American Journal of Botany 94(11): 1890–1899. 2007.

ASCOMA DEVELOPMENT AND PHYLOGENY OF AN APOTHECIOID DOTHIDEOMYCETE, *CATINELLA OLIVACEA*[1]

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Catinella olivacea is a discomycetous fungus often found fruiting within cavities in rotting logs. Because this habitat would lack the air currents upon which discomycete species normally rely for the dispersal of their forcibly ejected ascospores, we suspected an alternative disseminative strategy might be employed by this species. An examination of the development of the discomycetous ascomata in pure culture, on wood blocks, and on agar showed that the epithecium was gelatinous at maturity and entrapped released ascospores in a slimy mass. We interpreted this as an adaptation for ascospore disperal by arthropods. Developmental data also showed that *C. olivacea* was unusual among other discomycetes in the Helotiales (Leotiomycetes). For example, the ascoma developed from a stromatic mass of meristematically dividing cells and involved the formation of a uniloculate cavity within a structure better considered an ascostroma than an incipient apothecium. Furthermore, the ascus had a prominent ocular chamber and released its ascospores through a broad, bivalvate slit. These features, along with phylogenetic analyses of large subunit and small subunit rDNA, indicated that this unusual apothecial fungus is, surprisingly, more closely affiliated with the Dothideomycetes than the Leotiomycetes.

Key words: Alberta, Canada; apothecioid; ascolocular ontogeny; ascostroma; bitunicate; Dermateaceae; Dothideomycetes; Helotiales.

Catinella olivacea (Batsch) Boudier produces clusters of distinctive, flattened, dark olivaceous apothecia, up to 1.5 cm across, with a vertically furrowed, ochraceous margin (Boudier, 1907; Breitenbach and Kränzlin, 1981; Spooner and Legon, 1999). The fungus is easily identified and probably common, but reports are infrequent because apothecia are usually found only on the underside of moist, well-decayed logs of hardwood species or in places where the wood is covered by moss or soil (Pomerleau, 1980; Strödel, 1984; Keizer, 1990; Spooner and Legon, 1999). The forcibly ejected ascospores of apothecial fungi generally are assumed to be dispersed by wind, but air movement in these cryptic and protected habitats would be minimal and probably insufficient to transfer propagules of *C. olivacea* to new habitats. Some reliance on microfaunal cohabitants might explain how the fungus is dispersed from one rotting log to the next, but adaptations for this type of dispersal strategy are not evident in published descriptions of this species.

Boudier (1907) erected the genus *Catinella* to accommodate *Peziza olivacea* Batsch, and since then, its identity and taxonomic position have been the subject of some disagreement. The asci of *C. olivacea* appear inoperculate, and each contains a uniseriate file of eight darkly pigmented, one-celled ascospores. The hymenium is supported within a blackish-olive discoid receptacle that has an excipulum of large, thin-walled, deeply pigmented, subangular to globose cells. These characteristics, plus an ionomidotic reaction (dark brown-purple color change) of the excipular tissues in KOH and ascal tips that do not stain blue in iodine, supported its disposition in the Dermateaceae, a family in the Helotiales with darkly pigmented apothecia (Durand, 1922; Seaver, 1961; Korf, 1973; Nannfeldt, 1932 in Dennis, 1977; Eriksson et al., 2004). It has also been suggested that the taxon might be more appropriately disposed in the Leotiaceae, also a member of the Helotiales (Spooner and Legon, 1999), presumably because of the presence of a gelatinous layer that overlies the hymenium. Clements and Shear (1931) list the taxon as a synonym of *Karschia* Körb., a genus with similar, darkly pigmented ascomata and septate ascospores and having uncertain affinities within the Dothideomycetes (Eriksson et al., 2004). The discoid ascomata, i.e., apothecia, of the Helotiales, an order accommodated in the Leotiomycetes (Eriksson et al., 2004), and the apothecioid ascostromata of some taxa (e.g., Patellariales) in the Dothideomycetes can be superficially similar to one another but have a fundamentally different pattern of development (Korf, 1973; Kutorga and Hawksworth, 1997). However, *C. olivacea* is apparently known only from specimens collected in natural habitats, i.e., it has never been cultured, and there are no detailed reports of the developmental characteristics that would clarify its taxonomic position or its reproductive strategies.

Recent collections of *C. olivacea* found growing within cavities inside moist, decaying aspen logs allowed us to prepare cultures that we used to study in detail the development of the ascomata of this species and that served as a source of DNA for the examination of its phylogenetic affinities within the Ascomycota. Our objectives were to clarify the taxonomic position of *C. olivacea* using both morphological and molecular characters and to find evidence that might support our hypothesis that the species is dispersed from its cryptic environments by microfauna.

[1] Manuscript received 23 May 2007; revision accepted 13 September 2007.

The authors thank the Natural Sciences and Engineering Research Council of Canada for funding to R.S.C. through the Discovery Grants program and B. Wilson who brought this fungus to their attention. The authors would also like to thank T. Lumbsch for his advice on analyzing the molecular data and two anonymous reviewers for their comments.

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MATERIALS AND METHODS

Mature ascomata of *C. olivacea* were collected in July 2003 and September 2005 from wet, rotting logs of *Populus tremuloides* Michx. (aspen) located 2 km east of Elk Island National Park, in the southern boreal forest region of central Alberta, Canada. Pure cultures were prepared by germinating ascospores on malt extract agar (MEA; 15 g/L sterile distilled water, Difco malt extract; BD Biosciences, Mississauga, Ontario, Canada) and corn meal agar (CMA; 17 g/L sterile distilled water, Acumedia corn meal medium; Neogen, Lansing, Michigan, USA). Blocks of aspen wood, 5 × 5 × 2 cm, sterilized by autoclaving twice, were inoculated with mycelia from pure culture, incubated in glass deep culture plates (containing sterilized perlite covered with a sheet of filter paper) under ambient light and temperature on the laboratory bench or under black light with a 12 h light : dark photoperiod, and monitored weekly for ascomata formation. Material was moistened monthly with sterile water. The MEA and CMA plates were incubated at room temperature and observed daily to record cultural characteristics. Cultures on MEA were deposited in the University of Alberta Microfungus Collection and Herbarium (*UAMH 10679*). For observation with a light microscope (LM), fungal material (i.e., germinating ascospores, young ascomata, and sections of mature ascomata cut by hand with a razor blade) were mounted in lacto-fuchsin (0.1 g acid fuchsin in 100 mL 85% lactic acid) or were pretreated in 10% KOH and mounted in Melzer's reagent (Dennis, 1977) for observation and imaging using a Canon PowerShot A75 digital camera (Canon Canada, Mississauga, Ontario, Canada).

