Mycorrhiza (2006) 16: 241–244
DOI 10.1007/s00572-006-0040-2

ORIGINAL PAPER

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The mycorrhizal status of *Pseudotulostoma volvata* (Elaphomycetaceae, Eurotiales, Ascomycota)

Received: 4 May 2005 / Accepted: 21 December 2005 / Published online: 18 March 2006

Abstract *Pseudotulostoma volvata* (O. K. Mill. and T. W. Henkel) is a morphologically unusual member of the otherwise hypogeous Elaphomycetaceae due to its epigeous habit and exposed gleba borne on an elevated stalk at maturity. Field observations in Guyana indicated that *P. volvata* was restricted to rain forests dominated by ectomycorrhizal (EM) *Dicymbe corymbosa* (Caesalpiniaceae), suggesting an EM nutritional mode for the fungus. In this paper, we confirm the EM status of *P. volvata* with a combination of morphological, molecular, and mycosociological data. The EM status for *P. volvata* corroborates its placement in the ectotrophic Elaphomycetaceae.

Keywords Ectomycorrhiza · Neotropics · *Pseudotulostoma* · *Dicymbe* · *Elaphomyces*

Introduction

Pseudotulostoma volvata O. K. Mill. and T. W. Henkel (Elaphomycetaceae, Eurotiales, Ascomycota) was recently described as a monotypic genus from the tropical rain forests of Guyana (Miller et al. 2001). The taxon is unique among otherwise hypogeous, truffle-like Elaphomycetaceae in having an epigeous fruiting habit in which, during ascoma development, a spore-bearing gleba is raised several centimeters on a sterile stalk, rupturing the peridium, which is thus retained as a volva. Presumably, the spores of *P. volvata* are mechanically dispersed as raindrops batter the persistent gleba. This singular fruiting habit notwithstanding, *P. volvata* shares a number of phenotypic features with *Elaphomyces* spp., including a thick-walled double peridium, ornamented spores with similar ultrastructure at maturity, and abundant pseudocapillitium-like glebal hyphae. These features, along with 18S rRNA sequence data, confirmed the placement of *Pseudotulostoma* in the Eurotiales, in a sister position to *Elaphomyces* (Miller et al. 2001). Subsequently, a second species, *Pseudotulostoma japonicum* (Kawamura) Asai, H. Sato and Nara, was recognized from oak forests of Japan (Asai et al. 2004) and shown to be closely related to *P. volvata* (Masuya and Asai 2004).

Pseudotulostoma volvata was presumed to be ectomycorrhizal (EM) in nutritional habit due to its placement in the Elaphomycetaceae and the apparent restriction of its ascomata to rain forests dominated by the EM tree species *Dicymbe corymbosa* (Caesalpiniaceae) in Guyana (Miller et al. 2001; Henkel et al. 2002). In this paper, we confirm the EM status of *P. volvata* with a combination of morphological, molecular, and mycosociological data.

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Materials and methods

Ascoma and ectomycorrhiza collection Collecting expeditions were conducted in 1999 by TWH to the Upper Ireng River Basin along Guyana's western border with Brazil, in the south-central Pakaraima Mountains (general area: 5° 05′ N; 59°58′ W). Subsequent expeditions were conducted during May–July of 2000 by TWH, SLM, and MCA, and 2001–2004 by TWH and MCA to the Upper Potaro River Basin located approximately 40 km north of the Ireng site. Fungi were collected from an area of approximately 10 km^2 at each of the sites in forests dominated by EM

Dicymbe corymbosa Spruce ex Benth. (Caesalpiniaceae). Voucher specimens are maintained by the first author at Humboldt State University (HSU).

For collection of EM rootlets of *D. corymbosa* putatively associated with *P. volvata*, ascomata were located in the field and square sections of the litter/root mat and upper mineral soil, ~30 cm wide, were excavated around the ascomata. From these sections, ascomata and subtending roots were carefully removed and cleaned of soil particles and adhering humus. Care was taken to maintain direct connections, when present, between rootlets, hyphae, and ascomata bases.

Rootlets directly connected to *P. volvata* ascomata were assessed in the field for the presence of a fungal mantle and extramatrical hyphae; thin transverse and longitudinal sections were hand-made to determine mantle micromorphology and the presence of a Hartig net. Voucher specimens of ectomycorrhizas are maintained in formalin acetic-alcohol by the first author (HSU).

