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Evidence that the gemmae of *Papulaspora sepedonioides* are neotenous perithecia in the Melanosporales

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Abstract: *Papulaspora sepedonioides* produces large multicellular gemmae with several, thick-walled central cells enclosed within a sheath of smaller thin-walled cells. Phylogenetic analysis of the large subunit rDNA indicates *P. sepedonioides* has affinities to the Melanosporales (Hypocreomycetidae). The development of gemmae in *P. sepedonioides* was characterized by light and scanning and transmission electron microscopy and was similar to previous ontogenetic studies of ascoma development in the Melanosporales. However instead of giving rise to ascogenous tissues the central cells of the incipient gemma became darkly pigmented, thick walled and filled with lipid globules while the contents of the sheath cells autolysed, leaving them empty and deflated at maturity. Both central cells and pre-autolytic sheath cells produced both germ tubes and new gemmae primordia, suggesting microcyclic conidiogenesis occurs in this species. Mature gemmae were non-deciduous or seceded by schizolytic secession and appear to have both perennating and disseminative potential. The evolution of these neotenous perithecial propagules may be driven by life-history and ecological factors selecting for functional versatility.

Key words: aborted ascoma, ascoma development, boreal fungus, conidium development, microcyclic conidiogenesis, papulospore, ultrastructure

INTRODUCTION

The genus *Papulaspora* Preuss was erected in 1851 to accommodate fungi producing single, compound spores on prostrate fertile hyphae (Preuss 1851) and was redefined by Hotson (1912) as a form genus for species producing bulbils and lacking a described sexual state. Weresub and LeClair (1971) further refined the definition of *Papulaspora* by excluding fungi with basidiomycetous affinities and imposing a stricter definition of the propagules diagnostic of the genus. The current definition of *Papulaspora* accommodates ascomycetes producing asexual thallodic propagules that at some point in their development are heterogenous and differentiated into a core of enlarged, often darkly pigmented central cells that is surrounded by smaller, mostly hyaline sheathing cells (Weresub and LeClair 1971, Kirk et al 2001). The diagnostic propagules of *Papulaspora* have been referred to as bulbils, small sclerotia, conidia and papulospores (Weresub and LeClair 1971) but herein are classified under the generalized term ''gemmae'', in reference to their function as multicellular asexual reproductive structures.

The phylogenetic affinities of members of *Papulaspora* are largely unresolved, although *Papulaspora* anamorphs have been reported for species of *Melanospora* and *Ceratostoma* (Ceratostomataceae, Melanosporales sensu Hibbett et al 2007) (Bainier 1907, Hotson 1917, Weresub and LeClair 1971) and a species of *Chaetomium* (Chaetomiaceae, Sordariales) (Zang et al 2004). Isolation of species of *Papulaspora* from a wide variety of substrates including plant debris, pulp products, wood, dung, soil, other fungi and animal tissues has led to the speculation that these fungi act as saprobes, mycoparasites and opportunistic pathogens (Hotson 1912, Warren 1948, Crane 1975, Shadomy and Dixon 1989). However little is known about the life history of members of this genus and its ecological functions are not well understood. Although the function of *Papulaspora* gemmae is widely accepted as an asexual disseminative propagule, they also have been reported to be ascomata that have been aborted or arrested in early stages of development (Bainier 1907, Hotson 1912, Hotson 1917, Doguet 1955).

Given the phylogenetic and structural ambiguity associated with these complex asexual propagules and the availability of a fresh, readily sporulating isolate, we examined the development of the gemmae of *P. sepedonioides* with light and electron microscopy. Our observations were interpreted in the context of a phylogenetic analysis of the species based on 28S rDNA.

MATERIALS AND METHODS

Isolation.—Mature, fallen female cones of *Picea glauca* were collected along the shore of Moose Lake, Alberta, (16-6-64 W4) in Jan 2006. Cones were placed in moist chambers (Pyrex Petri dish [80 mm × 100 mm] containing 100 mL perlite overlaid with two sheets of 90 mm diam filter paper

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[Whatman, New Jersey] and moistened with 10 mL sterile distilled water), incubated at room temperature and ambient light and examined weekly for 6 wk. Developing gemmae were subcultured onto cornmeal agar (CMA: 17 g/L cornmeal agar [Acumedia Manufacturers, Baltimore, Maryland]), oatmeal agar (OA: 20 g/L agar [Invitrogen, Carlsbad, California], 20 g/L ground oatmeal) and polypore agar (PA: 20 g/L agar [Invitrogen, Calsbad, California], 6 g/L sterile shredded basidiome of *Fomes fomentarius*]) amended with 0.01% oxytetracycline (Sigma, St Louis, Missouri). A voucher specimen of *P. sepedonioides* has been deposited in the University of Alberta Microfungus Collection and Herbarium (UAMH 10707).