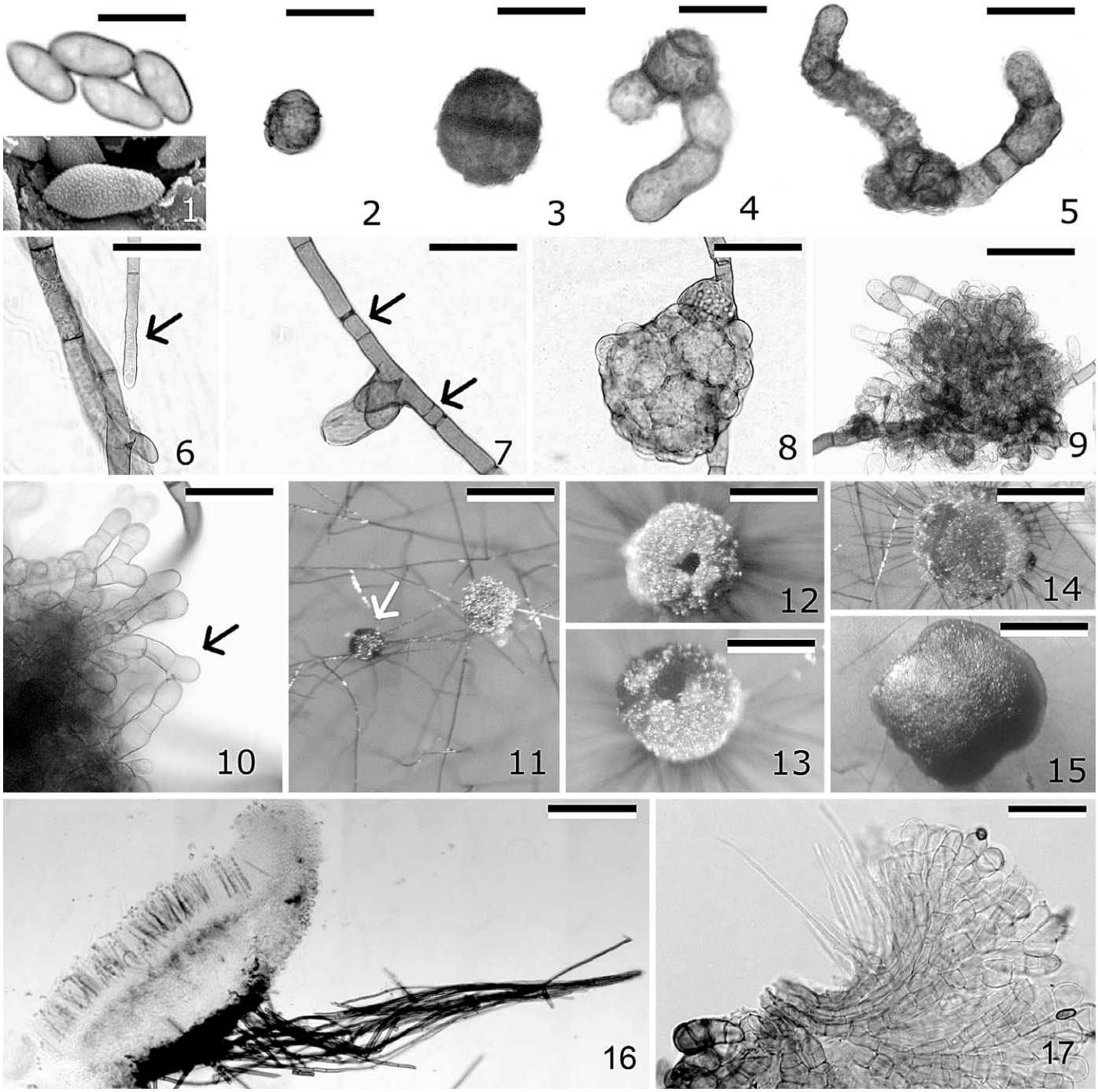
For observation with a scanning electron microscope (SEM), ascomata at various developmental stages on agar media and on wood blocks were excised, placed in phosphate buffer (pH 7.0), and fixed in glutaraldehyde in buffer for 2 h. Samples were rinsed in buffer and immersed in 2% tannic acid–2% guanidine hydrochloride solution for 3 h. Some ascomata at this stage in fixation were sectioned with a razor blade. All fungal material (sectioned and unsectioned) was rinsed in distilled water and post-fixed in 2% OsO_4 for 12 h at 5°C. Fixed material was dehydrated in an ethanol series, taken to amyl acetate, and critical point dried in a Polaron E-3000 dryer (Quorum Technologies, Ringmer, UK) using carbon dioxide. Dried samples were coated with gold and examined using a Hitachi S-510 scanning electron microscope (Hitachi, Tokyo, Japan).

The DNA sequences for the small subunit (SSU) and the large subunit (LSU) of the nuclear ribosomal DNA region were obtained from mature ascomata. The DNA was extracted following the method described by Cubero et al. (1999) with some modification. Approximately 100 mg of fungal tissue was ground in 2 × CTAB extraction buffer (10% CTAB, NaCl, 0.25 M EDTA, 1 M Tris-HCl pH 8.0, 2% polyvinyl pyrrolidine, and dH_2O). After 3 h at 65°C, genomic DNA was extracted using a chloroform : isoamyl alcohol (24 : 1 v/v) solution. Crude DNA was purified using a QIAquick DNA purification kit (Qiagen, Mississauga, Ontario, Canada). The SSU and LSU regions were amplified using the primer pairs NS1 and NS8 and BMBC-R and LR7, respectively (Lane et al., 1985; http://www.biology.duke.edu/fungi/mycolab/primers.htm). The PCR was run for 30 cycles in a PE GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, California, USA) set to the following parameters: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, then final extension at 74°C for 7 min followed by a cooldown stage at 4°C for 10 min. The amplicon was purified using the QIAquick DNA purification kit, and DNA concentration was determined using the Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, Delaware, USA). Cycle sequencing was done using primers NS1, NS2, NS4, NS6, and NS8 for the SSU gene and LROR, LR16, LR3, LR5, and LR7 for the LSU gene (White et al., 1990; http://www.biology.duke.edu/fungi/mycolab/primers.htm). Sequencing reactions were done using the BigDye terminator cycle sequencing kit (Applied Biosystems), and amplicons were run on an ABI 377 automated DNA sequencer (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). A consensus sequence was constructed and edited using Sequencher version 4.5 (Gene Codes, Ann Arbor, Michigan, USA). The newly determined sequences were then subjected to a BLAST search (Altschul et al., 1997) comparing our species with published sequences submitted to GenBank (www.ncbi.nlm.nih.gov).

