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Short title: *Grosmannia serpens* complex

# Phylogeny and taxonomy of species in the *Grosmannia serpens* complex

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***Abstract:*** *Grosmannia serpens* was first described from pine in Italy in 1936 and it has been recorded subsequently from many countries in both the northern and southern hemispheres. The fungus is vectored primarily by root-infesting bark beetles and has been reported to contribute to pine-root diseases in Italy and South Africa. The objective of this study was to consider the identity of a global collection of isolates not previously available and using DNA sequence-based comparisons not previously applied to most of these isolates. Phylogenetic analyses of the ITS2-LSU, actin, beta-tubulin, calmodulin and translation elongation factor-1 alpha sequences revealed that these morphologically similar isolates represent a complex of five cryptic species. *Grosmannia serpens* sensu stricto thus is redefined and comprises only isolates from Italy including the ex-type isolate. The ex-type isolate of *Verticicladiella alacris* was shown to be distinct from *G. serpens*, and a new holomorphic species, *G. alacris*, is described. The teleomorph state of *G. alacris* was obtained through mating studies in the laboratory, confirming that this species is heterothallic. Most of the available isolates,

including those from South Africa, USA, France, Portugal and some from Spain, represent *G. alacris*. The remaining three taxa, known only in their anamorph states, are described as the new species *Leptographium gibbsii* for isolates from the UK, *L. yamaokae* for isolates from Japan and *L. castellanum* for isolates from Spain and the Dominican Republic.

***Key words:*** bark beetle associates, *Grosmannia alacris*, insect/fungus symbiosis, *Leptographium castellanum*, *L. gibbsii*, *L. yamaokae*, multigene phylogeny, Ophiostomatales, pine-root disease

## INTRODUCTION

Species of *Leptographium* Lagerb. & Melin are anamorphic Ascomycota that are characterized generally by dark single and erect conidiophores terminating in complex conidiogenous apparatuses (Jacobs and Wingfield 2001, Kendrick 1962). *Leptographium* is well adapted for insect dispersal by producing conidia in slimy droplets at the apices of these conidiophores. More than 20 species of *Leptographium* are known to have teleomorphs accommodated in the genus *Grosmannia* Goid. (Zipfel et al. 2006), but the sexual states are not known for at least an additional 50 species.

*Leptographium* and *Grosmannia* have a worldwide distribution and the majority are known from conifer hosts (Grosmann 1931, Harrington 1988, Jacobs and Wingfield 2001, Paciura et al. 2010, Wingfield et al. 1988). Only a few species have been described from non-conifer hosts (Jacobs et al. 2006, Jacobs and Wingfield 2001, Paciura et al. 2010). It is widely believed that most *Leptographium* are native to the northern hemisphere where conifers are most common and that they were introduced to other parts of the world with their insect vectors (Harrington 1988, Jacobs and Wingfield 2001, Kendrick 1962, Wingfield et al. 1988, Zhou et al. 2001).

The majority of *Leptographium* species are saprophytes that cause blue stain of timber (Jacobs and Wingfield 2001, Seifert 1993). However some *Leptographium* can cause diseases

of trees that result in serious economic losses (Wingfield et al. 1988). One of the best examples is black-stain root disease caused by the three varieties of *L. wageneri* (W.B. Kendr.) M.J. Wingf. (Cobb 1988, Wagener and Mielke 1961). Other *Leptographium* species, such as *L. terebrantis* S.J. Barras & T.J. Perry and *L. procerum* (W.B. Kendr.) M.J. Wingf., have been associated with disease syndromes, although they are most likely not the primary cause of disease (Alexander et al. 1988, Jacobs and Wingfield 2001, Morrison and Hunt 1988, Wingfield et al. 1988). Of note, *L. procerum* recently was introduced to China with the red turpentine beetle *Dendroctonus valens* and this association has resulted in dramatic death of trees, which is atypical of the insect or fungus in their native North America (Lu et al. 2009a, b).

*Grosmannia serpens* Goid. first was described associated with a root disease of pines in Italy (Goidánich 1936, Lorenzini and Gambogi 1976), and it has been linked more recently to pine decline in USA (Eckhardt et al. 2007). In 1980 a disease similar to the one associated with *G. serpens* in Italy (Goidánich 1936) was reported on *P. radiata* and *P. pinaster* in South Africa and the causal agent was described as *Verticicladiella alacris* M.J. Wingf. & Marasas (Wingfield and Knox-Davies 1980, Wingfield and Marasas 1980). Based on comparisons of the morphological characters of *V. alacris* with the ex-type isolate of *G. serpens*, *V. alacris* subsequently was reduced to synonymy with *L. serpens* (Goid.) Siemaszko, the anamorph of *G. serpens* (Wingfield and Marasas 1981). Apart from Italy and South Africa *L. serpens* also has been recorded in USA, France, UK, Portugal, Spain and the Dominican Republic (Eckhardt et al. 2007, Harrington 1988, Jacobs and Wingfield 2001, Masuya et al. 2009, Wingfield and Gibbs 1991).

The many reports of *L. serpens* and *L. serpens*-like isolates (including those identified as *V. alacris*) and their association with a wide variety of hosts and insects has raised questions regarding their identity. The objective of this study was to reconsider the identity of isolates

originating from all the countries from which the fungus has been reported, applying comparisons of DNA sequences for five gene regions together with morphological characteristics.

## MATERIALS AND METHODS

*Isolates*.—Those used in this study are included herein (TABLE I).

*Morphology*.—Anamorphs were described from malt extract agar (MEA; 2% malt extract and 2% agar, Biolab, Midrand, South Africa). Agar blocks colonized by fungal isolates were transferred to 2% water agar, and heat-sterilized pine twigs were placed on the agar surface to promote conidiophore formation. Plates were incubated at 25 C in the dark 2–3 wk.

*Mating studies*.—Based on the production of anamorph structures as well as on geographic locations, 10 isolates grouping in the phylogenetic analyses with the ex-type culture of *V. alacris* were selected for mating studies (TABLE I). In addition all isolates from the other four lineages were used in the mating studies. Isolates were crossed with the technique described by Grobbelaar et al. (2010) but using pine twigs instead of hardwood twigs. Plates were incubated at 25 C in the dark for up to 3 mo and examined regularly for the presence of ascomata.

*Microscopy*.—Conidiophores and ascomata produced on pine twigs were mounted on microscope slides in 80% lactic acid. Microscopy was done as described by Kamgan Nkuekam et al. (2011). Colors were described with the charts of Rayner (1970).

*Growth studies*.—Optimal temperatures of all species were determined on MEA. Agar disks were removed from the actively growing edges of 5 d old MEA plates with a 5 mm cork borer and were placed mycelium side down in the centers of 90 mm plates containing MEA. Plates were incubated in the dark at 5–35 C at 5 C intervals. Colony diameters were measured daily at a fixed time and the measurements were discontinued when the mycelium reached the edges of the plates. Where possible three isolates per taxon with five replications at each temperature were included in the study. Mean colony diameters (± standard deviation) were determined. Tolerance to cycloheximide was tested by transferring each isolate to MEA containing 0.5 g/L cycloheximide. Incubation was at 25 C and measurements were done as described above.

*DNA extraction, PCR and DNA sequencing*.—Mycelium (ca. 100 mg) grown in YM broth (2% malt extract and 0.2 % yeast extract, Biolab, Midrand, South Africa) was placed in 1.5 mL Eppendorf tubes, to which 50 µL PrepMan Ultra reagent (Applied Biosystems, Foster City, California) had been added. The tubes were heated 5 min at 100 C after which a micro pestle was used to grind the mycelium. After an additional 5 min at 100 C the mixture was centrifuged 5 min at 12 000 rpm. The supernatant was collected and diluted five times with 10 mM

Tris-HCl pH 8.0. Of the diluted DNA solution, 2 µL was used as template for PCR reactions. The remainder was stored at −20 C.

Five gene regions were amplified for sequencing and phylogenetic analysis. The internal transcribed spacer region 2 and partial large subunit (ITS2-LSU) of the ribosomal DNA was amplified with primers ITS3 and LR5 (White et al. 1990). Part of the actin gene (ACT) was amplified with primers Lepact F and Lepact R (Lim et al. 2004). A portion of the β-tubulin gene (βT) was amplified with primers T10 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995), and part of the translation elongation factor-1 alpha (TEF-1α) was amplified with primers EF1-F and EF2-R (Jacobs et al. 2004). PCR with the calmodulin (CAL) primers CL1 and CL2a (O'Donnell et al. 2000) resulted in low amplicon concentrations for the isolates from Japan, while no amplification was obtained from the DNA of the other isolates. Novel CAL primers (CL2F [5′-GACAAGGAYGGYGATGGT-3′] and CL2R [5′-TTCTGCATCATGAGYTGSAC-3′]) thus were designed based on the genome sequence of *Grosmannia clavigera* (Rob.-Jeffr. & R.W. Davidson) Zipfel et al. (GenBank accession number ACYC01000232), *Neurospora crassa* Shear & B.O. Dodge (GenBank accession number AL807366) and partial CAL sequences of *Sporothrix schenckii* Hektoen & C.F. Perkins (GenBank accession number AM117437). These primers amplified the fragment spanning from the second exon to the last exon of the CAL gene. In cases where amplification was difficult, CL2R2 (5′-CTTCTCGCCRATSGASGTCAT-3′) was used in combination with CL2F, but this combination provided shorter fragments.

Reaction mixtures, 25 µL total volume, consisted of 2.5 µL 10× PCR reaction buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP, 0.2 µM each primer (0.4 µM each primer was used in the case of the CAL degenerate primers), 1 U FastStart *Taq* DNA Polymerase (Roche Applied Science, Mannheim, Germany) and 2 µL diluted genomic DNA solution. Amplifications were performed in an Eppendorf MasterCycler® gradient (Eppendorf, Hamburg, Germany) under these conditions: an initial denaturation step at 95 C for 5 min, followed by 35 cycles of 95 C for 30 s, 55 C annealing for 30 s, 72 C extension for 60 s and a final extension step at 72 C for 8 min. The sequencing was done with both forward and reverse primers as used in PCR. Products were purified with High Pure PCR Product Purification Kit (Roche Applied Science, Mannheim, Germany) following the manufacturer's protocols, sequenced with the Big Dye® Terminator 3.1 cycle sequencing premix kit (Applied Biosystems, Foster City, California) employing the forward and reverse primers in PCR and analyzed on an ABI PRISIM® 3100 Genetic Analyzer (Applied Biosystems). Consensus sequences were constructed with ContigExpress, a component of Vector NTI Advance 11 (Invitrogen, Carlsbad, California).

*Phylogenetic analyses*.—To examine the relatedness of *G. serpens*-like isolates with other species of *Grosmannia* and *Leptographium* ITS2-LSU sequences were compared with sequences of 66 species obtained from GenBank. For the final ITS2-LSU dataset only one isolate representing each of the *G. serpens*-like groups was included. Sequences of six species of *Ophiostoma* were used as outgroup. The ITS2 and LSU data were separated from each other and a partition homogeneity test (PHT) was conducted on the two datasets to test the congruence of the two regions with PAUP* 4.0b10 (Swofford 2003).

For the protein-coding genes (ACT, βT, CAL and TEF-1α) sequences of the *G. serpens*-like isolates were analyzed with *L. neomexicanum* M.J. Wingf., T.C. Harr. & Crous (CMW 2079) as outgroup, which was selected based on analyses of the ITS2-LSU data. Alignments were done with an online version of MAFFT 6 (Katoh and Toh 2008). To examine the possibility of combining ACT, βT, CAL and EF-1α data a PHT was conducted with PAUP* 4.0b10 (Swofford 2003). A heuristic search of 1000 replications was performed using the same software with parsimony default settings. Sequences for the protein-coding genes were analyzed separately as well as in a combined dataset. All datasets were subjected to maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) analyses as described below.