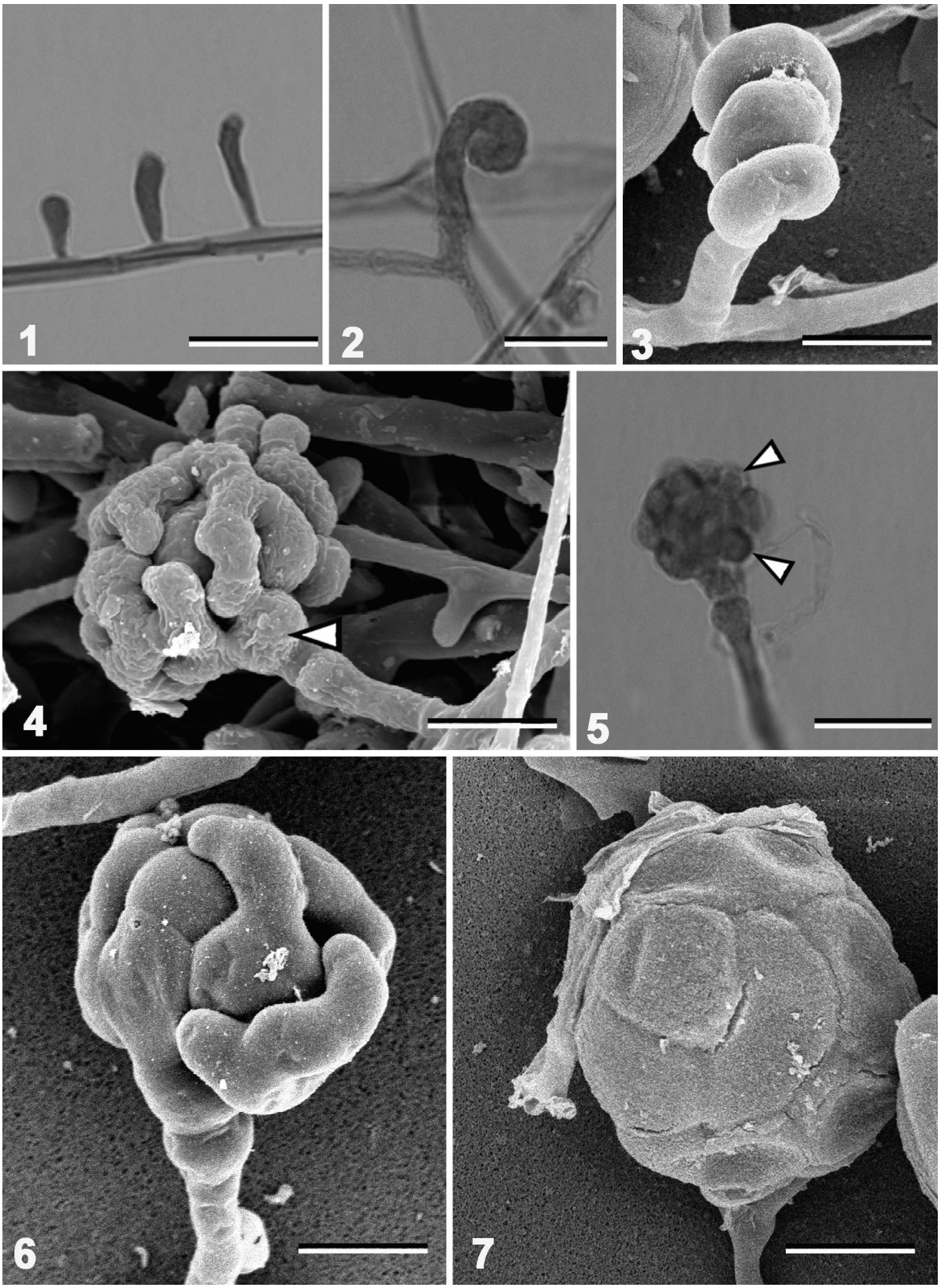
Electron microscopy.—*Papulaspora sepedonioides* was grown on OA or PA at 20 C for 4–7 wk in the dark. For scanning electron microscopy (SEM) 5 mm agar disks bearing gemmae were cut from cultures, washed in phosphate buffer (pH 7.0) and fixed in 2% glutaraldehyde (Sigma, St Louis, Missouri) for 2 h. Fixed disks were rinsed in buffer, placed in 2% tannic acid-2% guanidine hydrochloride (Sigma) solution for 4–5 h and postfixed overnight in 2% OsO_4 (Sigma) at 5 C. Fixed material was dehydrated in ethanol series, taken to amyl acetate and critical-point dried in a BAL-TEC CPD 030 dryer with carbon dioxide. Dried samples were coated with gold and examined with a Hitachi S-510 scanning electron microscope at 10 or 15 kV. For transmission electron microscopy (TEM) fungal materials were fixed in 2% glutaraldehyde, post-fixed in 2% OsO_4, dehydrated and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate (Tsuneda and Currah 2004). Photomicrographs of samples were taken at 75 kV with a Hitachi H-7000 electron microscope.

