Pseudotulostoma, a remarkable new volvate genus in the *Elaphomycetaceae* from Guyana

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Pseudotulostoma volvata gen. sp. nov. is described from the south-central Pakaraima Mountains of Guyana. *Pseudotulostoma volvata* is associated with ectomycorrhizal *Dicymbe corymbosa* trees (*Caesalpiniaceae*) and placed in the *Ascomycota*, *Eurotiales*, *Elaphomycetaceae*. Included are a description of the genus and species, illustrations of the macroscopic and microscopic features, and a discussion of the distinctive features and phylogenetic placement of this fungus.

INTRODUCTION

Recent collecting activities in the Pakaraima Mountains of western Guyana have revealed a rich macromycete biota, including many undescribed taxa (Henkel 1999, Henkel *et al*. 2000). Ongoing taxonomic investigations in this poorly explored region of the neotropics are centered on macromycetes associated with ectomycorrhizal rain forest trees of the genus *Dicymbe* (*Caesalpiniaceae*). This paper describes a new and interesting ascomycete from *Dicymbe*-dominated forests of Guyana.

The taxon at maturity is stalked and epigeous; the tough persistent volva is black with a white context. The black, fluted, stalk is equal and expands just beneath the enlarged, rounded, apical gleba. The apex has no sign of an ostiole and weathers away to expose a mazaedium which is gray.

This new ascomycete is a very unusual taxon and with the lack of an obvious hymenium or sexual cell, and with a very obvious woody volva, and large woody stock was placed at first by the authors in the *Basidiomycota*. A BLAST search of an initial rDNA sequence in 1999 most closely matched sequences near *Penicillium* and was thought due to a contamination. However, subsequent sequences obtained in 2000 again indicated ascomycete affinities, primarily with members of the *Eurotiales* and in particular the genus *Elaphomyces*. Subsequent field observations by T. H. and S. L. M. supported the hypothesis of a relationship with *Elaphomyces*. Molecular phylogenetic analysis clearly show that the new fungus is part of a clade of cleistothecial ascomycetes. A variety of morphological features in the new genus are congruent with *Elaphomyces*, most convincing of which is the similarity of ascospore ultrastructure. The related genus *Elaphomyces* has evanescent asci which disintegrate very early in the maturation of the ascomata, leaving a powdery gleba. We assume this to be the case with the new genus and therefore, although no asci have been seen in the material available, the spores are assumed to be ascospores. We therefore consider the fruiting bodies to be ascomata. Thus far the youngest buttons collected are too mature to demonstrate a structure in which or from which the spores develop.

MATERIALS AND METHODS

Collecting expeditions were conducted in 1998 and 1999 by T. H. to the Upper Ireng River Basin along Guyana's western border with Brazil, in the south-central Pakaraima Mountains (5°05′ N, 59°58′ W). A fourth expedition was conducted during May–July 2000 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.

Ascomata were examined in the field for their fresh characteristics. Colour characteristics were coded according to Kornerup & Wanscher (1978; 'Met' code noted in parentheses) and described subjectively. Ascomata were dried slowly over charcoal and subsequently placed in containers with silica gel to prevent spoilage in the excessively humid conditions. In addition, some ascomata were pickled fresh in FAA.

Microscopic anatomical details were determined from dried and pickled specimens with a Leitz Diaplan microscope with bright-field and phase-contrast optics. Features were observed in 3% KOH and Melzer's reagent. A total of 28 spores were measured from the holotype and each subsequent collection.

