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Phylogenetic origins of two cleistothecial fungi, *Orbicula parietina* and *Lasiobolidium orbiculoides*, within the operculate discomycetes

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Abstract: Parsimony, maximum-likelihood and Bayesian analyses of SSU rDNA sequences of representative taxa of Pezizomycetes, Eurotiomycetes, Dothideomycetes, Leotiomycetes and Sordariomycetes, all strongly support the cleistothecial fungi *Orbicula parietina* and *Lasiobolidium orbiculoides* to be of pezizalean origin. Previous hypotheses of close affinities with cleistothecial or highly reduced fungi now placed in the Thelebolales, Eurotiales or Onygenales are rejected. *Orbicula parietina* and *L. orbiculoides* are deeply nested within Pyronemataceae (which subsumes the families Ascodesmidaceae, Glaziellaceae and Otideaceae). LSU rDNA sequences suggest that *Orbicula* is nested within the apothecia-forming genus *Pseudombrophila* (including *Nannfeldtiella* and *Fimaria*) and that *L. orbiculoides* is closely related. *Ascodesmis* and *Lasiobolus*, which have been suggested as closely related to *Orbicula* and *Lasiobolidium*, are identified as a sister lineage to the *Pseudombrophila* lineage. Cleistothecial forms that have lost the ascus operculum and ability to discharge spores actively have evolved at least once in the *Pseudombrophila* lineage. Some species of *Pseudombrophila* produce subglobular ascomata initials that are closed early in development and open only in the mid-mesohymenial phase. We hypothesize that, in the *Pseudombrophila* lineage, ascomata forms that never open are derived from ascomata that open late in development. The placement of *O. parietina* and *L. orbiculoides* within *Pseudombrophila* is supported by morphological characters, ecology and temperature optima for fruiting.

Key words: ascoma evolution, molecular phylogenetics, Pezizales, *Pseudombrophila*, Pyronemataceae

INTRODUCTION

Morphological and molecular evidence show that cleistothecial fungi have evolved independently several times within apothecial and perithecial lineages of ascomycetes. Molecular phylogenetic analyses indicate one major group (composed of Ascosphaerales, Onygenales and Eurotiales) containing most cleistothecial ascomycetes but that other cleistothecial fungi fall within other ascomycete groups (Berbee and Taylor 1994, LoBuglio et al 1996). We investigate the placement of two cleistothecial fungi, *Orbicula parietina* and *Lasiobolidium orbiculoides*, that have been suggested to have pezizalean affinities.

The genus *Orbicula* Cooke (1871) produces epigeous, globose ascomata, up to 2 mm in diam, without an opening (FIGS. 1–3). At first sight it resembles, and is often mistaken for, a slime mold (a miniature *Lycogala*) (Hughes 1951). Asci lack an apical opening mechanism and disintegrate at maturity. The ascomata become filled with a dry mass of ascospores that are liberated when the peridium is disturbed and broken by an external force (FIG. 2). *Orbicula parietina*, the only accepted species, has been repeatedly described as new and assigned to nine genera, four in the myxomycetes (Hughes 1951). Moreover, the family Orbiculaceae and order Orbiculales (Ascomycota) were erected for *Orbicula* (Locquin 1974), but invalidly published (ICBN Art. 36.1). Malloch and Cain (1971) thought that *Orbicula* was closely related to members of the Thelebolaceae and placed it, along with other cleistothecial fungi—*Cleistothelebolus*, *Eoterfezia*, *Lasiobolidium*, *Microeurotium* and *Xeromyces*—in the cleistothecial family Eoterfeziaceae, which they considered Pezizales. Jeng and Krug (1976) transferred the genera of Eoterfeziaceae including *Orbicula* to the tribe Theleboleae sensu Korf (1972) of the Pyronemataceae, following the notion that closely related genera with exposed hymenia and cleistothecial genera are better accommodated in one rather than separate families. Benny and Kimbrough (1980) maintained *Orbicula* in Eoterfeziaceae, while Dennis (1981) placed it in Eurotiaceae (Plectascales). Arx (1981) treated *Orbicula* in the Pezizales and Campbell et al (1991) likewise suspected affinities with the operculate discomycetes. Malloch (in Dissing and Schumacher 1994) suggested that both *Orbicula* and *Lasiobolidium*, with clearly pezizalean characteristics, might better be included in Pyronemataceae or in the Pezizales without assignment rather than in Eoterfeziaceae. Currently *Orbicula* is placed in the Pyronemataceae, but its disposition is indicated tentatively with a question mark (Eriksson 2005).

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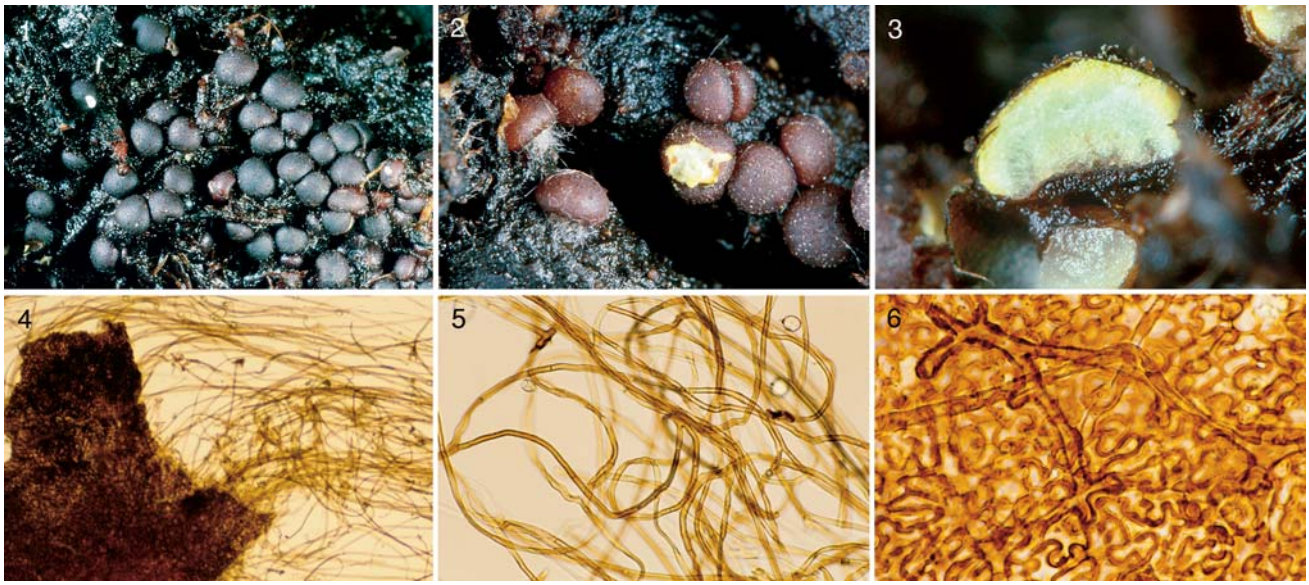