Light microscopy.—Gemmae were fixed, dehydrated, embedded in araldite, sectioned (about 1 μm) and stained with a slightly alkaline solution of toluidine blue (1%) in borax (1%) (Meek 1970). In addition 5–20 d old slide cultures grown on cereal agar (CA: 100 g/L Pablum, 20 g/L agar) were mounted in lacto-fuchsin (0.1 g lacto-fuchsin in 100 mL lactic acid), polyvinyl alcohol with acid-fuchsin (0.05 g acid-fuchsin in 10 mL lactic acid and 1 mL glycerine mixed with 1.66 g of polyvinyl alcohol dissolved in 10 mL H_20) or polyvinyl alcohol (1.66 g polyvinyl alcohol in 10 mL H_2O, 10 mL lactic acid, and 1 mL glycerine). Light micrographs of all preparations were taken with an Olympus BX50 microscope with a DP-12 digital camera.

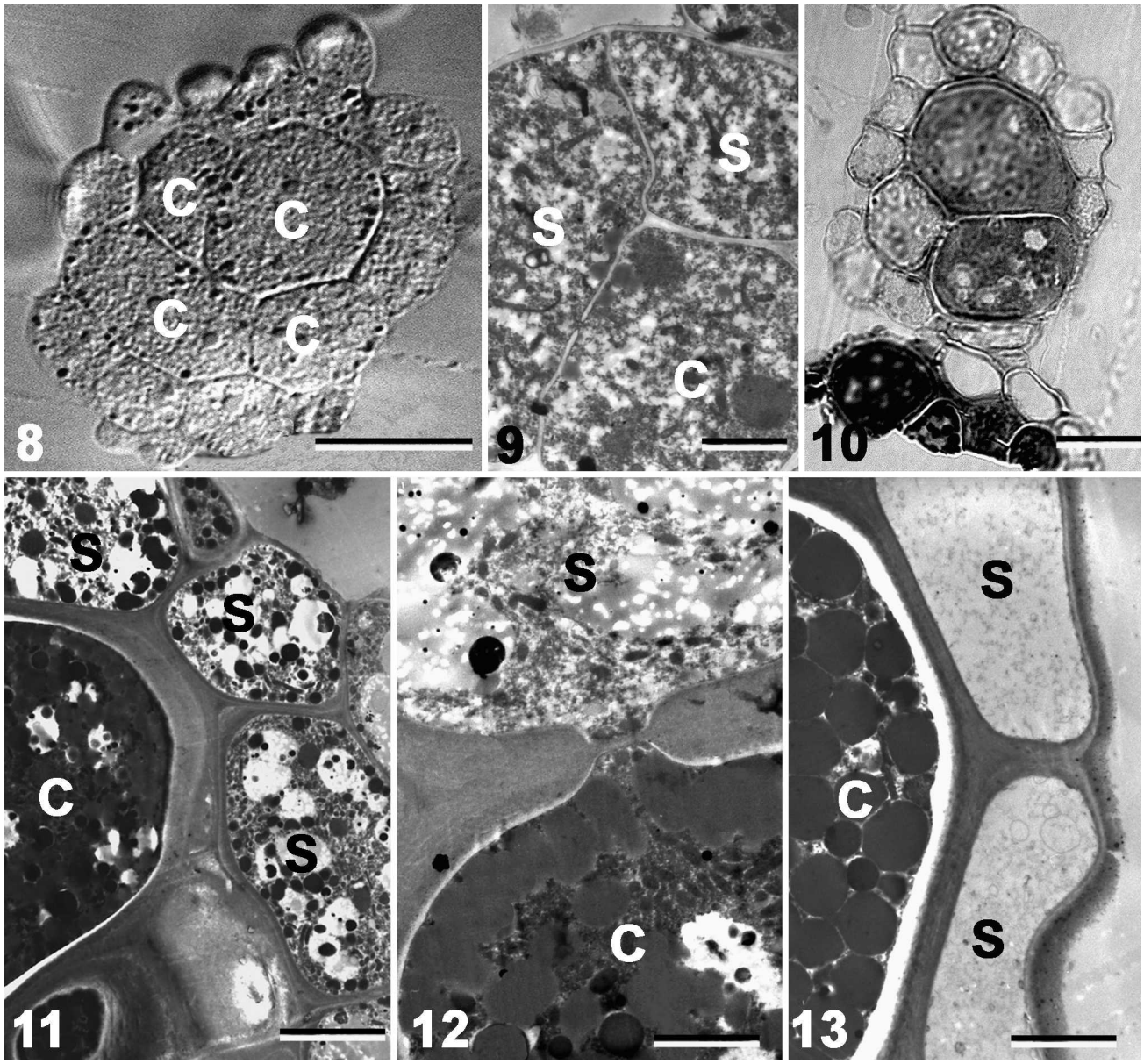
Phylogenetic analysis.—DNA extraction methods, PCR reaction parameters and automated sequencing protocols were as outlined in Hambleton et al (2005). Primers LROR (Bunyard et al 1994) and LR6 (Vilgalys and Hester 1990) were used both to amplify and sequence the LSU region. A consensus sequence was determined from overlapping sequence data with the software Sequencher™ (Gene Codes Corp., Ann Arbor, Michigan) and deposited in GenBank (accession No. EU518666). To examine phylogenetic relationships the new sequence and those of various taxa in the Hypocreomycetidae retrieved from GenBank were aligned manually with Se-Al v2.0a11 (available at http://evolve.zoo.ox.ac.uk/). This matrix was subjected to parsimony analysis with PAUP v.4.0b10 (Swofford 2003) with simple stepwise addition of taxa, tree bisection-reconnection (TBR) branch swapping and gaps treated as missing data. Support for branching topologies was evaluated with 1000 resamplings of the data by bootstrapping analysis (Felenstein 1985). All trees were scored for length in steps, CI, RC, RI and HI. The data matrix and resulting trees were deposited in TreeBASE (accession No. SN3854)

