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Epitypification of *Graphium penicillioides* Corda, with comments on the phylogeny and taxonomy of graphium-like synnematous fungi [1]

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Abstract: Graphium-like anamorphs have previously been known in three groups of ascomycetes, including the *Microascales* (*Graphium sensu stricto*), the *Ophiostomatales* (anamorphs now classified in *Pesotum*), and the *Chaetothyriales*. In this paper, the modern interpretation of the classical hyphomycete genus *Graphium* is fixed by epitypification of the type species, *G. penicillioides*, using a culture derived from the original host and near the original location where the holotype was collected more than 160 years ago. The epitype culture is described and illustrated, and a comparison is made with the remnants of the holotype specimen. Neighbor joining analyses of small subunit (SSU/18S) rDNA sequences confirm that the phylogenetic disposition of the epitype strain is near others identified as *G. penicillioides*, in the *Microascales* clade. Sequences of the internal transcribed spacer (ITS) region of the epitype and other strains identified as *G. penicillioides* confirm earlier results that this is a species aggregate, including at least four species. Comments on the phylogenetic relationships of some additional species sometimes referred to *Graphium* are included, and a fourth group of graphium-like anamorphs, phylogenetically related to the discomycetes, is briefly mentioned. The following new combinations are proposed: *Dendrostilbella smaragdina* (Alb. & Schw.) Seifert, *Exophiala calicioides* (Fr.) Okada & Seifert, *Graphium basitruncatum* (Mats.) Seifert & Okada, and *Pesotum erubescens* (Mathiesen) Okada (see Appendix).

Key words: *Microascales*, *Ophiostomatales*, *Chaetothyriales*, discomycetes, *Dendrostilbella*, *Exophiala*,

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[1] Part 12 in a series on the taxonomy of synnematous fungi by Gen Okada.

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Pesotum, ITS, SSU (18S) rDNA.

Introduction

The anamorph genus *Graphium* Corda (1837), lectotypified by *G. penicillioides* Corda (Hughes, 1958), has traditionally included species with darkly pigmented, determinate synnemata, percurrently proliferating conidiogenous cells and slimy, aseptate, hyaline to pale brown conidia (Ellis, 1971; Crane & Schoknecht, 1973). Although the teleomorph of the lectotype species *G. penicillioides* is unknown, the genus was long believed to have ophiostomatoid affinities (Goidànich, 1935; Upadhyay, 1981; Seifert & Okada, 1993). An historical overview of *Graphium*, including a morphological survey of representative species, and a discussion of their known and supposed teleomorph connections was presented by Seifert & Okada (1993). Their broad morphological generic concept, accommodating plasticity in conidium ontogeny and synnema pigmentation, incorporated species formerly disposed into much more narrowly defined genera. Similar conclusions were presented by Wingfield *et al.* (1991) and Mouton *et al.* (1993). However, as noted by Seifert & Okada (1993), even the most restrictive morphological generic concept for *Graphium* (limiting the genus to species with percurrently proliferating conidiogenous cells) includes anamorphs of three orders of the *Ascomycota*.

Okada *et al.* (1998) demonstrated that several cultures identified as *G. penicillioides* actually had phylogenetic affinities with *Graphium putredinis* (Corda) S. Hughes in the *Microascales*, based on phylogenetic analysis of 18S rDNA sequences. This necessitated the abandonment of the name *Graphium* for anamorphs of species of *Ophiostoma* Syd. & P. Syd. and the reassignment of the former morphological concept of this genus to the name *Pesotum* J. L. Crane & Schokn.. In addition, the phylogenetic affinities of *G. calicioides* (Fr.) Cooke & Massee were shown to be with the *Chaetothyriales*, not the *Chaetosphaeriaceae* as speculated earlier by Seifert & Okada (1993). Okada *et al.* (1998) provided provisional nomenclators for accepted species of *Graphium* and *Pesotum*.

Identification of *Graphium* and *Pesotum* species is difficult in the absence of teleomorphs, primarily because of the paucity of modern descriptions for most species. Morphologically similar sibling species (Brasier, 1993) exist for the teleomorphs of *Pesotum* species, but have rarely been critically compared using morphological techniques. The two best known species of *Graphium sensu stricto*, *G. penicillioides* and *G. putredinis*, were considered species aggregates by Seifert & Okada (1993) and Okada *et al.* (1998).

The correct application of the name *Graphium penicillioides* has been complicated by the suspicion that the name has been used for more than one species. The original description by Corda (1837) was based on a specimen collected in Prague on *Populus nigra* cv. *italica*. Ellis (1971) listed the fungus as occurring on *Populus* wood in Europe and North America. Sutton & Laut (1970) and Sutton (1973) described specimens identified as *G. penicillioides* as a common secondary colonizer of bark beetle tunnels in *Ulmus* trees killed by Dutch elm disease in Manitoba and Saskatchewan, and this fungus is still common in this niche in Canada (Seifert, unpublished). Meanwhile, Matsushima (1971) described *Stilbum basitruncatum* Mats. from a culture isolated from soil from the Solomon Islands; Sutton (1973) later synonymized this species with *G. penicillioides* (cf. Matsushima, 1975). Furthermore, the CBS culture collection catalogue (http://www.cbs.knaw.nl/database.html) lists two isolates from *Prunus armeniaca* in Tunisia (CBS 318.72, 319.72). Do all of these populations actually represent the same species, and if not, how should *G. penicillioides sensu stricto* be defined?

The holotype of *G. penicillioides* is depauperate and has been examined by two of the authors of this paper (K.A.S., M.J.W.) as well as by Hughes (1958), who deposited a slide in herb. DAOM, and Crane & Schoknecht (1973), who deposited a slide in herb. ILLS. A few synnemata remain on the holotype, enough to briefly characterize the conidiomata, the conidiogenous cells, and the conidia. Subsequent to the Tokyo version of the International Code of Botanical Nomenclature (Greuter *et al.*, 1994), the concept of epitypification (Art. 9.7) allows the designation of a specimen and/or a culture. Such material can serve as a proxy for the holotype in the determination of morphological, physiological or molecular characteristics that cannot be determined from the holotype. In 1998, one of us (T.K.) visited the Czech Republic and took core samples from living trees of *Populus nigra* cv. *italica*. Four cultures conforming with the morphological characters of the holotype of *G. penicillioides* were isolated. In this paper, one of these cultures is designated as epitype for this species, formally fixing the application of the name and allowing confirmation of its phylogenetic relationships.

Materials and Methods

Morphology and cultural characters

Colours of morphological structures and colonies were determined using the charts of Rayner (1970, numeric-alphabetic codes in the form 19'f) or Kornerup & Wanscher (1978, numeric-alphabetic-numeric codes in the form 26A2).