FIGS. 1–6. *Orbicula parietina*. 1. Cleistothecia on dung of dove (?) ×2.5. 2. Close-up of cleistothecia, globose with a flattened base, glabrous over the upper surface and with hyphoid hairs originating from the base. In one ascomata the thin outer excipulum is broken open to show the contents of the yellowish spores ×5. 3. Median, longitudinal section through a mature ascomata to show basal cushion on which asci and paraphyses are borne ×15. (FIGS. 1–3: JHP-01.013/PH01-001, C). 4. Light microscopic slide (LM) of part of lower cleistothecial wall in surface view, with long, hyphoid hairs coming from the base of the cleistothecia ×100. 5. LM of hyphoid, flexuous, pale brownish, thick-walled and remotely septate ascomatal hairs ×200. 6. LM of outer excipulum cells seen in surface view; cells thick-walled, small and irregularly lobed (puzzle-like) with intercellular, amorphous, brownish pigment. Hairs originating from outer excipulum cells, with forked bases ×400. (FIGS. 4–6: C F-24441). Photos: 1–3 J.H. Petersen. 4–6 K. Hansen.

Lasiobolidium orbiculoides was the second species described in *Lasiobolidium* (Malloch and Benny 1973). The specific epithet *orbiculoides* refers to its similarity to *Orbicula*; *O. parietina* and *L. orbiculoides* both have broadly oblate, uniseriate ascospores (Malloch and Benny 1973). *Lasiobolidium orbiculoides* differs from the type species of *Lasiobolidium*, *L. spirale*, by being fast-growing, producing ascomata with wavy to flexuous, septate appendages, an excipulum of one tissue type, cylindrical uniseriate asci and oblate ascospores (Moustafa and Ezz-Eldin 1989). *Lasiobolidium* was considered a cleistothecial counterpart of *Lasiobolus* (Thelebolaceae, Pezizales), but the genus was at first placed in the Eoterfeziaceae (Malloch and Cain 1971). Jeng and Krug (1976) transferred *Lasiobolidium* to the Theleboleae sensu Korf (1972) of the Pyronemataceae. Developmental studies of *L. orbiculoides* however, have shown little evidence of affinities with either *Lasiobolus* or Thelebolaceae, rather they support a relationship with other operculate discomycetes, especially with *Ascodemis* (Janex-Favre and Locquin-Linard 1979). The type genus of Thelebolaceae, *Thelebolus*, was found to be non-pezizalean based on ascoma development and ascus structure (e.g., Samuelson and Kimbrough 1978, Kimbrough 1981). Based on molecular phylogenetic analyses (Momol et al 1996, Landvik et al 1997) the Thelebolaceae was found to be closely related to Erysiphales and Leotiales, and was moved to the Leotiomycetes (Eriksson and Winka 1997). *Orbicula*, *Lasiobolus* and *Lasiobolidium* were maintained in the Pezizales, in Otideaceae (Eriksson 1999) (=Pyronemataceae [Eriksson et al 2001, Eriksson 2005]). Further studies by Landvik et al (1998) confirmed the relationship of Thelebolaceae with members of the Leotiomycetes, and grouped *Lasiobolus* with *Ascodesmis* in the Pezizales.

In the present study we performed phylogenetic analyses of the SSU rDNA to address the phylogenetic position of *O. parietina* and *L. orbiculoides* within the ascomycetes; and analyses of the LSU rDNA to address specific relationships of *O. parietina* and *L. orbiculoides* within the Pyronemataceae.

MATERIALS AND METHODS

Specimens.—Specimens sequenced and morphologically examined are listed (TABLE I). To test hypotheses regarding relationships of *Orbicula* and *L. orbiculoides*, the SSU rDNA sequences were analyzed with 61 sequences retrieved from GenBank from representative taxa of Pezizomycetes, Eurotiomycetes (Eurotiales and

TABLE I. Source of material examined and sequenced by the authors. Numbers in parentheses indicate multiple collections of a single taxon