DNA techniques

In the field, a tissue sample from inside the ascomatal stalk was aseptically transferred into 500 µl of DNA extraction buffer (Zolan & Pukkila 1986), and maintained at ambient temperature until returned to the lab. The tissue sample was then ground in buffer with a microcentrifuge pestle and incubated at 65 °C for 1 h. The sample was subject to two chloroform/isoamyl alcohol (24:1) extractions, and the DNA was precipitated overnight at −20 °. The gene encoding for small subunit rRNA was amplified in two portions using the primer combinations NS1/NS4 (White *et al*. 1990) and BMB-BR/5.8S (Lane *et al*. 1985, Vilgalys & Hester 1990) and amplification conditions according to Vilgalys & Hester (1990). Amplification products were purified using Ultrafree-MC filter units (Millipore, Marlborough, MA) and sequenced using dye terminator chemistry with the primers NS1, NS2, NS4, BMB-BR, SR1.5, and SR6 (White *et al*. 1990, James *et al*. 2000). Labeled fragments were separated on an ABI3700 DNA Analyzer (Applied Biosystems, Foster City, CA). The sequence of *Pseudotulostoma volvata* has been deposited in GenBank, accession no. AF308161.

Phylogenetic analysis

The DNA sequence alignment from the study of Berbee & Taylor (2000) was used as a starting point for identifying the phylogenetic affinity of *P. volvata* with other filamentous ascomycetes. Additional sequences from eurotialean/onygenalean fungi were added to the data set (Landvik *et al*. 1996, GenBank), and the alignment was readjusted manually (149 bases were considered nonalignable and excluded from analyses). GenBank accession numbers for taxa not included in Berbee & Taylor (2000) or Landvik *et al*. (1996) are: *Aspergillus niger* (D63697); *A. ochraceus* (AB008405); *A. zonatus* (AB008413); *Byssochlamys nivea* (M83256); *Cephalotheca sulfurea* (AF096173); *Chromocleista cinnabarina* (AB006747); *Monascus purpureus* (M83260); *Paecilomyces variotii* (Y13996); *Penicilliopsis clavariaeformis* (AB003946); *Penicillium chrysogenum* (M55628); *Talaromyces eburneus* (D88322); and *T. emersonii* (D88321). Phylogeny reconstruction using maximum parsimony was accomplished with PAUP* (Swofford 1998). Gaps were considered as informative, additional character states. Most parsimonious trees were found using 1000 replicate heuristic searches with the random-addition-sequence option. Support for nodes was assessed using 1000 bootstrap replicates (Felsenstein 1985).

TAXONOMY

Pseudotulostoma O.K. Mill. & T. Henkel, **gen. nov.** (Figs 1–5)

Etym.: 'Pseudo', false; appearing like the genus *Tulostoma*.

Fructificatio capitulo ovali in apice obtuso praedita, endoperidio atro-brunneo obtecta; endoperidium quum stipes evolvens abrasum, glebam griseam aperiens. Exoperidium atro-brunneum, siccum, exasperatum. Stipes longus, angustus, subaequus, siccus, usque ad endoperidium longistrorsum sulcatus. Volva alta, tenax vel lignosa, persistens, urceolata, ad basem obtusum decrescens, contextu albo in superficie interiore exterioreque atro-brunneo praedita. Peridium e hyphis parallelis, tenuitunicatis, materiam contentam griseo-brunneam continentibus compositum. Pseudocapillitium glebae abundans, raro ramosum, tenuitunicatum, hyalinum, flexuosum, interdum septatum, Asci non visi. Ascosporae globosae, reticulo irregulariter alti-crasso atque humili-tenui et intersectionibus incrassates in aspectu cavis ornatae, in 3% KOH pallide luteo-brunneae reagentes. Fibulae ignotae.

Typus: *Pseudotulostoma volvata* O. K. Mill. & T. Henkel.

Fruiting body with an oval head (4–) 9–11 mm × 9–14 mm; apex of head blunt, rounded; entirely covered by a dark brown endoperidium which erodes away, leaving a gray gleba. *Exoperidium* black-brown, roughened over the entire volva (use lens). *Stalk* 35–71 mm × 2–3 mm, sub-equal, dry, grooved, extending without interruption to the endoperidium. *Volva* deep, tough, persistent, and vase-like (Figs 1–2), 15–20 mm high, 9–14 mm broad at the apex narrowing to a blunt base 4–7 mm wide, context white, inner surface also black-brown, bruising reddish fresh. *Peridium* of mostly parallel to somewhat tightly interwoven, thin-walled hyphae 3.5–7.5 (–10) µm diam with gray-brown contents. *Mazaedium* with abundant, pseudocapillitium-like interwoven hyphae, seldom branched, thin-walled, flexuous, occasionally septate, hyaline with thin walls. *Ascospores* globose, 7–9.5 µm diam, with dense warts 0.8–1.2 µm high forming an incomplete reticulation, light yellow-brown in 3% KOH (Em = 1: E = 1).