MP analyses were performed with PAUP* 4.0b10 (Swofford 2003). One thousand random stepwise addition heuristic searches were performed with tree-bisection-reconnection (TBR) as the branch-swapping algorithm. All characters were unordered and of equal weight. Gaps were excluded. Branches of zero length were collapsed, and all equally parsimonious trees were saved. The robustness of resultant trees was evaluated by 1000 bootstrap replications. Tree length (TL), consistency index (CI), retention index (RI), homoplasy index (HI) and rescaled consistency index (RC) were calculated for the resulting trees.

ML analyses were performed with an online version of PhyML 3.0 (Guindon and Gascuel 2003). The best fit substitution models were determined with jModelTest 0.1.1 (Posada 2008). Confidence supports were estimated with 1000 replication bootstrap analyses.

BI analyses employing a Markov chain Monte Carlo method (MCMC) were performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Evolutionary models were determined with jModelTest 0.1.1 (Posada 2008) and manually converted to MrBayes models. Four MCMC chains were run simultaneously from a random starting tree for 5 000 000 generations. Trees were sampled every 100th generation. After the runs burn-in values were determined with Tracer 1.4 (Rambaut and Drummond 2007). Trees sampled at burn-in were discarded, and posterior probabilities were calculated from a majority rule consensus tree regenerated from the remaining trees.

## RESULTS

*Phylogenetic analyses*.—The number of characters, the substitution models used and other statistical values resulting from the different analyses of the respective datasets are presented (TABLE II). The LSU sequences of our isolates were identical, apart from the three UK isolates that differed by 1 bp. The ITS2 sequences of the UK isolates also differed by 2 bp from the other *G. serpens*-like isolates, and one of these differences was shared with isolates from Spain and the Dominican Republic. Because the PHT did not show that the nuclear rDNA datasets were incongruent ($P = 0.05$) the ITS2 (180–181 bp) and partial LSU sequences (383 bp) were analyzed together. ML (FIG. 1), MP and BI analyses of the ITS2-LSU dataset resulted in trees with similar topologies in the main clades with some variability within some of the subclades. In all trees the five *G. serpens*-like isolates formed a single, poorly supported lineage referred to here as the *G. serpens* complex. This complex formed part of a larger, more strongly supported lineage in *Grosmannia* that included seven other species including *L. neomexicanum* and the three varieties of *L. wageneri* (FIG. 1).

Sequences obtained for the four protein-coding genes (ACT, βT, CAL and TEF-1α) showed substantially more variation among our study isolates than was present in the ribosomal sequences. The majority of polymorphic sites for these gene regions were present in the introns (FIG. 2). ACT was the only exception, where five polymorphic sites were situated in the intron and seven in the exons. MP, ML and BI analyses of the separate protein-coding datasets resulted in trees with similar topologies (FIG. 3). In all trees the *G. serpens*-like isolates separated in five well supported lineages.

The PHT revealed no conflict ($P = 0.057$) among the sequences of the protein-coding genes, and these fragments were combined in a single dataset containing 2577 characters (TABLE II). Trees resulting from MP and BI analyses of this combined dataset had the same overall topology as the ML tree provided (FIG. 4). Similar to the results from the four separate gene regions (FIG. 3), the phylogenetic trees obtained from the combined dataset (FIG. 4)

showed five distinct, well supported lineages in the *G. serpens* complex. The first lineage in these trees, identified as *G. alacris* (FIGS. 3, 4), included the ex-type isolate of *V. alacris* and isolates from South Africa, USA, France, Portugal and some from Spain. The second lineage, identified as *G. serpens* (FIGS. 3, 4), included five isolates from Italy including the ex-type isolate of *G. serpens*. The third lineage, labeled *L. yamaokae*, included six isolates from Japan, while the fourth lineage, designated *L. gibbsii*, incorporated three isolates from the UK. The last lineage, specified *L. castellatum*, consisted of two isolates from the Dominican Republic and two from Spain.

*Morphology and growth in culture*.—Cultures of isolates belonging to the five lineages in the *G. serpens* complex were morphologically similar and all produced serpentine hyphae characteristic of this group. Although the dimensions of most structures that are taxonomically informative for *Leptographium* (Jacobs and Wingfield 2001) overlapped among these isolates, those from Japan could be distinguished by their longer conidiophores and relatively lighter colonies after 7 d on MEA. The optimal temperature for growth of isolates was 25 C, but the isolates from Japan grew slower than the others at this temperature. All isolates grew well on MEA containing cycloheximide with little reduction in growth after 5 d.

*Mating studies*.—Ascomata developed only in crosses between CMW 623 and the ex-type of *V. alacris* (CMW 2844) and between CMW 623 and all other isolates in the same lineage (TABLE I). Isolates acting as opposite mating types were labeled A and B (TABLE I).

## TAXONOMY

Based on DNA sequence analyses of five gene regions, morphology, colony characteristics and mating studies, the isolates formed five distinct lineages. The congruence of sequence data from unlinked loci confirmed that these five lineages represented distinct taxa. Two of these lineages included the ex-type isolates of *G. serpens* and *V. alacris* respectively and thus

represented known taxa for which the descriptions are emended and the nomenclature is updated. The remaining three lineages represented novel taxa that we propose as new species.

*Grosmannia serpens* Goid., Boll. Staz. Patol. Veg. 16:27. 1936. FIG. 5

≡ *Ophiostoma serpens* (Goid.) Arx, Antonie van Leeuwenhoek 18:211. 1952.

≡ *Ceratocystis serpens* (Goid.) C. Moreau, Rev. Mycol. (Paris), Suppl. Colon. 17:22. 1952.

*Anamorph: Leptographium serpens* (Goid.) Siemaszko, Planta Pol. 7:34. 1939.

≡ *Scopularia serpens* Goid., Boll. Staz. Patol. Veg. 16:42. 1936.

≡ *Verticicladiella serpens* (Goid.) W.B. Kendr., Can. J. Bot. 40:781. 1962.

≡ *Leptographium serpens* (Goid.) M.J. Wingf., Trans. Br. Mycol. Soc. 85:92. 1985. [superfluous combination]

MycoBank MB254673

Ascomatal bases black, globose and smooth, without ornamentation, 300–420 µm diam, necks black, cylindrical with a slight apical taper, smooth, 400–700 µm long, 33–65 µm wide at the base, 21–39 µm wide at the apex, ostiolar hyphae absent. Asci prototunicate, evanescent. Ascospores hat-shaped, hyaline, 3.3–4.8 × 1.8–2 µm (based on Goidánich 1936, Jacobs and Wingfield 2001).

Conidiophores occurring singly, arising directly from the mycelium, erect, macronematous, mononematous, (307–)453–722(–916) µm long, rhizoid-like structures present. Stipes dark olivaceous, not constricted, cylindrical, simple, 3–8-septate, (218–)354–623(–852) µm long, apical cell occasionally swollen, (12–)16–24(–28) µm wide at the apex, basal cell not swollen, (11–)14–19(–22) µm wide at the base. Conidiogenous apparatus (57–)81–118(–146) µm long, excluding the conidial mass, with multiple series of cylindrical branches. Primary branches dark olive, smooth, swollen at the apex, aseptate, (22–)28–45(–52) × (5.6–)9.5–19(–27) µm, arrangement of the primary branches on the stipe-type C (more than two branches with large central branch) (Jacobs and Wingfield 2001), secondary branches light olivaceous to hyaline, occasionally swollen, aseptate, (11–)15–26(–31) × (2.5–)3.1–7.0(–13) µm, tertiary branches hyaline, aseptate, (9.8–)12–19(–24) × (2.2–)2.4–4.2(–6.1)

µm, quaternary branches hyaline, aseptate, (7.0–)12–19(–28) × (2.0–)2.4–3.3(–4.2) µm. Conidiogenous cells discrete, 2–3 per branch, cylindrical, tapering lightly at the apex, (10.2–)11–18(–25) × (1.4–)1.8–2.3(–2.8) µm. Conidia hyaline, aseptate, oblong with truncate bases and rounded apices, (3.3–)3.8–5.5(–7.8) × (1.4–)1.8–2.5(–2.8) µm.

Colonies with optimal growth at 25 C on 2% MEA, covering 90 nm plate after 4 d incubation. Little growth below 5 C, no growth above 35 C and little reduction in growth on MEA containing 0.5 g/L cycloheximide after 5 d at 25 C in the dark. Colonies dark mouse gray (15’’’’’k). Colony margin effuse. Hyphae superficial and submerged in agar with no aerial mycelium, serpentine, septate, not constricted at septa.

*Specimens examined*. ITALY. From *Pinus sylvestris*, Jan 1953, *G. Goidánich* (HOLOTYPE, DAOM 34869; ex-holotype living culture CBS 141.36 = CMW 304 = CMW 305); From *P. pinea*, 1987, *P. Gambogi and G. Lorenzini* (CMW 289, CMW 290 = CBS 641.76 = ATCC 34322 = IMI 208636); From *P. pinea*, Nov 1986, *P. Capretii*, (CMW 191, CMW 192).

**Grosmannia alacris** T.A. Duong, Z.W. de Beer & M.J. Wingf. sp. nov. FIG. 6

MycoBank MB561907

*Anamorph: Leptographium*.

≡ *Verticicladiella alacris* M.J. Wingf. & Marasas, Trans. Br. Mycol. Soc. 75:22. 1980.

≡ *Leptographium alacre* (M.J. Wingf. & Marasas) M. Morelet, Annales de la S.S.NA. T.V. 40:44. 1988. [invalid, ICBN Art. 33.4]

Coloniae crescunt optime in 25 C in 2% MEA, usque ad 90 mm diam in 4 d. Margo coloniae effusa. Hyphae in agaro superciales immersaeque, sine mycelio aerio, serpentinae septatae in septis non constrictae. Bases peritheciorum nigrae globosae inornatae (195–)220–259(–283) µm diam. Colla peritheciorum nigra cylindrica apicem versus levuter angustata laeves (139–)369–667(–851) µm sine hyphis ostiolaribus. Asci prototunicati hyalini evanescentes. Ascosporae pileiformes non septatae, hyalinae non vaginatae (2.7–)3.2–3.8(–4.2) × (1.7–)2.0–2.4(–2.5) µm. Conidiophorae singulae proxime e mycelio exorientes erectae mecronematae mononematae (465–)653–925(–1128) µm longae, cum structuris rhizoidiformibus. Stipae atro-olivaceae non constrictae, cylindricae simplices 4–9-plo septatae (389–)580–838(–1041) µm longae. Conidia hyalina non septata, oblonga basibus truncatis apicibus rotundatis (4.5–)5.1–6.1(–6.8) × (1.8–)2.1–2.4(–2.6) µm.

Ascomatal bases black, globose and smooth, without ornamentation, (195–)220–259(–283) µm diam, necks black, cylindrical with a slightly apical taper, smooth, (139–)369–667(–851) µm long, (31–)39–51(–58) µm wide at base, (14–)17.2–27.7(–34.8) µm wide at the apex, ostiolar hyphae absent. Asci prototunicate, evanescent. Ascospores hat-shaped, aseptate, hyaline, without sheath, (2.7–)3.2–3.8(–4.2) × (1.7–)2.0–2.4(–2.5) µm.