The optimal growth temperature of *Graphium penicillioides* was determined by inoculating ten plates of 2% malt extract agar (MEA; 20 g Biolab malt extract, 20 g Biolab agar and 1000 ml distilled water) with 6 mm diameter agar disks taken from the actively growing margins of two-week-old isolates. The plates were incubated at temperatures ranging from 10 to 30°C at 5° C intervals. Cultural descriptions were made using colonies grown on MEA and homemade oatmeal agar (OA; Gams *et al.*, 1998).

Microscopic dimensions were based on 25 measurements and are given as arithmetic means ± standard error.

Cycloheximide tolerance was determined by inoculating 5 MEA plates amended with increasing concentrations of cycloheximide (0, 0.01, 0.05, 0.1, 0.5 g/l) and incubated at 25°C. Colony diameters were measured after eight days and mean growth was calculated.

For scanning electron microscopy (SEM), sporulating material from agar media was fixed and dehydrated using the methods of Cole & Samson (1979) or simpler methods of Nakagiri (1999). After critical-point drying with Hitachi HCP-2, the materials were coated with Pt-Pd (ca 100–200 Å thick) in an Eiko ion coater (IB-3) and observed with Hitachi (S-430 or S-2400) or JEOL (JSM-T20) scanning electron microscopes at 10–20 kV.

Strains used for DNA sequencing

In addition to the 18S rDNA sequences already determined by Okada *et al.* (1998) for *G. penicillioides* and other *Graphium* species, the following strains were used for DNA sequencing in this study. JCM = Japan Collection of Microorganisms, RIKEN, Saitama, Japan (http://www.jcm.riken.go.jp/). DDBJ/EMBL/GenBank accession numbers are shown in square brackets.

18S rDNA: *Graphium album* (Corda) Sacc. JCM 9744 (= CBS 276.54) [AB007657]; *G. erubescens* Mathiesen JCM 9747 (= CBS 278.54, ex-type) [AB007658]; *G. eumorphum* Sacc. JCM 9748 (= CBS 987.73) [AB007684]; *G. fructicola* El. & Em. Marchal JCM 9750 (= CBS 107.68) [AB007659]; *G. penicillioides* aggregate JCM 8083 (= G. Okada OFC 3534, ex soil in Japan) [AB038421], JCM 10496 (= T. Kirisits No. 1, ex *Populus nigra* cv. *italica* in the Czech Republic) [AF178009, not used in Fig. 1], JCM 10498 (= T. Kirisits No. 3, ex *Populus nigra* cv. *italica* in the Czech Republic) [AB038423, AF178010 (not used in Fig. 1)], JCM 10499 (= T. Kirisits No. 4, ex *Populus nigra* cv. *italica* in the Czech Republic) [AF178011, not used in Fig. 1]; *G. rubrum* Rumbold JCM 9751 (= CBS 210.34, ex-type according to CBS database) [AB007660]; *G. silanum* Goid. JCM 9752 (= CBS 206.37) [AB007661]; *G. tectonae* C. Booth JCM 9753 (= CBS 127.84, ex-type) [AB007662]; phialographium-like unidentified fungus JCM 8069 (= G. Okada OFC 3528) [AB038422].

ITS rDNA: *G. penicillioides* aggregate JCM 7440 (= CBS 506.86, ex *Ulmus procera* in the UK, representative of the European *Ulmus* population) [AB038424], JCM 8083 [AB038425], JCM 9299 (= CBS 470.71, ex *Fagus sylvatica* in Germany) [AB038426], JCM 9300 [= CBS 320.72 = ex-type of *Graphium basitruncatum* (Mats.) Seifert & Okada (see Appendix), ex forest soil in the Solomon Islands] [AB038427], JCM 9301 (= CBS 408.84, ex *Salix* sp. in the Netherlands) [AB038428], JCM 9331 (= CBS 781.85, South Africa (substratum not identified), tentatively identified as *Graphium pseudormiticum* M. Mouton & M. J. Wingfield) [AB038429], JCM 10496 [AB038430], JCM 10497 (= T. Kirisits No. 2, ex *Populus nigra* cv. *italica* in the Czech Republic) [AB038431], JCM 10498 [AB038432], JCM 10499 [AB038433].

DNA isolation, PCR amplification, and sequencing of ribosomal DNA

18S rDNA and ITS sequences for the four new isolates of *G. penicillioides* from the Czech Republic were determined independently in Japan and South Africa.

In Japan, the methods described by Okada *et al.* (1997) were used for isolation, amplification, cloning and sequencing of 18S rDNA of *G. album*, *G. erubescens*, *G. eumorphum*, *G. fructicola*, *G. penicillioides* aggregate (JCM 8083), *G. rubrum*, *G. silanum*, *G. tectonae* and the phialographium-like unidentified fungus. The primers shown in Table 1 of Okada *et al.* (1997) were used for amplifying and sequencing 18S rDNA. For JCM 10498 in *the G. penicillioides* aggregate, DNA was obtained by heating mycelium scraped from a slant culture in a lysing solution containing detergent (Makimura *et al.*, 1994) in a 1.5 ml microtube shaken on a vortex mixer with 0.34 mm diam sterile glass beads for 5-10 min. Following the methods of Sugita & Nakase (1999), the 18S rDNA and ITS-1 and ITS-2 regions were amplified by PCR using the universal primers P1 (cf. Suh & Nakase, 1995; Nishida & Sugiyama, 1993) and ITS4 (White *et al.*, 1990). The PCR product was purified using an E.Z.N.A.™ Cycle-Pure kit (Omega Biotek, Doraville, GA, USA), and directly sequenced using a Takara Ex Taq™ sequencing kit (Takara, Shiga, Japan) with the following primers (cf. Suh *et al.*, 1996; Sugita & Nakase, 1999) in addition to P1:

570 (5'–CGCGGTAATTCCAGCTCCA–3'),
934 (5'–CTGCGAAAGCATTTGCCAAGG–3'; Sugita & Nakase, 1999),
1315 (5'–CGATAACGAACGAGACCTT–3'),
U1 (5'–TGGAATTACCGCGGCTGCTGGCACC–3'),
U2 (5'–CCGTCAATTCCTTTAAGTTTCAGCC–3'),
U3 (5'–GACGGGCGGTGTGTACAAAGGGCAG–3'),
ITS2 (5'–GCTGCGTTCTTCATCGATG–3').

DNA sequence reactions were analyzed with an ABI PRISM 377 DNA sequencer (Perkin Elmer Applied Biosystems, CA, USA). ITS regions including 5.8S rDNA were directly sequenced using a SequiTherm™ Long-Read™ Cycle sequencing kit (Epicentre Technologies, Wisconsin, USA) or a Takara Ex Taq™ sequencing kit with the following primers: pITS-1 (Sugita *et al.*, 1998), ITS1 (5'–GTCGTAACAAGGTTTCCGTAGGTG–3') and ITS4 (White *et al.*, 1990). Other procedures or conditions

followed Sugita *et al.* (1998). DNA sequencing reactions were analyzed with an ALFexpress DNA sequencer (Pharmacia Biotech, Uppsala, Sweden) or an ABI PRISM 377 DNA sequencer.