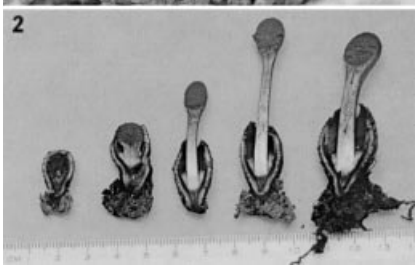
Pseudotulostoma volvata O. K. Mill. & T. Henkel, **sp. nov.** (Figs 1–5)

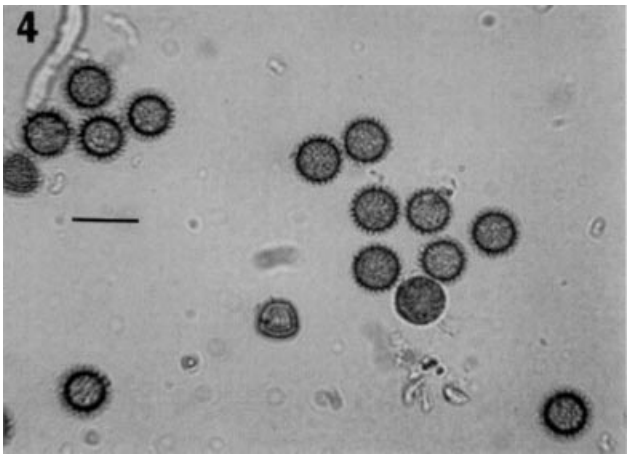
Etym.: 'volvata', possessing a volva.

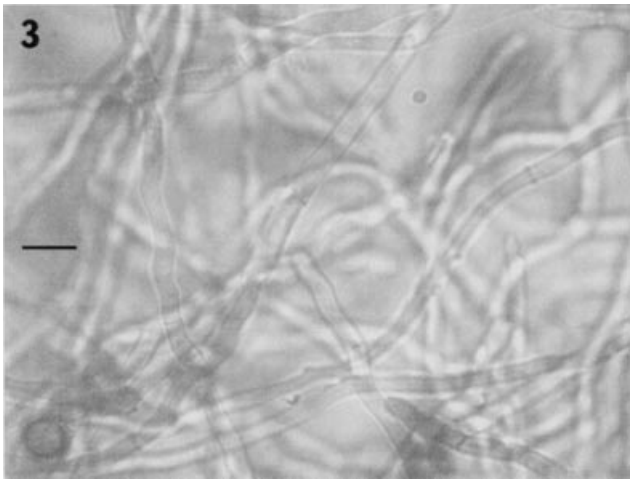
Fructificatio capitulo ovali 9–14 mm lato, 9–11 mm alto, ad apicem obtusa, rotundata; exoperidium atro-brunneum subabrasum, glebam griseam aperiens (Met 2D2 usque Met 4C2, vel Met 4D2), submaturitate pulveraceum. Peridium atro-brunneum (Met 6F2 usque Met 6F1), sub maturitate basem glebae ambiens. Stipes 35–71 mm longus, 2–3 mm latus, aequus, in superficie longistrorsum fossulatus atque alte sulcatus, glaber, atro-brunneus, peridio glebam ambiente continuus, lignosus. Volva alta, persistens, lignosa, urceolata, 15–20 mm alta, 9–14 mm lata, in superficie sub lente minute rimosa, interiore atque exteriore atro-brunnea, ad basem obtusa. Contextus glebae griseus (Met 8E 1–2), atque stipitis solidus et firmus obscure albus, atque volvae albus 0.4–1.2 mm crassus. Exoperidium e hyphis intertextis, tenuitunicatis 3.5–7.5 (–10) µm diam, materiam contentam griseo-brunneam continentibus compositum. Mesoperidium crassum, tenax, album, e hyphis 3–6 (–8) µm diam. In 3% KOH hyalinis reagentibus, compositum. Endoperidium tenue e hyphis (3.5–) 5–10 µm diam. Parum crassitunicatis (0.3–0.5 µm), parietibus in 3% KOH griseo-brunneis reagentibus, compositum. Stipitipellis e hyphis parallelis parum crassitunicatis 4–9 (12) µm, in 3% KOH hyalinis reagentibus composita. Pseudocapillitium glebae raro ramosum, tenuitunicatum, flexuosum, interdum septatum, hyphis 2–5 (–6) µm diam. Asci non visi. Ascosporae globosae, 7–9.5 µm diam (Em = 1, E = 1), reticulo 0.8 usque 1.2 µm alto irregulariter alti-crasso atque humili-tenui et intersectionibus incrassatis in aspectu cavis ornatae, in 3% KOH pallide luteo-brunneae reagentes. Fibulae ignotae.

