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Orbilia ultrastructure, character evolution and phylogeny of Pezizomycotina

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Abstract: Molecular phylogenetic analyses indicate that the monophyletic classes Orbiliomycetes and Pezizomycetes are among the earliest diverging branches of Pezizomycotina, the largest subphylum of the Ascomycota. Although Orbiliomycetes is resolved as the most basal lineage in some analyses, molecular support for the node resolving the relationships between the two classes is low and topologies are unstable. We provide ultrastructural evidence to inform the placement of Orbiliomycetes by studying an *Orbilia*, a member of the only order (Orbiliales) of the class. The truncate ascus apex in the *Orbilia* is thin-walled except at the margin, and an irregular wall rupture of the apex permits ascospore discharge. Ascus, ascogenous and non-ascogenous hyphae were simple septate, with septal pores plugged by unelaborated electron-dense, non-membranous occlusions. Globose Woronin bodies were located on both sides of the septum. Nuclear division was characterized by the retention of an intact nuclear envelope, and a two-layered disk-shaped spindle pole body. The less differentiated nature of the spore discharge apparatus and septal pore organization supports an earliest diverging position of Orbiliomycetes within the subphylum, while the closed nuclear division and disk-shaped spindle pole body are interpreted as ancestral state characters for Ascomycota.

Key words: Ascomycota, evolution, morphology, systematics, ultrastructure

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Introduction

Ascomycota is a monophyletic phylum (Lutzoni et al. 2004, James et al. 2006, Spatafora et al. 2006, Hibbett et al. 2007) comprising three subphyla, Taphrinomycotina, Saccharomycotina and Pezizomycotina (Sugiyama et al. 2006, Hibbett et al. 2007). Taphrinomycotina, according to the current classification (Hibbett et al. 2007), consists of four classes, Neolectomycetes, Pneumocystidiomycetes, Schizosaccharomycetes, Taphrinomycetes, and an unplaced genus, *Saitoella*, whose members are ecologically and morphologically highly diverse (Sugiyama et al. 2006). Soil Clone Group 1, poorly known from geographically widespread environmental samples and a single culture, was suggested as a fourth subphylum (Porter et al. 2008). More recently however the group has been described as a new class of Taphrinomycotina, Archaeorhizomycetes (Rosling et al. 2011), based primarily on information from rRNA sequences. The mode of sexual reproduction in Taphrinomycotina is ascogenous without the formation of ascogenous hyphae, and except for the enigmatic, apothecium-producing *Neolecta*, members lack an ascomatal or conidiomatal form. Saccharomycotina is characterized by fungi that reproduce asexually by individual budding yeast cells but also may form hyphae/pseudohyphae. Only a single order (Saccharomycetales) is accepted. The third subphylum Pezizomycotina is a large lineage that includes primarily filamentous fungi with morphologically distinct and complex ascomatal types. This ecologically highly disparate group includes an assortment of fungi that are saprobes, plant and animal pathogens and mutualists. The ascogenous hypha is characteristic of Pezizomycotina members and is not present in members of the other subphyla. The major clades within Pezizomycotina supported by molecular phylogenetic studies include the classes Sordariomycetes, Leotiomycetes, Lecanoromycetes, Lichinomycetes, Laboulbeniomycetes, Eurotiomycetes, Arthoniomycetes, Dothideomycetes, Pezizomycetes and Orbiliomycetes (Spatafora et al. 2006, Hibbett et al. 2007). Of these, Pezizomycetes and Orbiliomycetes are considered the two early diverging branches of Pezizomycotina (Spatafora et al. 2006). The early diverging position of Pezizomycetes, characterized by operculate asci, has been confirmed by molecular phylogenetic studies (Hansen and Pfister 2006, Spatafora et al. 2006). Among the two early diverging lineages, Orbiliomycetes, characterized by non-operculate, mainly non-poricidal asci with branched

bases, was indicated as the earliest diverging branch in molecular studies (Gernandt et al. 2001, James et al. 2006, Spatafora et al. 2006) but with only weak support. Ancestral state reconstruction efforts as part of a six-gene, 420-species phylogenetic study of Ascomycota (Schoch et al. 2009a) supported the hypothesis of Nannfeldt (1932) and Gargas and Taylor (1995) of a common ancestor of Pezizomycotina characterized by apothecial ascomata. However data from the studies seem inadequate to confidently resolve ancestry and distinguish which class is the earliest Pezizomycotina lineage (Spatafora 2007). Resolving this placement with high confidence is important to understand ascus evolution and is necessary for ancestral state reconstructions.

Orbiliomycetes is represented by a single order, Orbiliales, with the only family, Orbiliaceae, consisting of two accepted teleomorph genera, *Hyalorbilia* and *Orbilia* (Eriksson et al. 2003). *Pseudorbilia* was described (Zhang et al. 2007) as a third teleomorph genus to include a species that shares characteristics with both *Orbilia* and *Hyalorbilia*. The family has about 288 described species (Kirk et al. 2008). Orbiliomycetes members are characterized by small, translucent, waxy, disk-shaped apothecia that are formed in soil and wood. Apothecia are produced without stromata and have an ectal excipulum of globose, angular or prismatic cells. Small asci with truncate to hemispherical apex are intermixed with paraphyses that typically are swollen at the tip. Small hyaline ascospores usually possess an apical spore body that selectively stains in cresyl blue (Baral 1994). About 10 orbiliaceous anamorph genera are known (Zhang et al. 2007), and all are hyphomycetous. Certain of these anamorphic genera are predatory with known nematode-trapping abilities, and their evolution and phylogeny have been studied with nuclear and protein coding genes (Li et al. 2005, Yang et al. 2007). Based on synapomorphies of the sporocarp (apothecium) and mode of ascus dehiscence (inoperculate), orbiliaceous members were treated in Helotiales (= Leotiales) (Spatafora et al. 2006). However molecular studies (Pfister 1997, Gernandt et al. 2001) indicated their isolated position from the other taxa in Helotiales along with a suggested position outside the Leotiomycetes. Eriksson et al. (2003) erected a new order and class to recognize the separate lineage, and subsequent molecular studies (James et al. 2006, Spatafora et al. 2006, Wang et al. 2006) confirmed its monophyly and close relationship to Pezizomycetes. This phylogenetic placement of the class is reflected in the current supra-ordinal classification (Hibbett et al. 2007).

Ultrastructure has played a major role in ascomycete systematics. The ascus and the spore discharge apparatus in the ascus tip of a diverse group of ascomycetes have been the subject of many ultrastructural studies (Benny et al. 1978; Verkley 1992, 1993a, b, 1994, 1995; Leenurm et al. 2000; Landvik et al. 2003; Pärtel and Raitviir 2005) with systematic implications, and a review by Bellemère (1994) stressed the phylogenetic significance of these characters. Ascus dehiscence characters have proved to be most informative at class level within Pezizomycotina (Schoch et al. 2009a). Septal ultrastructure has been used to delimit various ascomycete groups, and its effectiveness in elucidating phylogenetic relationships has been well demonstrated (Curry and Kimbrough 1983, Kimbrough 1994). A series of studies (Schrantz 1964, Carroll 1967, Beckett 1981a, Curry and Kimbrough 1983, Kimbrough and Curry 1986a) on a number of ascomycete taxa (mainly pezizalean) revealed simple to complex structures associated with the septum in vegetative hyphae, ascogenous hyphae and the ascus base. These structures that partially or entirely occluded the septal pores had structures and distribution patterns that were shown to be consistent and specific to various ascomycete groups (Kimbrough 1991, 1994; Markham 1994). Nuclear division and spindle pole body characters also have been found to provide inherent phylogenetic signals at subphylum rank and to a limited extent at class rank within Ascomycota (Celio et al. 2006). In this study we analyzed ultrastructural characters of ascus, septal pores and nuclei from ascomata of an *Orbilia* species to assess phylogenetic relationships of the early diverging Pezizomycotina.