RESULTS

Development.—The isolate of *P. sepedonioides* produced mature gemmae within 96–144 h of inoculation onto OA, CMA and PA. Sporulation was sparse on CMA, moderate on OA and prolific on PA. Within 48–72 h of inoculation onto OA, numerous short, clavate lateral branches were initiated from the vegetative mycelium (FIG. 1). Branch apices subsequently recurved and coiled to form 1–3 complete turns and became septate, producing 1–4 celled hyaline, spiral primordia 8–12 × 6–10 μm borne on 1–2 celled stalks 5–15(–40) μm long (FIGS. 2–3). The basal cell of the spiral then produced several branches that were branched themselves once or twice and remained appressed to the surface of the spiral, ultimately creating a knot-like incipient gemma, 14–25 μm × 12–22 μm, composed of an investing layer of interdigitated hyphae surrounding the original cells of the primordium (FIG. 4). Less common branching of more distal cells of the primordium sometimes gave the appearance of blastic production of individual cells from the surface of the primordium (FIG. 5). As the gemma matured the interdigitated investing hyphae of the knot-like initial became inflated, tightly appressed to one another, flattened and finally septate and pseudoparenchymatous (FIGS. 6–7), ultimately forming the sheath cells of the mature propagule. The original cells of the primordium concurrently became greatly enlarged and angular and underwent 1–3 meristematic divisions, forming a core of 2–6(8) central cells (FIGS. 8–9) that became thick-walled and darkly pigmented after mature size was reached (FIGS. 10–13). The sheath and central cells were connected by adjoining perforations in the cell walls (FIG. 12). As the gemma matured further the contents of the hyaline sheathing cells autolysed and appeared empty and the central cells became packed with lipid globules (FIG. 13). Mature propagules were 37–66 × 35–58 μm and consisted of an outer sheath of thin-walled, empty, deflated hyaline cells surrounding a core of central cells that were thick-walled, darkly pigmented and contained many lipid globules (FIGS. 14–15). Mature gemmae underwent schizolytic secession at the simple septum of the basal cell (FIG. 16) or were nondeciduous (FIG. 17).



FIGS. 1–5. Development of gemma primordia in *Papulaspora sepedonioides*. 1. First stage in primordium development: initiation of short, clavate, lateral branches that will form gemma primordia. Bar = 10 μm. 2. Second stage in primordium development: formation of a hooked primordium. Bar = 10 μm. 3. Scanning electron micrograph of spiral gemma primordium with three complete turns and smooth primordium surface. Bar = 10 μm. 4. Scanning electron micrograph of



FIGS. 8–13. Maturation of gemmae in *Papulaspora sepedonioides*. 8. Thick section of a maturing gemma showing four central cells (C) that have been produced by two successive meristematic divisions of a single primordium cell. Bar = 15 μm. 9. Transmission electron micrograph of an immature gemma with thin-walled, living central (C) and sheath (S) cells. Bar = 2 μm. 10. Thick section of maturing gemma showing young central cells surrounded by investing hyphae that have become cellularized and pseudoparenchymatous. Bar = 10 μm. 11. Transmission electron micrograph showing contents of sheath cells (S) undergoing autolysis and central cells (C) packed with lipid globules. Bar = 2.5 μm. 12. Transmission electron micrograph showing adjacent cell wall perforations in central (C) and sheath (S) cells. Bar = 1 μm. 13. Transmission electron micrograph of mature gemma showing thick-walled, lipid globule-packed central cells (C) and thin-walled, empty, deflated, hyaline sheath cells (S). Bar = 2.5 μm.