The sequence data of *C. olivacea* were manually aligned by eye with sequences of 61 species retrieved from GenBank (www.ncbi.nlm.nih.gov) representing the Dothideomycetes (sensu Schoch et al., 2007), Eurotiomycetes, Sordariomycetes, Leotiomycetes, Arthoniomycetes, Lecanoromycetes, and Lichinomycetes where both SSU and LSU data were available (Table 1) using Se-Al version 2.0a11 Carbon (Rambaut, 2002). *Scutellinia scutellata* and *Orbilia auricolor* were used as outgroup taxa. A maximum parsimony analysis

TABLE 1. Fungal taxa used for phylogenetic analysis with GenBank accession numbers for large subunit (LSU) and small subunit (SSU) sequences.

Taxon	GenBank number	
	Gene	
	SSU	LSU
Arthonia dispersa	AY571379	AY571381
Arthopyrenia salicis	AY538333	AY538339
Aureobasidium pullulans	DQ471004	DQ470956
Botryosphaeria ribis	DQ678000	DQ678053
Botryosphaeria stevensii	DQ678012	DQ678064
Botryotinia fuckeliana	AY544695	AY544651
Bulgaria inquinans	DQ471008	DQ470960
Cainia graminis	AF431948	AF431949
Catinella olivacea	DQ915484	EF622212
Cercospora beticola	DQ678039	DQ678091
Chaetomium globosum	AB048285	AY346272
Cladosporium cladosporioides	DQ678004	DQ678057
Crinula caliciiformis	AY544729	AY544680
Cucurbitaria elongata	DQ678009	DQ678061
Davidiella tassiana	DQ678022	DQ678074
Delphinella strobiligena	AY016341	AY016358
Dendrographa leucophaea	AY548803	AY548810
Diatrype disciformis	DQ471012	DQ470964
Diploschistes thunbergianus	AF274112	AF274095
Discosphaerina fagi	AY016342	AY016359
Dothidea ribesia	AY016343	AY016360
Elsinoe centrolobii	DQ678041	DQ678094
Eremascus albus	M83258	AY004345
Eurotium rubrum	U00970	AY004346
Exophiala dermatitidis	DQ823107	DQ823100
Glyphium elatum	AF346419	AF346420
Guignardia bidwellii	DQ678034	DQ678085
Helicomyces lilliputeus	AY856942	AY856899
Helicomyces roseus	DQ678032	DQ678083
Hypocrea citrina	AY544693	AY544649
Hysterium pulicare	DQ678002	DQ678055
Hysteropatella clavispora	AY541483	AY541493
Lecanactis abietina	AY548805	AY548812
Lempholemma polyanthes	AF356690	AF356691
Lobaria scrobiculata	AY584679	AY584655
Lophium mytilinum	DQ678030	DQ678081
Microxyphium citri	AY016340	AY004337
Monilinia fructicola	AY544724	AY544683
Mycosphaerella punctiformis	DQ471017	DQ470968
Myriangium duriaei	AY016347	DQ678059
Neofabraea malicorticis	AY544706	AY544662
Neotestudina rosatii	DQ384069	DQ384107
Orbilia auricolor	DQ471001	DQ470953
Peltula auriculata	DQ832332	DQ832330
Peltula umbilicata	DQ782887	AF356689
Penicillium expansum	DQ912698	AF003359
Pezicula carpinea	DQ471016	DQ470967
Pleospora herbarum	DQ247812	DQ247804
Potebniamyces pyri	DQ470997	DQ470949
Ramichloridium anceps	DQ823109	DQ823102
Scorias spongiosa	DQ678024	DQ678075
Scutellinia scutellata	DQ247814	DQ247806
Setosphaeria monoceras	AY016352	AY016368
Sordaria fimicola	AY545724	AY545728
Stylodothis puccinioides	AY016353	AY004342
Trapelia placodioides	AF119500	AF274103
Trematosphaeria heterospora	AY016354	AY016369
Trimmatostroma abietis	DQ678040	DQ678092
Tubeufia cerea	AY856947	AY856903
Westerdykella cylindrica	AY016355	AY004343
Xylaria acuta	AY544719	AY544676
Xylaria hypoxylon	AY544692	AY544648



Figs. 1–17. Germinating ascospores and ascoma development in *Catinella olivacea*. (Fig. 1, scanning electron micrograph; Figs. 2–17, light micrographs.) **1.** Liberated mature ascospores were single-celled, melanized, minutely verrucose, navicular to cypriform, slightly swollen and pointed at one end, and narrower and rounded at the other. Bar = 10 μm. **2.** Germinating ascospore. Upon germination, ascospores swelled, changing shape from ellipsoidal to spherical. The outer cell wall also became coarsely roughened. Bar = 18 μm. **3.** Germinating ascospore divided by a cross wall. Bar = 18 μm. **4.** Germ tubes developing from germinated ascospore. Bar = 12 μm. **5.** Germ tubes elongated through a combination of tip growth and meristematic division to form short segments of rough-walled hyphae. Bar = 16 μm. **6.** Smooth, light brown, elongating hypha (arrow) and matured, thick-walled, darkly olivaceous, septate hyphae. Bar = 30 μm. **7.** The formation of short, subglobose branches from the side of a hyphal segment delimited by two sets of paired septa (arrows) was the first indication of ascoma development. Bar = 30 μm. **8.** Stroma composed of a dense cluster of polygonal cells derived from meristematically dividing, subglobose branches. Bar = 30 μm. **9.** Numerous broad, closely septate, and lightly pigmented hyphae developed from the ascoma. Bar = 40 μm. **10.** Hyphae on the surface of the ascoma anastomosed with one another and formed a compact tomentum (arrow). Bar = 30 μm. **11.** Newly formed (arrow) and expanding ascoma as observed under a dissecting microscope. Bar = 500 μm. **12.** Pore formed on the apical surface of the mature ascoma. Bar = 400 μm. **13.** Single unilocular cavity contained the developing hymenium. Bar = 400 μm. **14.** Apothecioid ascoma. The elevated remnants of the tomentum formed an ochre-yellow ring around the periphery of the ascoma. Bar = 800 μm. **15.** Mature apothecioid ascoma with upturned