MATERIALS AND METHODS

Morphological methods.—Fresh apothecia of an *Orbilia* species were collected 15 Aug 2009 at Lake Bronson State Park, Kittson County, NW Minnesota (voucher collection MIN 921434, Herbarium, University of Minnesota) from decaying wood and fixed with 2% glutaraldehyde, 2% paraformaldehyde and 1 mM calcium chloride in 0.1 M sodium cacodylate buffer, pH 7.2, 2.5 h at 4 C. Samples were rinsed in three changes of 0.1 M sodium cacodylate buffer 20 min each. Post-fixation was performed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1.5 h at 4 C and rinsed once in the same buffer 10 min followed by three distilled water washes, 10 min each. The samples were stained overnight with 0.5% aqueous uranyl acetate at room temperature in the dark and after three 5 min washes in distilled water were dehydrated in a graded ethanol series (25% 20 min, 50% 20 min, 75%, 95% 30 min each, 100% ethanol 1 h, four quick changes in 100% ethanol). Infiltration was in low viscosity Spurr's resin (Spurr 1969) prepared with the modified formulation of Ellis (2006). Samples were dish embedded (Mims et al. 2003) in Spurr's resin. The resin was polymerized at 74 C for 48 h. Semi-thin sections (2 μm) for light microscopy were obtained with a glass knife, collected on glass slides and stained with 0.05% toluidine blue O in borate buffer (pH 9) by gently warming

TABLE I. Characters and character states for data matrix

Ascus

1. Ascus apex non-truncate, 0; truncate, 1.
2. Ascus dehiscence non-poricidal, 0; poricidal, 1; operculate, 2; fissitunicate, 3; deliquescent, 4; rostrate, 5.

Septa

3. Septal pore occlusion in non-ascogenous hyphae absent, 0; non-lamellate, convex to somewhat flattened, dense, non-membranous occluding material, 1; granular lamellate structure, 2; subspherical occlusion, non-membrane-bound, lacking translucent finger-like extensions or plates, 3; subspherical occlusion, non-membrane-bound, with translucent finger-like extensions or plates, 4; osmiophilic material associated with reticulate (tubular) pattern, later associated with microbody derived crystal in pore plug, 5.
4. Septal pore occlusion in ascus and ascogenous hyphae non-lamellate, convex to somewhat flattened, dense, non-membranous occluding material, 0; convex or biconvex band or more complex hemisphere, 1; toroid occlusion, i.e., donut-like with central pore, 2; pore plug with reticulate pattern on both sides of the pore, no crystalline bodies, 3; subspherical pore cap membrane, 4.

over an alcohol flame and rinsing with distilled water. Sections were air-dried and mounted with cover slips in Eukitt (Caliberated Instruments Inc., Ardsley, New York) and observed with a Zeiss Axioskop equipped with a SPOT digital camera (Diagnostic Instruments, Michigan). Measurements of asci, paraphyses and spores are from fixed, stained sections 2 μm thick. Thin sections (96–100 nm) were cut with a diamond knife on a Reichert-Jung ultramicrotome and were collected on single slot formvar/carbon-coated copper grids (Electron Microscopy Sciences, Hatfield, Pennsylvania) and post-stained with 3% aqueous uranyl acetate 20 min and triple lead stain (Sato 1968) 3 min. Specimens were examined with a Philips CM 12 transmission electron microscope (TEM) operating at 60 kV.

The ascus and septal pore occlusion characters and their character states used for the morphological analysis are provided (TABLE I). Data for the analysis (except for *Orbilia* sp.) were obtained from the following sources: for *Neolecta irregularis* (AFTOL data unpubl), *Taphrina deformans* (Syrop and Beckett 1976), *Saccharomyces cerevisiae* (Schoch et al. 2009a), *Emericella nidulans* (Momany and Hamer 1997), *Herpomyces* species (Hill 1977), *Ascobolus immersus* (Kimbrough and Curry 1985), *Saccobolus portoricensis* (Kimbrough and Curry 1985), *Thecotheus pelletieri* (Kimbrough and Curry 1985), *Helvella crispa* (Kimbrough and Gibson 1989), *Helvella pezizoides* (Kimbrough and Gibson 1989), *Genea gardnerii* (Li and Kimbrough 1994), *Iodophanus carneus* (Kimbrough and Curry 1985), *Peziza quelepidotia* (Curry and Kimbrough 1983), *Acervus episparticus* (Kimbrough and Curry 1986a), *Aleuria cestrica* (Kimbrough and Curry 1986b), *Urnula craterium* (Li and Kimbrough 1995a), *Tuber maculatum* (Li and Kimbrough 1995b), *Pseudopeziza trifolii* (Meyer and Luttrell 1986), *Chaetomium brasiliense* (Rosing 1981), *Neurospora crassa* (Trinci and Collinge 1974, Read and Beckett 1996), *Sordaria fimicola* (Furtado 1971), *Geoglossum umbratile* (Verkley 1994), *Sporormiella australis* (Blanchard 1972), *Cladonia grayi* (Honegger 1983), *Peltula cylindrica* (Schultz et al. 2001), *Arthonia radiata* (Reynolds 1989). Data interpretations for characters 1 and 2 are ours, and data for characters 3 and 4 were coded based on the interpretation available in the Assembling the Fungal Tree of Life Structural and Biochemical Database (SBD) (http://aftol.umn.edu). We followed Schoch et al. (2009a) for definitions of ascus dehiscence types and for the tree structure used to plot character states. Character state reconstruction under parsimony criterion was performed with Mesquite 2.6 (Maddison and Maddison 2008). The character data matrix used for the morphological analysis is included (TABLE II).

TABLE II. Character data matrix used for the morphological analysis. See Table I for character definitions

Taxa	Character no. 1	2	3	4
Taphrina deformans	1	0	—	—
Neolecta irregularis	1	0	5	3
Saccharomyces cerevisiae	—*	4	—	—
Emericella nidulans	0	4	—	—
Herpomyces species	0	4	0	—
Ascobolus immerses	0	2	3	1
Saccobolus portoricensis	0	2	—	1
Thecotheus pelletieri	0	2	0	1
Helvella crispa	0	2	3	1
Helvella pezizoides	0	2	2	1
Genea gardnerii	0	4	2	1
Iodophanus carneus	0	2	2	1
Peziza quelepidotia	0	2	2	1
Aleuria cestrica	0	2	2	1
Urnula craterium	0	2	3	1
Tuber maculatum	0	4	2	1
Pseudopeziza trifolii	0	2	3	—
Chaetomium brasiliense	0	1	0	2
Neurospora crassa	0	1	4	4
Sordaria fimicola	0	1	3	—
Orbilia sp.	1	0	1	0
Geoglossum umbratile	0	1	—	—
Sporormiella australis	0	3	—	2
Cladonia grayi	0	5	—	—
Peltula cylindrical	0	5	—	—
Arthonia radiata	0	3	—	—

* Missing data are indicated by a minus symbol.

