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Two new ascomycetes with long gelatinous appendages collected from monocots in the tropics

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Abstract: Two new ascomycetes with long gelatinous appendages on brown 1- or 2-septate ascospores are described from monocots in the tropics. The new genus *Funiliomyces* with the single species *F. biseptatus* from a *Bromeliaceae* leaf from Brazil belongs to the *Amphisphaeriales,* according to morphology and phylogenetic reconstruction using LSU sequence data. It is characterised by the torpedo-shaped ascospores with two nearly central septa and one polar and one median appendage. The new species *Munkovalsaria appendiculata* from dead culms of *Zea mays* in Hong Kong is characterised by its long polar appendages. The genus *Munkovalsaria,* originally assigned to the *Dothideales,* clusters with high bootstrap support in the *Pleosporales,* and should consequently be assigned to that order. Both new species have a saprobic life style and are truly terrestrial. This is remarkable, because most ascomycetes with ascospores possessing long appendages occur in freshwater or in the marine environment.

Taxonomic novelties: *Funiliomyces biseptatus* Aptroot gen. et sp. nov., *Munkovalsaria appendiculata* Aptroot sp. nov.
Key words: *Amphisphaeriales,* ascomycetes, Brazil, *Bromeliaceae*, *Funiliomyces*, Hong Kong, ITS, LSU, monocots, *Munkovalsaria,* taxonomy, *Zea*

INTRODUCTION

Ascomycetes with extracellular, often gelatinous, appendages on the ascospores are mostly known from aquatic habitats. They are most abundant among marine fungi (Hyde & Pointing 2000), but also rather abundant among the freshwater aquatic fungi (Tsui & Hyde 2003). The appendages are usually thought to facilitate the movement of the ascospores in water. However, an increasing number of truly terrestrial ascomycetes with ascospores with appendages have recently been described, especially from palms (Aptroot 1995b, Hyde *et al.* 2000).

During recent collecting trips by the author to tropical countries, two undescribed ascomycetes with long gelatinous appendages on the ascospores were found on monocots. A collection from a *Bromeliaceae* leaf from Brazil is characterised by torpedo-shaped ascospores with two nearly central septa and one polar and one median appendage. It is described below as a new genus with uncertain affinities.

A new species of *Munkovalsaria* Aptroot was collected on dead culms of *Zea mays* L. in Hong Kong. It is characterised by long polar appendages. Both species were studied in detail because they show affinities with species of the genus *Didymosphaeria* Fuckel, which was revised by Aptroot (1995a) and found to contain only seven known species. Many of the over 550 taxa previously described in the genus were found to be synonyms of one of the more common saprobic species. However, a large proportion of the species was repositioned in other genera by Aptroot (1995b), including the new genus *Munkovalsaria* with two accepted species. Few species of *Didymosphaeria* have been described since the monograph by Aptroot (1995a, b). Only four of those, viz. *D. calamicola* Aptroot, J. Fröhl. & K.D. Hyde (Hyde *et al.* 1999), *D. berberidicola* (Rehm) Y.Z. Wang, A. Aptroot & K.D. Hyde (2004), *D. congruella* (P. Karst.) Y.Z. Wang, A. Aptroot & K.D. Hyde (2004), and *D. schizostachyi* (Rehm) Y.Z. Wang, A. Aptroot & K.D. Hyde (2004), fit the current generic concept.

MATERIAL AND METHODS

Isolates

Materials of the new species were collected in the field on dead plant materials, and subsequently air-dried. Cultures were made from the ascospores in the laboratory on oatmeal agar (OA) and 2 % malt extract agar (MEA) (Gams *et al.* 1998) and deposited in the CBS culture collection in Utrecht, the Netherlands. To increase changes of successful cultivation, cultures were subjected to different temperature conditions varying between 4 and 37 ºC under daylight conditions.

DNA isolation, amplification and phylogeny

The sequences of parts of LSU and ITS1 and ITS2 were obtained from the cultures following the standard method employed at CBS: Strains were trans-

ferred from agar cultures to 2 mL liquid medium (2 % malt extract) and incubated on a rotary shaker (300 rpm) for 3 wk at room temperature. Liquid cultures were transferred to 2 mL tubes, centrifuged and washed twice with sterile water. DNA was extracted using the FastDNA kit (Omnilabo 6050073, BIO 101, CA) according to the manufacturer's instructions. For ITS sequence analysis a part of the ribosomal RNA gene cluster was amplified by PCR using primer pairs V9G and LR5 (Vilgalys & Hester 1990). PCR was performed in 50 µL reaction volumes, each reaction containing 10–100 ng of genomic DNA, 25 pM of each primer, 40 µM dNTP, 1.0 unit Supertaq DNA polymerase and 5 µL 10× PCR buffer (SphaeroQ, Leiden, the Netherlands). PCR was performed in an Applied Biosystems (Foster City, CA) thermocycler with the following programme: 1 min 95 ºC, 30× (1 min 95 ºC, 1 min 55 ºC, 2 min 72 ºC), followed by a final extension of 5 min at 72 ºC. PCR products were cleaned with GFX columns (Amersham Pharmacia, NJ, 27-9602-01) and analyzed on a 2 % agarose gel to estimate concentrations. The primers ITS1 and ITS4 (White *et al*. 1990) and LR5 were used as internal sequencing primers for the ITS and LSU regions. The SSU region was sequenced using the PCR primers. Sequencing was performed with the BigDye terminator chemistry (Part number 403049, Applied Biosystems) following the manufacturer's instructions. The sequencing products were cleaned with G50 Superfine Sephadex columns (Amerham Pharmacia 17-0041-01), and separated and analyzed in ABI Prism 3700 DNA Analyzer (Applied Biosystems). Forward and reverse sequences were matched using SeqMan (DNAstar Inc., WI).