was performed using PAUP* version 4.0 b10 (Swofford, 1998). A heuristic search was done using parsimony as the optimality criterion. Gaps were treated as missing data. Starting trees were obtained at random via stepwise addition with tree-bisection-reconnection as the branch-swapping algorithm and the MulTrees option in effect. After 200 stepwise addition sequences were completed, confidence in the branches of the resulting trees was evaluated by bootstrap analysis (Felsenstein, 1985) using 2000 replicates. The resultant tree was visualized using PAUP* version 4.0 b10 (Swofford, 1998). A Bayesian analysis was conducted using MrBayes version 3.1.2 (Huelsenbeck et al., 2001), which approximates posterior probabilities of clades using a Markov chain-Monte Carlo (MCMC) method (Huelsenbeck and Ronquist, 2001). The data matrix was analyzed with the general time-reversible (GTR) model of substitution including estimation of invariant sites and assuming a discrete gamma distribution (GTR + I + G) with six rate categories provided for the nucleotide substitution model and for priors, with the chain temperature set at 0.2. The nucleotide substitution model was chosen by analyzing the data matrix using Modeltest version 3.7 (Posada and Crandall, 1998). Trees were sampled every 100 out of a total of two million generations, with the first 50 000 trees (25% of total) being deleted as "burn-in" of the chain. Only clades with posterior probability values $\geq$95% were considered to be phylogenetically informative. The resultant consensus tree was visualized using PAUP* version 4.0 b10 (Swofford, 1998). For testing the current systematic placement of *C. olivacea,* a hypothetical topology constraining this fungus with the Leotiomycetes, along with an unconstrained tree, was inferred using heuristic search settings in PAUP* with likelihood as the optimality criterion. Substitution rates were estimated via maximum likelihood, and among-site rate variation was estimated using the GTR + I + G model. Starting trees were obtained via neighbor-joining with tree-bisection-reconnection as the branch-swapping algorithm, MaxTrees set at 100, and the MulTrees option in effect. Starting branch lengths were obtained using the Rogers–Swofford approximation method. The resulting trees were then analyzed with the program TREE-PUZZLE version 5.3 (Schmidt et al., 2002) using the Shimodaira–Hasegawa test (Shimodaira and Hasegawa, 1999) and expected likelihood weight tests (Strimmer and Rambaut, 2002) to evaluate significance.

RESULTS

Mature, liberated ascospores were single-celled, guttulate, melanized, minutely verrucose, navicular to cypriform, slightly swollen and pointed at one end, narrower and rounded at the other, 7.6–9.6 × 3.6–4.6 μm (Fig. 1). Within 7–14 d on MEA, ascospores swelled until they were more or less spherical, 10–12 μm in diameter, and the outer wall became deeply pigmented and coarsely roughened to verrucose (Fig. 2). After one or two cross walls formed within the swollen ascospore (Fig. 3), germ tubes emerged from one or more cells (Fig. 4). Germ tubes elongated through a combination of tip growth and meristematic division to form short segments of rough-walled hyphae, 3–4 cells long and 5–6 μm in diameter (Figs. 4, 5). Smooth, pale brown hyphae, 4–6 μm in diameter, arose from the tips of these segments and became septate, broader (6–8 μm), and olivaceous as they matured (Fig. 6).

Growth on CMA, MEA, and wood blocks in moist chambers was sparse and consisted of deeply melanized subsurface and surface hyphae. An anamorph was absent, but ascomata developed on CMA and in moist chambers approximately 3 mo after inoculation. The first indication of ascoma formation was the appearance of a small cluster of short, subglobose branches from the side of a hyphal segment delimited by two sets of paired septa (Fig. 7). Meristematic division within the branches and cell enlargement led to the formation of a dense, stromatic mass, 60–200 μm in diameter and composed of polygonal cells (Fig. 8). The stromatic mass gave rise to numerous broad, closely septate and lightly pigmented hyphae (Fig. 9) that branched and anastomosed as they grew outward (Fig. 10). This radial proliferation resulted in the formation of a globose mass of densely interwoven hyphae that was 300–500 μm in diameter and green to orange-brown. The abundant, free, clavate hyphal tips, 6–10 μm thick, formed a uniform tomentum over the surface of the globose mass (Figs. 10, 11).

A minute pore appeared on the upper surface of the tomentum, revealing a single cavity containing the developing hymenium within the primordial ascoma (Figs. 12–14). The remnants of the tomentum surrounding the pore persisted as the ascoma flattened against the surface of the substrate to form a disk and remained as a prominent yellowish to ochre margin bearing numerous vertical fissures (Figs. 13–15). In culture, mature ascomata were 1–2 mm in diameter (Fig. 16). Straight, broad, and sparingly branched, darkly pigmented and cuticularized hyphae arose from the base of the ascoma, where they were 8–10 μm thick, and radiated outward, into and across the substrate (Figs. 12–14, 16), tapering to 4.8–7.2 μm at their apices (Fig. 16).

The newly exposed hymenium arose from a layer of globose cells that formed the outer excipulum, which was 120–160 μm thick (Figs. 16, 17, 19). The hymenium consisted of numerous slender, septate, sterile filaments, 70–105 × 0.7–1.6 μm, with slightly swollen apices (Figs. 17, 18). As the hymenial layer matured, an outer amorphous layer surrounding the tips of the sterile filaments appeared to dissolve and lift away from the wall (Fig. 18). A darkly pigmented, viscous liquid accumulated on the surface of the hymenium and formed a pseudoepithecial layer, up to 50 μm thick (Figs. 19, 20). Interspersed among the filaments were clavate asci, 62–84 × 4.8–6.0 μm, that in places protruded through the pseudoepithecial layer (Figs. 18–20). Ascus apices had markedly thickened walls and a prominent ocular chamber (Figs. 21, 22) and remained unstained with Melzer's reagent. Eight uniseriate ascospores were contained per ascus (Figs. 20, 21, 23) and were released through a broad bivalvate slit across the ascus apex (Fig. 24). Numerous ascospores remained entrapped in the gel of the pseudoepithecial layer (Fig. 25). Others landed close to the perimeter (within 1–2 mm) of the ascoma where they formed a halo (Fig. 26) of dark droplets (Fig. 27).