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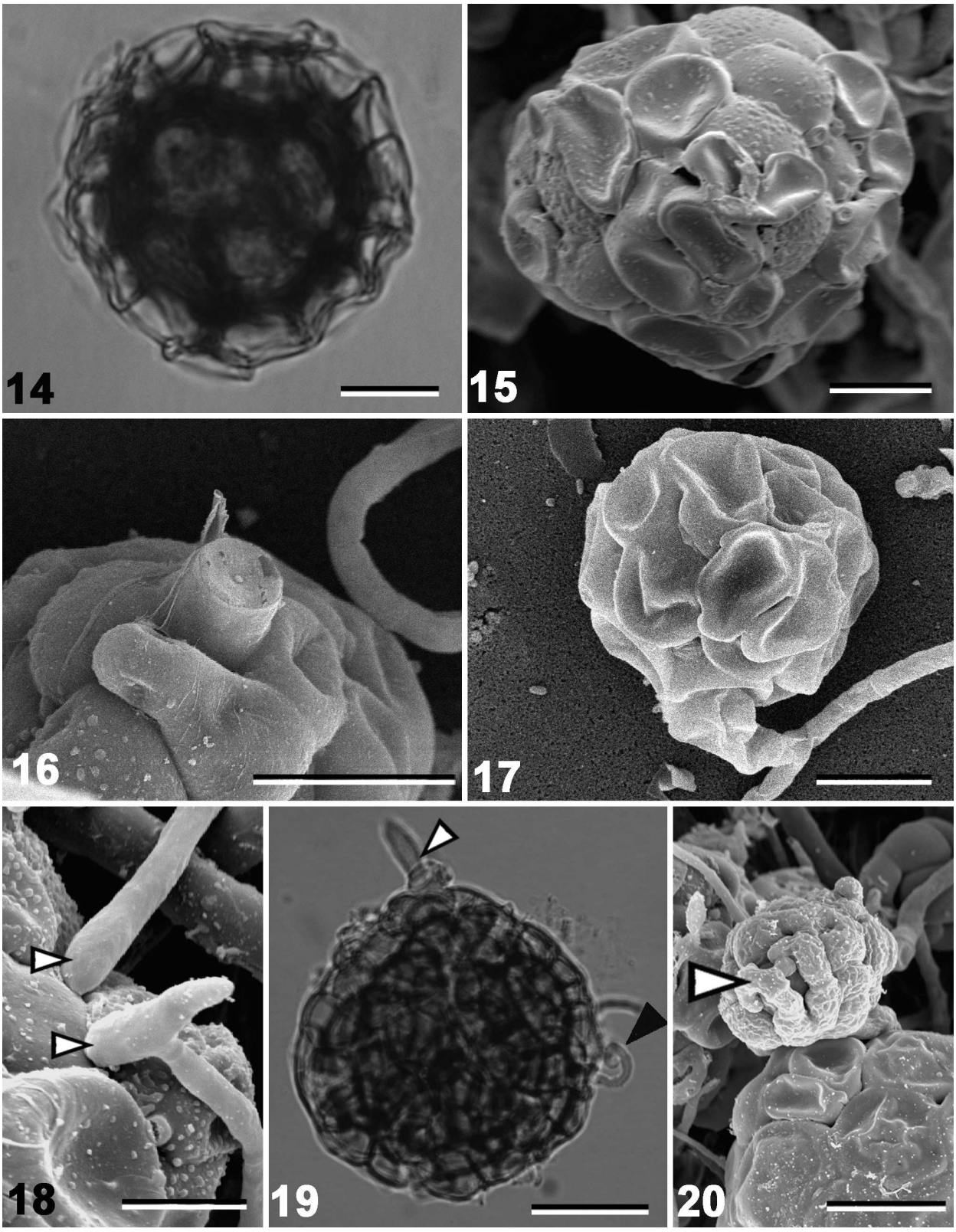
knot-like incipient gemma with branched, interdigitating investing hyphae originating from the basal cell (arrowhead). Bar = 10 μm. 5. Incipient gemma with short branches on distal cells of primordium, giving the appearance of blastically produced sheath cells (arrowheads). Bar = 15 μm. FIG. 6. Scanning electron micrograph of knot-like incipient gemma where interdigitating branches have become swollen and closely appressed to one another. Bar = 5 μm. FIG. 7. Scanning electron micrograph of young gemma with ensheathing hyphae that have become fused, flattened, septate and pseudoparenchymatous. Bar = 7 μm.