Conidiophores occurring singly, arising directly from the mycelium, erect, macronematous, mononematous, (465–)653–925(–1128) µm long, rhizoid-like structures present. Stipes dark olivaceous, not constricted, cylindrical, simple, 4–9-septate, (389–)580–838(–1041) µm long, apical cell occasionally swollen, (9.6–)13–18(–22) µm wide at the apex, basal cell not swollen, (11–)14–20(–23) µm wide at the base. Conidiogenous apparatus (43–)61–100(–125) µm long, excluding the conidial mass, with multiple series of cylindrical branches. Primary branches dark olive, smooth, swollen at the apex, aseptate, (16–)24–40(–51) × (5.9–)7.3–16(–27) µm, arrangement of the primary branches on the stipe-type C (more than two branches with large central branch) (Jacobs and Wingfield 2001), secondary branches light olivaceous to hyaline, occasionally swollen, aseptate, (12–)14–24(–35) × (3.3–)4.0–7.2(–14) µm, tertiary branches hyaline, aseptate, (12–)13–19(–22) × (2.3–)3.0–4.2(–4.9) µm, quaternary branches hyaline, aseptate, (8.8–)11–15(–18) × (2.0–)2.4–3.0(–3.8) µm. Conidiogenous cells discrete, 2–3 per branch, cylindrical, tapering lightly at the apex, (9.4–)11–17(–20) × (1.6–)1.7–2.1(–2.7) µm. Conidia hyaline, aseptate, oblong with truncate bases and rounded apices, (4.5–)5.1–6.1(–6.8) × (1.8–)2.1–2.4(–2.6) µm.

Colonies with optimal growth at 25 C on 2% MEA, covering 90 nm plate after 4 d incubation. Little growth below 5 C and no growth above 35 C. Cycloheximide tolerant with little reduction in growth on MEA containing 0.5 g/L cycloheximide after 5 d at 25 C in the dark. Colonies dark mouse gray (15’’’’’k). Colony margin effuse. Hyphae superficial and submerged in agar with no aerial mycelium, serpentine, septate, not constricted at septa.

*Specimens examined*. PORTUGAL. From *Pinus pinaster*, 1984, *Maria de Fatima Moniz* (HOLOTYPE, PREM 60635, dried culture obtained from cross between CMW 621 = CBS 128830 and CMW 623 = CBS 118621). SOUTH AFRICA. WESTERN CAPE: Tokai. From roots of *P. pinaster*, May 1978, *M. J. Wingfield* (HOLOTYPE of *V. alacris*, PREM 45483, dried culture of CMW 60 = CMW 2844 = CBS 591.79); Grabouw. From roots of *P. pinaster*, Feb 1978, *M. J. Wingfield* (PREM 45484); Lebanon State Forest. From roots of *P. pinaster*, Apr 1978, *M. J. Wingfield* (PREM 45485); Grabouw. From roots of *P. radiata*, Mar 1979, *M. J. Wingfield* (PREM 45486); Jonkershoek. From *P. radiata*, Apr 1984, *M. J. Wingfield* (PREM 56334, dried culture of CMW 310).

*Commentary:* Our results demonstrate that the earlier synonymy of this species with *L. serpens* (Wingfield and Marasas 1981) is incorrect. Morelet (1988) suggested that *V. alacris* represented the anamorph of *O. piceiperdum* (Rumbold) Arx [as '*piceaperdum*'], and suggested a new combination for this anamorphic species in the genus *Leptographium*. However the combination was invalid because the basionym of the species was not clearly indicated in the text (ICBN Art. 33.4, McNeill et al. 2006). Furthermore sequence data (Zipfel et al. 2006) confirmed that *G. piceiperda* represents a distinct species that is not part of the *G. serpens* complex.

**Leptographium gibbsii** T.A. Duong, Z.W. de Beer & M.J. Wingf. sp. nov. FIG. 7

MycoBank MB561915

*Etymology:* Named for Dr John Gibbs who supplied these isolates and in acknowledgement of his tremendous contribution to the discipline of forest pathology.

Coloniae crescunt optime in 25 C in 2% MEA, usque ad 90 mm diam in 4 d. Hyphae in agaro superciales immersaeque, sine mycelio aerio, serpentinae septatae in septis non constrictae. Conidiophorae singulae proxime e mycelio exorientes erectae mecronematae mononematae (438–)640–1067(–1424) µm longae, cum structuris rhizoidiformibus. Stipae atro-olivaceae non constrictae, cylindricae simplices 5–12-plo septatae (367–)545–964(–1279) µm longae. Conidia hyalina non septata, oblonga basibus truncatis apicibus rotundatis (3.8–)4.4–5.8(–6.8) × (1.9–)2.1–2.6(–3.0) µm.

Conidiophores occurring singly, arising directly from the mycelium, erect, macronematous, mononematous, (438–)640–1067(–1424) µm length, rhizoid-like structures

present. Stipes dark olivaceous, not constricted, cylindrical, simple, 5–12-septate, (367–)545–964(–1279) µm long, apical cell occasionally swollen, (13–)15–22(–28) µm wide at the apex, basal cell not swollen, (10.2 –)12–17(–22) µm wide at the base. Conidiogenous apparatus (62–)74–124(–169) µm long, excluding the conidial mass, with multiple series of cylindrical branches. Primary branches dark olive, smooth, swollen at the apex, aseptate, (16–)22–38(–49) × (4.3–)8.1–17.5(–27) µm, arrangement of the primary branches on the stipe-type C (more than two branches with large central branch) (Jacobs and Wingfield 2001), secondary branches light olivaceous to hyaline, occasionally swollen, aseptate, (8.5–)12–22(–32) × (3.4–)3.7–7.8(–15) µm, tertiary branches hyaline, aseptate, (10.5–)13–17(–20) × (2.5–)3.0–4.4(–5.9) µm, quaternary branches hyaline, aseptate, (8.9–)10.1–14(–18) × (2.3–)2.5–3.2(–3.6) µm wide. Conidiogenous cells discrete, 2–3 per branch, cylindrical, tapering lightly at the apex, (8.9–)10.6–14(–17) × (1.4–)1.7–2.1(–2.5) µm. Conidia hyaline, aseptate, oblong with truncate bases and rounded apices, (3.8–)4.4–5.8(–6.8) × (1.9–)2.1–2.6(–3.0) µm.

Colonies with optimal growth at 25 C on 2% MEA, covering 90 nm plate after 4 d incubation. Little growth below 5 C, no growth above 35 C and little reduction in growth on MEA containing 0.5 g/L cycloheximide after 5 d at 25 C in the dark. Colonies dark mouse gray (15’’’’’k). Colony margin effuse. Hyphae superficial and submerged in agar with no aerial mycelium, serpentine, septate, not constricted at septa.

*Specimens examined:* UNITED KINGDOM. ENGLAND: Hampshire, Yaterley Heath Wood. From *Hylastes ater* on *Pinus* sp., May 1988, *J. Gibbs* (HOLOTYPE, PREM 60636, dried culture of CMW 1376 = CBS 128695).

**Leptographium yamaokae** T.A. Duong, Z.W. de Beer & M.J. Wingf. sp. nov. FIG. 8

MycoBank M 561916

*Etymology:* Named for Dr Yuichi Yamaoka who first collected this species, and to acknowledge his contributions to the taxonomy of ophiostomatoid fungi and the understanding of bark beetle-fungus interactions.

Coloniae crescunt optime in 25 C in 2% MEA, usque ad 90 mm diam in 6 d. Hyphae in agaro superciales immersaeque, aliquando cum mycelio aerio, serpentinae septatae in septis non constrictae. Conidiophorae singulae proxime e mycelio exorientes erectae mecronematae mononematae (642–)874–1199(–1390) µm longae, cum structuris rhizoidiformibus. Stipae atro-olivaceae non constrictae, cylindricae simplices 5–11-plo septatae (583–)779–1084(–1297) µm longae. Conidia hyalina non septata, oblonga basibus truncatis apicibus rotundatis (3.3–)4.0–5.7(–8.3) × (1.7–)2.0–2.4(–2.8) µm.

Conidiophores occurring singly, arising directly from the mycelium, erect, macronematous, mononematous, (642–)874–1199(–1390) µm long, rhizoid-like structures present. Stipes dark olivaceous, not constricted, cylindrical, simple, 5–11-septate, (583–)779–1084(–1297) µm long, apical cell occasionally swollen, (9.2–)12–18(–22) µm wide at the apex, basal cell not swollen, (14–)16–22(–25) µm wide at the base. Conidiogenous apparatus (60–)84–127(–156) µm long, excluding the conidial mass, with multiple series of cylindrical branches. Primary branches dark olive, smooth, swollen at the apex, aseptate, (17–)26–54(–70) × (4.5–)6.6–14(–21) µm, arrangement of the primary branches on the stipe-type C (more than two branches with large central branch) (Jacobs and Wingfield 2001), secondary branches light olivaceous to hyaline, occasionally swollen, aseptate, (13–)16–26(–32) × (3.1–)3.3–7.0(–13) µm, tertiary branches hyaline, aseptate, (9.2–)12–20(–28) × (2.0–)2.4–3.8(–5.0) µm, quaternary branches hyaline, aseptate, (9.5–)10.4–15(–19) × (1.8–)1.9–2.7(–3.7) µm. Conidiogenous cells discrete, 2–3 per branch, cylindrical, tapering lightly at the apex, (8.3–)10.6–14(–15.5) × (1.2–)1.6–2.1(–2.4) µm. Conidia hyaline, aseptate, oblong with truncate bases and rounded apices, (3.3–)4.0–5.7(–8.3) × (1.7–)2.0–2.4(–2.8) µm.

Colonies with optimal growth at 25 C on 2% MEA, covering 90 nm plate after 6 d incubation. Little growth below 5 C, and no growth above 35 C and little reduction in growth on MEA containing 0.5 g/L cycloheximide after 5 d at 25 C in the dark. Colonies isabelline (19’’i) to dark mouse gray (15’’’’’k). Colony margin effuse. Hyphae superficial and

submerged in agar with occasional aerial mycelium, serpentine, septate, not constricted at septa.

*Specimens examined:* JAPAN. KOFU: Yamanashi. From dead tree of *Pinus densiflora*, Jul 1996, *H. Masuya* (HOLOTYPE, PREM 60637, dried culture of CMW 4726 = CBS 129732); Yamanashi. From dead tree of *P. densiflora*, Jul 1996, *Y. Yamaoka* (PREM 60638, dried culture of CMW 1944 = CBS 128696).

**Leptographium castellanum** T.A. Duong, Z.W. de Beer & M.J. Wingf. sp. nov. FIG. 9

MycoBank MB561918

*Etymology:* Name denotes the official language (Castilian; *castellano* in Spanish) of the two countries where this fungus was first collected.

Coloniae crescunt optime in 25 C in 2% MEA, usque ad 90 mm diam in 4 d. Hyphae in agaro superciales immersaeque, sine mycelio aerio, serpentinae septatae in septis non constrictae. Conidiophorae singulae proxime e mycelio exorientes erectae mecronematae mononematae (595–)735–1002(–1277) µm longae, cum structuris rhizoidiformibus. Stipae atro-olivaceae non constrictae, cylindricae simplices 7–12-plo septatae (525–)643–900(–1187) µm longae. Conidia hyalina non septata, oblonga basibus truncatis apicibus rotundatis (4.1–)4.6–5.4(–6.1) × (1.8–)2.0–2.4(–2.6) µm.

Conidiophores occurring singly, arising directly from the mycelium, erect, macronematous, mononematous, (595–)735–1002(–1277) µm long, rhizoid-like structures present. Stipes dark olivaceous, not constricted, cylindrical, simple, 7–12-septate, (525–)643–900(–1187) µm long, apical cell occasionally swollen, (13–)14–20(–25) µm wide at the apex, basal cell not swollen, (11–)14–20(–26) µm wide at the base. Conidiogenous apparatus (59–)75–120(–142) µm long, excluding the conidial mass, with multiple series of cylindrical branches. Primary branches dark olive, smooth, swollen at the apex, aseptate, (24–)30–51(–73) × (5.6–)9.5–20(–26) µm, arrangement of the primary branches on the stipe-type C (more than two branches with large central branch) (Jacobs and Wingfield 2001), secondary branches light olivaceous to hyaline, occasionally swollen, aseptate, (9.8–)17–28(–36) × (3.3–

)4.2–10.2(–16) µm, tertiary branches hyaline, aseptate, (6.7–)12–18(–22) × (2.1–)2.7–3.8(–5.6) µm, quaternary branches hyaline, aseptate, (9.0–)10.7–17(–21) × (1.4–)2.0–3.1(–3.7) µm. Conidiogenous cells discrete, 2–3 per branch, cylindrical, tapering lightly at the apex, (9.8–)13–19(–25) × (1.5–)1.9–2.7(–3.3) µm. Conidia hyaline, aseptate, oblong with truncate bases and rounded apices, (4.1–)4.6–5.4(–6.1) × (1.8–)2.0–2.4(–2.6) µm.