Phylogenetic analysis—The sequence alignment matrix using *C. olivacea* and 61 species of fungi yielded 2932 characters (including gaps) with the LSU sequences starting at bp 1 and the SSU sequences starting at bp 1281. In the Bayesian analysis, groups of species representing major classes/orders had high posterior probability support (Fig. 28). The Dothideomycetes sensu Schoch (Schoch et al., 2007) represented a clade comprising *C. olivacea*, the Myriangiales, Dothideales, Pleosporales, Tubeufiaceae, Hysteriales, and Capnodiales. *Catinella olivacea* formed part of a clade with 100% Bayesian support that contained the Myriangiales, Dothideales, and Capnodiales and that was distinct from other clades within the Dothideomycetes. The Dothideomycetes,

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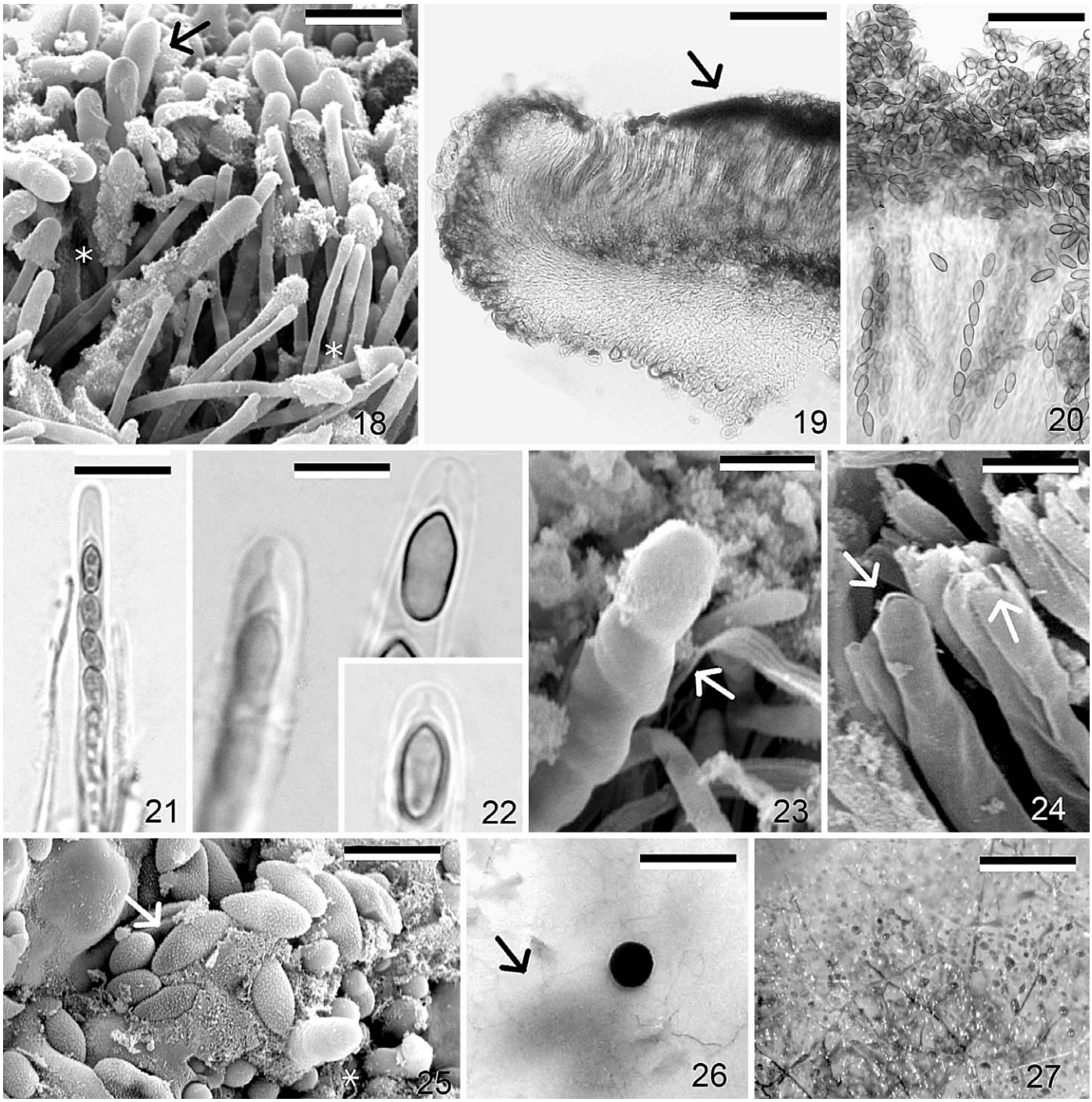
margin and applanate surface. Bar = 1 mm. **16.** Hand section through a maturing ascoma. The hymenium, composed of sterile filaments and asci, supported within an excipulum composed of globose cells. Sparingly branched anchoring hyphae emerged from the underside of fruiting structure. Bar = 150 um. **17.** Sterile filaments developed from the sides of the developing margin of the hymenium as the ascoma expanded. Bar = 30 μm.



Figs. 18–27. Ultrastructural details of the asci and pseudoepithecium of *Catinella olivacea*. (Fig. 18, 23, 24, 25, SEM; others, LM.) **18.** Tips of the sterile filaments surrounded by an outer amorphous layer. On some filaments this layer appeared to dissolve and lift away (*). Arrow indicates ascal tips that emerged through pseudoepithecial gel. Bar = 10 μm. **19.** Section of a mature ascoma. The hymenium was covered by a dark pseudoepithecium (arrow). Bar = 80 μm. **20.** Section through the hymenial layer and overlying pseudoepithecium showing numerous liberated ascospores caught in the gel. Bar = 30 μm. **21.** Ascus with a single wall layer and a thickened apex. Bar = 17 μm. **22.** Composite view of ascal tips under high magnification. Ocular chambers in tips were clearly visible. Bar = 7 μm. **23.** A mature ascus prior to dehiscence (arrow). Bar = 5 μm. **24.** Several asci showing a broad, bivalvate slit across the tip of the ascus (arrows). Bar = 6 μm. **25.** Surface view of mature ascoma. The gelatinous pseudoepithecium trapped large numbers of ascospores (arrow). Ascus visible protruding above the pseudoepithecium (*). Bar = 9 μm. **26.** Mature apothecioid ascoma with a dark halo of discharged ascospores (arrow). Bar = 2 mm. **27.** Close-up of the dark-colored droplets of gel and ascospores. Bar = 750 μm.