FIGS. 14–20. Mature and germinating gemmae of *Papulaspora sepedonioides*. 14. Mature gemma with darkly pigmented central cells and deflated, hyaline sheath cells. Bar = 15 μm. 15. Scanning electron micrograph of maturing gemma showing nonsynchronous deflation of sheath cells. Bar = 10 μm. 16. Gemma that has undergone schizolytic secession at the simple septum of the basal cell. Bar = 10 μm. 17. Nondeciduous mature gemma. Bar = 20 μm. 18. Scanning electron micrograph of a

Both central cells and pre-autolytic sheath cells could germinate, producing slender (1–2 μm diam) germ tubes and new spiral primordia (FIGS. 18–19). These secondary primordia developed into knot-like incipient gemmae (FIG. 20).

Phylogenetic analysis.—The aligned LSU data matrix consisting of sequences of *P. sepedonioides*, members of the Melanosporales and members of the Hypocreomycetidae sensu Eriksson et al (2003) was composed of 30 taxa and included 650 characters, of which 392 were constant, 58 were parsimony uninformative and 200 were parsimony informative. Parsimony analysis generated four equally parsimonious trees, each with 715 steps (CI = 0.526, RC = 0.399, RI = 0.759, HI = 0.474) whose topologies were consistent in their placement of *P. sepedonioides* at the family level. Results of the bootstrap analysis are shown on one most parsimonious tree (FIG. 21). *Papulaspora sepedonioides* forms a poorly supported clade (51%) with *Melanospora brevirostris*, *Sphaerodes fimicola* and *S. compressa* that is moderately supported (69%) as the sister group of the *Melanospora zamiae-M. tiffanii* clade and is nested within the strongly supported clade (100%) representing order Melanosporales. The Melanosporales clade is nested further within the strongly supported (100%) clade representing the Coronophorales, whose position was unresolved relative to other orders in the Hypocreomycetidae.

DISCUSSION

Phylogenetic analysis of the 28S rDNA places *Papulaspora sepedonioides* in close association with species of *Melanospora* and *Sphaerodes* within the strongly supported Melanosporales (sensu Zhang et al 2006), which forms a monophyletic clade with the Coronophorales. This relationship is supported further by reports of species in the Cerastomataceae (Melanosporales) having *Papulaspora* anamorphs (Bainier 1907, Hotson 1917, Doguet 1955, Weresub and LeClair 1971) and by reports of unidentified species of *Papulaspora* whose parasitic interactions with *Rhizoctonia solani* are anatomically similar to mycoparasitic structures observed in members of the Melanosporales (Jordan and Barnett 1978).

Several authors have suggested that the *Papulaspora* ''bulbils'' or gemmae produced by species of *Melanospora* are aborted ascomata instead of anamorphic vegetative propagules (Bainier 1907, Hotson 1917, Doguet 1955). The ontogenetic pattern we observed in gemmae of *P. sepedonioides* is consistent with the beginning stages of development of ascomatal initials and incipient ascoma formation in species of *Melanospora* (Doguet 1955, Goh and Hanlin 1994) supporting a homologous relationship between the two structures. Ascomatal initials of *Melanospora* species are evident first as clavate lateral branches that subsequently undergo septation and coil at the apex to form spirals (Doguet 1955, Goh and Hanlin 1994), resulting in a structure that is similar in form and size to the gemma initials of *P. sepedonioides*. In *Melanospora* incipient ascomata are formed when cells of the terminal spiral of the initial produce investing hyphae that branch extensively, grow against the surface of the initial and envelop it (Doguet 1955, Goh and Hanlin 1994). An identical pattern of hyphal proliferation occurs in *P. sepedonioides* to yield knot-like incipient gemmae. The investing hyphae of the incipient ascomata and gemmae of *Melanospora* and *P. sepedonioides* respectively become similarly differentiated into a continuous layer of hyaline to slightly pigmented pseudoparenchymatous cells that surround a core of enlarged cells derived from the original spiral (Goh and Hanlin 1994).