Molecular determination of mycobiont The 18S rRNA sequence and resulting phylogeny from an ascoma of *P. volvata* were described in Miller et al. (2001). We used similar methods to characterize the mycobiont of putative *P. volvata* ectomycorrhizas. In the field, cleaned EM rootlets attached directly to the ascomata of *P. volvata* were aseptically transferred into 500 µl of DNA extraction buffer (Zolan and Pukkila 1986) and maintained at ambient temperature until returned to the lab. Rootlets for DNA extraction were taken at least 1 cm away from the ascoma base to avoid possible contamination from the basal mycelium. The 18S rRNA gene was amplified from DNA extracts of EM rootlets using two overlapping primer pairs, NS1+NS4 (White et al. 1990) and BMB-BR+5.8S (Lane et al. 1985; Vilgalys and Hester 1990). Both PCR amplicons were sequenced on an ABI3700 using BigDye chemistry (Applied Biosystems) and compared with the sequence from the *P. volvata* ascoma using Sequencher v. 2.0 (Gene Codes).

Mycosociology of P. volvata ascomata As part of a multiyear mycosociology project at the Upper Potaro site (Henkel, Aime and S.L. Miller, unpublished), during the May–July rainy season of each year from 2000 to 2004, forest stands dominated by *D. corymbosa* and other mixed rain forests lacking EM trees were sampled systematically for macrofungi (see Henkel 2003 for details of plot system). Within three locally disjunct 1-ha plots in both the *D. corymbosa* forest and the mixed forest, during a given sampling event five randomly positioned 100-m^2 subplots per hectare were thoroughly sampled by five persons for the presence and number of fruiting bodies of macrofungal morphospecies present. Ascomata of *P. volvata* were counted accordingly. Each 1-ha plot was revisited from 4 to 10 times for each of the years 2000–2004. The number of 100 m^2 subplots sampled/forest type for the 2000–2004 period was 525, for a total sampling area of 52,500 m^2 for each forest type. For *P. volvata*, the total number of ascomata occurring in each forest type from 2000–2004 was determined and the frequency of occurrence in the 100-m^2 subplots calculated.

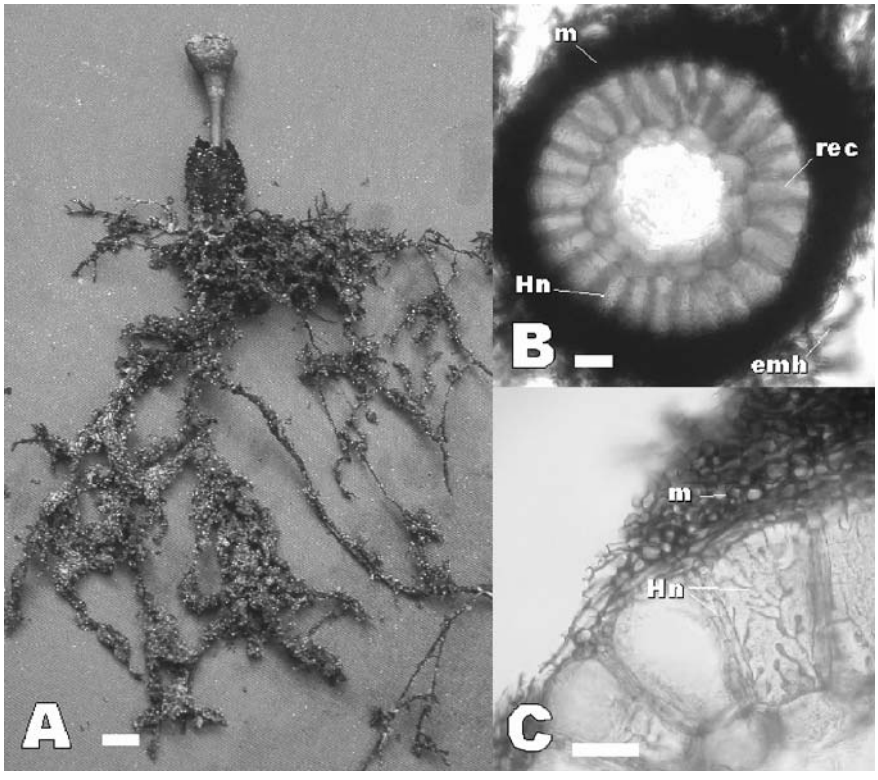

Fig. 1 *Pseudotulostoma volvata* ascoma and ectomycorrhizas; *emh* extramatrical hypha, *m* mantle, *rec* root epidermal cell, *Hn* Hartig net. **a** Ascoma arising from dense mass of ectomycorrhizas, and attached root network from mineral soil (Henkel 7022). Bar=1 cm. **b** Transverse section of ectomycorrhiza formed with *Dicymbe corymbosa*. Bar=50 µm. **c** Oblique transverse section of ectomycorrhiza showing the dense, plectenchymatous mantle and proliferation of the Hartig net. Bar=50 µm