Colonies with optimal growth at 25 C on 2% MEA, covering 90 nm plate after 4 d incubation. Little growth below 5 C, no growth above 35 C and little reduction in growth on MEA containing 0.5 g/L cycloheximide after 5 d at 25 C in the dark. Colonies dark mouse gray (15’’’’’k). Colony margin effuse. Hyphae superficial and submerged in agar with no aerial mycelium, serpentine, septate, not constricted at septa.

*Specimens examined:* DOMINICAN REPUBLIC. San José de las Matas. From *Pinus occidentalis*, 1991, *R. Webb* (HOLOTYPE PREM 60639, dried culture of CMW 2321 = CBS 128697); (PREM 60640, dried culture of CMW 2320 = CBS 128698).

## DISCUSSION

Multigene sequence data generated in this study provided strong phylogenetic evidence supporting five cryptic species in *G. serpens* complex. Of the five gene regions used, only ITS2-LSU data did not distinguish among the species. This was not unexpected however because authors (Lim et al. 2004, Paciura et al. 2010) failed to gain effective resolution of species in the *Grosmannia-Leptographium* complexes using this gene region. In contrast sequence data from the four protein-coding genes strongly supported the separation of the five species. In the case of the ACT dataset the type isolate (CMW 2844) of *G. alacris* was separated from the other *G. alacris* isolates (FIG. 4) due to transition at two base pair positions (FIG. 2). The low sequence variation in this gene region allows such small changes to affect the resulting trees. However this isolate grouped in the *G. alacris* lineage for all three of the other protein-coding genes and this was also true in the combined dataset. Among the four protein-coding genes used in this study, βT and TEF-1α are the most variable regions and thus

the most informative phylogenetically. Although the CAL gene was slightly less variable than βT and TEF-1α, the region complemented the other gene regions well. New primers developed in this study to amplify a portion of the CAL gene for this species complex might prove useful in studies of the phylogeny of other species complexes in *Grosmannia* and *Leptographium*.

Two of the five lineages identified in this study accommodated the ex-type isolates of *G. serpens* and *V. alacris* respectively and thus represented these species. For the latter species the sexual stage was induced to form in culture through pairing of different isolates. This provided material to revive the name of the fungus including a teleomorph state as *G. alacris*. Because no sexual state could be obtained for isolates in the remaining three lineages these isolates were described as novel species of *Leptographium*.

A mating compatibility experiment also confirmed heterothallism in *G. alacris*. This was a fortuitous discovery because only one of the 10 *G. alacris* isolates used in mating study was able to cross with the other nine isolates to form sexual structures. It is likely that the other four species in the complex are also heterothallic but that the limited number of available isolates represented only one mating type and thus led to the failure of the matings. This is probably the reason why the sexual state of *G. serpens* has never been observed in culture (Goidánich 1936, Hunt 1956).

The ex-type isolate of *G. serpens* grouped in a lineage including other isolates from Italy that were collected and reported as *L. serpens* in Gambogi and Lorenzini (1977), Lorenzini and Gambogi (1976) and Wingfield et al. (1988). The morphology of these isolates corresponded with the ex-type isolate that originally was described in the same paper as the genus *Grosmannia* (Goidánich 1936). The genus was treated later as a synonym of *Ceratocystis* (Hunt 1956, Moreau 1952, Upadhyay 1981) and then as *Ophiostoma* (Harrington 1988, von Arx 1952), resulting in *G. serpens* being treated in both these genera by different

authors. Zipfel et al. (2006) showed that *Grosmannia* (mostly with *Leptographium* anamorphs) and *Ophiostoma* (with *Sporothrix* and/or *Pesotum* anamorphs) are phylogenetically distinct and reinstated *Grosmannia* with *G. serpens* as one of 27 species in the genus. However sexual structures have never been observed for *G. serpens* subsequent to the first description of the species (Gambogi and Lorenzini 1977, Hunt 1956, Jacobs and Wingfield 2001, Siemazsko 1939, Upadhyay 1981, Wingfield and Marasas 1981).

The original collection of *G. serpens* was from *Pinus sylvestris*, and the fungus was described as an agent causing root disease in this tree species (Goidánich 1936). The species was discovered later also on stained *P. pinea* wood in Italy (Gambogi and Lorenzini 1977, Lorenzini and Gambogi 1976, Wingfield et al. 1988). More recently *G. serpens* was found in association with *Tomicus destruens* infesting *P. pinea* and *P. pinaster* (Sabbatini Peverieri et al. 2006) and in coarse woody debris in *P. pinea* forests in central Italy (Santini et al. 2008). In the latter two studies the fungus was identified based only on morphological characters, but it is probable that these isolates also represent *G. serpens*. Other reports of *L. serpens* based on morphology only from the Czech Republic (Kotýnková-Sychrová 1966) and Spain (Pestaña and Santolamazza-Carbone (2010) could represent any of the four cryptic species in the complex present in Europe. DNA sequence data from the original or fresh isolates will be required to confirm these reports.

*Verticicladiella alacris*, a species originally described in association with pine root disease in South Africa (Wingfield and Knox-Davies 1980, Wingfield and Marasas 1980), is member of a lineage in our tree (*G. alacris* in FIG. 4) that corresponds with two closely related lineages in a cluster analyses based on isozyme profiles containing the isolate of *V. alacris* (C297 = CMW 2844) and several other South African (C56, 297, 304, 306, 307, C141), USA (153, 169, 175) and Spanish (305) isolates (FIG. 1, Zambino and Harrington 1992). These two lineages were treated as *L. serpens* together with a third, more distant

lineage containing two Italian isolates (C30 = CMW 304, C79 = CMW 290), shown in our study to represent *G. serpens* (FIG. 4). Our results thus suggest that a more accurate position for delineating species would be around 0.9 on the x axis in the dendrogram presented by Zambino and Harrington (1992). This also would reflect more accurately the cut-off for species in the *G. clavigera* complex further down on their cladogram as delimited in several multigene and population studies (Alamouti et al. 2011, Lee et al. 2005, Roe et al. 2010, Six et al. 2011).

Of all species in the *G. serpens*-complex the newly described *G. alacris* has the widest distribution, including isolates from France, Portugal, Spain, South Africa and USA. Of these the European isolates were from *P. pinaster* while in South Africa the species is closely associated with the non-native pine-infesting bark beetles, *Hylastes angustatus* and *Hylurgus ligniperda* found on *P. elliottii*, *P. patula*, *P. pinaster* and *P. radiata* (Wingfield and Knox-Davies 1980, Wingfield and Marasas 1980, Zhou et al. 2001). This strongly implies a European origin for the South African fungus. USA isolates of *G. alacris* were from *P. strobus* in Virginia (Lackner and Alexander 1983) and *P. taeda* in Mississippi (Zambino and Harrington 1992). There are various root-feeding bark beetles native to USA that carry *Grosmannia*, so the fungus could be native in that country. Alternatively it could have been introduced into USA. This question has interesting and potentially important quarantine implications and will require further study.

Sequences for *G. alacris* exhibited a substantial level of variability within each of four protein coding gene regions (FIG. 4). This is most likely the consequence of widespread recombination resulting from sexual reproduction. The fact that the isolate from Portugal (CMW 623) crossed with isolates from South Africa, USA and Spain supports this hypothesis.

*Grosmannia serpens* has been reported (based on morphology) from native beetles on *P. taeda* and *P. palustris* from Alabama and Georgia respectively in USA (Eckhardt et al. 2007, Zanzot et al. 2010) and in low frequencies from the introduced bark beetle, *Hylurgus ligniperda*, on *P. halepensis* and *P. pinea* in California (Kim et al. 2010). Our results suggest the isolates reported as *G. serpens* in these studies might represent *G. alacris*. In the study of Kim et al. (2010) a partial ITS2-LSU sequence was produced for a single isolate. When compared with sequences generated in this study those of Kim et al. (2010) shared two unique base pairs in the LSU region with *L. castellanum*. However because sequences for this gene region do not distinguish among all five cryptic species in the complex sequence data from the protein-coding genes will be needed to identify the above-mentioned isolates from USA.

*Leptographium gibbsii*, one of the three novel taxa emerging from this study, was represented by only three isolates. These isolates originally were identified as *L. serpens* from *Hylastes ater* and *H. opacus* infesting billets of *P. sylvestris* in UK (Wingfield and Gibbs 1991). Neither *L. serpens* nor *V. alacris* has been reported from *H. ater* where the beetle has been examined for fungi elsewhere in the world (Harrington 1988, Jacobs and Wingfield 2001, Reay et al. 2005, Romón et al. 2007, Wingfield et al. 1988, Zhou et al. 2004). We also were unable to locate reports of fungal associates of *H. opacus* other than the study of Wingfield and Gibbs (1991), which was the source of isolates used in the present study. It thus appears that *L. gibbsii* is not a common associate of these two beetles.

Isolates of *L. yamaokae* were collected from *Pinus* in Japan (Masuya et al. 2003, 2009). In an extensive survey of ophiostomatoid fungi on *P. densiflora* its many bark beetle associates and their galleries *L. yamaokae* was isolated from only 10 of 20 *Hylastes plumbeus* galleries and from two of 48 beetles of this species (Masuya et al. 2009). Interestingly Aoshima (1965) listed several *Grosmannia* [as '*Ceratocystis*'] and *Leptographium* [as '*Verticicladiella*'] in his investigation on wood-staining fungi from Japan, but none of the descriptions of those species

match that of *L. yamaokae*. Neither *L. yamaokae* nor any fungus resembling *G. serpens* have been found in recent studies of ophiostomatoid fungi on pine in Korea (Kim et al. 2005) and China (Lu et al. 2009a, 2009b, Paciura et al. 2010). Considering the absence of this species from these studies in the Far East and the large number of beetle species investigated by Masuya et al. (2009), the association between *L. yamaokae* and *H. plumbeus* could be quite specific.

Isolates of *L. castellanum* described in this study originated from an unknown *Hylurgus* species in Spain and from *P. occidentalis* in the Dominican Republic. The presence of this fungus in these two countries might be linked to the fact that the Dominican Republic was a colony of Spain for three centuries, which would have allowed for the easy movement of wood or wood products between them.

In 1982 *L. galleciae* Fern. Magán [as '*Gallaeciae*'] was described from stressed *P. pinaster* trees with root damage in Galicia, northwestern Spain (De Ana Magán 1982). Although De Ana Magán described the morphology of the holomorph, only the anamorph state was provided with a Latin binomial because they could not induce a sexual state in culture. The teleomorph they described and illustrated from wood had hat-shaped ascospores similar to those known for some members of the *G. serpens* complex. However the species description was invalid because it lacked a formal Latin diagnosis (ICBN Art. 36.1, McNeill et al. 2006). In a subsequent paper the Latin diagnosis was provided (De Ana Magán 1983), yet the name remained invalid because no reference was made in either of the two papers to a holotype specimen (ICBN Art. 37.1, McNeill et al. 2006). The morphological description of "*L. galleciae*" overlaps largely with those of the two species reported in the present study from Spain, namely *G. alacris* and *L. castellanum,* except for the fact that the ascospores of "*L. galleciae*" are wider than those of *G. alacris* and the conidia of "*L. galleciae*" are smaller than those of both *G. alacris* and *L. castellanum*. Without material linked to the original

collection of De Ana Magán a neotype cannot be designated to validate "*L. galleciae*", and no comparisons are possible between that species and those from the *G. serpens* complex described here.

