

Aquatic fungi from peat swamp palms: *Phruensis brunneispora* gen. et sp. nov. and its phycomycete anamorph

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Abstract: *Phruensis brunneispora* is a new genus and species occurring on decaying trunks of the palm *Licuala longecalycata* in Sirindhorn Peat Swamp Forest, Thailand. We compare the genus with other aquatic ascomycetes with falcate septate ascospores: *Pseudohalonestria* and *Ophioceras*. Ascospores differ from species in these genera in being brown with lighter end cells. Also, the ascus pore is subapical, with a channel leading to the apex. *Lollipopaia minuta* differs from *Phruensis brunneispora* in that the ascomata are borne in a stroma, asci have an apical pore and the ascospores are hyaline. No genus was found to accommodate the new species. Molecular analysis of rDNA ribosomal 18S confirmed the exclusion of the new species from *Pseudohalonestria*, and *Ophioceras* and *Lollipopaia minuta* formed a sister group with it. *Phruensis brunneispora* and *Lollipopaia minuta* grouped in the Diaporthales with 100% bootstrap support. Therefore, both morphological and molecular evidence supports erecting a new genus to accommodate this taxon. A hyaline *Phialophora*-like anamorph was formed when single ascospores were plated out on agar. The taxon is described and illustrated with light micrographs.

Key words: freshwater ascomycete, palm, peat swamp, taxonomy

INTRODUCTION

While investigating decaying palm material in Sirindhorn Peat Swamp Forest, Thailand (Hyde et al 2002, McKenzie et al 2002, Pinruan et al 2002), we collected a *Pseudohalonestria*-like ascomycete with brown versicolorous ascospores and isolated it into axenic culture. The isolate resembles species in the genera *Pseudohalonestria*, *Ophioceras* (Sordariales incertae sedis) and *Lollipopaia* (Diaporthales). *Pseudohalonestria* was described from balsawood submerged in a freshwater lake in Japan (Minoura and Muroi 1978) with *P. lignicola* as the type species. Eight additional species subsequently have been described (Shearer 1989, Hyde et al 1999, Cai et al 2002). *Pseudohalonestria* is characterized by bright yellow to brown ascomata with erumpent, cylindrical, periphysate necks and a 3-layered peridium; asci are unitunicate, cylindrical to clavate, with a nonamyloid, thimble-shaped, refractive apical apparatus, and ascospores are cylindrical, smooth, hyaline to slightly colored and usually multiseptate (Shearer 1989). *Ophioceras* is similar to *Pseudohalonestria*, and both are referred to Sordariales incertae sedis based on phylogenetic analysis of rDNA restriction and sequence data (Chen et al 1995). These molecular data confirmed that the two genera are distinct and well supported by 80–86% bootstrap values.

The new ascomycete differs from *Pseudohalonestria* in that it lacks the characteristic yellow pigmentation of the ascomata in nature and in culture the peridium is 2-layered, the ascospores are versicolored and produce an anamorph with hyaline falcate phialidic conidia. *Lollipopaia minuta* shows similarities to the new taxon, especially in the falcate, septate ascospores. *Phruensis brunneispora* differs in that *L. minuta* has a weakly developed stroma, the ascus pore is apical, the ascospores are hyaline and no anamorph has been reported. In this study we use phylogenetic analyses to investigate whether this palm ascomycete is congeneric with *Pseudohalonestria*, *Ophioceras* or *Lollipopaia*, genera from freshwater habitats with similar septate cylindrical ascospores.

MATERIALS AND METHODS

Collection and isolation.—During May 2001, submerged palm material was collected from Sirindhorn Peat Swamp