Molecular methods.—Several fresh ascocarp segments were placed in 2× cetyl trimethylammonium bromide (CTAB) with sterile technique and stored at room temperature until extraction. Tissue was macerated in CTAB to which 0.25%

proteinase K was added and incubated at 65 C with 20% sodium dodecyl sulfate (SDS) 2 h. Genomic DNA was extracted with 25 : 1 chloroform : isoamyl alcohol and incubated with RNAse A (10 mg/mL) 2 h. DNA was precipitated with cold isopropanol, washed with cold 70% ethanol, air dried, resuspended in 50 µL 1M Tris EDTA buffer and a portion diluted 1 : 10 with sterile filtered water. This dilution (5 µL) served as the template for amplification of the internal transcribed spacer (ITS) region, including ITS1-5.8s-ITS2, of nuclear ribosomal DNA using the primer pair ITS1-f and ITS4 (Gardes and Bruns 1993, White et al. 1990) with a final concentration of 0.5 µM each, in a 25 µL reaction mix (total volume) of Hot Star Taq plus Master Mix (QIAGEN). Amplification took place in a DNA Engine PTC-200 Thermal Cycler (Bio-Rad Laboratories, Waltham, Massachusetts) programmed with this schedule: (i) 95 C for 15 min, (ii) 94 C for 1 min, (iii) 94 C for 30 s, (iv) 50 C for 30 s, (v) 72 C for 2 min, (vi) repeat steps 3–5 for 30 cycles, (vii) extend at 72 C for 2 min, (viii) hold at 4 C. The PCR product was checked by gel electrophoresis on a 1% agarose gel stained with 0.01% ethidium bromide that was run at 106 V for 25 min, and a band visualized under UV. The PCR product was cleaned with a 40% v/v addition of ExoSap-It (USB Products, Affymetrix Inc., Cleveland, Ohio), following manufacturer's directions and sequenced on an ABI 3730xl capillary sequencer (Applied Biosystems, Foster City, California) with ABI Big Dye Terminator 3.1 chemistry in the Biomedical Genomics Center at the University of Minnesota. A contig was assembled in Sequencher 4.10.1 (Gene Codes Corp., Ann Arbor, Michigan), the segments with ambiguous bases trimmed from the ends and similar sequences searched in GenBank with the BLAST algorithm. Similar sequences were uploaded onto Sequencher, assembled into an alignment and percent similarities calculated. The sequence for *Orbilia* sp. was deposited in GenBank with accession number JN088481.

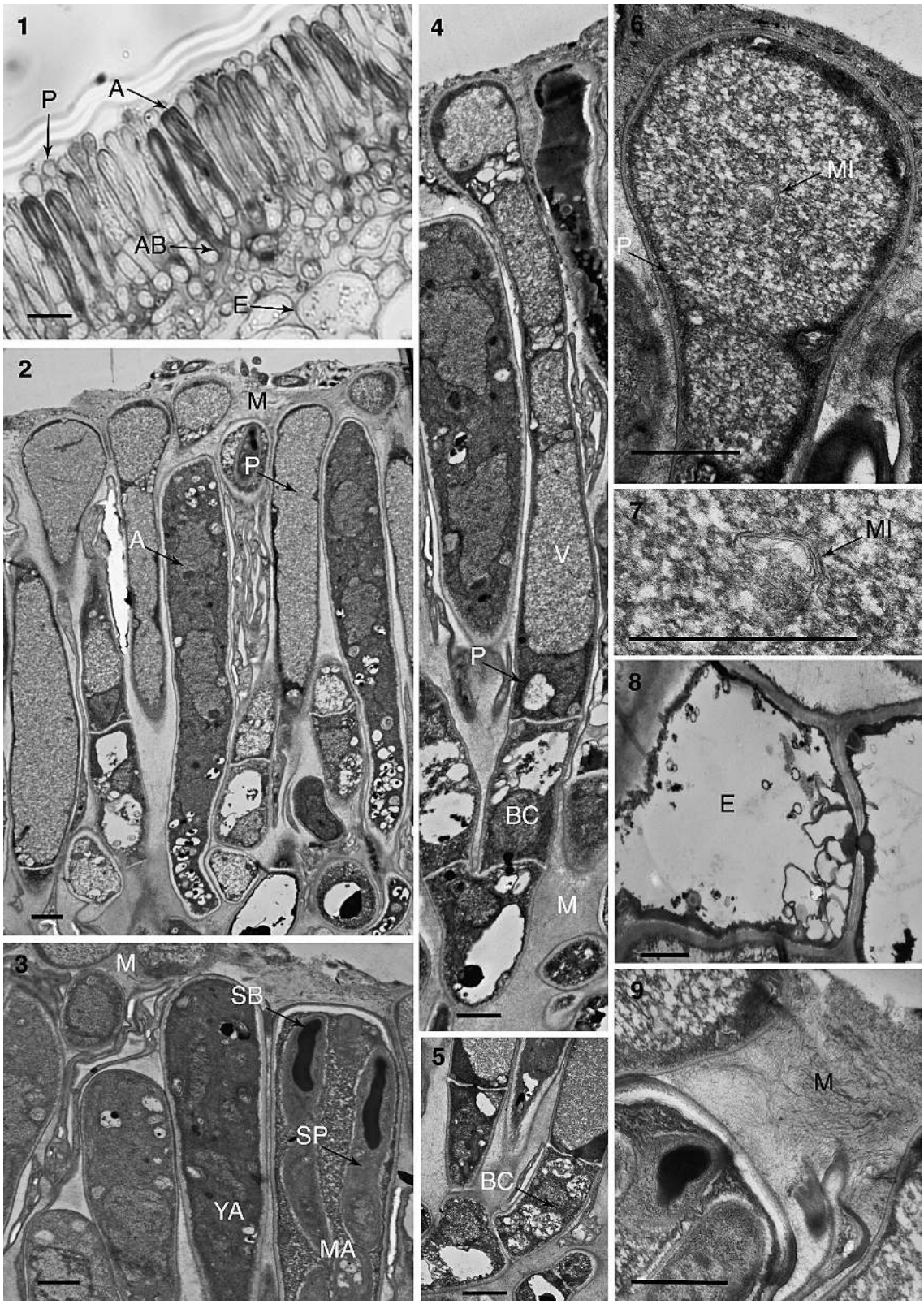
RESULTS

An undescribed species of *Orbilia* was collected in northern Minnesota on damp, rotting decorticated wood in bottomland deciduous woods of *Quercus macrocarpa* and *Prunus* sp., near a stand of *Pinus banksiana*. The gregarious apothecia were up to 1.3 mm diam, translucent white with a slight pink tinge, exciple and hymenium concolorous, smooth, stalkless, and centrally attached. The exciple was pseudoparenchymatous, of more or less isodiametric cells, mostly 2–4 cells thick along the disk, and thicker at attachment point. Asci eight-spored, clavate, tapered and sometimes forked at the base, 20.6–24 × 2.45–3.43 µm as measured in the hymenium. Spores were fusiform, hyaline, smooth, nonseptate, tapered at one end, 7.35–8.33 × 1.23 µm. Spore bodies were about 3 × 0.5 µm and the cytoplasm stained deep blue in toluidine blue O. Paraphyses were cylindrical, slightly longer than asci, with their tips swollen just beyond the ascus tips, 22–25.5 × 2–2.45 µm, swelling to 3–3.43 µm at the tips (FIGS. 1–4).