Table I The number and frequency of occurrence of *Pseudotulostoma volvata* ascomata in ectomycorrhizal *Dicymbe corymbosa*-dominated forest and arbuscular–mycorrhizal mixed rainforest in the Upper Potaro Basin, Guyana, 2000–2004[1]

Forest type		2000	2001	2002	2003	2004	Sum
Dicymbe corymbosa	No. of Ascomata (frequency[2])	81 (34)	95 (33)	38 (15)	106 (40)	127 (40)	447 (30)
Mixed	No. of Ascomata	0	0	0	0	0	0

[1]Designated sampling area = 3 ha (30,000 m^2) of permanent plots revisited annually (May–July) in each forest type; number of 100-m^2 subplots randomly sampled (total area sampled m^2) per year in each forest: 2000 – 150 (15,000); 2001 – 150 (15,000); 2002 – 60 (6,000); 2003 – 90 (9,000); 2004 – 75 (7,500); 2000–2004 total: 525 (52,500)
[2]Frequency = number of 100-m^2 subplots containing *P. volvata* ascomata/total number of 100-m^2 subplots sampled ×100

Specimens examined Guyana. Region 8, Potaro-Siparuni. Pakaraima Mountains. Upper Ireng River, western slope of Mt. Wokomung, along Suruwabaru Creek 2 km upstream from the confluence with Yuarka River, elevation 800 m, under *D. corymbosa*, 21 May 1999, Henkel 7022 (isotype – HSU); Upper Potaro River, 20 km east of Mt. Ayanganna, 3 km southeast of Potaro base camp, on *Dicymbe* plot 1, elevation 720 m, 19 May 2001, Henkel 8184 (HSU); Upper Potaro River, 20 km east of Mt. Ayanganna, 4 km southwest of Potaro base camp, on *Dicymbe* plot 3, elevation 720 m, 22 June 2002, Henkel 8481 (HSU).

Results

Field observations Soil excavations demonstrated that ascomata of *P. volvata* were invariably subtended by, and connected to, EM rootlets of *D. corymbosa* (Fig. 1a). These ectomycorrhizas, which proliferated extensively in the upper-leaf litter horizon but also descended into the mineral soil, were finely pinnately branched and covered with a dense, greyish-black mantle, and produced abundant dark greyish-brown extramatrical mycelium. The ascomata arose from dense masses of these ectomycorrhizas and extramatrical mycelium; sections of the lower volva revealed EM rootlets embedded in the peridium in a manner similar to that seen in ascomata of *Elaphomyces muricatus* (Miller and Miller 1984). Illustrations of *P. japonicum* provided by Asai et al. (2004, Figs. 1, 2 and 5) showed similar masses of fine rootlets around and embedded in the bases of ascomata.

Determination and description of ectomycorrhizas Hand sections of rootlets were consistent with EM micromorphology (Fig. 1 b,c; Agerer 1995). *Mantle* a moderately dense plectenchyma 50–70 µm thick, of 7–10 cell layers, gelatinous matrix inevident; hyphal elements periclinal to the root, dark grey, simple septate, uninflated, 5–10 µm broad, smooth, thin-walled; surface hyphae in scalp section with no discernable pattern. *Extramatrical hyphae* profuse, arising in the outer mantle from undifferentiated cells, hyphae 5–10 µm broad, concolorous, smooth, thin-walled, simple septate, occasionally branching. *Hartig net* surrounding elongated rootlet epidermal cells, 1–3 hyphal cells thick; hyphal elements elongated in transverse section, 3–7 µm broad.

Molecular identification of the mycobiont PCR amplification of the 18S rRNA gene from a dark ectomycorrhiza associated with a *P. volvata* ascoma produced single amplification products with both PCR primer pairs. Direct sequencing of these PCR amplicons produced uncontaminated DNA sequences identical to those obtained from the *P. volvata* ascoma reported in Miller et al. (2001; GenBank accession number AF308161), confirming the identity of the mycobiont as *P. volvata*.

Distribution of P. volvata ascomata During sampling from 2000 to 2004 at the Potaro site, ascomata of *P. volvata* were encountered only in forests dominated by EM *D. corymbosa*, and were entirely lacking from surrounding anectotrophic mixed rain forests. A total of 447 ascomata were encountered, occurring in 30% of the randomly positioned subplots within *D. corymbosa* plots over the 4-year sampling period (Table 1).