Species	Collection number (Herbarium), date and collector	LSU	SSU
Ascodesmis nigricans Tiegh.	CBS 389.68, Netherlands, Wageningen, 29.I.1986, G. Tichelaar	DQ168335[a]	—
Eleutherascus lectardii (Nicot) Arx	CBS 626.71, France, Moselle, I.1968, P. Lectard	DQ168334[a]	DQ062997
Geopyxis carbonaria (Alb. & Schwein.) Sacc.	C F-49793 (C), Denmark, Jutland, 13.XI.1982, T. Læssøe	DQ168336[a]	—
Geopyxis sp.	KH.04.48 (FH, DBG), USA, Colorado, 13.IX.2004, K. Hansen, V. Evenson	DQ062985	—
Glaziella aurantiaca (Berk. & Curt.) Cooke	PR-5954 (FH), Puerto Rico, Loquillo Mts., 11.VI.1998, N.C. Clum, D.J. Lodge.	—	DQ062996
Greletia reticulosperma Donadini, Riousset & G. Riousset	Part of isotype (herb. Roy Kristiansen), France, 1984, G. Riousset	AY500532	—
Lasiobolidim orbiculoides Malloch & Benny	CBS 344.73, USA, California, dung of deer, 10.VI.1953, G.L. Benny	DQ062995	DQ063000
Lasiobolus cuniculi Velen.	Rana 76.053 (C), Norway, Nordland, 9.IX,1976, H. Dissing	DQ168338[a]	—
Lasiobolus ciliatus (Berk.) Sacc.	KS-94-005 (C), Denmark, Møn, 5.IV.1994, K. Hansen, S.K. Sandal	DQ167411[a]	—
Orbicula parietina (Schrad.) S. Hughes	C F-24441 (C), Denmark, Zealand, on rush mat, 1988, H.F. Gøtzsche	DQ062988	DQ062998
Otidea onotica (Pers.) Fuckel	KH-98-107 (C), Denmark, Zealand, 21.VII.1998, K. Hansen	AF335121	—
Otidea umbrina (Pers.) Bres.	KH.01.09 (C), Denmark, Bornholm, 30.IX.2001, C. Lange	AY500540	—
Paurocotylis pila Berk.	Trappe 12583 (OSC), New Zealand, South Island, 24.IX.1993, M. Amaranthus	DQ168337[a]	—
Pseudombrophila guldeniae Svrcek (1)	Kongsv. 85.10B (C), Norway, Oppdal, 23.VIII.1985. H. Dissing, S. Sivertsen	DQ062993	DQ063001
Pseudombrophila guldeniae (2)	s.n. (FH, part in C and TRH), Norway, Oppdal, 13.VI.1985, S. Sivertsen, I. Dissing, H. Dissing	DQ062994	—
Pseudombrophila merdaria (Fr.) Brumm. (1)	s.n. (FH), USA, ME, on manured soil, 30.VII.1994, D.H. Pfister	DQ062990	—
Pseudombrophila merdaria (2)	s.n. (FH), USA, VE, on composted silage at edge of hay field, VI.1979, M. Shemluck	DQ062991	—
Pseudombrophila merdaria (3)	s.n. (FH), USA, IA, no date, T.J. Farrell	DQ062992	
Pseudombrophila theioleuca Rolland	C F-70057 (C), Denmark, on deer dung, 25.IX.1982, H. Knudsen	DQ062989	DQ062999
Pulvinula constellatio (Berk. & Broome.) Boud.	KH.03.64 (FH), Norway, Nordland, 22.VIII.2003, K. Hansen, C. Lange	DQ062987	—
Pulvinula convexella (P. Karst.) Pfister	KH.01.020 (C), Denmark, Fyn, 5.X.2001, K. Hansen	DQ062986	—
Smardaea amethystina (W. Phillips) Svrcek	KH-97-132 (C), Denmark, Zealand, 1997, C. Lange, K. Hansen	AF335176	—
Tarzetta catinus (Holmsk.) Korf & J.K. Rogers	KS.94.10A (C), Denmark, Møn, 11.V.1994, K. Hansen, S.K. Sandal	DQ062984	—
Tarzetta pusilla Harmaja	KH.03.66 (FH), Norway, Nordland, 22.VIII.2003, K. Hansen, C. Lange	DQ062983	—

[a] Sequences from manus in prep.

Onygenales), Dothideomycetes (Pleosporales), Leotiomycetes (Helotiales, Erysiphales and Thelebolales) and Sordariomycetes (Sordariales and Hypocreales): *Ascobolus carbonarius* P. Karst., AY544720; *Ascodesmis sphaerospora* W. Obrist., U53372; *Ascozonus woolhopensis* Renny, AF010590; *Barssia oregonensis* Gilkey, U42657; *Byssonectria terrestris* (Alb. & Schwein.:Fr.) Pfister, Z30241; *Caccobius minusculus* Kimbr.; *Chaetomium elatum* Kunze, M83257; *Chalazion helveticum* Dissing, AF061716; *Cheilymenia stercorea* (Pers.:Fr.) Boud., U53375; *Chorioactis*

geaster (Peck) Kupfer, AF104340; *Cookeina speciosa* (Fr. : Fr.) Dennis (= *C. sulcipes* (Berk.) Kuntze), U53376; *Desmazierella acicola* Lib., AF104341; *Discina macrospora* Bubák, U42651; *Elaphomyces maculatus* Vittad., U45440; *Erysiphe orontii* Castagne, AB033483; *Eurotium herbariorum* (F.H. Wigg.) Link, AB008402; *Geopyxis carbonaria* (Alb. & Schwein. : Fr.) Sacc., AF104665; *Gymnoascus reessii* Baran., AB015774; *Gyromitra montana* Harmaja, U42652; *Hymenoscyphus ericae* (D.J. Read) Korf & Kernan, AY524847; *Iodophanus carneus* (Pers. : Fr.) Korf, U53380; *Lamprospora kristiansenii* Benkert, AF121075; *Lasiobolus papillatus* (Pers. : Fr.) Sacc., AF010588; *Monascus ruber* Tiegh., AB024048; *Morchella elata* Fr. : Fr., U42641; *Nectria cinnabarina* (Tode : Fr.) Fr., AB003949; *Neottiella rutilans* (Fr.) Dennis, AF061720; *Neurospora crassa* Shear & B.O. Dodge, X04971; *Helvella lacunosa* Afzel. : Fr., U53378; *Leucangium carthusianum* (Quél.) Paol., U42647; *Onygena equina* (Willd. : Fr.) Pers., U45442; *Paurocotylis pila* Berk., U53382; *Peziza succosa* Berk., U53383; *Phyllactinia guttata* (Wallr. : Fr.) Lév., AF021796; *Plectania rhytidia* (Berk.) Nannf. & Korf, AF104344; *Pleospora herbarum* (Pers. : Fr.) Rabenh., U05201; *Pulvinula archeri* (Berk.) Rifai, U62012; *Pyronema domesticum* (Sowerby : Fr.) Sacc., U53385; *Reddellomyces donkii* (Malençon) Trappe et al, U42660; *Rhizina undulata* Fr., U42664; *Sarcosphaera coronaria* (Jacq.) Boud., AY544712; *Saccobolus* sp., U53393; *Sarcoscypha austriaca* (Berk.) Boud., AF006318; *Sarcosoma globosum* (Schmidel : Fr.) Casp., U53386; *Sclerotinia sclerotiorum* (Lib.) de Bary, L37541; *Scutellinia scutellata* (L. : Fr.) Lambotte, U53387; *Sordaria fimicola* (Roberge ex Desm.) Ces. & De Not., AY545724; *Sporormia lignicola* W. Phillips & Plowr., U42478; *Talaromyces flavus* (Klöcker) Stolk & Samson, M83262; *Tarzetta catinus* (Holmsk. : Fr.) Korf & J.K. Rogers, U53389; *Thecotheus holmskjoldii* (E.C. Hansen) Chenant., AF010589; *Thelebolus crustaceus* (Fuckel) Kimbr., U53394; *Thelebolus stercoreus* Tode : Fr., U49936; *Trichophaea hybrida* (Sowerby.) T. Schumach., U53390; *Tuber gibbosum* Harkn., U42663; *Wilcoxina mikolae* (Chin. S. Yang & Wilcox) Chin. S. Yang & Korf, U62014; *Xeromyces bisporus* L.R. Fraser, AB024049; *Zopfia rosatii* (Segretain & Destombes) D. Hawksw. & C. Booth, L76623.