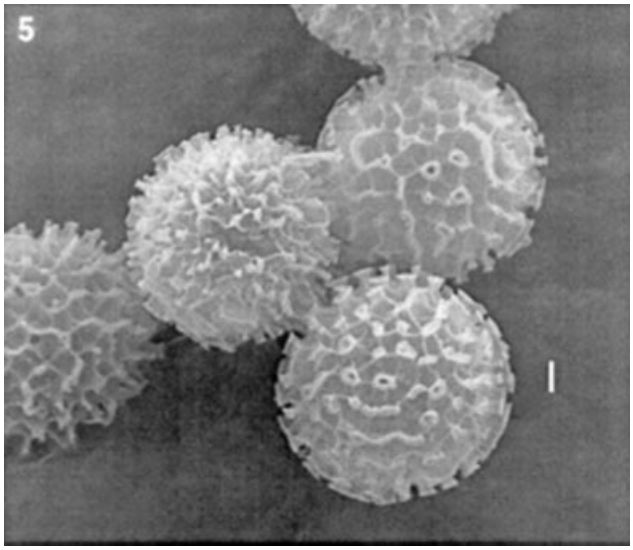
Typus: **Guyana**: *Region* 8, *Potaro-Siparuni*: Pakaraima Mountains, upper Ireng River, western slope of Mt Wokomung, along Suruwabaru Creek 2 km upstream from confluence with Yuarka River, alt. 800 m, under *Dicymbe corymbosa*, 21 May 1999, *Henkel 7022* (BRG-holotypus; K, DUKE, VPI-isotypi).

Fruiting body with an overall head 9–11 mm × 9–14 mm; apex blunt, rounded; where the exoperidium has eroded away









Figs 1–5. *Pseudotulostoma volvata* (*Henkel 7022*). **Fig. 1.** Ascomata Bar = 1 cm. **Fig. 2.** Longitudinal sections showing ascomata development. Bar = 1 cm. **Fig. 3.** Pseudocapillitium with simple septa. Bar = 10 µm. **Fig. 4.** Ascospores. Bar = 10 µm. **Fig. 5.** Scanning electron micrograph of ascospores, × 5000. Bar = 1 µm.