Of the five species in the complex, *G. serpens* and *G. alacris* have been found associated with root diseases of pines. *Grosmannia serpens* was isolated from symptomatic *P. pinea* trees with a root disease in Italy (Lorenzini and Gambogi 1976) and *G. alacris* was isolated from diseased roots of *P. pinaster* and *P. radiata* in South Africa (Wingfield and Knox-Davies 1980, Wingfield and Marasas 1980). Studies also showed that *G. alacris* can cause lesions when inoculated in pine seedlings and mature trees (Eckhardt et al. 2004, Zhou et al. 2002). None of these authors suggested that this fungus is a serious primary pathogen (Eckhardt et al. 2004, Matusick et al. 2008, Zhou et al. 2002). Yet among the fungi associated with conifer root-infesting bark beetles those in the *G. serpens* complex are among the most pathogenic. Because many of their vectors mature while feeding on healthy roots, these fungi might contribute to disease development in some situations. In this regard they deserve to be studied further.

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## LEGENDS

FIG. 1. ML tree derived from analysis of ITS2-LSU dataset. Bold branches have posterior probabilities ≥ 95. Bootstrap values above 75% are indicated at nodes as ML/MP. * = bootstrap values < 75%.

FIG. 2. Polymorphism sites in the protein coding gene regions of the five species in *G. serpens* complex. Numbers above column indicate the relative positions in the alignments. Sites indicated by * form part of spliceosomal introns, while sites in exons are indicated with –. Numbers in brackets present the number of polymorphism sites/total number of characters. Only one isolate per haplotype is listed.

FIG. 3. ML trees derived from analyses of ACT, βT, CAL and EF-1α datasets. Bold branches have posterior probabilities ≥ 90. Bootstrap values above 70% are indicated at nodes as ML/MP. * = bootstrap values < 70%.

FIG. 4. ML tree derived from analysis of combined data set (ACT, βT, CAL and EF-1α). Bold branches have posterior probabilities ≥ 99. Bootstrap values above 75% are indicated at nodes as ML/MP. * = bootstrap values < 75%.

FIG. 5. *Grosmannia serpens* (CMW 290). A. Conidiophore. B. Conidiogenous apparatus. C. Conidia. D. Serpentine hyphae. E. Conidiogenous cells. Bars: 1, 2, 4 = 50 µm; 3, 5 = 10 µm.

FIG. 6. *Grosmannia alacris* (CMW 6187). A. Conidiophore. B. Conidiogenous apparatus. C. Conidia. D. Serpentine hyphae. E. Conidiogenous cells. F, G. Ascoma with globose base, with conidiophore in the background (CMW 621 x CMW 623). H, I. Hat-shaped ascospores. J. Ostiole with ascospores. Bars: 1, 2, 4, 6, 7 = 50 µm; 3, 5, 8, 9, 10 = 10 µm.

FIG. 7. *Leptographium gibbsii* (CMW 1376). A. Conidiophore. B. Conidiogenous apparatus. C. Conidia. D. Serpentine hyphae. E. Conidiogenous cells. Bars: 1, 2, 4 = 50 µm; 3, 5 = 10 µm.

FIG. 8. *Leptographium yamaokae* (CMW 4726). A. Conidiophore. B. Conidiogenous apparatus. C. Conidia. D. Serpentine hyphae. E. Conidiogenous cells. Bars: 1, 2, 4 = 50 µm; 3, 5 = 10 µm.

FIG. 9. *Leptographium castellanum* (CMW 2321). A. Conidiophore. B. Conidiogenous apparatus. C. Conidia. D. Serpentine hyphae. E. Conidiogenous cells. Bars: 1, 2, 4 = 50 µm; 3, 5 = 10 µm.

## FOOTNOTES

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TABLE I. Fungal isolates used in this study

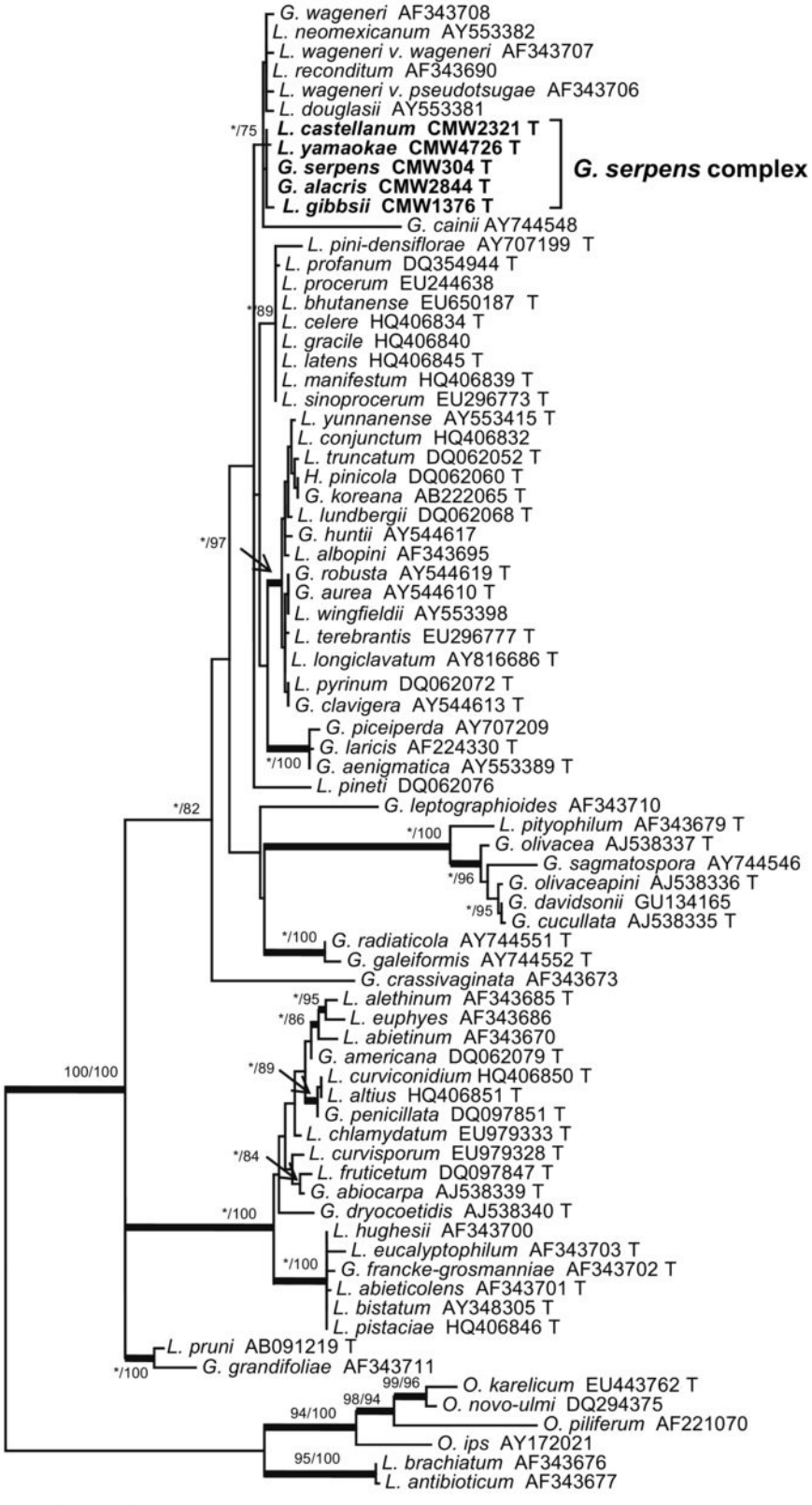
| Species | Isolate number | | Mating type | Host | Origin | GenBank accession numbers | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | [a]CMW | [b]Other | | | | ITS2-LSU | ACT | βT | CAL | TEF-1 α |
| *G. alacris* | [c]CMW 60 = CMW 2844 | CBS 591.79 | A | *Pinus pinaster* | South Africa | JN135313 | JN135320 | JN135329 | JN135296 | JN135304 |
| | CMW 310 | | NT | *P. radiata* | South Africa | | =JN135321 | =JN135327 | = JN135296 | = JN135304 |
| | [T]CMW 621 | CBS 128830 | A | *P. pinaster* | Portugal | | JN135318 | JN135327 | = JN135296 | JN135305 |
| | [T]CMW 623 | CBS 118621 | B | *P. pinaster* | Portugal | | =JN135321 | =JN135327 | = JN135295 | JN135306 |
| | CMW 746 | | A | *P. pinaster* | France | | =JN135321 | =JN135329 | = JN135296 | = JN135302 |
| | CMW 1136 | C 153 | A | *P. taeda* | USA | | JN135321 | JN135328 | JN135295 | JN135302 |
| | CMW 1137 | C 169 | A | *P. strobus* | USA | | JN135319 | =JN135327 | = JN135296 | JN135303 |
| | CMW 6187 | | A | *Hylastes angustatus* | South Africa | | =JN135321 | =JN135329 | = JN135296 | = JN135304 |
| | CMW 6188 | | A | *H. angustatus* | South Africa | | =JN135321 | =JN135329 | = JN135296 | = JN135304 |
| | CMW 7700 | | NT | *H. angustatus* | South Africa | | =JN135321 | =JN135329 | = JN135296 | = JN135304 |
| | CMW 7726 | | NT | *H. angustatus* | South Africa | | =JN135321 | =JN135329 | = JN135296 | = JN135304 |
| | CMW 25936 | | A | *P. pinaster* | Spain | | =JN135318 | =JN135327 | = JN135295 | = JN135303 |
| | CMW 25937 | | A | *P. pinaster* | Spain | | =JN135318 | =JN135327 | = JN135295 | = JN135303 |
| *G. serpens* | CMW 191 | | | *P. pinea* | Italy | | =JN135325 | =JN135334 | = JN135300 | = JN135307 |
| | CMW 192 | | | *P. pinea* | Italy | | =JN135325 | =JN135334 | = JN135300 | = JN135307 |
| | CMW 289 | | | *P. pinea* | Italy | | =JN135325 | =JN135334 | = JN135300 | = JN135307 |
| | CMW 290 | CBS 641.76 | | *P. pinea* | Italy | | =JN135325 | =JN135334 | = JN135300 | = JN135307 |
| | [T]CMW 304 = CMW 305 | CBS 141.36 | | *P. sylvestris* | Italy | JN135314 | JN135325 | JN135334 | JN135300 | JN135307 |
| *L. castellanum* | CMW 1988 | | | *Hylurgus* sp. | Spain | | =JN135324 | =JN135333 | = JN135299 | = JN135310 |
| | CMW 1989 | | | *Hylurgus* sp. | Spain | | =JN135324 | =JN135333 | = JN135299 | = JN135310 |
| | CMW 2320 | CBS 128698 | | *P. occidentalis* | Dominican Republic | | =JN135324 | =JN135333 | = JN135299 | = JN135310 |
| | [T]CMW 2321 | CBS 128697 | | *P. occidentalis* | Dominican Republic | JN135317 | JN135324 | JN135333 | JN135299 | JN135310 |
| *L. gibbsii* | CMW 853 | CBS 347.90 | | *H. ater* | UK | =JN135322 | =JN135330 | = JN135297 | JN135312 | =JN135322 |
| | [T]CMW 1376 | CBS 128695 | | *H. ater* | UK | JN135316 | JN135322 | JN135330 | JN135297 | JN135308 |
| | CMW 36371 | | | *H. opacus* | UK | =JN135322 | =JN135330 | = JN135297 | = JN135312 | =JN135322 |
| *L. neomexicanum* | CMW 2079 | CBS 168.93 | | *P. ponderosa* | USA | AY553382 | JN135326 | AY534930 | JN135301 | AY536176 |
| *L. yamaokae* | CMW 1935 | | | *Pinus* sp. | Japan | | =JN135323 | JN135331 | = JN135298 | JN135309 |
| | CMW 1944 | CBS 128696 | | *Pinus* sp. | Japan | | =JN135323 | =JN135331 | = JN135298 | = JN135309 |
| | [T]CMW 4726 | CBS 129732 | | *P. densiflora* | Japan | JN135315 | JN135323 | JN135332 | JN135298 | JN135311 |
| | CMW 4727 | | | *P. densiflora* | Japan | | =JN135323 | =JN135332 | = JN135298 | = JN135311 |
| | CMW 4728 | | | *P. densiflora* | Japan | | =JN135323 | =JN135332 | = JN135298 | = JN135311 |
| | CMW 4729 | | | *P. densiflora* | Japan | | =JN135323 | =JN135332 | = JN135298 | = JN135311 |

[a]CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
[b]CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands ; C = Culture Collection of TC Harrington, Department of Plant Pathology, Iowa State University, Ames, Iowa, USA.
[c]Ex-type isolate of *Verticicladiella alacris.*
[T] = ex-type isolates.