TABLE I. Sequences from this study and those obtained from GenBank

Classification	Taxon	GenBank accession numbers
Order Diaporthales	<i>Apioplagiostoma aceriferum</i> (Cooke) M.E. Barr	AF277111
	<i>Apiosporopsis carpineae</i> (Fr.) Traverso	AF277110
	<i>Cryphonectria cubensis</i> (Bruner) Hodges	L42439
	<i>Cryphonectria parasitica</i> (Murrill) M.E. Barr	U78541
	<i>Endothia gyrosa</i> (Schwein.) Fr.	U78540
	<i>Gnomonia padicola</i> (Lib.) Kleb.	AF277112
	<i>Leucostoma persoonii</i> (Nitschke) Höhn.	M83259
	<i>Lollipopaia minuta</i> Inderbitzin	AF301534
	<i>Phruensis brunneispora</i> strain 1 (BCC11169)	AY580160
	<i>Phruensis brunneispora</i> strain 2 (BCC14138)	AY581944
	<i>Valsa ambiens</i> Nitschke	AF277120
Order Sordariales Incertae Sedis	<i>Ophioceras tenuisporum</i> Shearer, J.L. Crane & W. Chen, CS 652-1	AF050475
	<i>Pseudohalonectria falcata</i> Shearer, CS 617-2C	AF050477
	<i>Pseudohalonectria lignicola</i> Minoura & T. Muroi, J13-21	AF050478
Order Ophiostomatales	<i>Ophiostoma pilliferum</i> (Fr.) Syd. & P. Syd., CBS 129.32	AJ243295
	<i>Ophiostoma ulmi</i> (Buisman) Nannf.	M83261
Order Halosphaerales	<i>Halosphaeria appendiculata</i> Linder	U46872
	<i>Nais inornata</i> Kohlm.	AF050482
Order Microascales	<i>Nimbospora effusa</i> Jørg. Koch	U46877
	<i>Microascus cirrosus</i> Curzi	M89994
Order Hypocreales	<i>Petriella setigera</i> (Alf. Schmidt) Curzi	U43908
	<i>Hypocrella</i> sp. GJS 89-104	U32409
Order Phyllachorales	<i>Hypomyces polyporinus</i> Peck	U32410
	<i>Nectria haematococca</i> Berk. & Broome	U32413
	<i>Collectotrichum gloeosporioides</i> (Penz.) Penz. & Sacc.	U76339
Order Sordariales	<i>Glomerella septospora</i> Sivan. & W.H. Hsieh	U78779
	<i>Chaetomium globosum</i> Kunze	U20379
Order Xylariales	<i>Sordaria fimicola</i> (Roberge ex Desm.) Ces. & De Not.	X69851
	<i>Xylaria carpophila</i> (Pers.) Fr.	Z49785
Order Pezizales	<i>Xylaria</i> sp. PF 1022	AB014042
	<i>Helvella terrestris</i> (Velen.) Landvik	AF046216
	<i>Morchella esculenta</i> (L.) Pers.	U42642

Forest, Narathiwat, Thailand. The material was returned to the laboratory, incubated in plastic boxes on damp tissue paper and examined within 4 wk. Single spore isolations were made on cornmeal agar (CMA), with added antibiotics to suppress bacterial growth, following the method of Choi et al (1999). Two strains of the fungus from different collections (Wah 113.1, Wah 113.2) were used for the molecular study. All observations, including photographic documentation and measurements of the fungus growing on wood, were of material mounted in water and examined with a differential interference microscope. Other collections subsequently have been made and treated in the same manner.

Growth of fungi, DNA extraction, amplification and sequencing.—Stock cultures of the fungus were maintained on CMA at 25 C. The fungus was grown in liquid GYP (glucose, yeast extract, peptone; Abdel-Wahab et al 2001) broth on a rotary shaker at 200 rpm at 25 C. Fungal biomass was harvested by vacuum filtration and washed with sterile distilled water. The biomass was frozen in liquid nitrogen and

ground with a mortar and pestle. DNA was extracted using a NucleoSpin® Plant DNA extraction kit (MACHEREY-NAGEL, Catalogue No. 740 590. 50). The small subunit ribosomal DNA (rDNA) was amplified using FINNZYMES, DyNAzyme™ II DNA Polymerase Kit (MACHEREY-NAGEL, product code F-551S), in a Perkin Elmer thermal cycler. Primers NS1, NS4, NS5 and NS6 were used to amplify the small subunit rRNA (White et al 1990). The amplification cycles were performed following White et al (1990). The PCR product was purified using a NucleoSpin® Plant DNA purification kit (MACHEREY-NAGEL, Catalogue No. 740 570. 50), then sequenced automatically by the BIOTEC Service Unit (BSU) laboratory using primers NS1, NS3, NS5 and NS6 (White et al 1990).

