description by Mouton et al. (1994).

Habitat: Associated with *Orthotomicus erosus* on *Pinus* spp., *Tomicus minor* on *P. sylvestris*, and *Ips sexdentatus* and *O. laricis* on *Larix* sp.

Known distribution: Germany, Austria and South Africa.

Material examined: CHINA, Dali, Yunnan province, from a *Pissodes* sp. on *T. dumosa*, January 2005, D. Paciura (CMW12285).

Graphium carbonarium Paciura, Z.W. de Beer, X.D. Zhou & M.J. Wingf. **sp. nov**

(Figs. 21–33)

MycoBank: MB 512644.

Etymology: Referring to the dark conidiophores produced in culture with texture similar to coal.

Conidiophorae in synnemate singulae vel aggregatae orientes, stipa basi fuliginea, apicem versus avellanea vel hyalina, 134–225(-300) µm longa, medio 31–36(-43) µm, apice (40-)46–58(-60) µm latae. Structurae rhizoidiformes abundantes. Conidiophorae cum ramis biseriatis vel quadriseriatis, cellulis 2–3 in quoque loco ramificationis, 15–18 (-24) µm longis, 1–3 µm latis, annelationibus noduliformibus. Conidia non septata, hyalina falcata cylindrica basi truncata 4–6(-7) µm longa×1–3 µm lata, in massa hyalina mucosa in apicibus synnematum aggregata, juventute hyalino-nivea, aetate atrantia. Synapomorpha Scedosporiiformis adest, conidiophoris erectis simplicibus vel ramosis, conidiis obovoideis brunneo-olivaceis, parietibus crassis, 7–8 µm longis, 4–6 µm. Coloniae in OMA atrogriseo-olivaceae. Crescunt optime in 25–35°C, in 5–10°C non crescunt.

Colonies dark grayish olive (21‴″k) on Oat meal Agar with abundant synnemata concentrated at the center of the plate, reverse dark mouse grey (15‴″k), in PDA the colony is light buff (17′f) with smooth aerial mycelium, reverse buff yellow (19d). Mycelium mostly aerial and immersed in the agar, with hyphae hyaline, septated, 2–3 µm wide. Conidiophores arranged in synnemata that sometimes arise singly, generally in groups, composed of a stipe dark grayish brown (5‴″k) at the base, becoming hazel (11′k) and hyaline toward the apex, 134–225(-300) µm long, 31–36(-43) µm wide at the center, and (40-)46–58(-60) µm wide at the apex. Rhizoid-like structures abundant. Conidiophores with two to four series of branches, with 2–3 conidiogenous cells per branch point, conidiogenous cells 15–18(-24) long and 1–3 µm wide, with nodular annelations. Conidia aseptate, hyaline, curved, cylindrical with truncated bases, 4–6(-7) µm×1–3 µm, aggregate in a hyaline mucilaginous mass at the apices of the synnematas, bright transparent white when young, becoming darker with age. *Scedosporium*-like synanamorph with erect, simple or branched conidiophores and conidia obovoid, brownish olive (19″m), thick walled, 7–8 µm long and 4–6 µm wide.

Teleomorph: not observed.

Synanamorph: *Scedosporium*-like.

Habitat: Associated with a *Pissodes* sp. on *Salix babylonica*.

Known distribution: Lijiang, Yunnan, China.

Material examined: CHINA, Lijiang, Yunnan province, from *Pissodes* sp. on *T. dumosa*, January 2005, D. Paciura, (PREM60013 [Holotype], CMW12420 = CBS 123610 [Ex-holotype culture]; PREM60014 [Paratype], CM W12418 = CBS 123611 [Ex-paratype culture])

Note: *Graphium carbonarium* is most closely related to *G. basitruncatum*. The optimum growth temperature for both species is 25–30°C. However, *G. carbonarium* isolates were able to grow at 35°C reaching 12 mm of growth after 8 days of incubation, while *G. basitruncatum* did not grow at this temperature. On Oat agar medium, *G. carbonarium* sporulated at 15°C to 35°C while *G. basitruncatum* only sporulated at 25°C and 30°C. On Malt extract agar the *Scedosporium*-like stage was present for both species at 15–30°C. *Graphium carbonarium* had larger synnemata, conidia and *Scedosporium*-like conidia than *G. basitruncatum*. The latter species is characterized by conidiophores (70-)72–131(-158) µm in length, conidiogenous apparatus (19-)24–45(-56) µm wide, conidia of 5–6×1–2 µm and *Scedosporium*-like conidia of 5–6 (-7)×3–5 (-7) µm in size.