In South Africa, DNA was extracted from two-week-old cultures grown in malt extract broth (ME). A small amount of mycelium was ground to a fine powder in liquid nitrogen and 1.0 µl extraction buffer (1% CTAB) was added, followed by incubation in a 60°C waterbath for 1 hour. Proteins were removed with phenol and chloroform (1:1), followed by a series of chloroform steps, until the interface was clean. DNA was precipitated with cold 100% ethanol, left overnight at –20°C, pelleted at 13000 rpm for 30 min, washed with cold 70% ethanol and dissolved in 100–200 µl sterile water. Part of the 18S rDNA were amplified using a Hybaid™ Touchdown Thermocycling system (Ashford, UK) using primers shown in Table 1 of Okada *et al.* (1997). Reactions were done in 100 µl containing 10 µl 10X PCR buffer, 20 µl of 25 mM $MgCl_2$, 10 mM dNTPs, 20 pmol of each primer, 0.5 µl DNA and 1.75U Expand Taq polymerase (Boehringer Mannheim, Germany). The PCR conditions were as follows: 2 min at 94°C, annealing at 48°C for 1 min, 10 s at 62°C, 2 min at 72°C with an increase of 5°C/s, 40 cycles with a final elongation step at 72°C for 8 min. The resulting products were purified with the High Pure™ PCR product purification kit (Boehringer Mannheim, Germany). Sequencing was performed on an ABI 377 automated sequencer using the Thermo Sequenase Dye Terminator Cycle Sequencing Pre-Mix kit (Perkin Elmer Applied Biosystems, CA, USA) with the primers listed in Table 1 of Okada *et al.* (1997). Sequence data were edited in Sequence Navigator (Perkin Elmer Applied Biosystems, CA, USA). The ITS-2 region and part of the LSU rDNA were amplified with the primers ITS3 and LR3 (White *et al.*, 1990) and sequenced with the primers ITS3, LR3 and 404X (5'–CCCTTTCAA CAATTTCAC–3'). Other procedures or conditions were identical to those for used for 18S rDNA.

MOLECULAR PHYLOGENETIC ANALYSIS

The newly determined 18S rDNA sequences, excluding introns, were aligned with sequences mentioned by Okada *et al.* (1998) using the multiple sequence alignment program CLUSTAL W version 1.74 (Thompson *et al.*, 1994) and adjusted manually. The following additional sequences from the nucleotide sequence databases (GenBank, EMBL and DDBJ) were included in the alignment: *Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltoir M83264; *Byssochlamys nivea* Westling M83256; *Cryphonectria radicalis* (Schw.) Barr L42442; *Ctenomyces serratus* Eidam U29391; *Gymnoascoideus petalosporus* Orr, Roy & Ghosh U29392; *Hypocrea lutea* (Tode) Petch D14407; *Leucostoma persoonii* (Nitschke) Höhnel M83259; *Renispora flavissima* Sigler, Gaur, Lichtwardt & Carmichael U29393; *Saccharomycopsis fibuligera* (Lindner) Klöcker X69841; *Sordaria fimicola* (Roberge) Ces. & De Not. X69851; *Talaromyces flavus* (Kloecker) Stolk & Samson M83262; *Thermoascus crustaceus* (Apinis & Chesters) Stolk M83263; *Uncinocarpus reesii* Sigler & Orr U29394; *Xylaria carpophila* (Pers.) Fr. Z49785. The alignment file has been deposited in TreeBASE (http://www.herbaria.harvard.edu/treebase/index.html).

The aligned data sets for the 18S rDNA were analyzed using CLUSTAL W with options set to exclude gaps and correct for multiple substitutions. Phylogenetic trees were constructed using the neighbour joining method (NJ; Saitou & Nei, 1987) based on the comparison of 1485 sites in the aligned 18S rDNA data set. To evaluate the statistical significance of the resulting NJ trees, bootstrap tests of 1000 random resamplings were performed (Felsenstein, 1985), with identical sequences pruned from the data set.

Phylogenetic analyses of ITS rDNA of ten strains of the *G. penicillioides* aggregate were done using heuristic searches with PAUP version 3.1.1 (Swofford, 1993), using *Pseudallescheria boydii* (GenBank AF181558) as an outgroup, using default settings. Confidence intervals were determined by 1000 bootstrap replicates using heuristic searches.

Sequence similarities in the ITS-1 and ITS-2 regions in rDNA were determined visually in pairwise alignments using CLUSTAL W (Sugita *et al.*, 1999). The alignment was also deposited in TreeBASE.

Results

MORPHOLOGY OF GRAPHIUM-LIKE SYNNEMATOUS FUNGI

The new isolates from the type host and locality (JCM 10496–10499) were morphologically typical (Figs 3–17, 21) of the *Graphium penicillioides* aggregate (Figs 3–25) and possessed similar characters to those that can be determined from the holotype (see Table 4). These isolates also produced mononematous conidiophores abundantly, in which conidial sizes were considerably broader than in the synnematous conidiomata. The new isolates from the Czech Republic were quite similar to JCM 9301 (= CBS 408.84, isolated from *Salix* in the Netherlands; cf. Figs 18–20), and produced orange to brownish colonies, especially on the reverse, on PDA (Difco or Nissui, Fig. 21) and straight (rather than curved) conidia with a truncate base (Figs 3, 4–7, 14–17; cf. Figs 18–20). Although it was not always clear using light microscopy (Figs 3, 8–10), both dense (Figs 15, 17–19) and nodular (Figs 15, 16, 18, 19) annellations were observed in these cultures using SEM. The thick-walled pale brown conidia produced by these strains are more pigmented than the typically hyaline, thin-walled conidia of *Pesotum* species, and rather similar in pigmentation and wall structure to the conidia of species of the *G. putredinis* aggregate. A complete description of *G. penicillioides sensu stricto*,

based on the epitype specimen and culture, is provided below. Supplemental morphological observations on other members of the *Graphium penicillioides* and *G. putredinis* aggregates, ophiostomatoid *Pesotum* species, and other graphium-like hyphomycetes are also provided below.

Cultural characters of *Graphium penicillioides sensu stricto*

The results of the temperature and cycloheximide studies using the Czech strains of *G. penicillioides sensu stricto* are shown in Tables 1 and 2, respectively. The fungus has an optimal growth temperature of 25–30°C. All strains tolerated cycloheximide and grew at 0.5 g/l, although some strains were more inhibited than others at this high concentration.