Phylogenetic analysis.—Sequences of *Ph. brunneispora* (Accession numbers: AY580160, AY581944) were analyzed with other sequences obtained from the GenBank database (TABLE I). *Morchella esculenta* and *Helvella terrestris* served as outgroups. Sequences were aligned in Clustal W 1.6 program (Thompson et al 1994) and refined visually in Bioedit

TABLE II. Results of Kishino-Hasegawa maximum likelihood tests on alternative topologies

Analysis	No. of trees	Tree length (steps)	Consistency indices (CI)	Retention indices (RI)	Rescaled indices (RC)	–ln likelihood	Difference –ln L	P-value
Unweighted parsimony	2	869	0.666	0.730	0.485	7430.47546	2.00008	0.000*
Weighted parsimony-ti:tv	1	1014.2	0.671	0.736	0.496	7429.15521	0.67983	0.000*
Maximum likelihood	1	—	—	—	—	7428.47538	(best)	

* A significant difference at $P < 0.05$.

version 5.0.6 (Hall 2001) and Se-AI v1.0a1 (Rambaut 1996). The alignment was entered into PAUP* 4.0b10 (Swofford 2002) and MacClade 3.08 (Maddison and Maddison 2001). Phylogenetic trees were generated using unweighted parsimony, weighted parsimony and maximum likelihood criteria on 32 taxa. For the unweighted maximum parsimony analysis, we used a heuristic search with a stepwise starting tree, a random stepwise addition of 100 replicates and tree-bisection-reconnection branch-swapping algorithm, with gaps treated as missing data. Weighted parsimony analysis was performed using a stepwise matrix to weight nucleotide transformations based on the transition:transversion (ti:tv) ratio, estimated from the dataset using maximum likelihood score in PAUP* (Swofford 2002). Maximum likelihood parameters: ti:tv ratio, proportion of invariable sites, gamma distribution shape parameter and base frequency, also were estimated from the dataset using maximum likelihood score in PAUP*. For the maximum likelihood heuristic searches, we used these settings: stepwise addition of sequence, as-is stepwise addition sequence and TBR branch-swapping algorithm. Tree topologies from unweighted parsimony, weighted parsimony and maximum likelihood analyses were tested with the Kishino-Hasegawa (K-H) maximum likelihood test (Kishino and Hasegawa 1989) to find the most likely tree for the dataset. Bootstrap analysis (Felsenstein 1985) based on unweighted parsimony was performed with full heuristic searches on 1000 replicates, stepwise addition of sequence, 10 replicates of random addition of taxa, tree-bisection-reconnection branch swapping algorithm. Alignments were deposited in TreeBase: accession no. 51059, matrix accession number = M1806.

RESULTS

Phylogenetic analyses.—Maximum parsimony analysis of unweighted characters yielded two most parsimonious trees. Of the 1832 characters, 180 were variable (9.82%) and 279 parsimony informative (15.2%). The two trees differed by the position of taxa within the Hypocreales. The K-H test was applied to these trees, which resulted in the best tree for unweighted characters. The weighted parsimony analysis, with the assignment of weight 1.4 to transversion and 1 to transition based on the estimation of ti:tv ratio (ti:tv ratio = 1:1.47441), resulted in one tree with the same topology as the best tree of unweighted parsimony