leaving a gray exposed gleba (i.e. spore mass) (Met 2D2 to Met 4C2, or Met 4D2), powdery when mature. *Peridium* surrounding the base of the gleba blackish brown (Met 6F2 to Met 6F1), with no clear demarcation between gleba and stipe tissue. *Stipe* 35–71 mm × 2–3 mm, sub-equal, expanding and sulcate at the base of the gleba, surface with longitudinal furrows and deeply grooved, glabrous, black-brown and continuous with the peridium surrounding the gleba, woody in texture, *Volva* deep and persistent, vaselike, 15–20 mm × 9–14 mm, woody; surface minutely rimose (use lens), black outside and inside; with a blunt base. *Context* of gleba gray (Met 8E 1–2); stipe context dull white, solid and firm; interior of volva also white, bruising reddish when fresh 0.4–1.2 mm thick, *Exoperidium* of tightly interwoven, thin-walled, hyphae 3.5–7.5 (–10) µm diam with gray-brown contents, occasionally branched in 3% KOH. *Mesoperidium* thick, tough, white, of interwoven hyphae 3–6 (–8) µm in diam, hyaline in 3% KOH. *Endoperidium* thin, sloughing off at apex when mature, hyphae (3.5–) 5–10 µm diam, slightly thickwalled (0.3–0.5 µm), gray-brown walls and contents in 3% KOH. *Surface of stalk* composed of parallel, slightly thick-walled hyphae, 4–9 (–12) µm wide, hyaline in 3% KOH. Mazaedium of abundant interwoven hyphae, 2–5 (–6) µm diam, seldom branched, thin-walled, flexuous, occasionally septate. *Asci* not seen, *Ascospores* globose, 7–9.5 µm diam (Em = 1.0: E = 1), with dense warts 0.8–1.2 µm high, forming a thick higher reticulum and a thin, uneven lower reticulum with thickened intersections which appear hollow (Fig. 3), light yellow-brown in 3% KOH.

Habit, habitat and distribution: Single to several on the root mat around the base of *Dicymbe corymbosa* trees; known only from the type locality in the Upper Ireng River Basin, and the adjacent Upper Potaro Basin, of Guyana.

Other specimens examined: **Guyana:** *Region* 8, *Potaro-Siparuni*: Pakaraima Mountains, Upper Ireng River, 3 km east of mouth of Sukabi River, toe slopes of Mt Kukuinang alt. 800 m, under *Dicymbe corymbosa*, 23 May 1998, *Henkel 6561* (BRG, DUKE); Upper Ireng River, 3 km east of mouth of Sukabi River, alt. 800 m, under *D. corymbosa*, 5 May 1998, *Henkel 6615* (BRG, DUKE, VPI); Upper Potaro River, south bank, 3 km upstream from Ayanganna airstrip, alt. 750 m, under *D. corymbosa*, 24 July 2000, *Henkel 7878* (BRG, DUKE).

Observations: *Pseudotulostoma volvata* is easily recognized in the field by the stalked ascomata 35–70 mm tall with an exposed apical gleba and woody volvate base (Figs 1–2), this