Neolecta Speg. and *Taphrina* Fr. were used to root the SSU trees (*Neolecta vitellina* [Bres.] Korf & J.K. Rogers, Z27393; *Neolecta irregularis* [Peck] Korf & J.K. Rogers, Z47721; *Taphrina pruni* Tul., AJ495828). Representatives of Saccharomycetes were also included (*Kluyveromyces lactis* (Boidin, Abadie, J.L. Jacob & Pignal) Van der Walt, AY790534; *Saccharomyces cerevisiae* E.C. Hansen, V01335; *Pichia* sp., AY227899).

Ingroup taxa in the LSU data set, in addition to the *Pseudombrophila* lineage, were selected based on preliminary results of a large-scale molecular study of the Pyronemataceae (in prep). In that study, based on analyses of LSU rDNA sequences, the type species of *Lasiobolidium*, *L. spirale*, is placed in a different lineage of Pyronemataceae distant from *L. orbiculoides* (data not shown). Therefore, *L. spirale* is not included in the present study. The genus *Pseudombrophila* sensu Brummelen (1995) includes two sections, *Pseudombrophila* and *Nannfeldtiella*. The type species of both sections, *P. merdaria* and *P. guldeniae*, were included. *Smardaea* and *Greletia* were used to root the LSU trees, based on the results of our large-scale Pyronemataceae study (in prep.), which resolved these taxa in a clade basal to the ingroup of this investigation. The effect of additional, alternative outgroups was explored (*Pyronema* and *Byssonectria*).

Molecular methods and Analyses.—Laboratory techniques generally followed procedures outlined in Hansen et al (2002). SSU rDNA was amplified using the PCR with primer pair SL1 (Landvik et al 1997) and NS8 (White et al 1990). In addition to the primers used for PCR, internal primers SL122 and SL344 (Landvik et al 1997), and NS2, NS4 and NS6 (White et al 1990) were used for sequencing. The 5′ end of the LSU-rDNA was amplified using the primer pairs LROR and LR5 (Moncalvo et al 2002). These primers and two internal primers, LR3 and LR3R (Moncalvo et al 2002), were used for sequencing. Electrophoresis and data collecting were done on an ABI PRISM® 3100 or 3730 DNA sequencer (Perkin-Elmer/ABI). To verify the LSU rDNA sequence of *Orbicula*, DNA was extracted and sequenced twice, on two different occasions, from coll. no. F-24441 (C). Sequences were edited using Sequencher 3.0 (Gene Codes, Ann Arbor, Michigan). Sequences are deposited in GenBank (TABLE I). Nucleotide sequences were aligned by hand using the software program Se-Al v. 2.0a11 (Rambaut 2002). Alignments are available from TreeBase (http://www.treebase.org/treebase/) as accessions M2365 (SSU) and M2366 (LSU). Analyses were performed using PAUP* 4.0b 10 for Unix (Swofford 2002) and MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001) on a G5 Macintosh computer. Maximum parsimony (MP), Bayesian and Maximum likelihood (ML) analyses were performed as in Hansen et al (2005), except MP analyses of the LSU data used branch-and-bound searches, ML model parameter values were estimated on the LSU data set, and Bayesian MCMC were run for 2 000 000 generations. To select the model of nucleotide substitution with the least number of parameters best fitting the SSU data set, hierarchical likelihood ratio tests were performed as implemented in the program MrModeltest 2.2 (Nylander 2004). In Bayesian analyses of the SSU and LSU data, the first 2000 trees and 1000 trees were deleted respectively as the ''burn-in'' period of the chain. In addition to parsimony bootstrap proportions (BP) and Bayesian posterior probabilities (PP), ML bootstrap proportions (ML-BP) were generated using 100 bootstrap replicates of ''fast'' stepwise sequence addition for the SSU data, and using 500 bootstrap replicates of heuristic searches, with 10 random addition sequences and TBR branch swapping for the LSU data. The model parameters estimated for the ML analyses were entered manually into PAUP for the ML-BP searches. Topologically constrained MP and ML analyses of the LSU data set were used to evaluate if the cleistothecial form originated once or twice from apothecia-forming taxa

with loss of active spore discharge. MacClade 4.05 (Maddison and Maddison 2002) was used to construct a constraint tree with *O. parietina* and *L. orbiculoides* forced to be monophyletic. Parsimony and ML analyses were performed under the constraint, using the same settings as specified above. For the ML analyses the estimated model parameters were used. The Kishino-Hasegawa test (Kishino and Hasegawa 1989) was used to compare the trees under the constrained and unconstrained topologies in PAUP.

RESULTS

Morphological features.—The material of *O. parietina* used for DNA extraction (C F-24441) agrees with the description by Hughes (1951) and Udagawa and Furuya (1972). This collection is fully mature; asci have completely disintegrated and the dry ascoma is filled with loose, powdery, yellow spores. We want to highlight the following characters in *O. parietina*. Dried mature ascomata show no signs of breaking open (FIG. 1), but a light touch ruptures the thin and brittle outer excipular wall (FIG. 2). Ascomata are glabrous and loosely covered at the base with pale brown hyphal hairs, that are up to 120 μm long or possibly longer, up to 5 μm wide, flexuous, thick-walled (1.2 μm) and remotely septate (FIGS. 4 and 5). The hairs originate from the base of the ascoma, are rounded at the tips, abundant and often form a loose ''mat'' at the base of the ascoma, almost like a subiculum. The cells of the outer excipulum are, in surface view, brown, thick-walled, small and irregularly lobed in outline (puzzle-like) (FIG. 6).