Phylogeny of graphium-like synnematous fungi in the Ascomycetes based on 18S rDNA sequences

The NJ analysis, using representatives of the Hemiascomycetes as the outgroup (Fig. 1), leads to the following conclusions: (i) The newly sequenced strains of *the G. penicillioides* aggregate belong to the same clade as the other members of this species aggregate sequenced previously (Okada *et al.*, 1998), supported with a 100% bootstrap value; (ii) In the *G. penicillioides* clade, some subclustering is evident (i.e., JCM 9301, 10498 and JCM 8083, 9300, supported with 89% and 71% bootstrap values, respectively; (iii) *Graphium eumorphum* (JCM 9748), *G. fructicola* (JCM 9750) and *G. tectonae* (JCM 9753, ex-type) belong to the *G. putredinis* aggregate subclade (Okada *et al.*, 1998; see also Issakainen *et al.*, 1997) of the *Microascales* clade, supported by a 99% bootstrap value; (iv) The *G. penicillioides* aggregate and *G. putredinis* aggregate clades are sister groups, with 100% bootstrap support, as shown previously by Okada *et al.* (1998); (v) *Graphium album* (JCM 9744) and *G. erubescens* (JCM 9747, ex-type) are included in the *Ophiostomatales* clade, supported with a 100% bootstrap value; (vi) *Graphium rubrum* (JCM 9751, ex-type), *G. silanum* (JCM 9752) and a phialographium-like fungus (JCM 8069) belong to the discomycete clade supported with a 82% bootstrap value; (vii) *Graphium calicioides* clusters with some species of *Capronia* Sacc. and *Exophiala* J. W. Carmich., supported by a 100% bootstrap value, as noted previously (Okada *et al.*, 1998).

Thus, at present, graphium-like synnematous fungi occur in four phylogenetically different groups, the *Microascales* (*Graphium*), *Ophiostomatales* (*Pesotum*), *Chaetothyriales* (*Exophiala*, see below) and probably the discomycetes (undescribed genus/genera, or *Dendrostilbella*).

Sequences of the ITS rDNA of the *Graphium penicillioides* aggregate

The gene tree for the ITS of ten sequenced strains of the *G. penicillioides* aggregate is shown in Fig. 2, with *Pseudallescheria boydii* as an outgroup. The data set included 519 characters, of which 35 were phylogenetically informative in the ingroup. The phylogenetic tree is one of six equally parsimonious trees 52 steps long (CI = 0.971, HI = 0.029, RI = 0.914, RC = 0.888).

In all the examined strains of the *G. penicillioides* aggregate, the 5.8S rDNA sequences were identical and 158 bp long. In ITS-1 and ITS-2, the sequences were identical or very similar among JCM 9301, 10496–10499 (ITS-1: 148 bp in JCM 9301, 149 bp in JCM 10497, 150 bp in JCM 10496, 10468, 10499. ITS-2: 175 bp in JCM 10496–10499, 174 bp in JCM 9301). Sequences were identical in JCM 7440, 9299 (ITS-1: 167 bp. ITS-2: 163 bp), although the colonies of these strains on PDA were considerably different in colour and growth rate (Fig. 21). In JCM 8083 and 9300 the lengths of ITS-1 and ITS-2 were the same (132 bp ITS-1 and 172 bp ITS-2), but there was one bp difference in the ITS-2 sequences. ITS rDNA sequence similarities are shown in Table 3 and % similarity values in additional combinations are as follows. ITS-1: 99.3% (149/150 bp) between JCM 10496/10498/10499 and 10497, 98.7% (148/150 bp) between JCM 9301 and 10496/10498/10499, 99.3% (148/149 bp) between JCM 9301 and 10497. ITS-2: 98.9% (173/175 bp) between JCM 9301 and 10496/10497/10498/10499, 99.4% (171/172 bp) between JCM 8083 and 9300. Based on the sequences of the ITS regions mentioned above, the examined strains in *G. penicillioides* aggregate were divided into four groups.

The four groups that could be visually extracted from the alignment (cf. TreeBASE) correspond with the four clades marked in Fig. 2. These clades may represent phylogenetically distinct species, three of which can presently be named (see Fig. 2, Table 3 and Appendix). The clustering in the ITS gene tree is consistent with the less finely dissecting subclustering in the 18S neighbour joining tree (see Fig. 1). The low bootstrap support in Fig. 2 reflects the relatively few phylogenetically informative sites available for subsampling. However, the clades can be considered relatively robust because of the high consistency index, low homoplasy index, and their occurrence in the strict consensus tree.