TABLE II. Parameters used and statistical values resulting from the different phylogenetic analyses of individual datasets

| | Dataset → | ITS2-LSU | ACT | βT | CAL | TEF-1a | Combined |
|---|---|---|---|---|---|---|---|
| **Number of characters** | **Total** | 632 | 815 | 365 | 657 | 740 | 2577 |
| | **Variable** | 60 | 7 | 12 | 31 | 34 | 84 |
| | **Constant** | 405 | 798 | 339 | 614 | 687 | 2438 |
| **MP** | **PIC** | 167 | 10 | 14 | 12 | 19 | 55 |
| | **Number of trees** | 36 | 1 | 2 | 4 | 3 | 7 |
| | **Tree length** | 634 | 11 | 23 | 18 | 62 | 118 |
| | **CI** | 0.621 | 0.914 | 0.921 | 0.899 | 0.945 | 0.883 |
| | **HI** | 0.379 | 0.087 | 0.078 | 0.101 | 0.055 | 0.117 |
| | **RI** | 0.907 | 0.986 | 0.981 | 0.984 | 0.99 | 0.979 |
| | **RC** | 0.563 | 0.901 | 0.908 | 0.885 | 0.936 | 0.865 |
| **ML & BI** | **Substitution model** | TrN+G | TrN+G | TrN | TrN+G | TrN+G | TrN+G |
| | **Gamma** | 0.214 | 0.011 | - | 0.286 | 0.25 | 0.013 |
| **BI** | **Burn-in** | 250 | 300 | 300 | 300 | 300 | 500 |