At this developmental stage the ontogenetic patterns of melanosporalean ascoma development and gemma development of *P. sepedonioides* diverge. In *Melanospora* the enlarged central cells of the incipient ascoma produce hyphae that give rise to both the centrum prosenchyma and ascogenous tissues (Goh and Hanlin 1994). Conversely in *P. sepedonioides* the enlarged central cells undergo meristematic growth and become thick-walled, darkly pigmented and full of lipid globules, ostensibly letting the central cells act as UV-resistant nutrient storage structures.

Mature gemmae of *P. sepedonioides* are nondeciduous or secede by schizolytic secession at the simple septum of the basal cell of the gemma, suggesting they fulfill a dual life-history function with liberated gemmae acting as disseminative propagules that disperse to new substrates and nondeciduous gemmae acting as perennating structures that ensure persistence through adverse conditions once a suitable substrate has been colonized. Both pre-autolytic

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germinating gemma that has produced two germ tubes (arrowheads). Bar = 10 μm. 19. Germinating gemma that has produced both a germ tube (white arrowhead) and a new gemma primordium (black arrowhead). Bar = 15 μm. 20. Scanning electron micrograph of a knot-like incipient gemma (arrowhead) developing from a mature gemma. Bar = 10 μm.

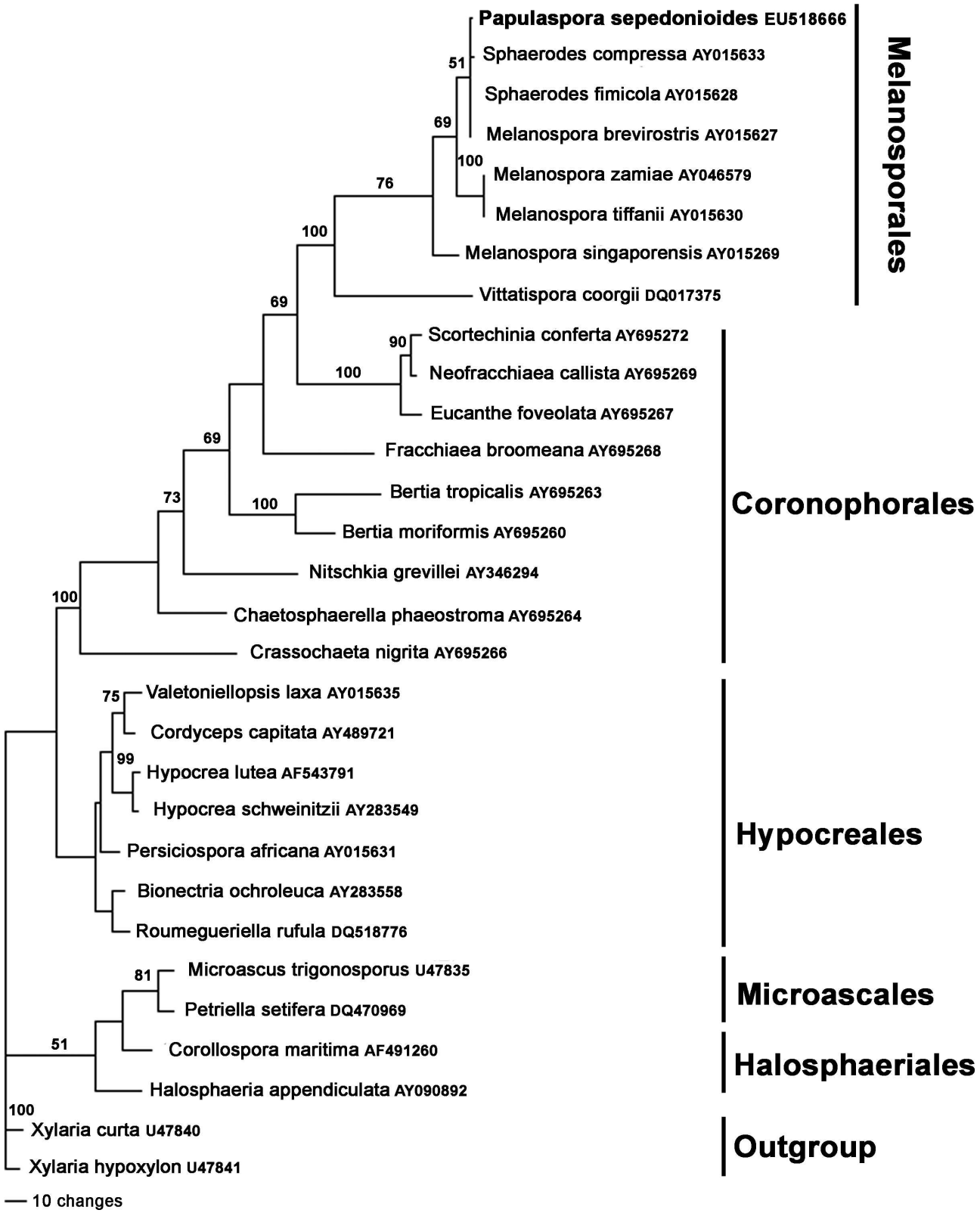

FIG. 21. One of four equally parsimonious trees (715 steps, CI = 0.526, RI = 0.759, RC = 0.399, HI = 0.474) inferred from a MP analysis of large subunit rDNA sequences showing the placement of *Papulaspora sepedonioides* among classes in the Hypocreomycetidae. BS values greater than 50% calculated from 1000 replicates are given above the branches. *Xylaria curta* and *Xylaria hypoxylon* are outgroup taxa. Accession numbers for sequences retrieved from GenBank are given after species name.

sheath cells and central cells of seceded gemmae of *P. sepedonioides* could germinate in concurrence with reports by Dodge and Laskaris (1941) but contrary to Weresub and LeClair (1971) who describe the sheath cells as ''purportedly sterile''. Germination of gemmae produces either slender germ tubes or new primordia that develop into gemma initials and presumably mature gemmae. This microcyclic conidiogenesis life-cycle stage has not been described previously in *P. sepedonioides*. Microcyclic conidiogenesis has been found to increase conidium survival and persistence rates (Fargues and Robert 1985) and to affect population dynamics (Arroyo et al 2005) in other fungi, suggesting that the process also might play similar roles in the life history and ecology of *P. sepedonioides*.