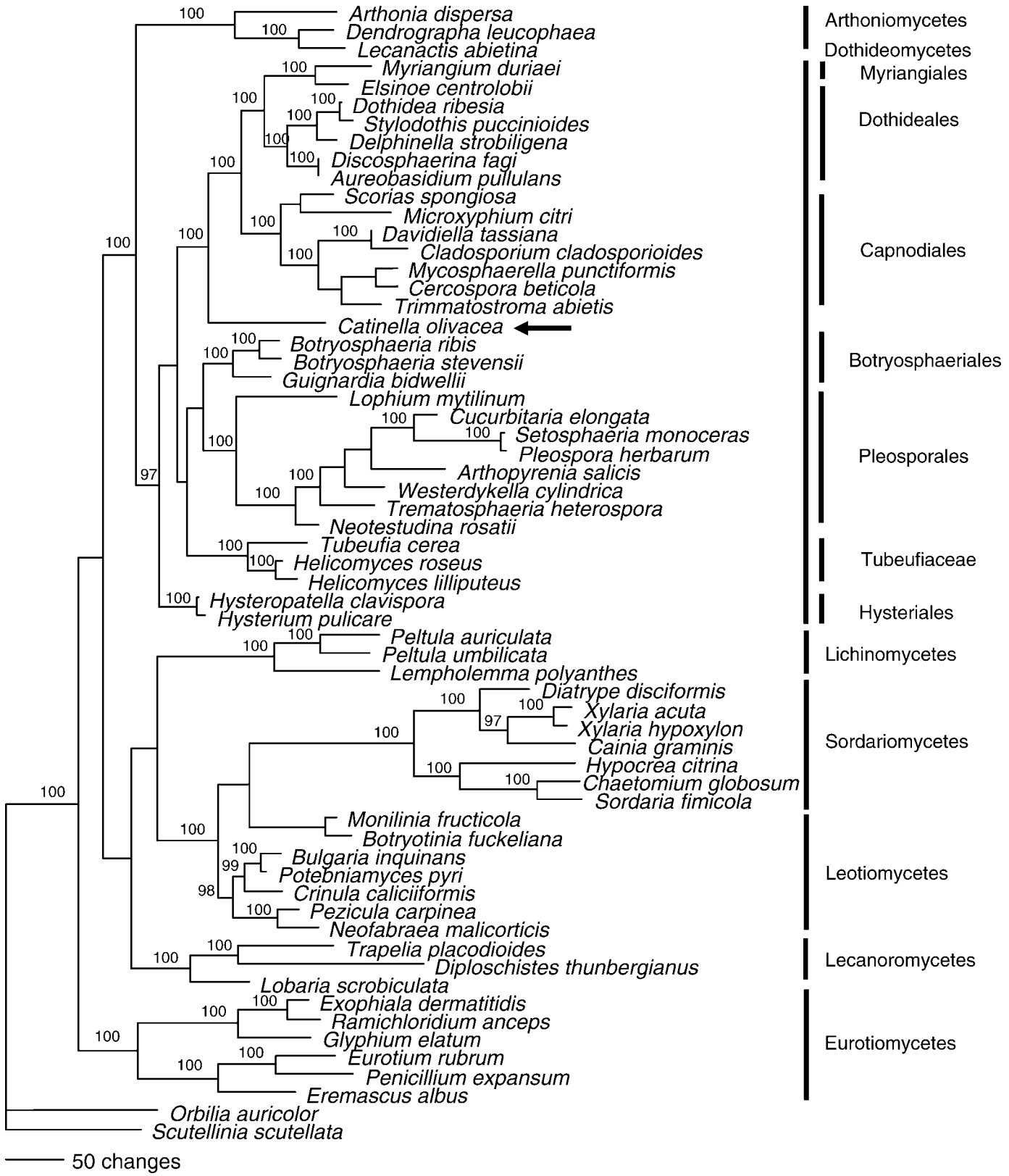


Fig. 28. Consensus tree obtained from Bayesian inference showing the position of *Catinella olivacea* among ascomycete taxa based on large subunit and small subunit rDNA sequence data. Posterior probability values ≥95% derived from a Bayesian analysis are shown above branches. *Catinella olivacea* (arrow) is in a clade containing the Myriangiales, Dothideales, and Capnodiales.

including *C. olivacea*, together with taxa representing the Arthoniomycetes, formed a clade with 100% Bayesian support distinct from an unsupported clade containing the Leotiomycetes, Sordariomycetes, Lecanoromycetes, and Lichinomycetes. These clades were distinct from the Eurotiomycetes. The maximum parsimony analysis yielded a single tree with less support and a different overall topology than the tree generated from the Bayesian analysis (Fig. 29) and with a consistency index of 0.394 and a retention index of 0.605. In particular, the Lichinomycetes and Arthoniomycetes formed a weakly supported clade, and the Eurotiomycetes formed an unsupported clade with Leotiomycetes, Sordariomycetes, and the Lecanoromycetes as a basal lineage. Within the Dothideomycetes, the Tubeufiaceae formed a clade with the Myriangiales, Dothideales, Capnodiales, and *C. olivacea*. However, the Dothideomycetes, Lichinomycetes, and Arthoniomycetes were separated from other taxa with high bootstrap support. *Catinella olivacea* was in a weakly supported clade with the same topology as seen in the Bayesian analysis except for the placement of *Microxyphium citri* and the addition of the Tubeufiaceae (Figs. 28, 29). The statistical analysis of the two maximum likelihood trees using the Shimodaira–Hasegawa and expected likelihood weight tests revealed that constraining *Catinella* with the Leotiomycetes produced a significantly worse tree (for both tests $P < 0.0001$).

DISCUSSION

The LM and SEM observations of morphological and developmental characters and the phylogenetic analysis of the LSU and SSU rDNA genes indicate that *C. olivacea* is dothideomycetous and does not have affinities with the Leotiomycetes, as others have suggested (Seaver, 1961; Korf, 1973; Nannfeldt, 1932 in Dennis, 1977; Spooner and Legon, 1999). The earlier placement of *C. olivacea* in the Leotiomycetes (Dermateaceae) was based on observations of mature, field-collected apothecia and the structure of the ascal tip, which can be ambiguous in older specimens. Our research shows the importance of using living material at different stages of development to observe accurate taxonomic characters.