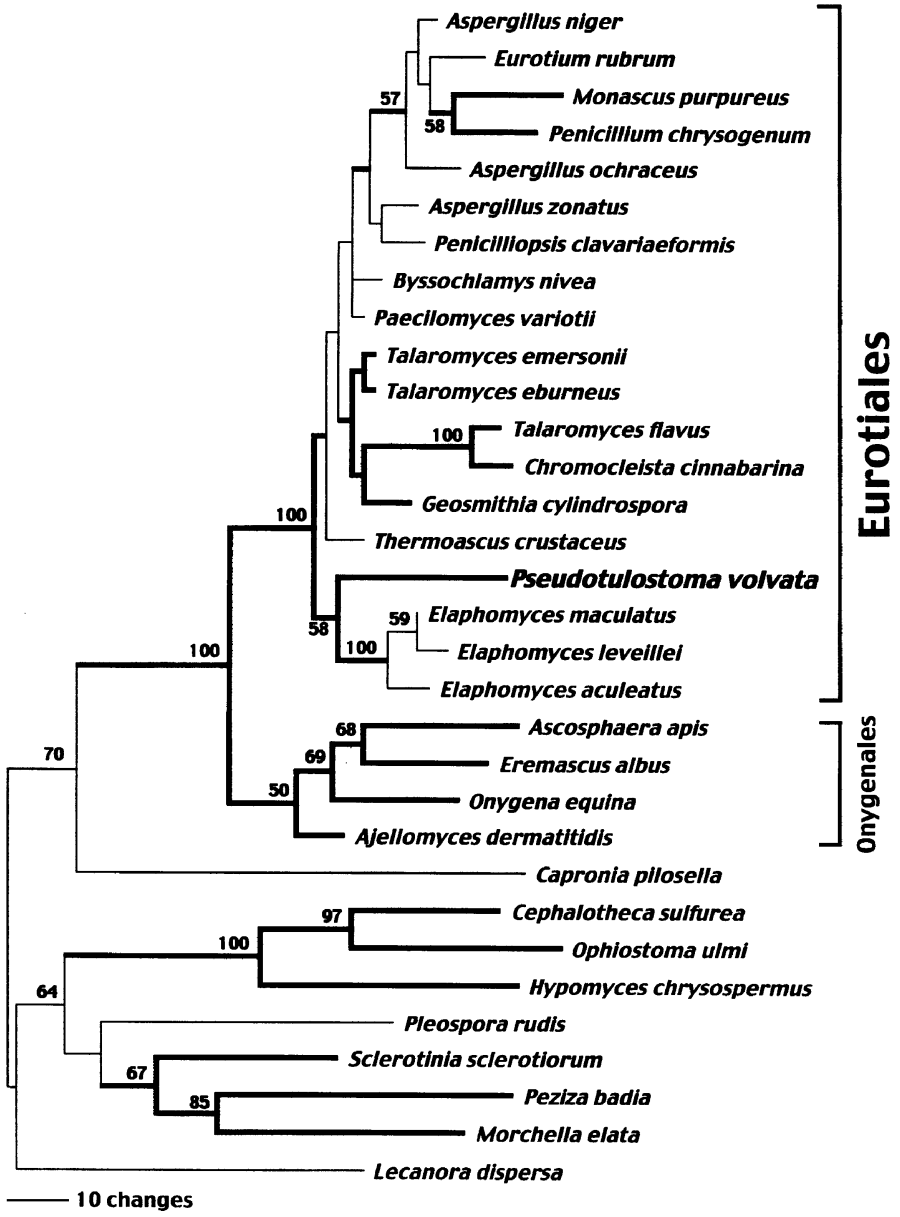


Fig. 6. Phylogenetic placement of *Pseudotulostoma volvata* among the filamentous ascomycetes using 18S rDNA sequences. Shown is one of 24 most parsimonious trees (length = 841; CI = 0.637). Branches in bold indicate those which are present in the strict consensus of the 24 trees. Numbers above nodes indicate percent bootstrap support.

gross morphology being suggestive of the basidiomycete genus *Tulostoma*. This suite of distinctive macromorphological features and size as seen in *P. volvata* has no counterpart in the *Ascomycota*. However, small fruiting bodies are known in the *Trichocomataceae* as treated by Malloch & Cain (1972), Benney & Kimbrough (1980) and illustrated by Kominami *et al*. (1952) also referred to the *Eurotiales*. The usual anamorphs of this family belong to *Penicillium*, *Aspergillus*, and *Paecilomyces*, and the taxa are saprobes or parasites. Based upon the currently available morphological and developmental information, *Pseudotulostoma* is best referred to the *Elaphomycetaceae* (Benny & Kimbrough 1980, Landvik *et al*. 1996). Phenotypic features shared between *P. volvata* and *Elaphomyces* spp. include a thick, tough gleba; thick-walled, ornamented spores (Figs 4–5) with similar ultrastructure at maturity; and the presence of abundant pseudocapillitium-like glebal hyphae (Fig. 3). The thick-walled double peridium in the unopened *Pseudotulostoma* ascomata has a strong resemblance to that found in *Elaphomyces* (Fig. 2).

The spore ornamentation of *Pseudotulostoma* has striking similarities to that found in species of *Elaphomyces*. The spores of *Pseudotulostoma*, as revealed by scanning electron microscopy (Fig. 5), have a distinctive episporium with deep, uneven reticulations and thickened protruding intersections which are hollow. A very similar morphology for *Elaphomyces* spores is illustrated by Samuelson *et al*. (1987). It can be noted in Figs 12 and 16 of Samuelson *et al*. (1987) that hollow intersections are formed by the ridges in both *Elaphomyces persoonia* and *E. granulatus*. The authors attributed this to a breakdown of perisporial ground material in mature ascospores. Under light microscopy, *Pseudotulostoma* spores appear partially reticulate with warts (Fig. 4).