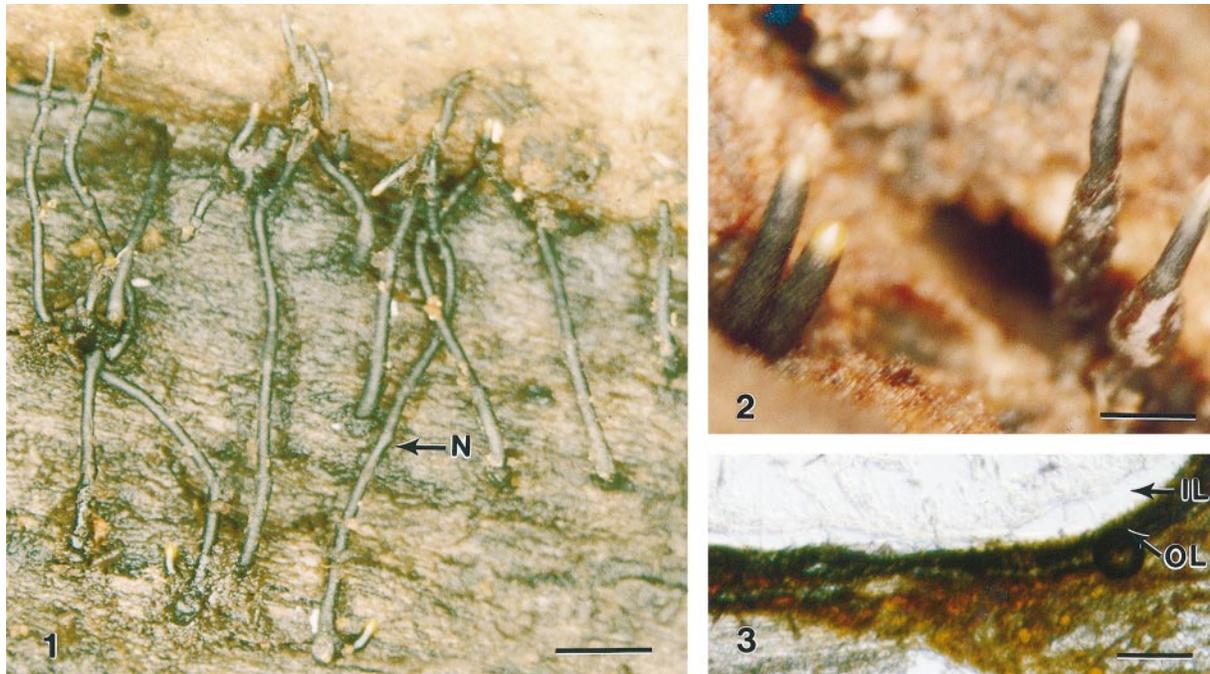
analysis. The result of the K-H test for all trees generated from unweighted, weighted parsimony and maximum likelihood analysis, and the tree generated by the latter method gave the best phylogenetic hypothesis for the dataset (TABLE II). The trees produced by all analyses were significantly different from the most likely tree ($P < 0.05$). However, the tree topology was the same. The maximum likelihood tree (FIG. 17) has given the –ln likelihood score of 7428.47538. The two strains of *Ph. brunneispora* sequenced are monophyletic and supported by 100% bootstrap value. Phylogenetic analyses grouped taxa into three clades. *Xylaria* species (Xylariales, subclass Xylariomycetidae) formed a basal clade to the subclass Sordariomycetidae. Clade 2 comprises the orders Halosphaerales, Microascales, Phyllachorales, Hypocreales and Sordariales with high bootstrap support. Clade 3 comprised the Diaporthales, Ophiostomatales and Sordariales incertae sedis, the latter containing various *Pseudohalonectria* and *Ophioceras* species. *Phruensis brunneispora* is well supported in the Diaporthales and groups with *Lollipopaia minuta* (FIG. 17) with which it shares some morphological characteristics.

TAXONOMY

Phruensis pinruan Ascomata immersa, subglobosa, coriacea, nigra, ostiolata, collo longis cylindrica. Peridium compositum ex 2 cellis laminae. Asci cylindrici vel fusiformes, unitunicati, pedecellati, J-, apparatus subapicale praediti. Ascospores cylindricae, laevis vel curvae, verrucosae, brunneae, 9–11-septatae. Anamorph similis *Phialophora*.

Etymology. from the Thai Phru, meaning peat swamp and Latin *-ensis* meaning “pertaining to.”