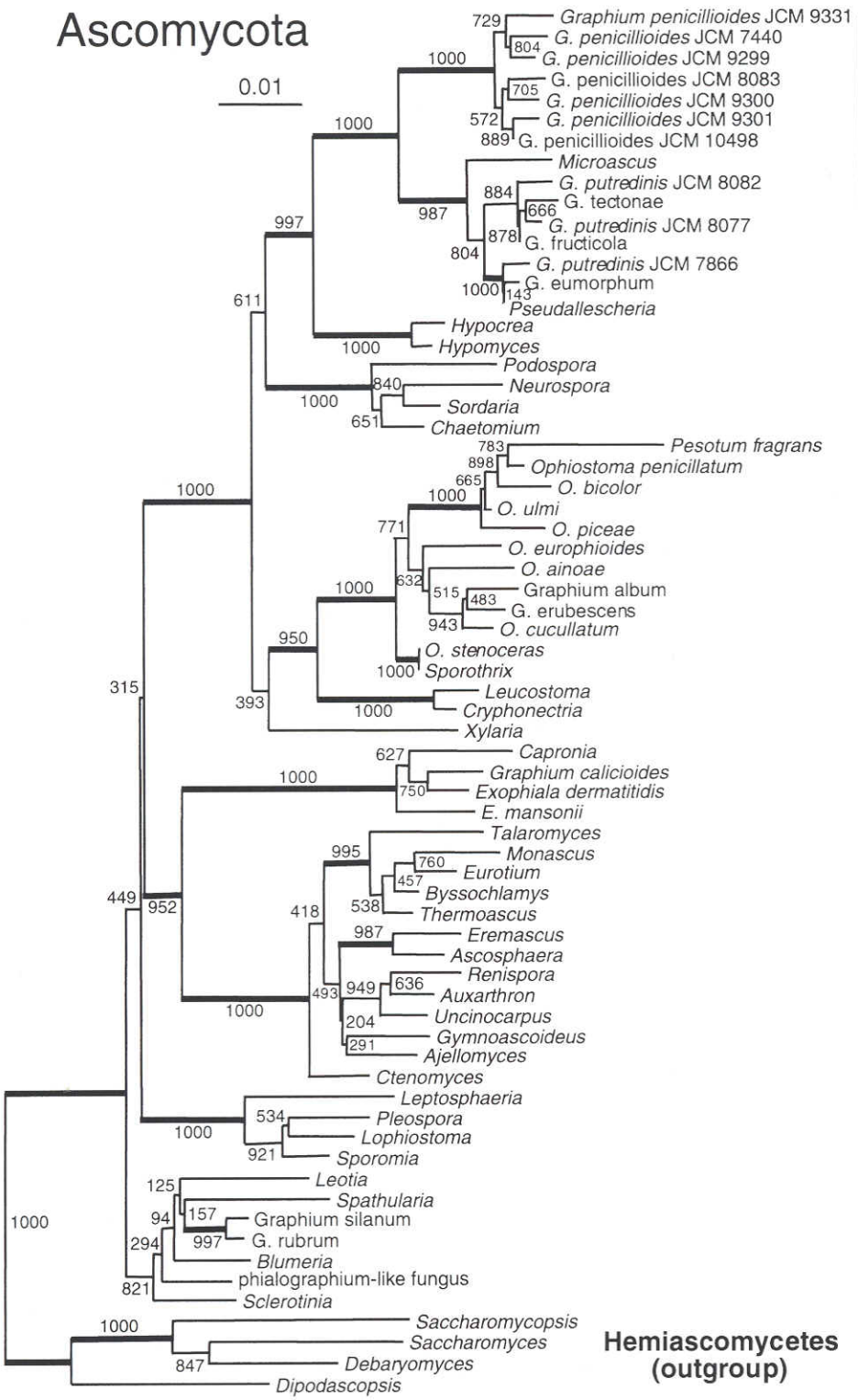


Fig. 1. 18S rDNA sequence-based phylogenetic tree derived using neighbour-joining, showing the disposition of graphium-like synnematous fungi in the *Ascomycota*. Non-italicized Latin names indicate DNA sequences newly determined in this study. The scale bar indicates one base change per 100 nucleotide positions. Bootstrap values were calculated from 1000 replications. Bold lines indicate lineages with more than 95% bootstrap support. *Ajellomyces* = *A. capsulatus*, *Ascosphaera* = *A. apis*, *Auxarthron* = *A. zuffianum*, *Blumeria* = *B. graminis*, *Byssochlamys* = *B. nivea*, *Capronia* = *C. pilosella*, *Chaetomium* = *C. elatum*, *Cryphonectria* = *C. radicalis*, *Ctenomyces* = *C. serratus*, *Debaryomyces* = *D. hansenii*, *Dipodascopsis* = *D. uninucleata*, *Eremascus* = *E. albus*, *Eurotium* = *E. rubrum*, *Gymnoascoideus* = *G. petalosporus*, *Hypocrea* = *H. lutea*, *Hypomyces* = *H. chrysospermus*, *Leotia* = *L. lubrica*, *Leptosphaeria* = *L. bicolor*, *Leucostoma* = *L. persoonii*, *Lophiostoma* = *L. crenatum*, *Microascus* = *M. cirrosus*, *Monascus* = *M. purpureus*, *Neurospora* = *N. crassa*, *Pleospora* = *P. rudis*, *Podospora* = *P. anserina*, *Pseudallescheria* = *P. boydii*, *Renispora* = *R. flavissima*, *Saccharomyces* = *S. cerevisiae*, *Saccharomycopsis* = *S. fibuligera*, *Sclerotinia* = *S. sclerotiorum*, *Sordaria* = *S. fimicola*, *Spathularia* = *S. flavida*, *Sporormia* = *S. lignicola*, *Sporothrix* = *S. schenckii*, *Talaromyces* = *T. flavus*, *Thermoascus* = *T. crustaceus*, *Uncinocarpus* = *U. reesii*, *Xylaria* = *X. carpophila*.

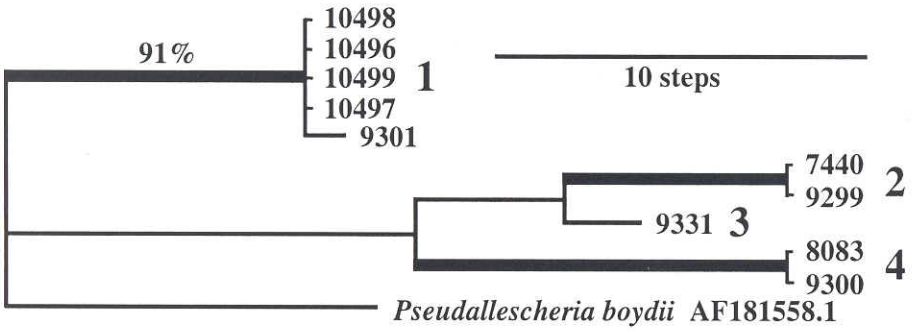


Fig. 2. ITS rDNA (including 5.8S rDNA) sequence-based phylogenetic tree using maximum parsimony, showing a possible arrangement of the *Graphium penicillioides* aggregate into species, with *Pseudallescheria boydii* as an outgroup, one of six equally parsimonious trees. Numbers at the ends of branches represent JCM numbers. Clade 1 represents *Graphium penicillioides sensu stricto*, clade 2 a potentially undescribed species, clade 3 possibly *G. pseudormiticum* and clade 4 *G. basitruncatum*. Heavy lines represent strict consensus branches, with the % figure indicating the only branch supported by bootstrap values greater than 50%.

Taxonomy

The four strains isolated from wood cores of different trees of *Populus nigra* cv. *italica* in České Budějovice, Czech Republic, are almost identical in morphology and in ITS rDNA sequences and are thus considered to represent one species. Because they agree well with the brief, original description of *G. penicillioides* (Corda, 1837), we arbitrarily designate one of them, JCM 10498 (= T. Kirisits No. 3), as epitype strain of *G. penicillioides*. A dried culture grown on *Populus* twigs, the epitype specimen, has been deposited as PRM 842988 and epitype strains have been deposited in Centraalbureau voor Schimmelcultures (CBS 102632) and Japan Collection of Microorganisms (JCM 10498). The species description provided below is based entirely on the epitype specimen and culture of *G. penicillioides*.

Graphium penicillioides Corda, Ic. Fung. 1: 18. 1837.

Colonies on OA after 7 days at 25°C in the dark 1.4–1.7 cm diam, olive-brown (4DF6–7) to dark brown (5F5–7), planar, aerial mycelium sparse, appearing minutely glandular because of synnemata, lacking soluble pigments, margin more or less invisible, reverse brownish gray to grayish brown (5DE2–3). *Colonies on MEA* after 8 days at 25°C up to 1.2 cm diam, buff (19'f) becoming darker with age. Hyphae immersed in medium with sparse aerial mycelium, hyaline to light to olivaceous, smooth, (0.5–)3–4 μm diam.

Synnemata on water agar with *Populus* twigs (50–)75–167 (95.4 ± 3.8) μm tall, scattered but abundantly produced, generally single, sometimes in pairs or triplets, arising from the agar or twig surface, sometimes from aerial mycelium on twigs, with cylindrical, dark brown to black stipes 10–15 μm wide, and divergent light brown to grey capitula, surmounted by watery conidial masses, at first colourless, then white, but quickly becoming olive-brown to almost black, 25–100(–250) μm, becoming confluent, especially near the inoculum. Synnemata sometimes originating from one or two single, clavate hyphae, giving rise to whorls of hyphae that comprise the synnema stipe; other synnemata arising from multiple hyphae and lacking basal clavate hyphae. *Hyphae of stipe* 2–3.5 μm wide, to 5 μm at the base, olivaceous to dark olivaceous, frequently constricted at the septa, the walls thin to slightly thickened; rhizoids absent. *Conidiophore branching* generally biverticillate, with whorls of 2–4 metulae, 10–11.5 × 1.5–2.5 μm, with the apex swollen up to 5 μm diam; basal cells of the branching apparatus slightly brown. *Conidiogenous cells* in whorls of 2–4, 7–18(–26.5) (13.7 ± 0.9) μm long, 1–2 μm wide, cylindrical to subulate, straight or sometimes gently curved, the conidiogenous zone up to about 5 μm long, annellations inconspicuous, sometimes with one or two geniculations. Conidia 3–4(–6) × 1–1.5(–2.5) (3.7 ± 0.1 × 1.5 ± 0.1) μm, L/W ratio 2–3(–4), hyaline, aseptate, cylindrical to obovoid, with rounded apices and subtruncate to truncate bases.