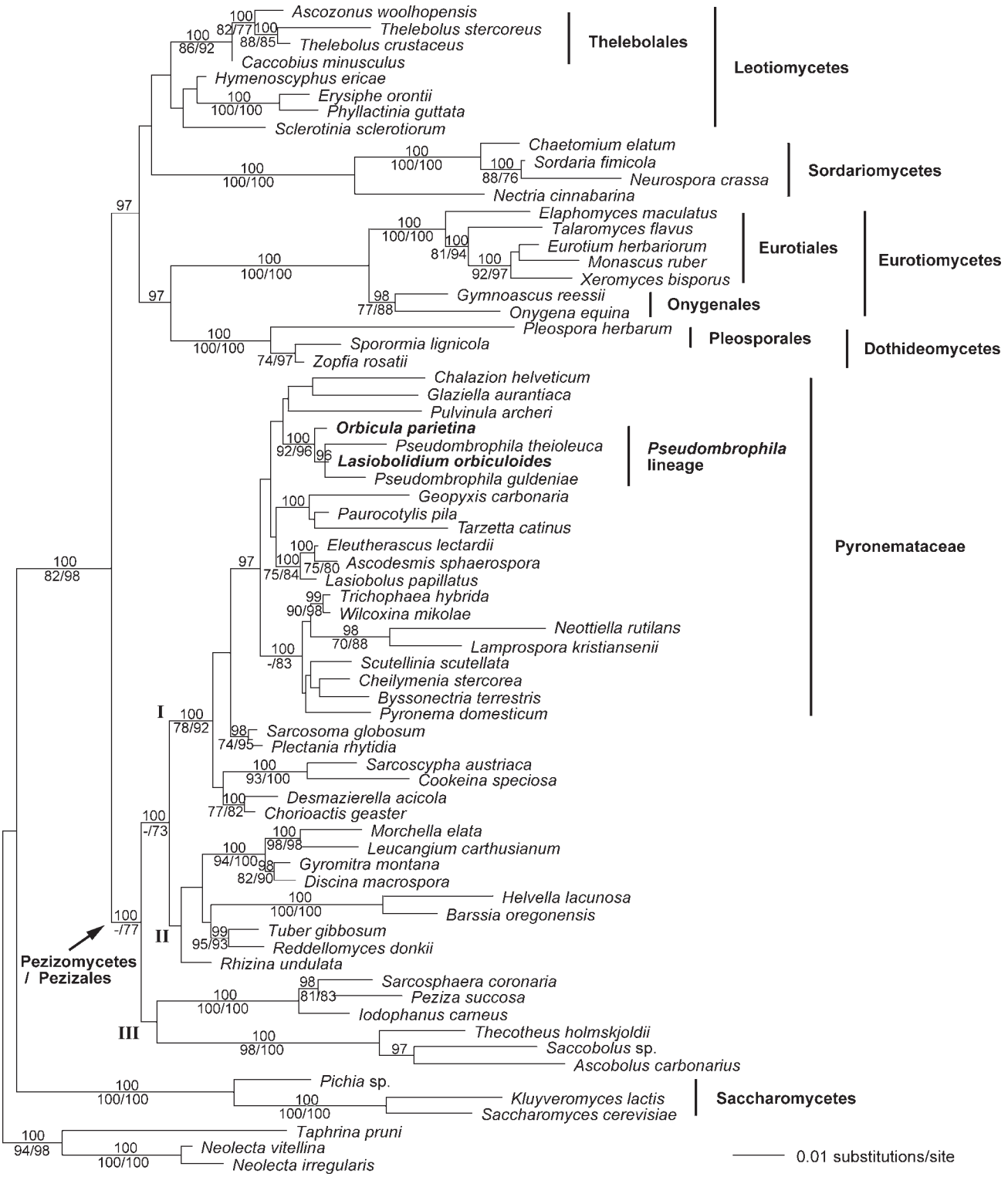
Phylogenetic position of Orbicula *and* L. orbiculoides *among the Ascomycetes; the SSU phylogeny.*—-The SSU rDNA data set included 1721 characters with 398 being parsimony informative. Parsimony analysis resulted in 16 MPTs (1667 steps, CI = 0.496, RI = 0.702). ML analysis resulted in one optimal tree (−lnL = 11 857.5723, FIG. 7) under the GTR + I + G model of sequence evolution selected by MrModeltest with base frequencies A = 0.2593, C = 0.2042, G = 0.2645, T = 0.2720, and the substitution model: [A–C] = 1.2438, [A–G] = 2.3987, [A–T] = 1.5819, [C–G] = 0.8876, [C–T] = 4.9308, [G–T] = 1.0000, with a proportion of invariable sites I = 0.4510, and a gamma distributed shape parameter = 0.5648.

The Pezizomycetes form a moderately supported monophyletic group in the MP and Bayesian analyses (BP 77%, PP 100%), as a sister group to all other Euascomycetes sampled (including Thelebolales) (PP 97%, FIG. 7). Although relationships between classes are resolved with only weak support (BP < 50%), the Dothideomycetes, Eurotiomycetes and Sordariomycetes are highly supported (all 100%). Leotiomycetes is resolved as monophyletic in all analyses but with low support. The orders, Thelebolales and Erysiphales are strongly supported as monophyletic (BP 92–100%, ML-BP 86–100%, PP 100%) within the Leotiomycetes.