Discussion

Six ophiostomatoid species with synnematous anamorphs were isolated and identified from bark beetles in China. These fungi include *Ophiostoma quercus*, *O. piceae*, *O. setosum, Pesotum fragrans*, *Graphium pseudormiticum* and a novel *Graphium* sp., and were characterized using morphology and DNA sequence comparisons. The results confirmed for the first time the occurrence of *O. setosum* and *P. fragrans* in China based on DNA sequence data. *Graphium pseudormiticum* is also reported for the first time from China, and a novel taxon, *G. carbonarium* sp. nov. is described.

Of the fungi collected and identified in this study, four reside in the Ophiostomatales. Three of these, *O. quercus, O. piceae* and *O. setosum* reside in the *O. piceae*-complex based on DNA sequences and the presence of both *Pesotum* and *Sporothrix* synanamorphs. The fourth species, *P. fragrans*, lacks a *Sporothrix* anamorph and is not considered part of the *O. piceae*-complex (Harrington et al. 2001).

Ophiostoma quercus is widely distributed in many parts of the world (Harrington et al. 2001; De Beer et al. 2003; Lin et al. 2003; Geldenhuis et al. 2004; Thwaites et al. 2004; Zhou et al. 2004; Carlier et al. 2006; Kim et al. 2007; Kamgan Nkuekam et al. 2008; Linnakoski et al. 2008, 2009; Grobbelaar et al. 2009). It appears to be a casual associate of

many tree wound-inhabiting insects (Geldenhuis et al. 2004; Kamgan Nkuekam et al. 2008), and is primarily known as a sap-staining fungus associated with dead or stressed hardwood trees (Luque et al. 2000; Harrington et al. 2001). It has been suggested to exhibit some pathogenicity (Luque et al. 2000; Geldenhuis et al. 2004), and has for example been linked to Chinese fir wilt, associated with *Crypturgus* infestations in Taiwan (Lin et al. 2003). *Ophiostoma quercus* has also been isolated from *Betula* and *Quercus* in Japan (De Beer et al. 2003; Chung et al. 2006) and logs of *P. densiflora* and *P. koraiensis* in Korea (Kim et al. 2007). Finding the fungus in *Pissodes* and *Tomicus* galleries from conifers and hardwoods such as *Pinus yunnanensis*, *Tsuga dumosa*, and *Salix babylonica*, in China, is therefore not surprising. However, the Chinese isolates of *O. quercus* exhibited a wide range of morphotypes and showed substantial variation especially among the β-tubulin sequences. It thus seems likely that these isolates represent a species complex. Multi-gene sequence analyses and population genetic studies will be necessary to elucidate taxonomic relationships of the lineages present within the complex.

Ophiostoma piceae is a common associate of conifer-infesting bark beetles (Seifert 1993; Harrington et al. 2001). In this study, *O. piceae* was associated with *Ips subelongatus*, a secondary pest (Stauffer et al. 2001) infesting *Larix olgensis* in China. The fungus is known to occur with the non-aggressive beetle, *Ips cembrae* on *L. kaempferi*, and the much more aggressive beetles, *I. typographus* var. *japonicus* on *Picea jezoensis*, in Japan (Yamaoka et al. 1997, 1998), and *Dendroctonus valens* on *Pinus tabuliformis* in China (Lu et al. 2009a). Other conifer hosts of *O. piceae* in Asia are *Abies veitchii* infested by *Dryocoetes autographus*, *A. homolepis* infested by *Polygraphus proximus* and *D. striatus* (Yamaoka et al. 2004), and *P. densiflora* and *P. koraiensis* in Korea (Kim et al. 2007).