Ascomata immersed, subglobose, black, coriaceous, ostiolate, with long central cylindrical neck. Peridium composed of 2 layers, outer layer parenchymatous, intensely brown and merging with the host cells, inner layer, cells elongate and hyaline. Paraphyses hyaline, broad, septate and attached at the base of the centrum. Asci cylindrical to fusiform, unitunicate, apedicellate, apically rounded, with a re-



FIGS. 1–3. Light micrographs of *Phruensis brunneispora* (from holotype). 1, 2. Ascogonia embedded in the substratum with long necks, tips bright orange. 3. Horizontal section of ascogonium with outer perithecial wall (arrow OL) dark brown to black, inner layer (arrow IL) of hyaline elongate cells. Scale bars: 1 = 10 mm; 2 = 5 mm; 3 = 30 μm .

fractive, J-, subapical ring. Ascospores cylindrical, straight or curved, versicolorous, brown with hyaline or pale brown end cells, transseptate. Anamorph similar to *Phialophora*.

Typus species. *Phruensis brunneispora* Pinruan.

***Phruensis brunneispora* Pinruan, sp. nov.** FIGS. 1–16

Ascogonia 950–980 μm alta, 1.6–1.7 mm diametro, immersa, subglobosa, nigra, coriacea, ostiolata. Collum usque ad 7500 μm longum, 500 μm diametro, cylindricum, nigrum. Peridium usque ad 250 μm crassum, compositum ex 2 cellis laminae. Paraphyses usque ad 12.5–15 μm lata ad basim, Asci 260–275 \times 45–50 μm , 8-sporei, cylindrici vel fusiformes, unitunicati, pedicellati, J-, apparatus subapicalis 5–6.3 μm alta, 3.75–4.3 μm diametro praediti. Ascospores 115–120 \times 7.5–8.8 μm , 4-seriatae vel fasciculatae, cylindricae, laevae vel curvae, brunneae, 9–11-septatae. Anamorph: cf. *Phialophora* sp.

Holotypus. THAILAND. NARATHIWAT: Sirindhorn Peat Swamp Forest, on dead trunk of *Licuala longecalycata*, 12 May 2001, U. Pinruan (Wah 113.1) in BIOTEC Bangkok Herbarium (BBH). Culture of type isolate: BCC 11169.

Entymology. From brunneispora, in reference to the brown versicolorous ascospores.

Ascogonia 950–980 μm high, 1.6–1.7 mm diam, deeply immersed, subglobose, black, coriaceous, ostiolate, scattered (FIG. 1). Neck up to 7.5 mm long, 0.5 mm diam., central, cylindrical, black, orange at

apex (FIGS. 1, 2). Peridium up to 250 μm thick, comprising 2 layers, outer layer (40–45 μm) parenchymatous, intensely brown and merging with the host cells, inner layer, cells elongate and hyaline (25–30 μm) (FIGS. 3, 4). Paraphyses up to 11.2–15 μm wide at the base, hypha-like, tapering distally, not embedded in a gelatinous matrix (FIG. 9). Asci 260–275 \times 45–50 μm (\bar{x} = 265 \times 48 μm , n = 25), 8-spored, cylindro-clavate to fusiform, unitunicate, apedicellate, apically rounded, with a refractive, J-, cuboid subapical ring, 5–6.2 μm high, 3.7–4.2 μm diam, with a faint channel leading to the apex (FIGS. 5–8). Ascospores 115–120 \times 7.5–8.7 μm (\bar{x} = 116.5 \times 7.8 μm , n = 25), 4-seriate to fasciculate, cylindrical, straight or curved, versicolored, brown with hyaline to pale brown end cells, 9–11-septate, smooth-walled, with minute ephemeral mucilaginous material at the ends (FIGS. 10–12).