The *Orbilia* collection yielded an ITS1-5.8s-ITS2 nrDNA contig 606 base pairs long after the ends were trimmed of segments with ambiguous bases. A BLAST query in GenBank produced a 99% similarity hit to a sequence (GenBank accession number GU188276) from an *Orbilia* from China referred to as ''*O.* aff. *luteorubella*'' in Yu et al. (2011). Direct comparison between these two sequences showed six base pair differences. The next most similar ITS sequence (GenBank accession number U72607) was only 96% similar, from an *Orbilia* collected in Massachusetts (Pfister 1997), also referred to as ''*O.* aff. *luteorubella*'' (Yu et al. 2011). The nomenclatorial status of the isolate we studied and that of Yu et al. (2011) is not clear. Therefore we refer here to the MN specimen in our study as *Orbilia* sp.

Ascomata and ascus tip.—Asci interspersed with paraphyses in a regular hymenium were distinguished readily based on shape and cytoplasmic organization of the cells. Compared to paraphyses, asci stained darker with toluidine blue O (FIG. 1) when viewed under the light microscope and were found to have an organelle-rich cytoplasm (FIG. 2) under the TEM. Asci were cylindrical-clavate with a more or less truncate, thin-walled apex with shoulder-like edges (FIGS. 1, 2, 3) and were borne on branched bases (FIG. 1). Each mature ascus possessed eight fusoid to elliptical spores, each with a uniformly highly electron-dense apical spore body (FIGS. 3, 11). Paraphyses, also arising from branched bases (FIGS. 4, 5), were distinct with characteristic inflated apices that usually extended beyond the asci. Paraphyses were septate with a lower basal cell (stalk cell) that had fewer vacuolate cytoplasmic contents (FIGS. 4, 5) and a terminal cell with large vacuoles filled with homogenous granular contents (FIGS. 4, 6). Membranous inclusions (FIGS. 6, 7) sometimes were observed within the granular contents. It was difficult to confirm whether the paraphyses arose from the ascogenous hyphae or non-ascogenous hyphae because the hyphae that gave rise to the asci and paraphyses were tightly packed and intertwined. The characteristic granular contents in the terminal cells of paraphyses helped differentiate them from the asci. Asci and paraphyses were covered in a gelatinous matrix, which was up to 2.5 µm thick over the hymenium (FIGS. 2, 3, 4, 9). Isodiametric ectal excipulum cells (FIGS. 1, 8) with scanty cytoplasmic contents subtended the hymenial and subhymenial layers.

The ascus wall consisted of two layers: an outer almost uniformly thickened electron-dense layer (20–40 nm thick) and an inner less electron-dense amorphous layer (20–80 nm thick) (FIGS. 10, 11). The inner wall layer of a mature ascus was thickened



FIGS. 1–9. Asci and paraphyses in the hymenium of *Orbilia* species. 1. Light micrograph of hymenium showing paraphyses interspersed with asci (A) arising from forked bases (AB) and excipulum cells (E); paraphysis (P). 2–9. Transmission electron micrographs. 2. Hymenium showing young asci (A) and paraphyses (P). The shape of the ascus apex differs according to whether the section is median or peripheral through the apex. A granular matrix (M) covers the hymenium (also seen in

(120–160 nm) at the slightly protruding shoulder-like flanks (FIG. 11) and sometimes was thinner toward the central region of the flattened apex. Ascospores were arranged in a single row spanning the length of the ascus in young asci, while they were arranged in clusters of 2–3 in the apical end of mature asci. An electron-light zone was found between the plasma membrane and the inner wall layer of the ascus apex (FIG. 11) and became prominent as the ascus matured. This zone was almost absent or little developed in very young asci. In mature asci the electron-light zone (up to 200 nm thick) was evident until ascospore discharge by an irregular wall rupture through the flat ascus apex between the thickened areas (FIGS. 12, 13). Ascus wall layers remained intact until spore release. Dehisced asci (FIGS. 12, 13) with a thickened inner wall layer at the shoulder-like edges and the ruptured portion of the ascus apex persisted among the paraphyses and younger asci after spore discharge.

Septal pores.—All asci/ascogenous hyphae and non-ascogenous hyphae observed were simple septate, with a strikingly similar septal pore organization (FIGS. 14–18). The plasma membrane was continuous through the single pore, and the pores were approximately 160–200 nm wide. Septal pores had an unelaborated margin. A more or less convex, non-membranous, electron-opaque band plugged the septal pores (FIGS. 14–18). The single convex electron-dense band was simple, spanned the width of the pore, appeared to be connected to the plasma membrane across the pore and lacked substructure (FIG. 18). The globose membrane-bound Woronin bodies were rarely located on both sides of the septum in the ascus base (FIG. 14) but were common on both sides in ascogenous hyphae (FIG. 15), paraphyses (FIG. 16) and excipulum cells (FIG. 17). Woronin bodies (FIGS. 14–17, 19) were more frequent in the vegetative hyphae than in the ascogenous hyphae and were common near the septa of the basal and terminal cells of the paraphyses and near the septa of excipular cells. They were 200–350 nm diam. A crystal lattice was visible in the Woronin bodies (FIG. 19).

Nuclear division and spindle pole body.—Premeiotic interphase nuclei (number of nuclei observed per stage of nuclear division from all thin sections examined under TEM (n = 17) were oriented along the long axis of the ascus (FIG. 20), were often oblong (2–3.7 μm long) and contained a homogenous nucleoplasm with fine granules and fibrils, part of which might represent condensed chromatin. A nucleolus with a granular texture was visible in many sections, often oriented toward the distal end of the nucleus (FIG. 21). The extranuclear bar-shaped spindle pole body (SPB) with an outer electron-dense layer and an inner less electron-dense layer lay within a depression or on a flattened region of the nuclear envelope (FIGS. 21–23). Interphase nuclei possessed two spindle pole body disks (100–220 nm long × 100–120 nm high), connected by a middle piece (FIG. 23). Prophase I nuclei (n = 6) were larger (4–5.2 μm long) than interphase nuclei, and synaptonemal complexes were evident (FIG. 24).

In metaphase-anaphase I (n = 2) spindle microtubules were aligned at oblique to perpendicular angles to the long axis of the nucleus and did not form a compact central spindle. The spindles were surrounded by bundles of condensed chromatin (FIG. 25). The nuclear envelope remained intact in all nuclei observed in interphase and metaphase-anaphase. The extranuclear SPB (300–350 nm long × 80–100 nm high) was located within a depression or next to a flattened region of the nuclear envelope adjacent to the intranuclear SPB. Spindles radiated from the intranuclear SPB element, which was an amorphous granular region that is more electron dense than the rest of the non-nuclear nucleoplasm and was present at the inner surface of the nuclear envelope opposite the extranuclear SPB. The extranuclear SPB was flattened and disk-shaped with an outer dense and an inner light zone (FIG. 26).