Single-celled ascospores are less common than septate ones in the Dothideomycetes (Eriksson, 1981; Sivanesan, 1983; Barr, 1987; Barr and Huhndorf, 2001) but the melanized, minutely verrucose walls, previously alluded to by Strödel (1990), and the clear evidence of meristematic growth during germination are more indicative of a relationship with this class than with the Leotiomycetes. Ascomata arose from mycelia derived from single germinating ascospores, indicating that the fungus is homothallic and did not involve the prior formation of an ascogonial coil. Instead, ascomata developed following a pattern typical among Dothideomycetes in which the tissues that will enclose the ascogenous system form before the appearance of gametangia and ascogenous initials (Luttrell, 1973; Eriksson, 1981; Barr, 1987; Barr and Huhndorf, 2001; Lindemuth et al., 2001). In contrast, ascoma formation among inoperculate and true perithecial taxa is initiated by the formation of an ascogonial coil (from which asci eventually arise) that is then enveloped by fungal tissue derived from adjacent vegetative hyphae (Emmons, 1932; Greif et al., 2004; Tsuneda and Currah, 2004).

The majority of Dothideomycetes are unilocular (Barr, 1987), and fruiting bodies of many apothecioid taxa in the Myriangiales, Hysteriales, and Dothideales develop a pore or vertical slit that facilitates exposure of the hymenium and release of ascospores (Luttrell, 1973; Eriksson, 1981). In *C. olivacea*, the marginal tissue surrounding the initial pore persisted to form the distinctive fissured, ochraceous margin composed of textura prismatica-like excipulum, sensu Kutorga and Hawksworth (1997), as the fruiting body took on its characteristic apothecioid form similar to the ascomata produced by taxa in the Patellariales (Bezerra and Kimbrough, 1982; Kutorga and Hawksworth, 1997). The prominent cuticularized "anchoring hyphae" radiating from the base of the excipulum is a feature also reported in some taxa in the Patellariales (Kutorga and Hawksworth, 1997). The anchoring hyphae in *C. olivacea* may represent vestiges of subicular tissue that is more prominent in other dothideomycete taxa (Dennis, 1977; Eriksson, 1981), but their function is unknown.

The apparently unitunicate ascus and nonblueing reaction in the ascal tip were important in classifying *C. olivacea* as an inoperculate taxon (Durand, 1922; Korf, 1973; Keizer, 1990; Spooner and Legon, 1999). Asci with a single wall layer do occur in some Dothideomycetes (Eriksson, 1981; Barr and Huhndorf, 2001), but their apices differ from those of unitunicates in the Leotiomycetes. The presence of an ocular chamber along with the prominent bivalvate split that develops across the ascus apex during dehiscence are strong indicators of affiliation with bitunicate taxa (Eriksson, 1981; Eriksson et al., 2004). Without a second ascal wall layer, ascospore ejection does not involve a "jack-the-box" mechanism common to most other Dothideomycetes (Eriksson, 1981; Barr, 1987).

Depictions of the sterile filaments (i.e., "paraphyses") in Dennis (1977: plate 28, fig. M) show that an outer layer or coating on the apex of these structures lifts away from tip as the hymenium matures. Our SEM observations confirm this phenomenon and, further, show that as this outer layer sloughs off it contributes to the formation of the amorphous, brown, gelatinous pseudoepithecium. The production of pseudoepithecial gels in other taxa has been suggested as a water retention mechanism to aid in spore maturation and ejection (Ingold, 1959; Moore, 1965) and as a type of antifreeze (Sherwood, 1981). Given the types of habitats in which *C. olivacea* is found, protection from desiccation is not likely a function for this material. Both the sticky spore droplets that accumulate in the vicinity of mature ascomata and the gelspore matrix that accumulates on the surface of the hymenium may be an adaptation favoring dispersal by passing microfauna. Fungi that are adapted for such cryptic habitats often exhibit a range of morphological mechanisms that utilize arthropods and other animals for spore dispersal. For example, the Myxotrichaceae (Helotiales), common on decaying wood (Currah, 1985; Lumley et al., 2001), produce burrlike fruiting bodies that are capable of attaching to arthropods (Greif and Currah, 2003). Many species of *Ophiostoma*, a taxon that occurs in excavated beetle galleries in conifers, produce elongate perithecia that exude ascospores in slimy droplets that adhere to passing arthropods. Discoid, dung-inhabiting species in the Pezizales also produce spores enveloped in a gelatinous outer layer, ostensibly to facilitate attachment to arthropods for dispersal (Pfister and Kimbrough, 2001). Isolation of *C. olivacea* from cohabiting arthropods would provide support for our hypothesis concerning the function of the pseudoepithecial layer and surrounding spore-rich droplets. The apparent absence of an anamorph would make the