Orbicula parietina and *L. orbiculoides* form a highly supported group with two species of the apothecial genus *Pseudombrophila* in all analyses (BP 96%, ML-BP 92%, PP 100%, FIG. 7). The *Pseudombrophila* lineage is deeply nested within a highly supported lineage of members of Pyronemataceae s.l., Sarcoscyphaceae and Sarcosomataceae (BP 92%, ML-BP 78%, PP 100%) (lineage I) within the Pezizales. A few potential sister lineages are supported e.g., the *Ascodesmis-Lasiobolus* lineage (BP 84%, ML-BP 75%, PP 100%) and the *Geopyxis-Tarzetta* lineage (PP 100%). The Pyronemataceae as sampled here, including Ascodesmidaceae and Glaziellaceae, are monophyletic and supported by Bayesian analyses (PP 97%, FIG. 7). The families Discinaceae, Helvellaceae, Morchellaceae, Rhizinaceae and Tuberaceae are highly supported (BP 90–100%, PP 98–100%) and form a weakly supported sister group (II) to the lineage I (FIG. 7). Lineages I and II form a moderately supported group by MP and Bayesian analyses (BP 73%, PP 100%). Ascobolaceae and Pezizaceae are strongly supported (each BP and PP 100%, ML-BP 98–100%) and form a weakly supported lineage (III, BP 65%) that is sister to the lineages I and II.

Relationships of the Pseudombrophila lineage within Pyronemataceae; the LSU phylogeny.—Branch and Bound searches resulted in 2 MPTs (501 steps, CI = 0.653, RI = 0.749) produced from 915 total characters, of which 182 are parsimony informative. ML analysis resulted in one optimal tree (−lnL = 3797.8522, FIG. 8) under the GTR + I + G model with base frequencies A = 0.2519, C = 0.2051, G = 0.3044, T = 0.2386, and the substitution model: [A–C] = 0.6839, [A–G] = 3.0327, [A–T] = 1.9889, [C–G] = 0.6644, [C–T] = 6.2107, [G–T] = 1.0000, with a proportion of invariable sites I = 0.5745, and a gamma distributed shape parameter = 0.7125. The trees recovered by the different analyses of the LSU data did not posses any conflict. Trees obtained in analyses with *Pyronema* and *Byssonectria* as alternative outgroups (not shown) were identical to trees obtained with *Smardaea* and *Greletia* as outgroup.

Confirming the SSU data, *O. parietina* and *L. orbiculoides* form a highly supported group with *Pseudombrophila theioleuca*, *P. merdaria* and *P. guldeniae*, in all analyses (BP 90%, ML-BP 77%, PP 100%, FIG. 8). *Orbicula* groups with *P. theioleuca* with



high support (BP 96%, ML-BP 93%, PP 100%), otherwise relationships within the *Pseudombrophila* lineage are not supported. A clade of three lineages, the *Geopyxis-Tazzetta*, *Ascodesmis-Lasiobolus* and *Pulvinula* lineages, is resolved as the sister group to the *Pseudombrophila* lineage in all analyses, and highly supported by MP bootstrap (BP 86%). The *Geopyxis-Tazzetta*, *Ascodesmis-Lasiobolus* and *Pulvinula* lineages are all highly supported (BP 92–100%, ML-BP 97–100%, PP 100%), but relationships between the lineages are unresolved in the strict consensus tree of the MPTs and by Bayesian analyses, and with only

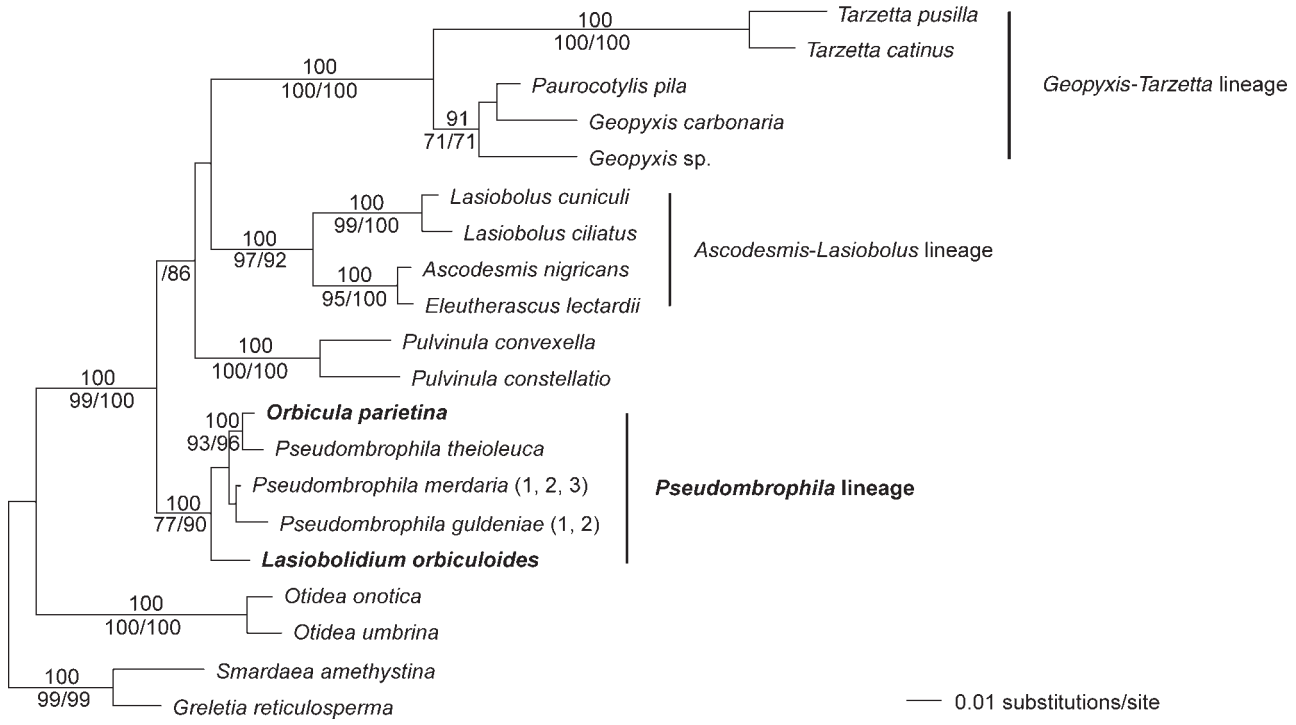


FIG. 8. Phylogenetic relationships of *Orbicula* and *Lasiobolidium orbiculoides* among members of the Pyronemataceae (taxa selection are based on a large-scale molecular study of the Pyronemataceae (in prep.) inferred from LSU rDNA sequences. The tree with the highest likelihood (−lnL = 3797.8522) obtained from maximum likelihood analyses. Branch length corresponds to genetic distance (expected nucleotide substitutions per site). Numbers above branches are posterior probabilities (PP ≥ 95%), obtained from the 50% majority rule consensus tree of the 19 000 trees sampled from a Bayesian MCMC analysis. Numbers below branches that are before the backslash are ML bootstrap proportions (ML-BP > 70%) and numbers after the backslash are MP bootstrap proportions (BP > 70%).

low ML-BP support (<50%, FIG. 8). *Otidea* forms a separate lineage, sister to the highly supported clade of the *Pseudombrophila*, *Geopyxis-Tarzetta*, *Ascodesmis-Lasiobolus* and *Pulvinula* lineages (99–100%).