Character state reconstruction.—Resolved and ancestral state changes for morphological characters are plotted on branches of the tree (FIG. 27) that includes taxa belonging to the three subphyla of Ascomycota. Representative taxa of the 11 classes recognized in Pezizomycotina (Schoch et al. 2009a) have been included. The character codes plotted present state changes of the ascus and septal pore occlusion characters. Many taxa lack complete information for septal pore occlusion characters. The non-poricidal mode of ascus dehiscence (clade A) is ancestral for Taphrinomycotina and Orbiliomycetes but changes to other modes of ascus dehiscence in other Pezizomycotina. The truncate ascus apex also is

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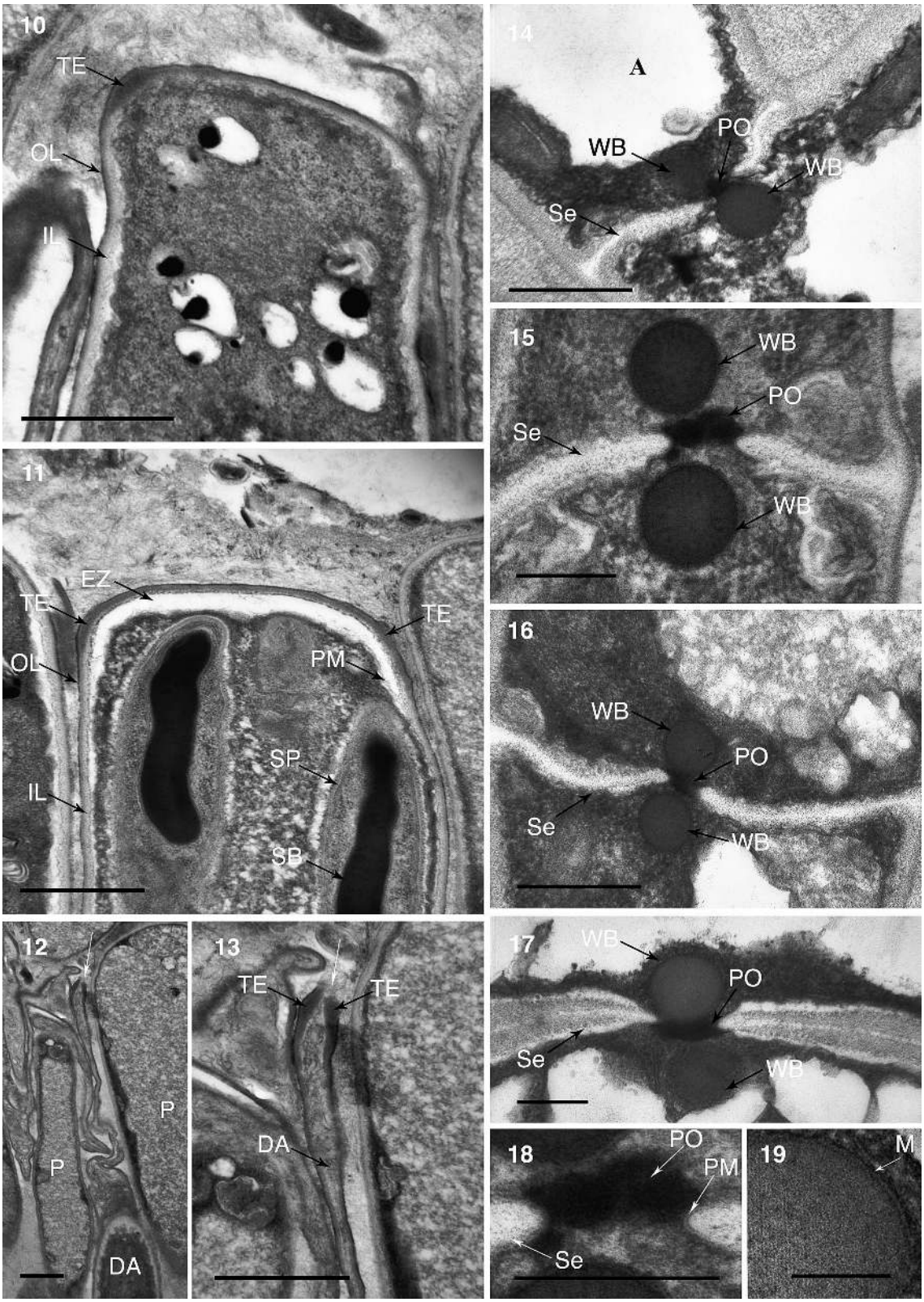
FIGS. 3, 4, 9). 3. A young (YA) and a mature (MA) ascus. The latter has mature spores (SP) with prominent electron-opaque spore bodies (SB). 4. Longitudinal section of paraphysis showing a basal cell (BC) and terminal cell, the latter containing large vacuoles (V) with granular contents of unknown composition. 5. Forked base of paraphyses. Basal cells of the paraphyses have less vacuolate cytoplasmic contents. 6. Paraphysis apex showing a large vacuole with uniform granular contents and membranous inclusions (MI). 7. Magnified view of the paraphysis vacuolar contents. 8. Excipulum cell with sparse cytoplasm and plugged septal pore. 9. Granular matrix covering the hymenium. Bars: 1 = 5 μm, 2–9 = 1 μm.



FIGS. 10–13. Transmission electron micrographs of longitudinal sections of ascus apex of *Orbilia* sp. 10. Apex of a young ascus (prefusion nuclear stage) with a thin electron-dense outer wall layer (OL) and a thicker less electron-dense inner wall layer (IL), which is conspicuously thickened at the protruding edge of the ascus apex (TE). 11. Truncate, mature ascus apex showing the wall layers with characteristic thickening of the inner layer at the edges. An electron-light zone (EZ) that becomes

present in Taphrinomyotina and Orbiliomycetes, but this character is unresolved in clades B and C making it unclear whether it is a shared character. Although data are incomplete for the Saccharomycetes, a shift from a non-poricidal to a deliquescent mode of ascus dehiscence is shown. The septal-pore occlusion in *Orbilia* sp. differs from the *Neolecta* pattern and is a simple non-membranous electron-dense band in both non-ascogenous and ascogenous hyphae. Whether the unique pore occlusion is characteristic of the class needs confirmation from additional taxa. Except for Orbiliomycetes, all other Pezizomycotina classes are characterized by asci with non-truncate apices. Pezizomycetes are characterized by an operculate mode of ascus dehiscence and by a septal pore occlusion in ascus and ascogenous hyphae that is a convex or biconvex band or more complex hemisphere. Within the Pezizomycetes there has been a change in the pore occlusion of the non-ascogenous hyphae from a spherical occlusion to other types in some species. The septal pore occlusions of ascus and non-ascogenous hyphae are supporting characters of Pezizomycetes. The ascus and septal pore occlusion characters seem to have evolved multiple times in the other classes of the Pezizomycotina. The ancestral state of the septal pore occlusion of ascogenous hyphae in Pezizomycotina other than Orbiliomycetes and Pezizomycetes (Clade D) is resolved as a toroid occlusion, but this is based on only two taxa in this lineage and data are absent for most taxa (TABLE I).