Discussion

The combination of DNA sequence data, mycorrhizal macro- and micromorphology, and ascoma distribution indicated that *P. volvata* is EM in nutritional habit. The placement of *P. volvata* in the Elaphomycetaceae is further supported by its EM status, as the sister genus of *Pseudotulostoma*, *Elaphomyces*, has long been considered EM based on ascoma distribution (Trappe 1962, 1971; North and Greenberg 1998), in vitro synthesis of *Elaphomyces* ectomycorrhizas (Miller and Miller 1984; Theodorou and Reddell 1991), as well as molecular determination from field-sampled ectomycorrhizas (Bird and McCleneghan 2001). Additionally, the dense plectenchymatous mantle showing no discernable pattern in longitudinal section, as well as the common Hartig net type of *P. volvata* ectomycorrhizas, was consistent with EM micromorphology characterizing the Elaphomycetaceae (Miller and Miller 1984; Agerer 1995). Interestingly, while *Elaphomyces* ectomycorrhizas are typically found adhering to the ascoma and embedded in the peridium, with no obvious aggregations in the surrounding soil, *P. volvata* combines the adhering ectomycorrhizas with an extensive EM mat connecting to the ascomata. While asci were not observed in the original specimens of *P. volvata*, Asai et al. (2004, Figs. 12, 14 and 15) demonstrated oval, thin-

walled, eight-spored asci in immature ascomata of *P. japonicum*; these are nearly identical to those illustrated for *Elaphomyces* by Pegler et al. (1993, Fig. 4). With further exploration in Guyana, we expect to find *P. volvata* ascomata sufficiently immature to exhibit the presumably evanescent asci. While strikingly different from *Elaphomyces* spp. in macromorphology of the mature ascomata, *P. volvata* conforms well in other respects with the Elaphomycetaceae, the only EM clade within the largely saprotrophic Eurotiales (Geiser and LoBuglio 2001).

Based on 18S rRNA gene phylogenies, *Pseudotulostoma* was the sister taxon to *Elaphomyces*, rather than being derived from within a clade of hypogeous *Elaphomyces* species (Miller et al. 2001; Masuya and Asai 2004). Because of this sister relationship, it is unclear whether the epigeous habit with exposed gleba of *Pseudotulostoma* was a novel adaptation derived from sequestrate, *Elaphomyces*-like ancestors, or whether *Elaphomyces* represents a reduction of the *Pseudotulostoma* form. We expect additional *Pseudotulostoma* spp. to be discovered, particularly as tropical, and possibly temperate, ectotroph-dominated forests are further explored. Additionally, species previously assigned to the Tulostomataceae should be re-examined, as some may actually be *Pseudotulostoma* species (e.g., *Battarrea japonica=Pseudotulostoma japonicum*; Asai et al. 2004). With more detailed phylogenetic analyses involving more taxa, the ancestral ascoma form of the Elaphomycetaceae may be inferred.

Pseudotulostoma volvata and several undescribed species of *Elaphomyces* (Henkel, Aime, and S.L. Miller, unpublished) form a conspicuous ascomycetous component of the largely basidiomycetous EM fungal guild associated with *Dicymbe* spp. in the Pakaraima Mountains of Guyana (Henkel et al. 2002). These are the first records for the Elaphomycetaceae from South American tropical rainforests (J. M. Trappe, personal communication), as well as for their association with leguminous hosts. The ecological importance of *Pseudotulostoma* and *Elaphomyces* in *Dicymbe* forests is unknown but may be significant, as ectomycorrhizas likely contribute to the competitive abilities of *Dicymbe* species when occurring in monodominant stands (Henkel 2003; Henkel et al. 2005; Mayor and Henkel 2005). Further studies, including in vitro synthesis and experimentation with *P. volvata* ectomycorrhizas, would be fruitful in this respect.

Acknowledgements This work was made possible by grants to TWH from the Smithsonian Institution's Biological Diversity of the Guiana Shield Program, the National Geographic Society's Committee for Research and Exploration, and the A.W. Mellon Foundation, and NSF DEB 0315607 and USDA 2003-01542 to SLM. The authors wish to thank Mimi Chin and Christopher Andrew for expert field assistance in Guyana. The Guyana Environmental Protection Agency and the Ministry for Amerindian Affairs provided research permits. Early observations by Gary Samuels on *Pseudotulostoma* in Guyana are belatedly acknowledged, as are helpful discussions with Scott Redhead. This is paper number 103 in the Smithsonian Institution's Biological Diversity of the Guiana Shield Program publication series.

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