Phialophora-like anamorph: Colonies (BCC 11169) on PDA reaching 2 cm diam in 7 d at room temperature (22–24 C), effuse, brown mycelium partly immersed, nonstromatic. Conidiophores up to 5 μm wide at the base, semimacronematous, mononematous, branched, straight or slightly flexuous, pale brown to brown, smooth. Conidiogenous cells monophialidic, determinate, with small collarettes (FIGS. 13, 14). Conidia 11.5–14 \times 1.5 μm (\bar{x} = 12 \times 1.5 μm , n = 25), aggregated in slimy heads, semi-endog-



FIGS. 4–16. Light micrographs of *Phruensis brunneispora* (from holotype) and *Phialophora*-like anamorph. 4. Cross section of neck with fine paraphyses. 5, 6. Asci cylindrical-clavate, ascospores 4-seriate. 7, 8. Cuboid subapical ring (arrows). 9. Hypha-like paraphyses. 10–12. Ascospores with 12–13-septae, central cells brown, end cells hyaline to pale brown. 13, 14. Conidiophores with small collarettes (arrows). 15, 16. Conidia. Scale bars: 4 = 40 μm ; 5–12 = 20 μm ; 13–16 = 10 μm .

enous, straight or curved, oblong, colorless, smooth, 0-septate (FIGS. 15, 16). Ascomata not formed in culture.

Other collections: THAILAND. NARATHIWAT: Sirindhorn Peat Swamp Forest, on dead trunk of *Licuala longecalycata*, 2 April 2003, U. Pinruan (Wah 113.2) in BBH. Culture from this collection deposited in BCC 14138.

DISCUSSION

Phylogenetic analyses showed that *Phruensis brunneispora* belongs in the order Diaporthales. This is supported by various characters such as saprobic on decaying plant material, bilayered ascomata, long and periphysate necks, paraphysate, and unitunicate asci with a refractive, apical J-ring (Barr 1991). *Ph.*