PIC = number of parsimony informative characters; CI = consistency index; HI = homoplasy index; RI = retention index; Subst. model = best fit substitution model; Gamma = Gamma distribution shape parameter.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L. douglasii AY553381
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H. pinicola DQ062060 T
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L. lundbergii DQ062068 T
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G. piceiperda AY707209
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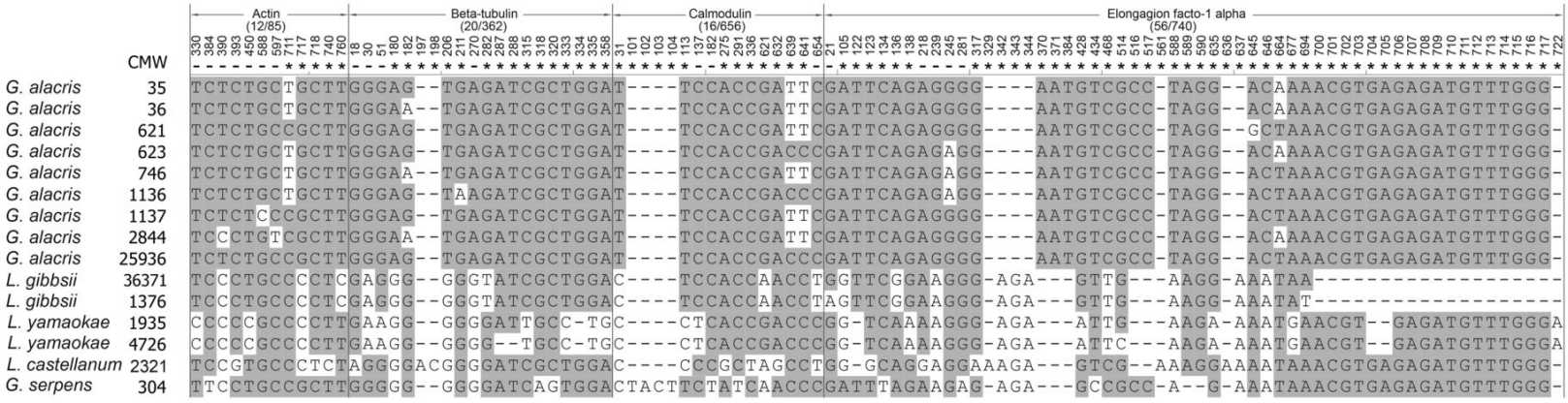
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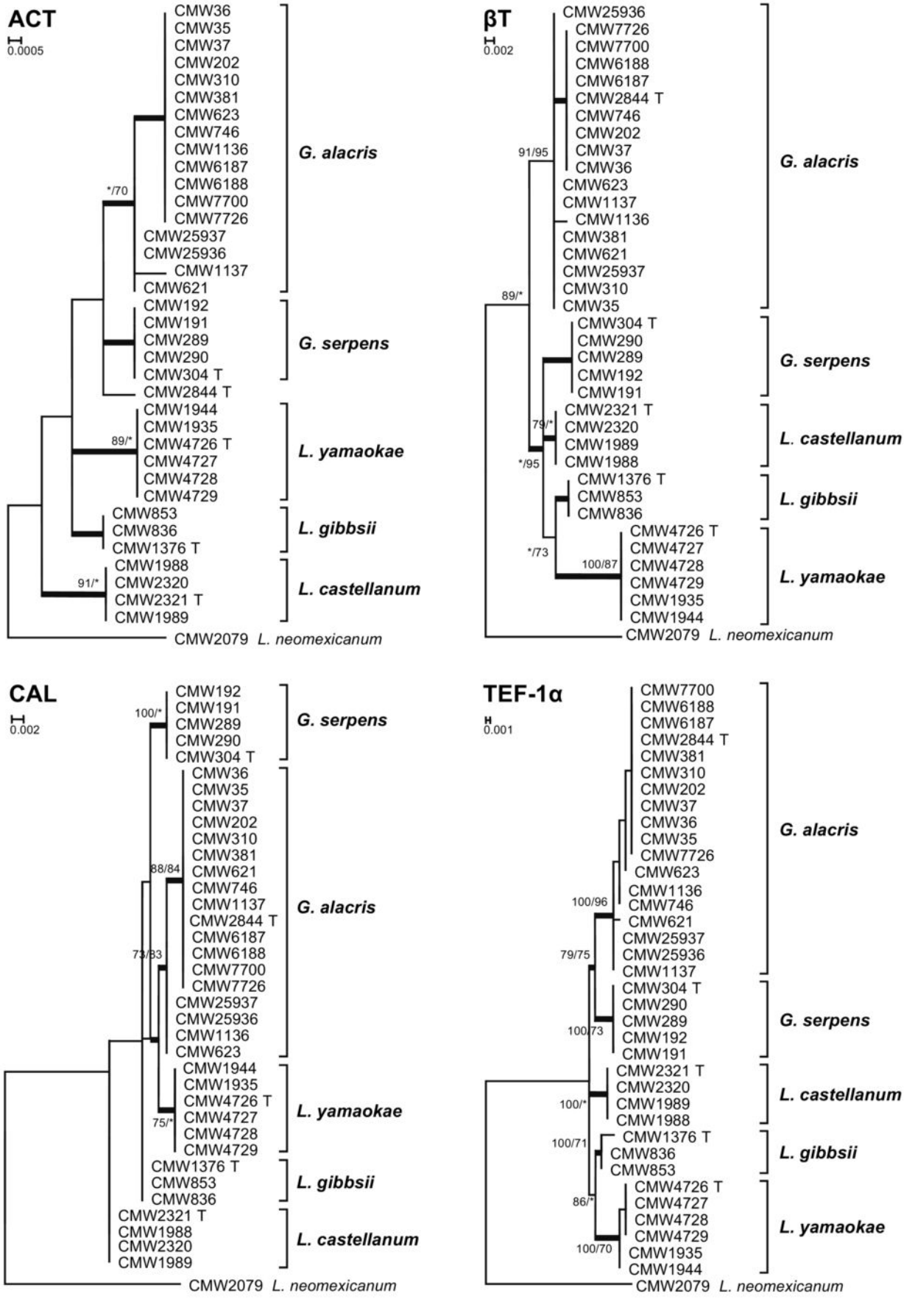
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CMW1137
CMW621
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G. serpens
L. yamaokae
L. gibbsii
L. castellanum
βT
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L. castellanum
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CAL
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G. serpens