Ophiostoma setosum was first described from *Tsuga heterophylla* logs in Canada (Uzunovic et al. 2000), and has since been reported on various species of *Pinus*, *Pseudotsuga*, and *Picea* from Canada, USA, New Zealand and Korea (Harrington et al. 2001; Thwaites et al. 2004; Kim et al. 2007). In the present study, this fungus was isolated from *Pissodes* galleries on *T. dumosa* and an *Abies* sp. in Yunnan, China. To the best of our knowledge, this is the first time that the fungus has been linked to a possible insect vector but this is not unusual as some *Pissodes* spp. act as vectors of Ophiostomatoid fungi (Nevill and Alexander 1992). However, it will be necessary to isolate *O. setosum* from *Pissodes* to confirm its association with the beetle in China.

Pesotum fragrans, for which no teleomorph is known, belongs to the Ophiostomatales but is only distantly related to the other *Ophiostoma* species collected in this study (Okada et al. 1998; Harrington et al. 2001; Jacobs et al. 2003b). Based on ITS sequence data our results suggest that the closest relative of *P. fragrans* is *O. rectangulosporum* (Ohtaka et al. 2006). *Pesotum fragrans* was initially described from *Pinus sylvestris* infested by *Ips sexdentatus* and *Orthotomicus proximus* in Sweden (Mathiesen-Käärik 1954), and has since been reported from various bark beetle species in other countries of Europe, North America, Australasia and Africa (Harrington et al. 2001; Jacobs et al. 2003b; Thwaites et al. 2004; Zhou et al. 2006; Romón et al. 2007; Kamgan Nkuekam et al. 2008). The only report of the fungus in Asia is from stained *Pinus densiflora* logs in Korea (Kim et al. 2007).

The two *Graphium* species (Microascales) found in this study share similar ecological niches with those of species in the Ophiostomatales. They produce black and brown staining on wood and are also associated with bark beetles (Mathiesen-Käärik 1954; Okada et al. 1998, 2000; Stauffer et al. 2001; Geldenhuis et al. 2004). *Graphium pseudormiticum* is known as an associate of the pine-infesting bark beetle, *Orthotomicus erosus*, in South Africa (Mouton et al. 1994). The fungus has since been found associated with *Crypturgus, Dryocoetes, Hylurgops, Polygraphus, Trypodendron, Pityogenes* and *Ips* species on spruce, and *Ips* and *Orthotomicus* species on pine trees in Germany (Kirschner 1998). Jacobs et al. (2003a) found that species in the *G. pseudormiticum* complex have host preference, and suggested that *G. pseudormiticum* is restricted to pine, *G. fimbriasporum* (M. Morelet) K. Jacobs, Tirisits & M.J. Wingf. to spruce, and *G. laricis* K. Jacobs, Tirisitis & M.J. Wingf. to larch. This study represents the first report of *G. pseudormiticum* from Asia. The single isolate from a *Pissodes* gallery on *Tsuga dumosa* does not, however, provide sufficient evidence to draw conclusions regarding new host or insect associations for this species.

Graphium carbonarium, described in this study, is closely related to *G. basitruncatum*. The latter was first described from forest soil in the Solomon Islands as *Stilbum basitruncatum* Matsush. (Matsushima 1971). It was later placed in the *G. penicillioides* complex (Okada et al. 1998, 2000), typified by the presence of *Graphium* and *Scedosporium*-like synanamorphs, with no known teleomorphs. This species aggregate is phylogenetically related to the *G. putredinis* aggregate that includes opportunistic human pathogens such as *Pseudallescheria* and *Scedosporium* species. *Graphium basitruncatum* has been isolated from a patient with leukemia in Canada, confirming that this species can act as an opportunistic human pathogen (Deepali et al. 2007).

Given the limited available knowledge regarding the ophiostomatoid fungi in China, it is not surprising that

many species, including the new taxon belonging to this group have emerged from surveys such as the one reported here. There is no doubt that more detailed work and sampling in other areas of China will reveal many novel species. Knowledge regarding these fungi will be valuable in terms of the protection of biodiversity in the region and it will also help in promoting appropriate quarantine measures which will be required for the trade in timber that is likely to emerge in China in the future.

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