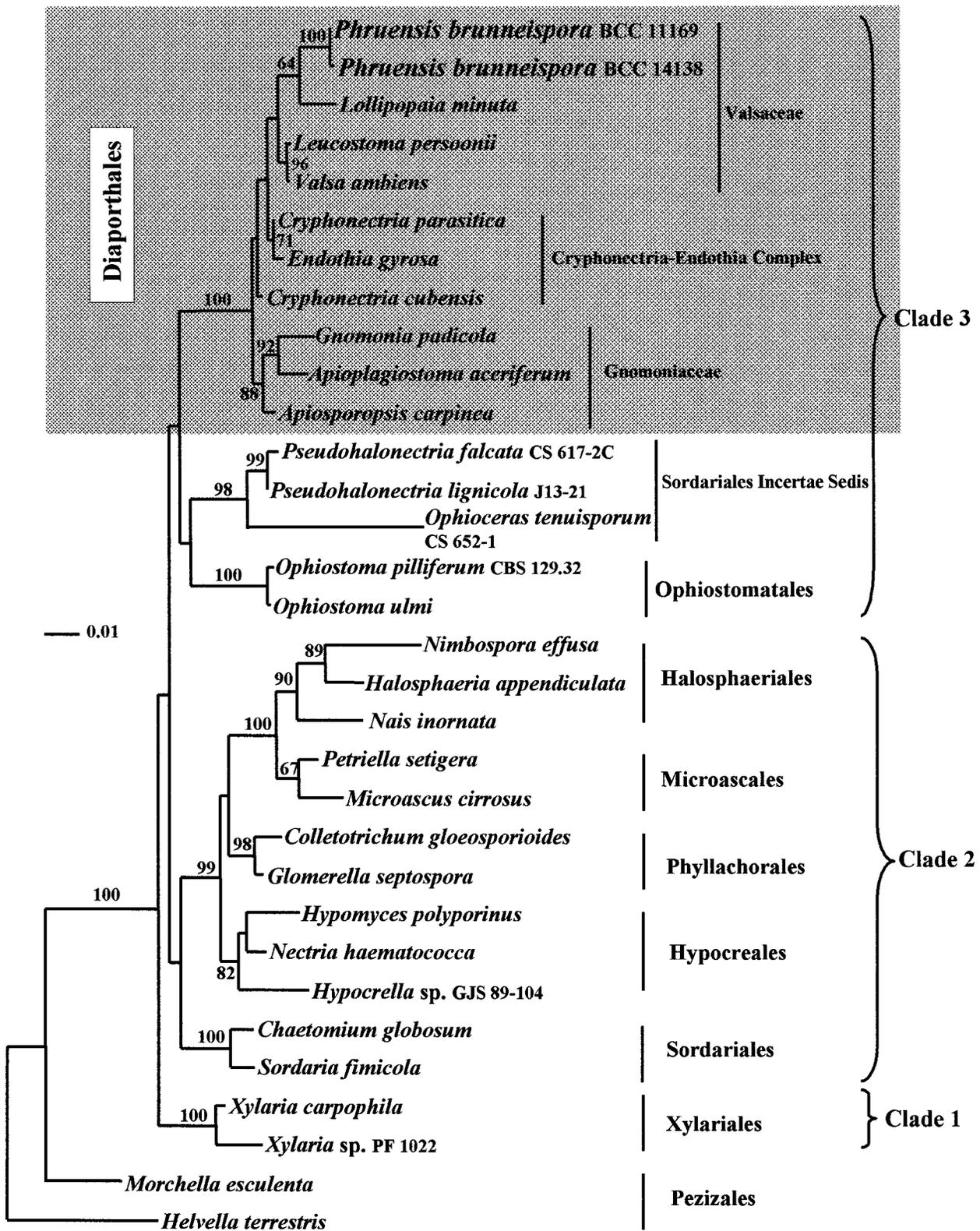


FIG. 17. A single most likely tree inferred from maximum likelihood analysis ($-\ln$ likelihood score = 7428.47538), from partial 18S rDNA sequences. Bootstrap values higher than 50% from maximum parsimony analysis are given on the branches. Scale bar indicates one base substitution per 100 nucleotides.

brunneispora differs from other members of the Diaporthales in a number of respects: ascomata are not formed in a stroma, although they may be grouped together in the substratum; paraphyses are wide, hypha-like and persistent; and ascospores are falcate with numerous septa. Other genera in the Diaporthales also may have hyaline, falcate ascospores (e.g., *Linospora*, *Sillia*).

Phruensis brunneispora most closely resembles *Lollipopaia minuta*, which also differs morphologically from other taxa of the Diaporthales (Inderbitzin and Berbee 2001). *Phruensis brunneispora* differs morphologically from *L. minuta* in that its ascomata are not stromatic, the peridial walls comprise two tissues types and asci have a cuboid subapical, J-ring. In addition, the ascospores of *Ph. brunneispora* are wider, longer, versicolorous with obvious septa and have hyaline to pale brown end cells. In contrast, *Lollipopaia* has a weakly developed stroma, asci with a noncuboid apical ring and hyaline ascospores with indistinct septa. Inderbitzin and Berbee (2001) did not refer *L. minuta* to a family, merely commenting that it could be placed in the Gnomoniaceae sensu Barr (1990) or Valsaceae sensu Hawksworth et al (1995). Similarly, our data do not allow assignment of *Phruensis* and *Lollipopaia* to a family within the Diaporthales.

Phruensis brunneispora is similar in appearance to species of *Ophioceras* and *Pseudohalonestria* (Minoura and Muroi 1978, Shearer 1989, Chen et al 1995, 1999) but differs in having versicolored ascospores. In addition, the ascus rings in *Ophioceras* and *Pseudohalonestria* differ in being thimble-shaped and apical rather than cuboid and subapical. Because of their morphological similarity, we included three species of *Ophioceras* and two species of *Pseudohalonestria* in our phylogenetic analyses. The analyses supported the morphological data that, although *Ph. brunneispora* and the genera *Ophioceras* and *Pseudohalonestria* are similar, they are distinct. *Phruensis* clustered with 100% bootstrap support (FIG. 17) within the Diaporthales, while *Ophioceras* and *Pseudohalonestria* clustered in the Sordariales incertae sedis. This confirms the observations of Inderbitzin and Berbee (2001) that *Lollipopaia* and *Ophioceras/Pseudohalonestria* species have little in common and indicates that the latter genera are better placed in the Magnaporthaceae, Sordariales incertae sedis. Chen et al (1999) sequenced a greater number of *Ophioceras* and *Pseudohalonestria* species and showed they grouped with *Gaeumannomyces*, whereas Shearer et al (1999) drew attention to the morphological similarity of these genera with those of the Magnaporthaceae.

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