DISCUSSION

Nomenclature.—The focus of our study was on phylogenetically informative ultrastructure in *Orbilia*. The Orbiliaceae are undergoing revision by H.-O. Baral (Baral pers comm). Because species identifications in the genus are difficult (Yu et al. 2011) and the revision of the family is not yet available we turned to the ITS region of nrDNA, which has served as an approximation of species at 97% similarity in a wide variety of fungi (Smith et al. 2007). Our taxon nests within the current concept of the genus and, based on the 97% ITS species approximation, appears to be conspecific with the specimen from which GU188276 was sequenced, ''*O.* aff *luteorubella*'' of Yu et al. (2011).

Ascomata and ascus tip.—Apothecioid sporocarp morphology with an exposed hymenium is hypothesized to be the ancestral sporocarp state of Pezizomycotina (Schoch et al. 2009a). Based on information available from the literature and including data obtained during this study, a basal most ancestral status seems to fit the Orbiliomycetes lineage with taxa possessing apothecioid sporocarps that lack stromata and characters that appear to be ancestral within the subphylum. The asci in *Orbilia* are unique among members of Pezizomycotina in that they arise from branched bases and have truncate apices resembling the simple ''préarchaeascé'' type of asci found in *Neolecta* and *Taphrina* species (Landvik et al. 2003) that belong to the basal ascomycete group Taphrinomycotina. This type of ascus is considered to be an ancestral type from which other ascus types are derived (Chaudefaud and Galinou 1953, Landvik 2003). Bundles of 2–3 asci are produced from branched hyphae in *Neolecta*. The slightly conical or horn-like projections at the ascus apices in *Orbilia* also are similar to those observed in *Neolecta* (Landvik 2003).

No definite mechanism of ascospore release has been observed in *Orbilia* sp. and the thin-walled ascus apex ultrastructure agrees with that of *O. luteorubella* described by Benny et al. (1978), except for the electron-light zone in mature asci in the former species. This condition is in contrast to the complex ascus apical apparatus and spore-release mechanism in other non-operculate Pezizomycotina taxa reported from ultrastructural studies of the Helotiales (Bellemère 1977; Benny et al. 1978; Verkeley 1992, 1993a, b, 1994, 1995; Pärtel and Raitviir 2005) and Geoglossales (Verkeley 1994). The thickened ascus apex in these non-operculate members was characterized by distinct cylinder and plug-like structures believed to aid spore release. The operculate ascomycetes, restricted to Pezizomycetes, differ by an apical or subapical operculum that opens in a characteristic pattern (Schoch et al. 2009b) with about eight different ascus dehiscence mechanisms observed in Pezizales (Kimbrough and Curry 1986a). Of interest, the ascus apex

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prominent as the ascus matures is evident between the inner wall layer and the plasma membrane (PM). SB = spore body, SP = spore. 12, 13. Dehisced ascus (DA). Note the thickening of the outer wall, indicating that wall rupture occurred in the center region of the ascus apex (arrow). FIGS. 14–19. Transmission electron micrographs of septal pore organization in *Orbilia* sp., showing electron-dense, non-membranous pore occlusions (PO) and globose Woronin bodies (WB) on both sides of the septum (Se). 14. Septal pore structure at the ascus base. Note Woronin body inside the ascus cytoplasm. 15. Septal pore organization in ascogenous hypha. 16. Septum at base of terminal cell of paraphysis (P). 17. Septum in an excipulum cell. 18. Non-membranous electron-dense septal pore occlusion connecting both sides of the pore in an ascogenous hyphal septum. Plasma membrane (PM) is continuous through the pore. 19. Part of a membrane bound Woronin body (M) with a crystal lattice structure. Bars: 10–13 = 1 μm; 14, 16 = 0.5 μm; 15 = 0.25 μm; 17, 18 = 0.2 μm; 19 = 0.05 μm.

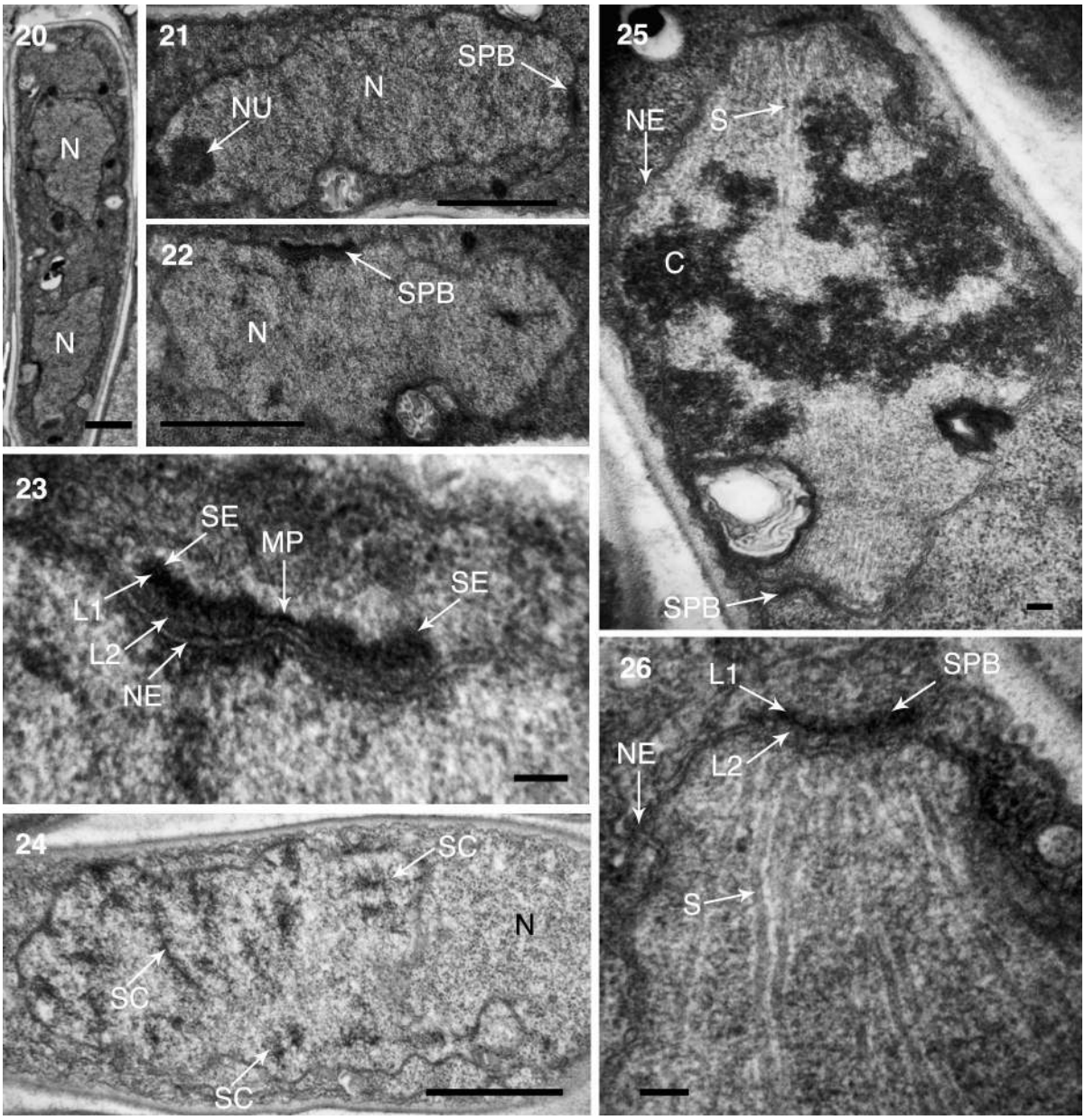
ultrastructure and dehiscence mechanism in *Orbilia* spp. is more like the simple apical apparatus found in *Neolecta* (Landvik et al. 2003) and *Taphrina* (Syrop and Beckett 1976). In *Taphrina* and *Neolecta*, both members of the Taphrinomycotina, no differentiation of ascus apex has been observed and spores actively escape through an irregular slit. A similar mechanism of spore release that results from tearing of the ascus apical wall appears to function in *Orbilia* sp. and *O. luteorubella* (Benny et al. 1978). The electron-light zone observed at the mature ascus apex in *Orbilia* sp. may play a role in forcible spore discharge.