Degenerate synnemata or mononematous conidiophores present, especially on MEA, with conidiophores and conidia basically identical in shape to those found in synnemata, but much more variable in conidial dimensions.

Optimal growth on MEA at 25 or 30°C (Table 1). Resistant to high concentrations of cycloheximide with a 50% reduction in growth on 0.5 g/l cycloheximide in MEA at 25°C (Table 2).

Teleomorph unknown, but the species has affinities to the *Microascales* based on 18S rDNA sequences (Fig. 1).

ITS-1 (uppercase) – 5.8S rDNA (lowercase) – ITS-2 (uppercase) sequences of the epitype culture:
CCGAGTGTTTCACTCCAAACCCACTGTGAACC-
TTACCACTGTCGTTGCTTCGGCGGGCGAAACC-
CCCCCCCGGGGGGCCAGCCCGCCGGCGGCAC
CCAAACTCTTATATCTTACCTAGCGTCTCTTCT
GAGTACAAAAGACAAACAATCAaaactttcaacaacg
gatctcttggctgtggcatcgatgaagaacgcagcgaaatgcgataactaa
tgtgaattgcagaattcagtgaatcatcgagtctttgaacgcacattgcgccc
gctggtattccggcgggcatgcctgtccgagcgtcatttCGTCCCTCA
AGCCCCCGCGCTTGGTGTTGGGCACCCCGCGA
ACCCCCCCGGGGGTCGGCGGGCGCCCCAAAT
GCATCGGCGGTCCCGCCTGGCGGCTCCCTGCGT
AGTAGAACCTCTTCTCGCATCGGGTCCCGGGC
GCGCGGCCGGCCGCTAAACCCCCCAATCGTAC
CAACGG.

HOLOTYPE: *Graphium penicillioides*, Prague, on "*Populus it.*" (= *P. nigra* cv. *italica fide* Holubová-Jechová *in litt.* to K.A.S., 14 Dec. 1988) (PR 155518). Isotype, a slide from the holotype (DAOM 51800).

EPITYPE: PRM 842988 [JCM 10498 = CBS 102632 = T. Kirisits No. 3); epitype designated herewith], Czech Republic, České Budějovice, isolated from wood core of *Populus nigra* cv. *italica*, 3 Sep. 1998, T. Kirisits.

Table 1. Colony diameters of the Czech strains of *Graphium penicillioides* on MEA after eight days at temperatures ranging from 10 to 30°C*.

	Growth temperature (°C)				
Strain	**10**	**15**	**20**	**25**	**30**
JCM 10496	2.1 (± 0.1)	4.9 (± 0.2)	13.9 (± 0.1)	19.2 (± 0.2)	17.2 (± 0.5)
JCM 10497	2.6 (± 0.1)	5.9 (± 0.1)	13.8 (± 0.2)	19.3 (± 0.3)	20.1 (± 0.2)
JCM 10498	2.1 (± 0.1)	4.8 (± 0.1)	12.5 (± 0.6)	17.5 (± 0.4)	16.4 (± 0.3)

* Colony diameters shown in mm ± standard error represent the means of eight measurements in ten plates, excluding the minimum and the maximum measurements.

Table 2. Colony diameters of the Czech strains of *Graphium penicillioides* after eight days at 25°C on MEA amended with increasing concentrations of cycloheximide*.

	Concentrations of cycloheximide (g/l)				
Strain	**0**	**0.01**	**0.05**	**0.1**	**0.5**
JCM 10496	12.3 (± 0.2)	10.9 (± 0.1)	10.2 (± 0.1)	9.8 (± 0.2)	5.9 (± 0.1)
JCM 10497	11.8 (± 0.3)	9.4 (± 0.1)	9.2 (± 0.1)	8.4 (± 0.2)	3.4 (± 0.1)
JCM 10498	11.8 (± 0.4)	10.5 (± 0.2)	10.0 (± 0.1)	9.3 (± 0.1)	6.1 (± 0.1)
JCM 10499	2.7 (± 0.1)	2.9 (± 0.1)	2.9 (± 0.1)	3.0 (± 0.2)	3.2 (± 0.1)

* Colony diameters represent the means of ten measurements in five plates, shown in mm ± standard error.

ADDITIONAL SPECIMENS AND STRAINS EXAMINED.— JCM 10496 (= CBS 102630 = T. Kirisits No. 1), JCM 10497 (= CBS 102631 = T. Kirisits No. 2), JCM 10499 (= CBS 102633 = T. Kirisits No. 4), Czech Republic, České Budějovice, isolated from wood cores of *Populus nigra* cv. *italica*, 3 Sep. 1998, T. Kirisits. JCM 9301 (= CBS 408.84), Netherlands, Zuidelijk Flevoland, Almeerderhout, isolated from wood of *Salix* sp., 1 Apr. 1984, H. A. van der Aa.

EXCLUDED STRAINS TENTATIVELY TREATED AS OTHER *GRAPHIUM* SPECIES IN THE *G. PENICILLIOIDES* AGGREGATE.— JCM 7440 (= CBS 506.86), JCM 9299 (= CBS 470.71); JCM 8083, JCM 9300 (= CBS 320.72); JCM 9331 (= CBS 781.85). Other strains are discussed in the Appendix.

Discussion

The overall phylogenetic relationships of graphium-like fungi demonstrated here conform with those shown by Okada *et al.* (1998), namely:

a) *Graphium penicillioides* and *G. putredinis* form sister clades allied with the *Microascales*. Because this

Table 3. Number of nucleotide differences in ITS-1 and ITS-2 in the *Graphium penicillioides* aggregate[1].