Some insights as to whether these structures originated through neoteny (i.e. as aborted perithecia) or merely represent large multicellular conidia can be obtained through the examination of similar structures in other taxa. The conversion of structures associated with sexual reproduction into asexual propagules or conidiomata has been observed in a taxonomically diverse suite of fungi, including the *Phoma* state of some *Leptosphaeria* species (Samuels 1980), the *Rhacophyllus* state of *Coprinus clastophyllus* (Redhead et al 2000), the *Decapitatus flavidus* (Cooke) Redhead & Seifert anamorph of *Mycena citricolor* (Buller 1934, Redhead et al 2000) and the *Tilachlidiopsis racemosa* anamorph of *Collybia racemosa* (Stalpers et al 1991). The argument for neoteny in *Papulaspora sepedonoides* is well supported by developmental comparisons; while the development of *Papulaspora* gemmae is apparently homologous to the initial stages of perithecial development in the Melanosporales it does not share developmental characters with other multicellular asexual propagules. The large prosenchymatous propagules of *Pulvinella* (Ramaley 2001) and *Verdipulvinus* (Ramaley 1999), the pseudoparenchymatous asexual bulbils or conidia of species of *Burgoa*, *Minimedusa* (Weresub and LeClair 1971), *Monodictys* (Day et al 2006), and *Taxomyces* (Strobel and Stierle 1993), and the specialized coiled, interwoven conidiogenous hyphae of *Clathrosporium* (Nawawi and Kuthubutheen 1987, Hennebert 1998) and *Spirosphaera* (Voglmayr 2004) that can be considered most similar in structure and function to the gemmae of *P. sepedonioides* all are of multihyphal origin, lack the specialized cell differentiation observed in the gemmae and(or) do not exhibit mixed thallic and meristematic growth.

The cell wall perforations in adjacent sheath-central cells observed by Weresub and LeClair (1971) have been suggested to represent simple septal pores at points of origin of the investing hyphae that form the sheath cells. Given the phylogenetic affinities of *P. sepedonioides* to the Melanosporales and Coronophorales and its apparent neotenous origin, these perforations instead may be analogous to the Munk pores observed in the peridial cells of members of the Coronophorales and less frequently other members of the Sordariomycetes (Nannfeldt 1975a, b; Cannon 1995; Huhndorf et al 2004). Cannon (1995) suggested these peridial pores might allow for efficient nutrient translocation between pseudoparenchymatous cells. If this is the case it is possible that as the sheath cells of *Papulaspora* gemmae senesce and undergo autolysis the lysed cell contents are translocated to the central cells, where they are stored as additional nutrients for the propagule. Translocation of materials through peridial pores or the cell wall perforations of *Papulaspora* gemmae has not been demonstrated, and further research would be required to confirm this putative function of sheath cells.

Factors leading to the evolution of the gemmae of *P. sepedonioides* remain largely unknown, and cytological studies are needed to determine whether *P. sepedonioides* gemmae are of dikaryotic origin, as are perithecia in the Melanosporales. Similarly ecological and life-history studies that determine whether *P. sepedonioides* is mycoparasitic and exploits ephemeral substrates could provide insight into the ecological pressures that might have contributed to the evolution of these functionally versatile neotenous perithecial propagules.

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