The most parsimonious interpretation of the molecular LSU phylogeny suggests that the cleistothecial form originated twice in the *Pseudombrophila* lineage (FIG. 8). Nevertheless, constraint MP and ML analyses forcing *O. parietina* and *L. orbiculoides* into a monophyletic group could not be rejected using the Kishino-Hasegawa test ($P < 0.05$). Forced monophyly of the cleistothecial taxa did not yield trees that were significantly less likely or longer than the unconstrained trees (MLT: −lnL = 3806.9008, difference in −LnL = 9.0486; MPT: 505 steps, 4 steps longer than the unconstrained MPTs).

DISCUSSION

Evolutionary relationships.—Molecular phylogenetic analyses confirm the evolutionary origins of *O. parietina* and *L. orbiculoides* within Pyronemataceae (Pezizales) as suggested by Malloch (in Dissing and Schumacher 1994). Other hypotheses, such as close affinities with cleistothecial or highly reduced fungi now placed in the Thelebolales,

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FIG. 7. Phylogenetic placement of *Orbicula* and *Lasiobolidium orbiculoides* within the ascomycetes inferred from SSU rDNA sequences. The tree with the highest likelihood (−lnL = 11 857.5723) obtained from maximum likelihood analyses. Branch length corresponds to genetic distance (expected nucleotide substitutions per site). Numbers above branches are posterior probabilities (PP ≥ 95%), obtained from the 50% majority rule consensus tree of the 18 000 trees sampled from a Bayesian MCMC analysis. Numbers below branches that are before the backslash are ML ''fast'' bootstrap proportions (ML-BP > 70%) and numbers after the backslash are MP bootstrap proportions (BP > 70%). Selected classifications are indicated on the tree for discussion. The lineages I, II and III represents the pezizalean families: Pyronemataceae (including Ascodesmidaceae and Glaziellaceae), Sarcoscyphaceae and Sarcosomataceae (I); Discinaceae, Helvellaceae, Morchellaceae, Rhizinaceae and Tuberaceae (II); and Ascobolaceae and Pezizaceae (III).

FIGS. 9–10. Apothecia of *Pseudombrophila ripensis* (E.C. Hansen) Brumm. developing from large sclerotia, on old dung of cow (JHP-95.104, C). 9. Apothecia at first sub-globular and closed, here deeply cup-shaped to urnulate; the excipular roof over the hymenium has opened and left the margins raised and ragged ×1. 10. Apothecia fully expanded, flattened to curved-lobate, showing dentate to irregular laciniate prominent margin from the disruption of the excipular roof, and receptacles covered with woolly hairs ×1. Photos: J.H. Petersen.

Eurotiales or Onygenales are rejected (FIG. 7). The position within the Pezizales is supported by morphology; the genus *Orbicula* and *L. orbiculoides* possess some clearly pezizalean characteristics such as uniseriate, narrowly clavate to cylindrical asci and unicellular, large, hyaline, thin-walled ascospores (Hughes 1951, Udagawa and Furuya 1972, Malloch and Benny 1973). For the first time, a close relationship between species of *Pseudombrophila* and *O. parietina* is suggested. Furthermore, a close relationship between *O. parietina* and *L. orbiculoides*, as indicated by Malloch and Benny (1973), is shown.

Evolution of cleistothecial forms.—The cleistothecial form, with loss of active spore discharge, has evolved at least once in the *Pseudombrophila* lineage (FIG. 8). Species of *Pseudombrophila* produce disc- to cup-shaped apothecia with an exposed hymenium at maturity (FIGS. 9, 10) and forcibly discharging, operculate asci. Epigeous, open apothecia of various shapes with forcible discharge are the most common ascoma form in the Pezizales and presumably the ancestral state. Cain (1956a, b, 1961) and Malloch (1979, 1981) were among the first to suggest that certain cleistothecial ascomycetes were derived from apothecial and perithecial forms, a concept now widely accepted. Cain (1956a) believed that the cleistothecial fungi represented a large number of unrelated and highly evolved lineages, arguing that these fungi were adapted to passive spore dispersal in very specific ecological niches. Malloch (1979, 1981) placed several cleistothecial taxa in the Pezizales, of which only *Cleistothelebolus*, *Warcupia* and *Orbicula* are still retained in the order. Recent molecular phylogenetic analyses, especially within perithecial lineages, have strengthened the evidence that the cleistothecial ascoma with concomitant loss of active spore discharge has arisen independently from ostiolate forms multiple times (e.g., Berbee and Taylor 1992, Rehner and Samuels 1995, Suh and Blackwell 1999).

An analogous situation within the Pezizales is the repeated evolution of hypogeous or semi-hypogeous, closed ascomata, with loss of active spore discharge. Truffle and truffle-like forms evolved independently at least 10 times in six different families (e.g. Trappe 1979, O'Donnell et al 1997, Landvik et al 1997, Percudani et al 1999, Hansen et al 2001 and unpublished). Molecular data supports the view (e.g. Trappe 1979) that the truffles are all derived from epigeous apothecial ancestors, through selection forces related to animal mycophagy, reduction in water loss (Thiers 1984, Bruns et al 1989), or selection in mycorrhizal fungi for deposition into the soil spore-bank (Miller et al 1994).