Strain groups (species)	No. of nucleotide differences (bp length)			
	ITS-1	ITS-2	ITS-1 + 2	% similarity of ITS-1 + 2 in each strain group
G. penicillioides				
JCM 10498 [2]	– (150)	– (175)	– (325)	–
JCM 10496	0 (150)	0 (175)	0 (325)	100
JCM 10499	0 (150)	0 (175)	0 (325)	100
JCM 10497	1 (149)	0 (175)	1 (324)	99.7
JCM 9301	2 (148)	2 (174)	4 (322)	98.8
Unidentified *Graphium* species				
JCM 7440 [3]	– (167)	– (163)	– (330)	–
JCM 9299 [3]	0 (167)	0 (163)	0 (330)	100
G. basitruncatum				
JCM 9300 [4]	– (132)	– (172)	– (304)	–
JCM 8083	0 (132)	1 (172)	1 (304)	99.7
"G. pseudormiticum" [5]				
JCM 9331	– (175)	– (165)	– (340)	–

[1] The nucleotide sequences in 5.8S rDNA are identical and 158 bp long in all the strains used.
[2] Ex-epitype strain.
[3] Colonies on PDA were considerably different each other in colour and growth rate.
[4] Ex-type strain.
[5] Tentatively identified as *G. pseudormiticum*.

Table 4. Comparison of the epitype (growing on *Populus* twigs) and holotype specimens of *Graphium penicillioides*. All measurements in μm, based on 25 measurements for the epitype and variable numbers of measurements for the holotype.

	Epitype (PRM 842988)	**Holotype** (PR 155518)
Synnemata		
height	(50–)75–167 (95.4 ± 3.8)	up to 250
width	10–15	10–25(–75)
Hyphae of stipe		
width	2–3.5	1.5–2
Conidiogenous cells		
length	7–18(–26.5) (13.7 ± 0.9)	15–26 (19.4 ± 2.7, n = 4)
width	1–2	1.5–2
Conidia		
shape	cylindrical to obovoid	cylindrical to obovoid
apex	rounded	rounded
base	truncate	truncate
length	3–4(–6.0) (3.7 ± 0.1)	4–5.5 (4.4 ± 0.1, n = 18)
width	1–1.5(–2.5) (1.5 ± 0.1)	1.5–2 (1.6 ± 0.03, n = 18)
L/W	2–4	2.25–3.7

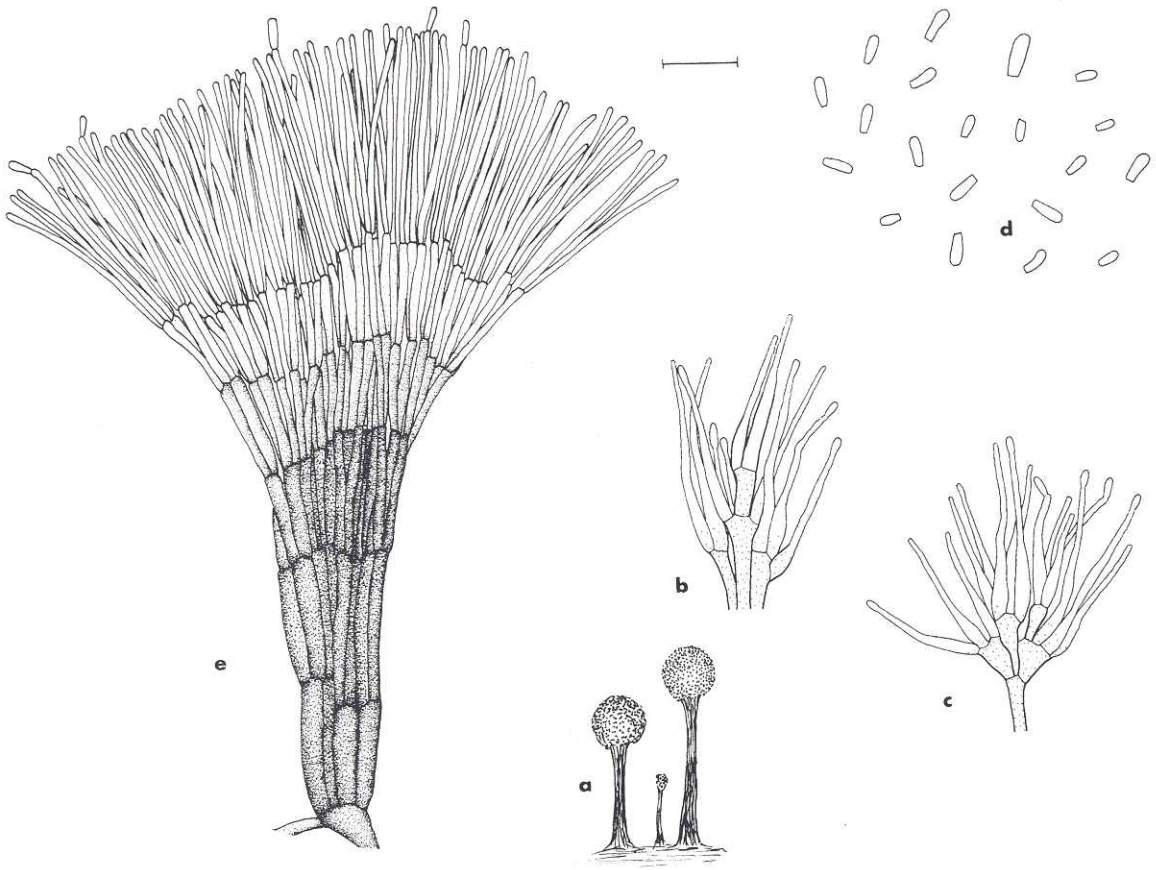


Fig. 3. Line drawings of *Graphium penicillioides* epitype strain on *Populus* twigs in water agar (except e). a. Habit of synnemata (not to scale). b, c. Details of branching of conidiophores dissected out from synnemata. d. Conidia. e. Reduced synnema on MEA. Scale bar = 10 μm.