The role of the electron-dense spore body present in mature spores of *Orbilia* spp. was not determined during this study, although data were obtained that support the Benny et al. (1978) elucidation of its development from a mitochondrion present inside the spore. A function as a storage product for use during germination was suggested as the most likely role of the spore body (Benny et al. 1978), and the dense staining of the cytoplasm with toluidine blue O suggests the possibility that it contains RNA (Feder and O'Brien 1968). Speculation by Benny et al. (1978) that the reserve is required until the germ tube comes into contact with an algal associate however lacks proof. In fact an algal presence was never detected in our material, although Benny et al. (1978) reported a blue-green algal association in *O. luteorubella* and in at least one other *Orbilia* species, probably *O. pulviscula* (Benny et al. 1978). On this basis they attributed lichenization to these taxa and proposed a transfer of these species from Helotiales to the lichens (Lecanorales). This transfer proved to be an artificial placement based on current knowledge (James et al. 2006, Spatafora et al. 2006, Wang et al. 2006) that confirmed the placement of the group outside Lecanoromycetes and Lichinomycetes. The report of possible lichenization in at least some species of *Orbilia* (Benny et al. 1978) however is interesting in that it would represent an exception to the statement by Schoch et al. (2009a) that ''lichens remain unreported in extant lineages of Taphrinomycotina, Saccharomycotina, and the earliest diverging Pezizomycotina classes (Orbiliomycetes and Pezizomycetes).'' In light of hypotheses including the independent origins of lichenization (Gargas and Taylor 1995), lichenization in early Ascomycota evolution (Lutzoni et al. 2001) and the inference that some extant non-lichenized lineages evolved from lichenized lineages (Schoch et al. 2009a), clarification of algal-associated *Orbilia* spp. is required to further refine evolutionary hypotheses on the origins of lichenization among Ascomycota.

The gelatinous matrix covering the hymenium in our specimens appears to correspond to the material that Pfister (1997) noted as holding the asci and paraphyses together in *Orbilia*. It also was observed with TEM in *O. luteorubella* (Benny et al. 1978).

Septal pores.—*Orbilia* sp. has the simple septum characteristic of the ascomycetes with globose Woronin bodies situated on both sides of the septum. Globose Woronin bodies have been reported to be commonly associated with vegetative septa and rarely with the ascogenous hyphal septa of the Pezizales (Li and Kimbrough 1995b). In contrast to their presence in vegetative and ascogenous hyphae, Woronin bodies were observed only rarely in the ascus cytoplasm in *Orbilia* sp. Wells (1972) reported the absence of Woronin bodies in asci. However our observations support those of Kimbrough (1981) who noted their presence in the ascus cytoplasm of several *Thelebolus* species (Leotiomycetes).

A simple septum has been reported in the vegetative hyphae of *Arthrobotrys dactyloides* (Heintz and Pramer 1972) and *Dactylaria brachopaga* (Dowsett et al. 1977), two *Orbilia* anamorphs. However the single convex electron-dense pore occlusions observed in *Orbilia* sp. were not reported in these studies. Kimbrough (1994) identified several types of septal pore plugs unique to different families within Pezizales. These range from the simplest convex to biconvex structures in asci and ascogenous hyphae in Pezizaceae to intricate hemispherical plugs in Ascobolaceae, Ascodesmidaceae and Pyronemataceae and complex structures with V-shaped striations in Morchellaceae and Helvellaceae. Kimbrough (1994) noted that the increasing complexity of septal pore occlusion-related structures among the pezizalean groups possibly reflects an evolutionary trend. The unelaborated basic dome-shaped pore occlusions observed in the ascogenous, ascal and non-ascogenous hyphal septa in *Orbilia* sp. resemble the basic Pezizaceae type, except for the lamellate structure noted (Kimbrough 1994) to be characteristic of taxa of Pezizales. Septal pore structures in *Orbilia* clearly differ from the ''*Neurospora*-septal type'' (Shatkin and Tatum 1959, Trinci and Collinge 1973, Curry and Kimbrough 1983, Kimbrough 1994), which is found in a variety of ascomycetes, usually outside Pezizales. The ''*Neurospora*-septal type'' is characterized by a subspherical occlusion that is non-membrane-bound, begins as translucent finger-like extensions from the pore margin and matures into a multilayered plate surrounded by amorphous material, and its related types as observed by Saito (1974) in *Sclerotinia sclerotiorum* (Kimbrough 1994). The septal pore plugs in the ascogenous hyphae and asci generally were observed to be more elaborate than and varied from those in the vegetative cells (Curry and Kimbrough



FIGS. 20–26. Transmission electron micrographs of longitudinal sections of asci of *Orbilia* sp. showing nuclei and nuclear division. 20. Ascus with a pair of premeiotic interphase nuclei (N). 21, 22. Prefusion interphase nuclei with granular nucleolus (NU) and a spindle pole body (SPB). 23. Premeiotic interphase SPB located in a depression in the nuclear envelope (NE) before SPB separation, showing two SPB disks (SE) connected by an electron-dense middle piece (MP). Each SPB disk consists of an upper electron-dense layer (L1) and lower electron-light layer (L2). 24. A fusion nucleus with synaptonemal complexes (SC). 25, 26. Metaphase-anaphase I of two nuclei with a flattened extranuclear SPB appressed to the intact nuclear envelope (NE) and spindle microtubules (S). Spindle microtubules surrounded by bundles of condensed chromatin (C) can be seen in FIG. 25. Bars: 20–22, 24 = 1 μm; 23, 25, 26 = 0.1 μm.