Uecker & Burdsall (1977: Fig. 6) illustrated the spore

morphology of *Tuber spinoreticulatum* with a scanning electron micrograph. The reticulate-spored species of the *Tuberales* appear to have a very different ascospore ornamentation from that of either *Elaphomyces* or *Pseudotulostoma*.

The apparent restriction of *P. volvata* ascomata to stands dominated by ectomycorrhizal *D. corymbosa* trees (Henkel unpubl.), as well as its placement in the ectotrophic *Elaphomycetaceae*, suggests that *P. volvata* may be ectomycorrhizal in its nutritional habit. Studies are currently underway, using molecular and morphological methods, to determine the mycorrhizal status of *P. volvata*.

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REFERENCES

Benny, G. L. & Kimbrough, J. W. (1980) A synopsis of the orders and families of *Plectomycetes* with keys to the genera. *Mycotaxon* **12**: 1–91.

Berbee, M. L. & Taylor, J. W. (2000) Fungal molecular evolution: gene trees and geologic time. In *The Mycota. Vol. VIIB. Systematics and Evolution* (D. J. McLaughlin & E. McLaughlin, eds): 229–246. Springer-Verlag, Berlin.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Henkel, T. W. (1999) New taxa and distribution records for *Tylopilus* from *Dicymbe* forests of Guyana. *Mycologia* **91**: 655–665.

Henkel, T. W., Aime, M. C. & Miller, S. L. (2000) Systematics of pleurotoid *Russulaceae* from Guyana and Japan, with notes on their ectomycorrhizal status. *Mycologia* **92**: 1119–1132.

James, T. Y., Porter, D., Leander, C. A., Vilgalys, R. & Longcore, J. E. (2000) Molecular phylogenetics of the *Chytridiomycota* supports the utility of ultrastructural data in chytrid systematics. *Canadian Journal of Botany* **78**: 336–350.

Kominami, K., Kobayasi, Y. & Tubaki, K. (1952) Is *Trichocoma paradoxa* conspecific with *Penicillium luteum*? *Nagoa* **2**: 16–23.

Kornerup, A. & Wanscher, J. H. (1978) *Methuen Handbook of Color*. Eyre Methuen, London.

Landvik, S., Shailer, N. F. J. & Eriksson, O. E. (1996) SSU rDNA sequence support for a close relationship between the *Elaphomycetales* and the *Eurotiales* and *Onygenales*. *Mycoscience* **37**: 237–241.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. & Pace, N. R. (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences, USA* **82**: 6955–6959.

Malloch, D. & Cain, R. F. (1972) The *Trichocomataceae*: ascomycetes with *Aspergillus*, *Paecilomyces*, and *Penicillium* imperfect states. *Canadian Journal of Botany* **50**: 2613–2628.

Samuelson, D. A., Benny, G. L. & Kimbrough, J. W. (1987) Ultrastructure of ascopore ornamentation in *Elaphomyces* (Ascomycetes). *Mycologia* **79**: 571–577.

Swofford, D. L. (1998) *PAUP*. Phylogenetic Analysis Using Parsimony* (** and Other Methods*). Version 4. Sinauer Associates, Sunderland, MA.

Uecker, F. A. & Burdsall, H. H. (1977) *Tuber spinorectorum*, a new truffle from Maryland. *Mycologia* **69**: 626–630.

Vilgalys, R. & Hester, M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.

White, T. J., Burns, T. D., Lee, S. B. & Taylor, J. W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a guide to methods and applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 315–321. Academic Press, New York.

Zolan, M. E. & Pukkila, P. J. (1986) Inheritance of DNA methylation in *Coprinus cinereus*. *Molecular and Cellular Biology* **6**: 195–200.

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