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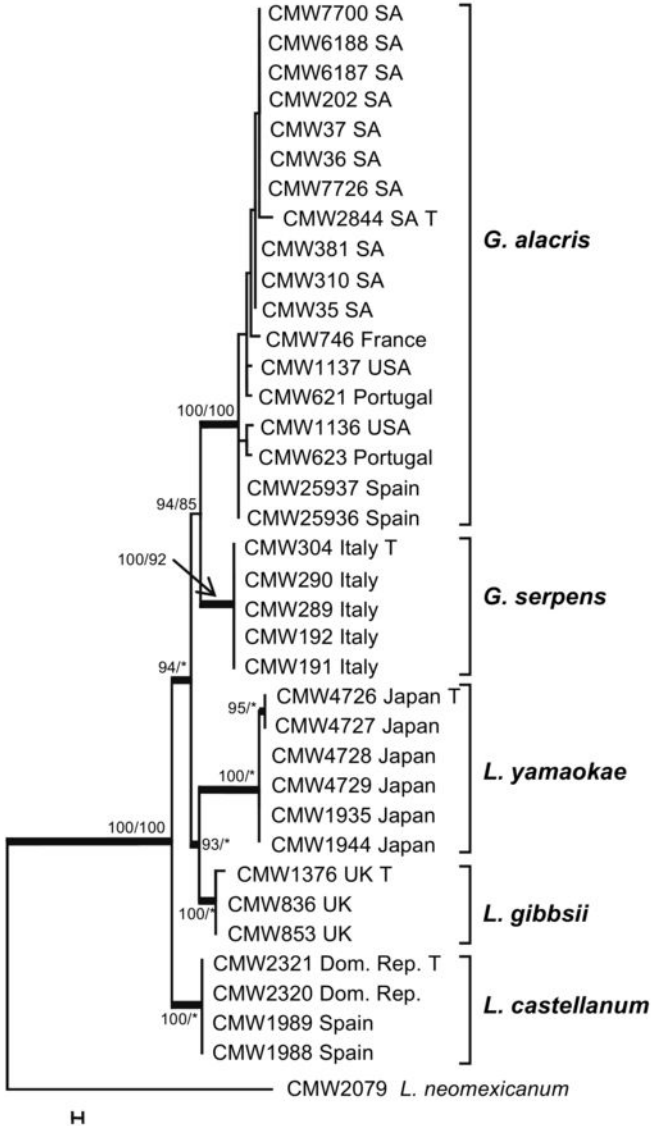
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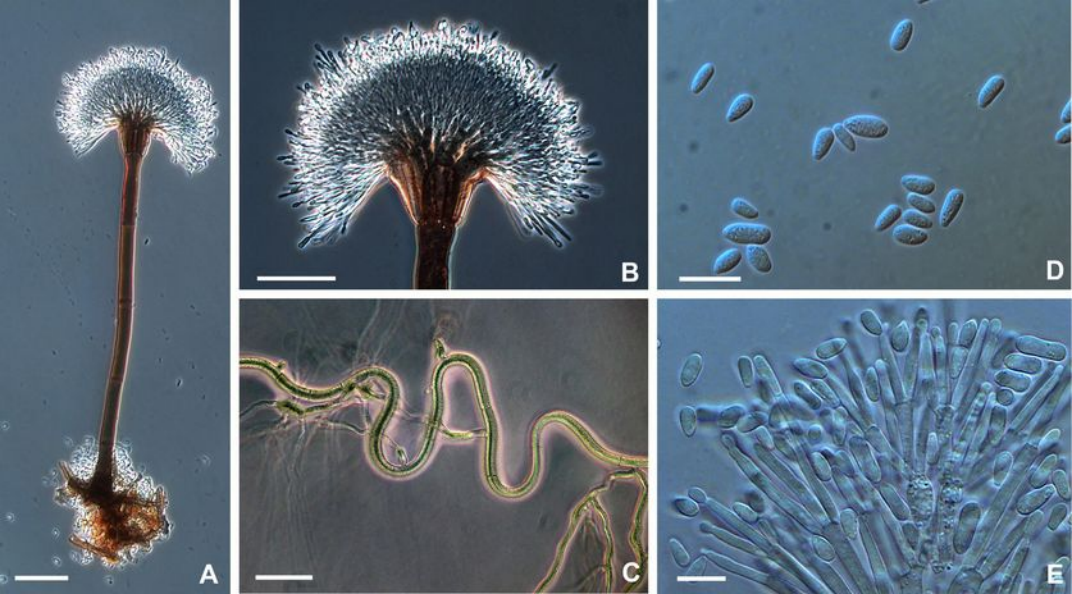
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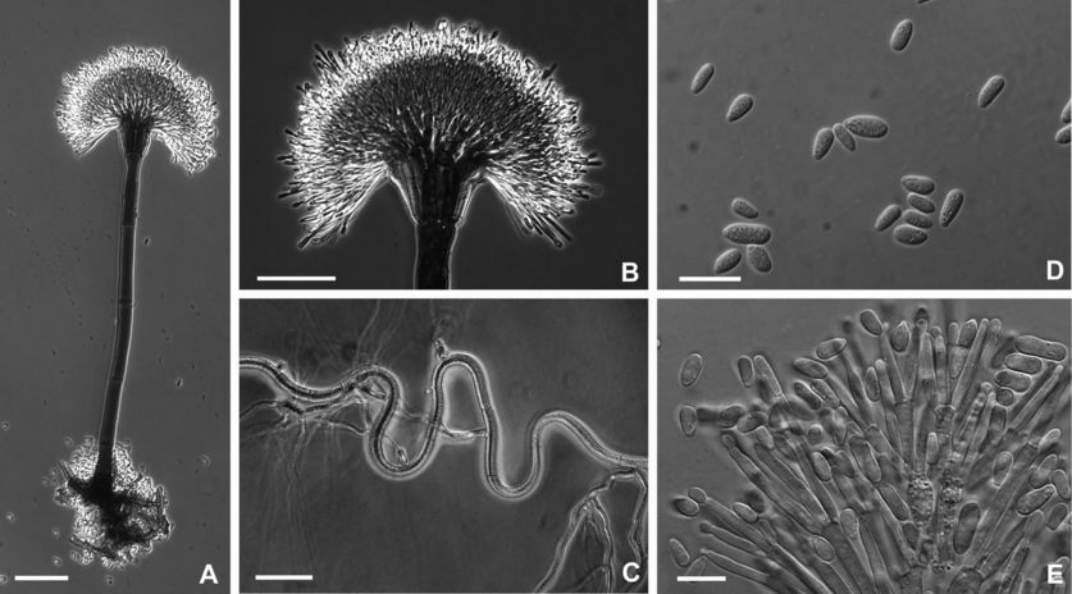
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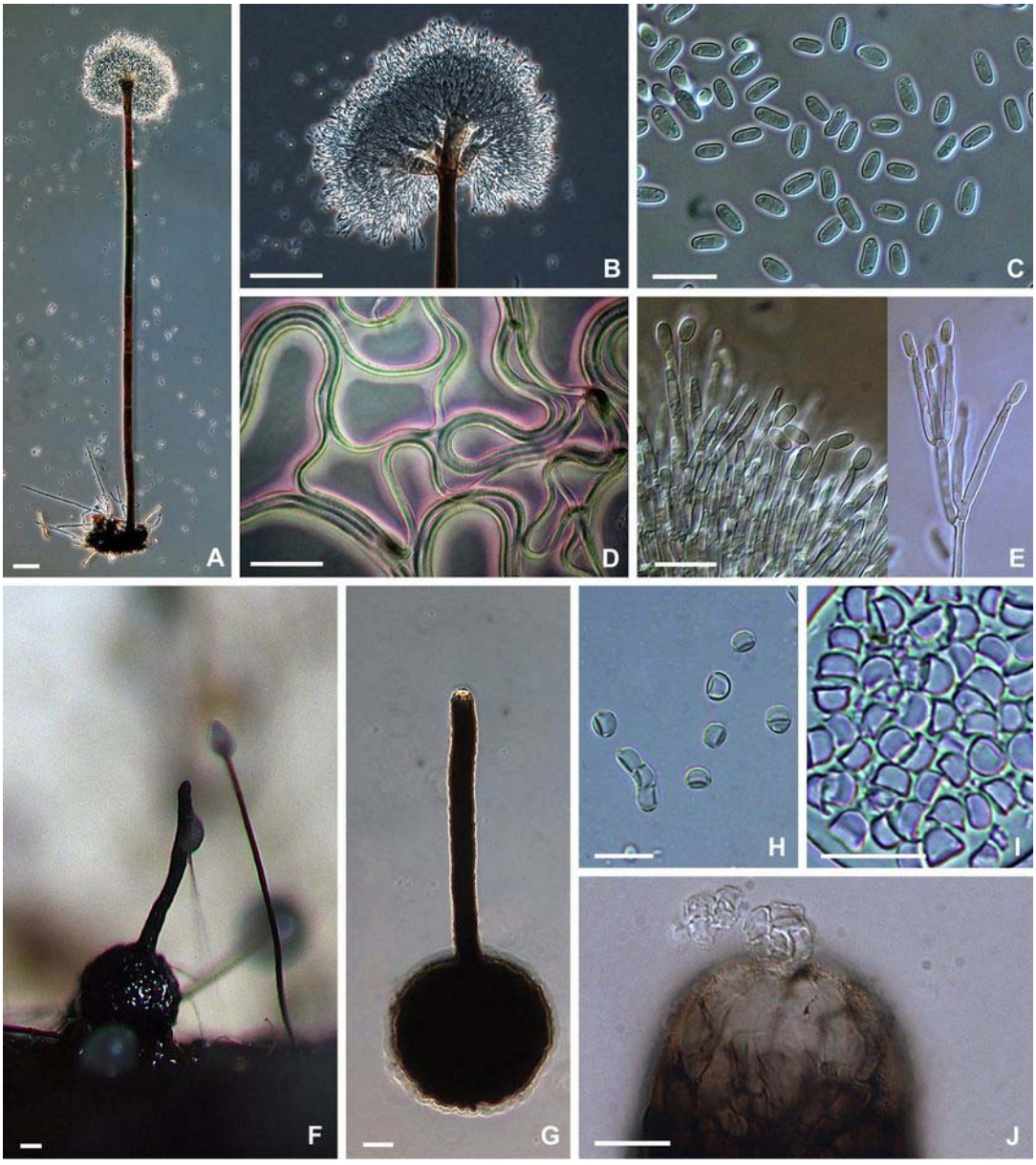
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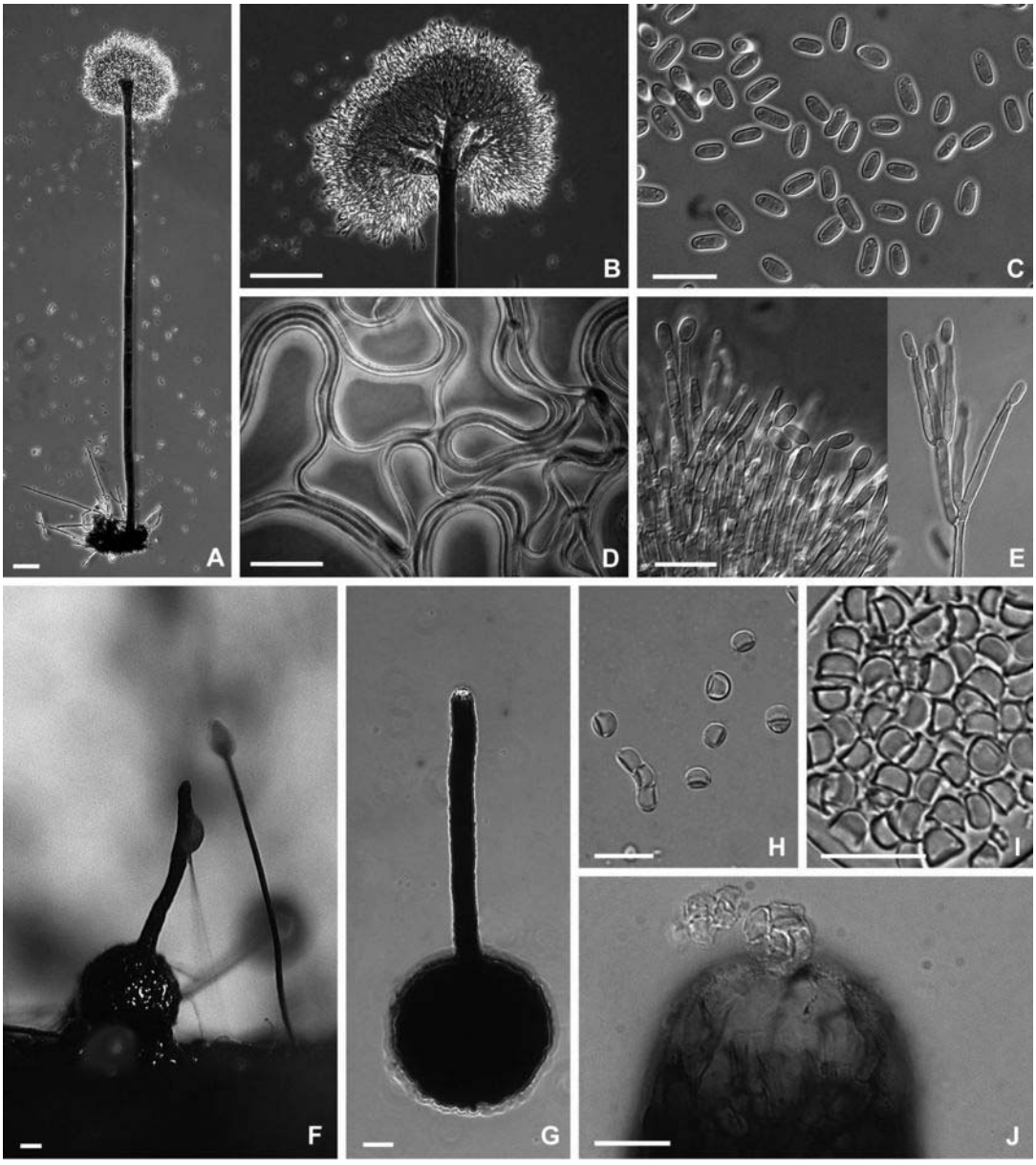
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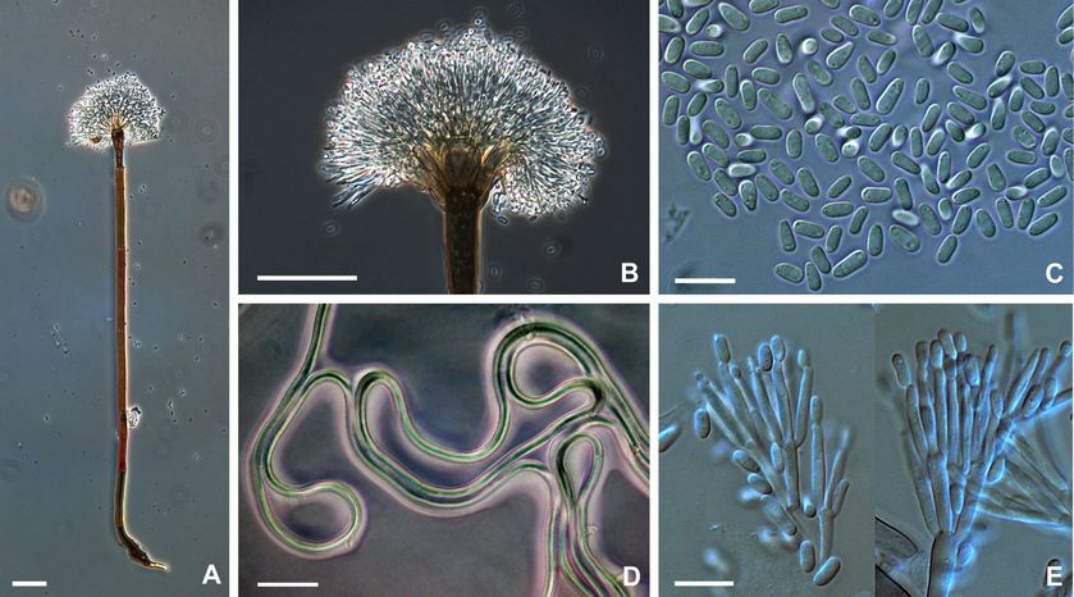
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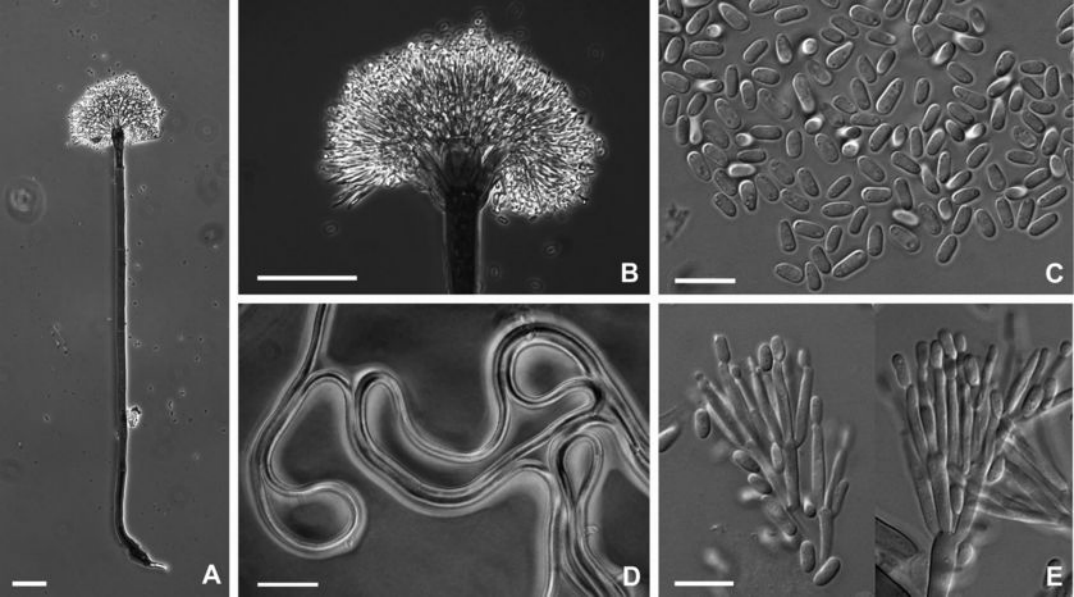
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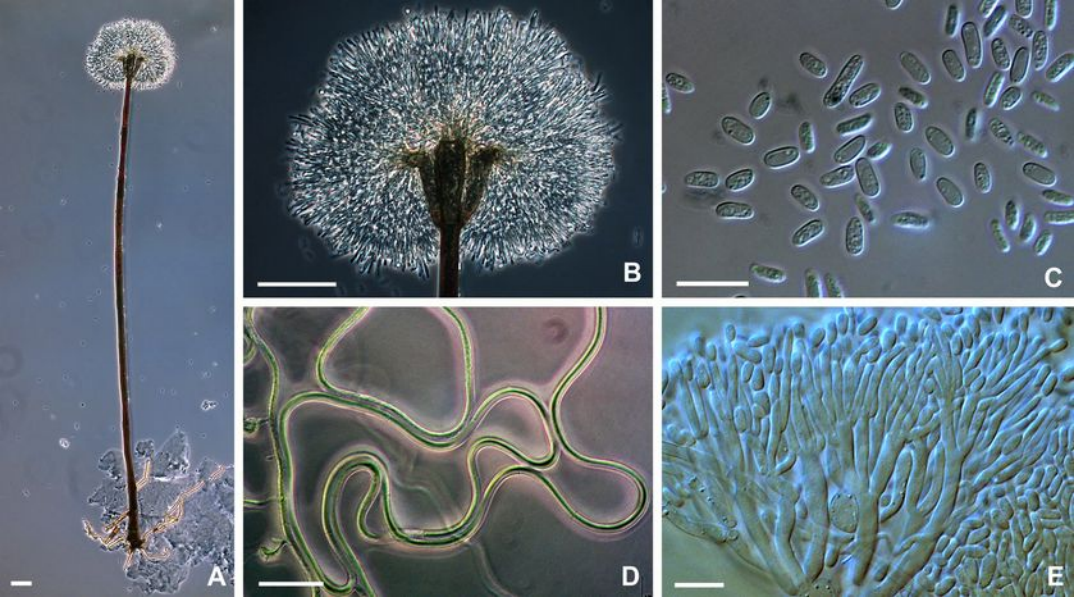
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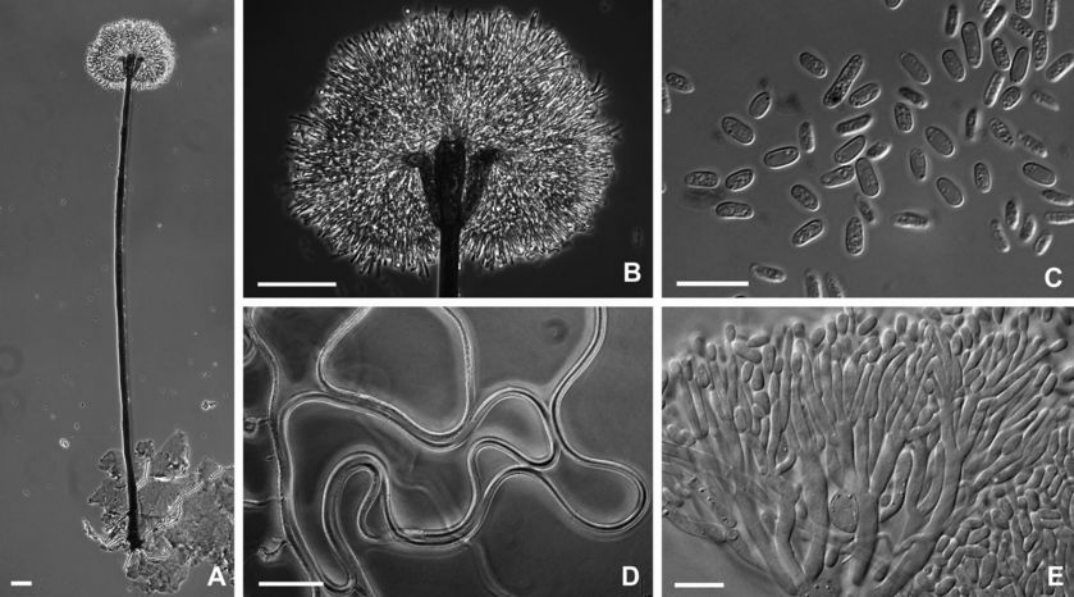
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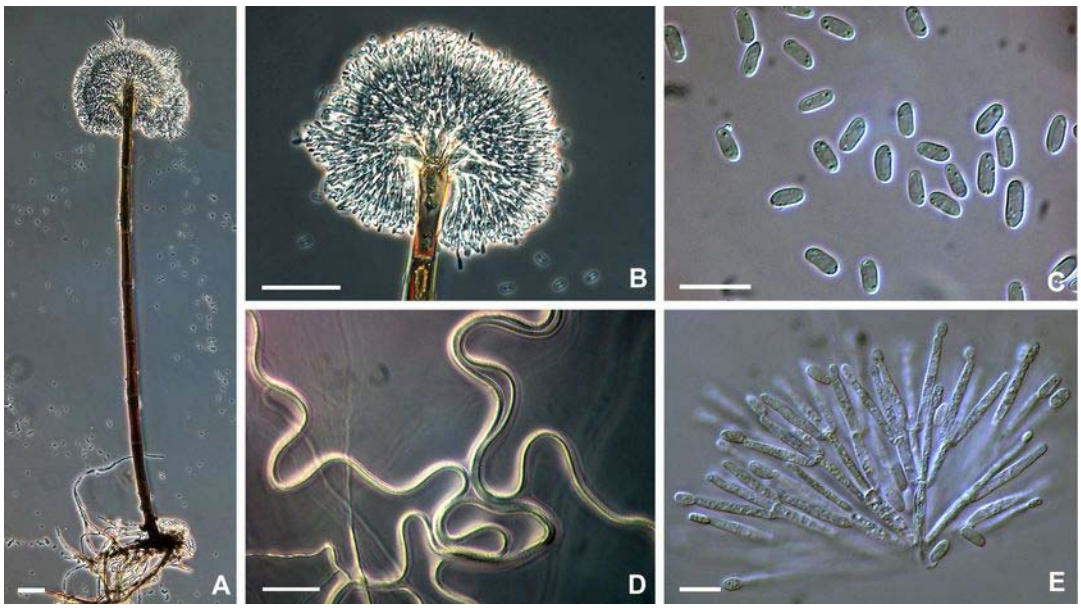
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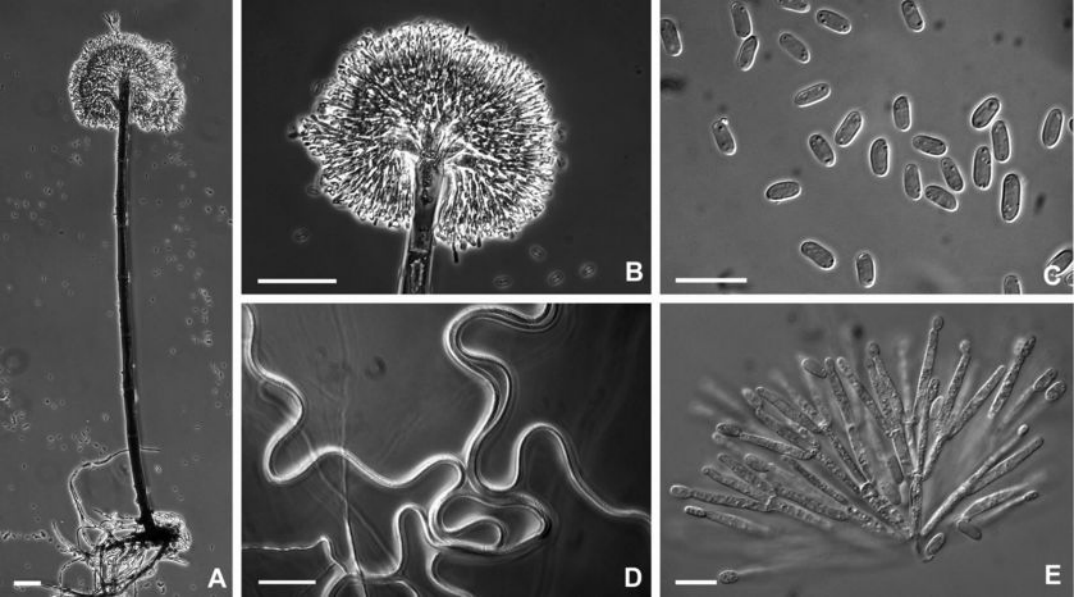
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