1983, Kimbrough 1994, Markham 1994), and such differences, according to Gull's postulate (1978), reflect differences in cell differentiation (Curry and Kimbrough 1983). In *Peziza* spp., considered to have the least elaborate septal pore structures of all the pezizalean taxa studied so far, Curry and Kimbrough (1983) observed convex and bi-convex bands occluding the pores that became covered with electron-opaque amorphous material or by additional secondary wall as they developed. A general differentiation pattern was evident with the presence of convex bands in reproductive cells and lamellate structures in the septa of somatic cells of *Peziza* spp., although with less variation in the differentiation pattern and some

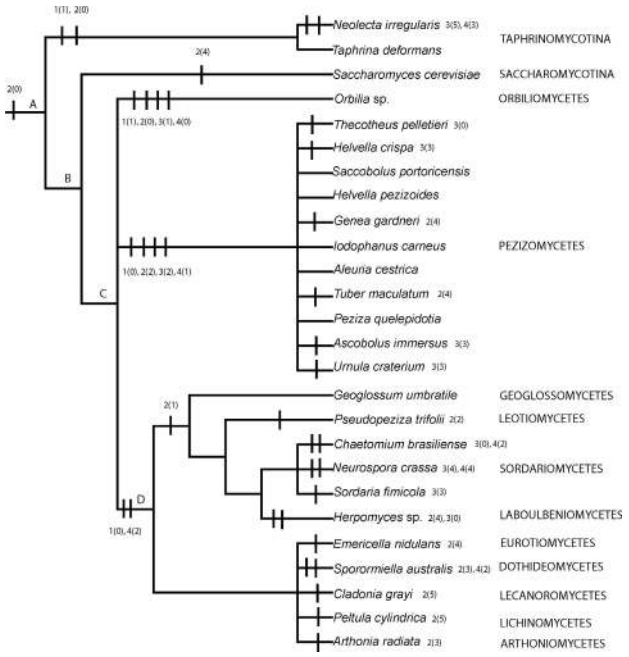


FIG. 27. Mesquite tree showing ancestral state reconstruction. Numbers indicate character state changes.

possible overlap between the two cell types (Curry and Kimbrough 1983). Lamellate structures were absent in septa of the vegetative and reproductive hyphae of *Orbilia* sp. Of interest, the relative lack of differentiation of septal pore structures observed in the different hyphae of *Orbilia* sp. at different stages of their development is consistent with its interpretation as the ancestral state in comparison with those observed in Pezizomycetes.

Nuclear division and spindle pole body.—Characters associated with nuclear division and the SPB in ascomycetes have been the subject of many reviews (Zickler 1970, Heath 1981, Beckett 1981b, Read and Beckett 1996), and an assessment of their homology at the phylum and lower taxonomic rank (Celio et al. 2006) identified characters distinctive to Ascomycota, Saccharomycotina and Pezizomycotina. Retention of an intact nuclear envelope throughout nuclear division is characteristic of Pezizomycotina. *Orbilia* sp. possesses Pezizomycotina-like nuclear division/SPB characteristics and shows characters that are in close agreement with those observed in pezizomycete taxa. The nuclear envelope has been observed to be intact in the metaphase-anaphase nuclei of *Orbilia* sp. A two-layered disk-shaped SPB, as in *Orbilia* sp., has been reported in Pezizomycetes (*Peziza michelii*, Schrantz 1970) and also in Dothideomycetes (*Cochliobolus sativus*, Huang et al. 1975). This structure differs from the unlayered plaque or disk-shaped SPB observed in members of Eurotiomycetes (Robinow and Caten 1969, Kanbe and Tanaka 1985), Leotiomycetes (McKeen 1972) and Sordariomycetes (Beckett and Crawford 1970, Zickler 1970, Aist and Williams 1972, van Winkle, et al. 1971, Aist and Berns 1981). SPBs in Pezizomycotina always are located close to the intact nuclear envelope. SPBs of Saccharomycotina are distinct from those of Pezizomycotina in having a multilayered (7–9) disk, which is connected directly to the spindle microtubules and situated in small polar fenestra in the nuclear envelope (Celio et al. 2006). Nuclear division studies on Taphrinomycotina members (Heath et al. 1982, Tanaka and Kanbe 1986) do not provide characters that are apomorphic for the subphylum (Celio et al. 2006). However SPBs formed in gaps in the nuclear envelope are a feature distinct from those observed in Pezizomycotina members, including *Orbilia*. The present nuclear division study of *Orbilia* sp. does not present a comprehensive elucidation of the whole nuclear division cycle because it was beyond the scope of this study.

Ancestral character state reconstruction.—From the morphological analysis, a truncate ascus apex may be the ancestral character state of the basal Ascomycota while the non-poricidal dehiscence mechanism is resolved as the ancestral character state for *Neolecta*, *Taphrina* and *Orbilia*. This suggests the relatively early diverging position of the Orbiliomycetes from other Pezizomycotina classes, including the Pezizomycetes,

which is considered to be among the earliest diverging classes in this subphylum (Schoch et al. 2009a). A shift in the septal pore occlusion characters of the Orbiliomycetes from those of Taphrinomycotina is suggested by the analysis. Although semiglobose structures on both sides of the septum have been reported in *N. flavovirescens*, they are known only from light microscopic studies (Landvik 2003) and hence lack the ultrastructural details needed to include them in the analyses. In a study of septal pore ultrastructure in *N. vitellina* (Landvik 2003) hexagonical structures appeared to block the septal pore and these were interpreted as Woronin bodies. However our interpretation of the ultrastructural data for *N. irregularis* (AFTOL data unpubl) suggests that Woronin bodies are not present. Although septal pore structures and a hexagonal crystal plug the septal pore, there is no evidence of a membrane surrounding these structures that is characteristic of Woronin bodies. Furthermore searches of the NCBI GenBank database revealed that the HEX-1 protein, a membrane-specific marker for Woronin bodies, is known only from members of Pezizomycotina and has not been detected in *Neolecta* or any other Taphrinomycotina. It is intriguing to imagine that the structures observed in the septal pores of *Neolecta* may be one of the steps leading to the evolution of Woronin bodies, but more complete morphological and molecular data from additional taxa of the Taphrinomycotina and other species of Orbiliomycetes are necessary to support such a conclusion.

Important aspects of the SBD are (i) clarifying which characters have been studied for specific taxa and (ii) improving the accuracy of character state comparisons (Celio et al. 2006). For example it was suggested earlier that Agaricomycotina shared a septal character state with some Pezizomycotina. However analysis of septal pore organization in *Orbilia* sp. revealed that the pore plug was different from the type found in some taxa of Agaricomycotina and necessitated a redefinition of a character state to separate them. Our analysis also highlights the missing data for septal pore organization in Taphrinomycetes, a critical gap in our knowledge of the Ascomycota. This study also reveals how poorly studied these characters are in most classes of the Pezizomycotina, with data absent for six classes and incompletely known for two additional classes. Although this subphylum constitutes the majority of known fungal species, missing data create significant problems in understanding character evolution.

CONCLUSION

Structural data on ascus apex, nuclear division/SPB and septal pore plugs obtained during this study coupled with data from a few earlier morphology-based studies on the group support the prediction that Orbiliomycetes possess some of the ancestral character states of Pezizomycotina. The basic ascus type with a simple spore dehiscence mechanism similar to that of early diverging ascomycetes (i.e. *Taphrina* and *Neolecta*) and the least differentiation of septal pore associated structures in relation to those of other Pezizomycotina lineages support the early diverging position of the group. Nuclear division/SPB characters and membrane-bound Woronin bodies in *Orbilia* sp. are very similar to those found in other pezizalean members and indicate its advanced status when compared with taxa in Taphrinomycotina and Saccharomycotina. The ultrastructural evidence obtained during this study strongly supports the placement of Orbiliomycetes as the earliest diverging lineage of Pezizomycotina.

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