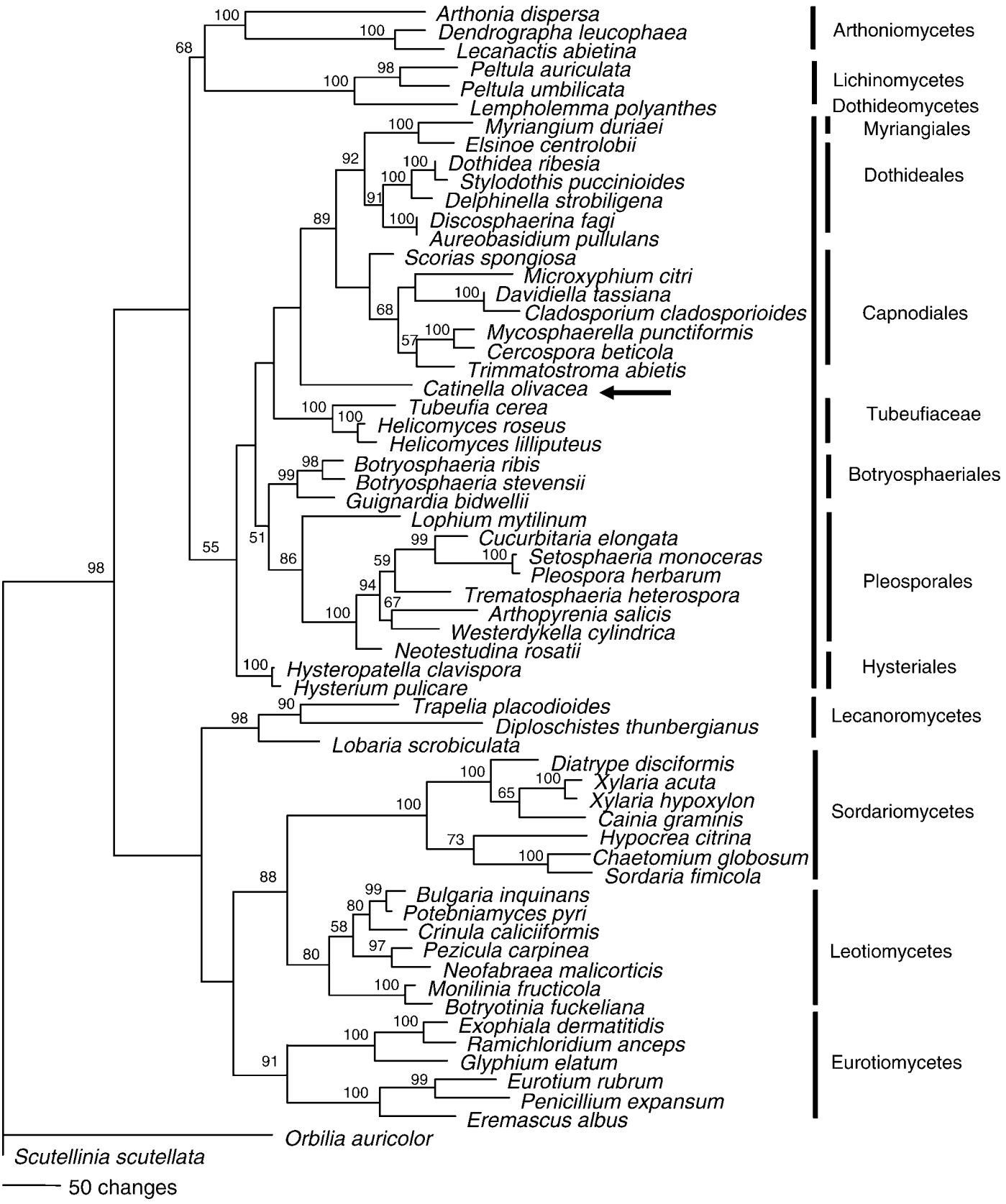


Fig. 29. Consensus tree obtained from maximum parsimony showing the position of *Catinella olivacea* among ascomycete taxa based on large subunit and small subunit rDNA sequence data. Bootstrap support values ≥50% are shown above branches. *Catinella olivacea* (arrow) is in a clade containing the Myriangiales, Dothideales, Capnodiales, and the Tubeufiaceae.

successful transfer of meiotic propagules critical to the survival and dispersal of this species.

The analyses of the LSU and SSU sequence data of *C. olivacea* (Figs. 28, 29) indicate an affiliation with the Dothideomycetes rather than the Leotiomycetes and thus agree with conclusions based on observations of apothecial development and morphology. The overall arrangement of the Bayesian analysis, such as a close relationship between the Chaetothyriomycetidae and Eurotiomycetidae within the Eurotiomycetes, agree with previous findings (Spatafora et al., 1995; Berbee, 1996; Silva-Hanlin and Hanlin, 1999; Lindemuth et al., 2001; Lumbsch et al., 2005), although the Arthoniomycetes has been shown to be sister to either the Sordariomycetes (Tehler et al., 2003) or the Dothideomycetes (Lumbsuch et al., 2005; Spatafora et al., 2007), and the relationship between the Lichinomycetes and Lecanoromycetes differs from that seen by others (Spatafora et al., 2007). Of particular importance is that both analyses show a similar placement for *C. olivacea*. The maximum parsimony tree differed in overall arrangement from the Bayesian tree and other published phylogenies (Lumbsch et al., 2005; Spatafora et al., 2007), but was similar in the overall arrangement of orders and taxa within the Dothideomycetes to both the Bayesian analysis and other studies (Schoch et al., 2007; Spatafora et al., 2007).

The ordinal placement of *C. olivacea* within the Dothideomycetes was not resolved (Figs. 28, 29). However, this could be because a number of dothideomycetous taxa currently lack SSU and/or LSU sequence data in GenBank (i.e., Patellariales and Jahnulales) and were not included in the data matrix. A putative relationship with the Patellariales (Boudier, 1907; Durrand, 1922; Korf, 1973; Nannfeldt, 1932 in Dennis, 1977) has been suggested, but sequence data for this order at the time of writing were only available for the SSU gene for *Rhytidhysteron rufulum* (Spreng.) Speg. Clements and Shear (1931) and others (e.g., Dennis, 1977) have previously noted similarities between *C. olivacea* and the type species of *Karschia*, a taxon that is listed as incertae sedis in the Dothideomycetes (Eriksson et al., 2004). *Karschia lignyota* (Fr.) Sacc. has flat, stalkless, olive-black ascomata, cylindrical-clavate, melanized ascospores, asci with thickened apices, paraphyses that produce a brownish gel, and similar proclivities to fruit on damp, rotten wood. It differs in having two-celled ascospores, in an irregular biseriate pattern, in asci that turn blue at the apices in iodine (Dennis, 1977). While *Karschia lignyota* and *C. olivacea* are currently unrepresented in GenBank, their relationship should also be reexamined when DNA sequences and fertile cultures are obtained. A previous analysis using only the SSU rDNA gene and additional taxa in the Jahnulales and Patellariales placed *C. olivacea* in a clade containing the Jahnulales and Tubeufiaceae (data not shown). *Catinella olivacea* differs morphologically from the Jahnulales and Tubeufiaceae in having apothecioid rather than perithecioid ascomata and in having aseptate rather than septate ascospores (Scheuer, 1991; Pang et al., 2002), but whether these characters are reliable indicators of relationship is questionable. Previous studies of various taxa of the Dothideomycetes have shown that classifications based on traditional morphological characters are unsupported or in conflict with topologies based on analyses of rDNA genes (Silva-Hanlin and Hanlin, 1999; Lumbsch et al., 2005; Kruys et al., 2006). Additional sequence data and the inclusion of taxa currently unrepresented should allow for a more robust analysis of the taxonomic affiliation of *C. olivacea* within the Dothideomycetes.

In conclusion, the morphological, developmental, and molecular characters reported here show that *C. olivacea* is not a member of any taxon within the Leotiomycetes and that it would be most appropriately disposed in the Dothideomycetes, although affinities within specific orders within this class remain unresolved. The production of a sticky pseudoepithecial mass of ascospores and the formation of sticky multispored droplets in the vicinity of mature ascomata suggest that microfauna would likely play an important role in the dispersal of this fungus. The results of this research indicate the complementary value of morphological data when combined with molecular analysis. The placement of *C. olivacea* in the Dothideomycetes would have appeared controversial without the support of morphological characters derived from cultured material.

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