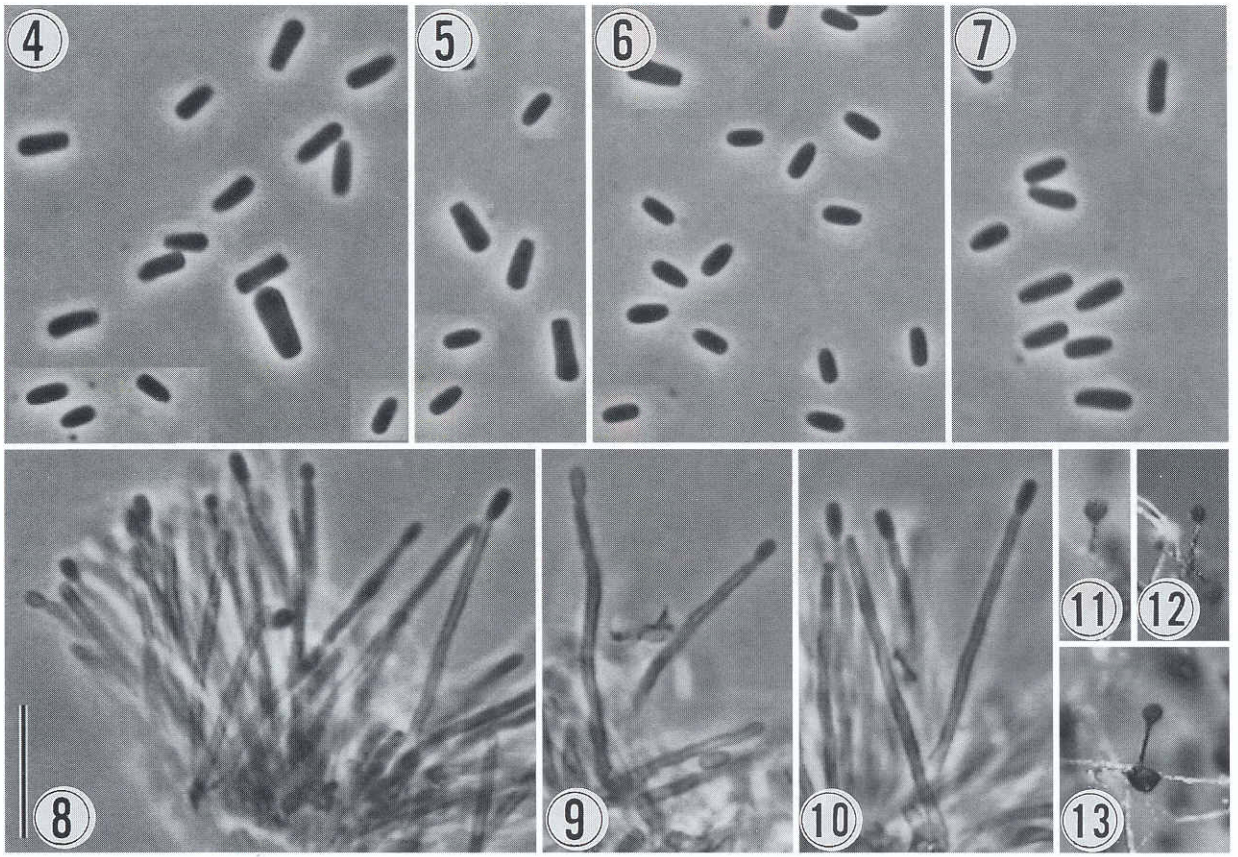
includes the type species of *Graphium*, the generic name should be restricted to fungi related to this ascomycete order.

b) The synnematous anamorphs of *Ophiostoma* species are phylogenetically unrelated to *Graphium sensu stricto*, and should be referred to the anamorph genus *Pesotum*.

c) The phylogenetic affinities of *Graphium calicioides* are with the *Chaetothyriales*.

To this overall pattern, we can add a fourth clade of graphium-like synnematous anamorphs that is probably related to the discomycetes. These includes *Graphium rubrum* (JCM 9751 = CBS 210.34, extype), *G. silanum* (JCM 9752 = CBS 206.37, authentic strain isolated and identified by G. Goidànich) and a phialographium-like unidentified fungus (JCM 8069, common on rotten wood in Japan), which are related to the discomycetes based on their 18S rDNA sequences. In these strains, typical phialidic conidiogenesis was observed using SEM (Figs 33, 34) or light microscopy. This cannot be distinguished morphologically from the conidiogenesis of *Pesotum sagmatosporum* (Upadhyay & Kendrick) Okada & Seifert (originally described in *Phialographium*), the anamorph of *Ophiostoma sagmatosporum* (Wright & Cain) Solheim. Synnematous anamorphs are well-known, but rather sparsely dispersed in the discomycetes. Graphium-like synnematous anamorphs are classified in *Crinula* Fries (teleomorphs in *Holwaya* Sacc.; Seifert & Okada, 1993), *Coryne* Nees (teleomorphs in *Ascocoryne* J. W. Groves & D. E. Wilson; Seifert, 1989) and *Dendrostilbella* Höhnel (teleomorphs in *Claussenomyces* Kirschst.; Seifert, 1985). In fact, one of these species, *Dendrostilbella smaragdina* (Alb. & Schw.) Seifert (see Appendix), with dark green synnemata and phialidic conidiogenous cells, has often been referred to as *Graphium smaragdinum* (Alb. & Schw.) Sacc.

The convergent evolution demonstrated by these four groups of synnematous anamorphs is remarkable. As we have observed in the past, the anamorph of *Ophiostoma columnare* is very similar in micromorphological characters to *G. penicillioides*, differing primarily in the pigmentation of the synnemata (Seifert & Okada, 1993). However, if fasciculation of conidiophores is a banal evolutionary event, then the occurrence of such similar anamorphs in different



Figs 4–13. Light micrographs of the *Graphium penicillioides* epitype culture growing on *Populus* twigs (= epitype specimen). 4–7. Conidia. 8–10. Conidiogenous cells. 11–13. Synnemata. Scale bar in Fig. 8 = 10 μm for Figs 4–10.

clades of the Ascomycetes is unsurprising. As for conidium ontogeny, similar convergent evolution also exists in graphium-like hyphomycetes. Conidiogenous cells in *Graphium sensu stricto* exhibit percurrent proliferation, in common with other members in the *Microascales* (e.g. species of *Scopulariopsis* Bainier, *Cephalotrichum* Link). In *Pesotum* species, intermediate modes between percurrent and sympodial proliferations and phialidic conidiogenesis are frequently observed. *Graphium calicioides*, when grown in culture, is quite reminiscent of the so-called 'black yeasts', producing sessile pustules of slime on the agar surface, and the conidiogenous cells have a characteristic narrowing before percurrent proliferations begin that is quite similar to the conidiogenous cells of *Exophiala* species. Many anamorphs of discomycetes have percurrently proliferating or phialidic conidiogenous cells, which are similar to those illustrated here.

Okada *et al.* (1998) provided preliminary generic diagnoses to distinguish the synnematous anamorphs of two of the three phylogenetic groups recognized. They emphasized differences in conidium ontogeny between the ophiostomatalean and microascalean anamorphs. Pale brown conidia in *G. penicillioides* and the *G. putredinis* aggregate possibly reflect their phylogenetic relationships to the *Microascales*. The colours of conidia, conidiogenous cells, conidiophores and agar colonies sometimes reflect phylogenetic affinities in anamorphic fungi (Okada *et al.*, 1997, 1998). In all the examined strains of the *G. penicillioides* aggregate, we have observed nodular annellations (Figs 15, 16, 18, 19, 22–25), as well as dense annellations (Figs 15, 17, 18, 19, 25). Nodular annellations were frequently observed especially at the base of the percurrently elongating part of the conidiogenous cells (Figs 15, 18, 19). In some strains, dense annellations were more frequently observed than nodular ones. Nodular annellations do not always suggest affinities to the *Microascales* (cf. *Remersonia thermophila* (Fergus) Seifert & Samson related to the *Sordariales*; Seifert *et al.*, 1997).

The morphological data presented here support our previous contention (Seifert & Okada, 1993; Okada *et al.*, 1998) that *G. penicillioides* should be regarded as a species aggregate. Sugita *et al.* (1999), working with species of *Trichosporon* Behrend, observed that conspecific strains have less than a 1% overall nucleotide difference in both the ITS-1 and ITS-2 regions. As indicated clearly in Fig. 2 and Table 3, the examined