The grouping of *O. parietina* and *L. orbiculoides*, taxa that produce completely closed ascomata, with *Pseudombrophila*, is not as surprising as it first might seem. All species of *Pseudombrophila* produce subglobular ascomatal primordia and in *P. hepatica*, *P. leporum*, *P. cervaria* and *P. theioleuca* these are closed at early stages. The excipular roof over the hymenium opens in the mid-mesohymenial phase when the asci are ripening (Brummelen 1995). In most species of *Pseudombrophila* section *Pseudombrophila* the evidence of the tearing of the excipular roof is seen in mature ascomata, by a ragged rim of the excipular margin (FIGS. 9, 10). We hypothesize that in the

Pseudombrophila lineage, ascomata forms that never open (FIG. 1) are derived from ascomata that open late in development (FIGS. 9, 10). Once the opening of the ascomata is lost, relaxation of selection for forcible spore discharge may permit loss of some or all of the accompanying morphological traits, such as the loss of the operculum and/or loss of a distinct hymenial layer (Malloch 1981). In a transition from open apothecia to cleistothecial forms, *Orbicula* may be considered a morphological intermediate and *L. orbiculoides* a more derived form. In *O. parietina* asci arise in a hymenium at the base of the ascoma accompanied by paraphyses, whereas in *L. orbiculoides* asci are not arranged in a parallel layer but rather arise in small clusters at several points within the ascoma and paraphyses are lacking (Malloch and Benny 1973).

Morphology and ecology.—The excipular hairs found in *Orbicula* (FIGS. 4, 5) are of the same type as found in some species of *Pseudombrophila*, most notably in species previously placed in *Fimaria*, e.g. *P. theioleuca*, *P. hepatica* and *P. dentata* (Brummelen 1962, Jeng and Krug 1977 with *P. dentata* as *Fimaria trochospora* Jeng & Krug, Pfister 1984). All hairs in *Pseudombrophila* are pale brown, hyphoid and originate from the outermost layer of the excipulum from base to margin (Brummelen 1995). Ascomatal hairs of *L. orbiculoides* also originate from the outermost excipular cells, are flexuous to wavy, remotely septate, thick-walled, very long (2 to 3 mm; Malloch and Benny 1973), and scattered all over the ascoma.

In *Pseudombrophila* species the outer excipulum is differentiated from the underlying medullary excipulum (Brummelen 1995) and is comparable to the excipulum of *O. parietina* and *L. orbiculoides*. It is composed of thick-walled cells, subglobose to irregularly lobed in outline in surface view (consisting of closely compacted oblong or isodiametric cells) (FIG. 6). *Orbicula parietina* has a more differentiated pezizalean excipulum than *L. orbiculoides*, which has a simple one-celled excipulum (Malloch and Benny 1973). In *O. parietina* the outermost cells of the excipulum are thick-walled, small and brown (FIG. 6), compared to the inner cells, which are thin-walled, large and hyaline (Hughes 1951). In all species of *Pseudombrophila* and in *O. parietina* the pigment is intercellular and amorphous (Brummelen 1995, and FIG. 6).

The similarities in ecological requirements and substrate also support the close relationships between species of *Pseudombrophila*, *O. parietina* and *L. orbiculoides*. All are likely saprobes, fruiting on dung or well-rotted material, and species of *Pseudombrophila* and *O. parietina* fruit in cold conditions. The optimal temperature for developing fruit-bodies of *Pseudombrophila* is between 10 and 15 C, and thus they are found more frequently in spring and in mild winters (Brummelen 1995). Arx (1981) listed *O. parietina* as psychrophilous and Campbell et al (1991) reported *Orbicula* fruiting in early spring on *Pseudocercosporella*-inoculated oat kernels left on sand over winter, and considered it to be psychrophilic. Species of *Pseudombrophila* are strictly coprophilous (FIGS. 9 and 10); occur on soil or vegetable debris contaminated with dung, urine or urea; on decaying stems and leaves of plants; or on rotting materials (Brummelen 1995). *Orbicula parietina* is most commonly reported on a variety of rotting substrates, including damp paper, cardboard, straw, willow baskets and leaves, or directly on dung (Hughes 1951, Udagawa and Furuya 1972, FIGS. 1–3). Hughes (1951) found the best medium for fruiting of *Orbicula* to be tapwater agar with ground weathered rabbit pellets. *Lasiobolidium orbiculoides* occurs on dung but also has been reported from soil (in Yaguchi et al 1996).

Conclusions and taxonomic directions.—Based on LSU rDNA sequence data, morphology and ecology we consider *Orbicula* and *Pseudombrophila* sensu Brummelen (1995) to be congeneric. The genus *Pseudombrophila* sensu Brummelen (1995) includes the formerly recognized genera *Fimaria* and *Nannfeldtiella*. Further sampling of species of *Pseudombrophila* for molecular phylogenetic study may reveal that these genera represent separate lineages that could deserve recognition. *Orbicula* groups with *P. theioleuca*, a species placed in *Fimaria* by Brummelen (1962). The type species of *Fimaria*, *P. hepatica*, was not sampled. Ascomata that open late in development and similarities in excipular hair morphology supports the placement of *O. parietina* in *Fimaria*. In the *Pseudombrophila* lineage *Orbicula* (Cooke 1871) is the earliest generic name and thus has priority. If this lineage is recognized as a single genus, we will propose to conserve *Pseudombrophila* against *Orbicula*, because *Pseudombrophila* includes a larger number of species (28 total, Brummelen 1995). *Lasiobolidium orbiculoides* also may be considered congeneric with *Pseudombrophila*. However, based on LSU rDNA sequences *L. orbiculoides* is not unambiguously nested within *Pseudombrophila* (FIG. 8) and therefore no combination will be made. To further understand phylogenetic relationships in the *Pseudombrophila* lineage, and to determine the number of times the cleistothecial form has arisen, a larger sampling of taxa within the lineage is needed. Detailed developmental

studies of *Pseudombrophila* species and *L. orbiculoides* have been done (Brummelen 1995, Janex-Favre and Locquin-Linard 1979), but comparable studies with *O. parietina* are lacking. Studies of ascomatal development in *O. parietina* would provide data that could further clarify the evolution of closed forms in the apothecia-forming operculate lineage.

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