Parsimony analysis was performed on a selection of taxa representing the two new taxa, the closest relatives found by BLAST search in GenBank, and representatives of the major ascomycete lineages. The parsimony programme PAUP v. 4.0b10 was used. The heuristic search was performed with the following parameters: characters unordered with equal weight, *Peziza* L. as outgroup, alignment gaps were treated as fifth state, after deletion of the most variable regions of which the alignment was not unambiguous. Parsimony bootstrap analyses were performed using the full heuristic search option, and 1000 replicates, with maxtrees set at 100.

Sequences of the following taxa were used in the analyses (with GenBank accession numbers): *Amphisphaeria umbrina* (Fr.) De Not. AF452029, *Arecophila* K.D. Hyde sp. AF452039, *Bimuria novae-zelandiae* D. Hawksw. *et al.* AY016356, *Bipolaris papendorfii* (Aa) Alcorn AF163980, *Byssothecium circinans* Fuckel AY016357, *Capronia semiimmersa* Cand. AF050280, *Chaetomium globosum* Kunze : Fr. AF286403, *Cochliobolus hawaiiensis* Alcorn AF163979, *Didymella curcurbitacearum* A.J. Roy AY293792, *Dothidea sambuci* Pers. : Fr. AY544681, *Eurotium herbariorum* (Wigg. : Fr.) Link AY176751, *Fusarium lichenicola* (Speg.) Sacc. & Trotter AY097320, *Hyponectria buxi* (D.C.) Sacc. AY083834, *Letendraea helminthicola* (Berk. & Broome) E. Müll. & Arx AY016362, *Oxydothis frondicola* K.D. Hyde AY083835, *Peziza proteana* (Boud.) Seaver AY452029, and *Sordaria macrospora* Auersw. AY346301. Sequences generated for the new taxa were also deposited at GenBank, namely *Funiliomyces biseptatus* AY772015, and *Munkovalsaria appendiculata* AY772016.

Morphology

Hand sections were made through mature fruiting bodies on the natural substratum. Sections were observed in tap water and in IKI (10 %) and KOH (10 %). Morphological observations were made with an Olympus BX compound microscope. Illustrations were made from sections in tap water.

RESULTS

Phylogeny

Two equally most parsimonious cladograms were obtained from a phylogenetic analysis of the Large Subunit with characters unordered with equal weight, *Peziza* as outgroup, alignment gaps treated as fifth state, after deletion of the most variable regions of which the alignment was ambiguous. Parsimony bootstrap values are added on branches with over 50 % support.

The phylogenetic tree leaves no doubt that the new genus *Funiliomyces* belongs to the *Amphisphaeriaceae,* which is supported by a bootstrap value of 85 %. The two most parsimonious trees differ only in topology inside the *Amphisphaeriales*. The new genus *Funiliomyces* is therefore not assigned to any family within the *Amphisphaeriales*.

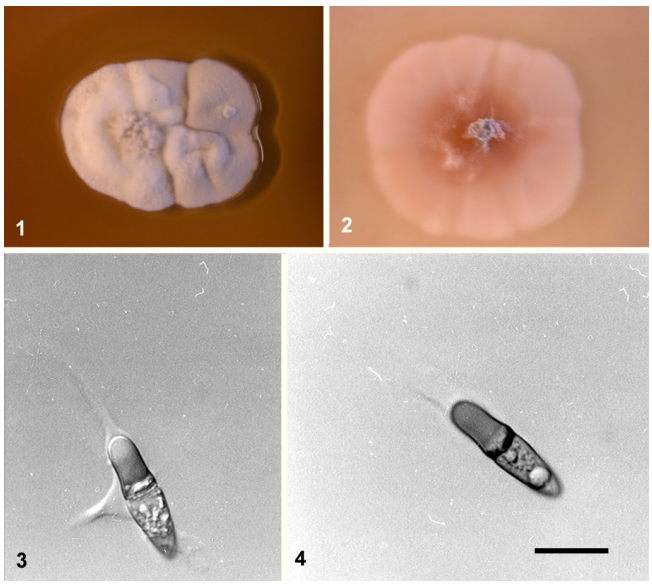
The new species of the genus *Munkovalsaria* appears within the *Pleosporales* clade, with a bootstrap value of 85 %. The whole *Pleosporales* clade is also well-supported, with a bootstrap value of 69 %. The genus *Munkovalsaria* was originally thought to belong to the *Dothideales* (Aptroot 1995b) and is currently classified in the *Dothideales* and *Chaetothyriales incertae sedis* (Eriksson *et al.* 2004).

The remaining genera in the phylogenetic reconstruction are representatives of the remaining main (sub)orders in the subphylum *Pezizomycotina*.

Morphology

Funiliomyces biseptatus Aptroot, **gen. et sp. nov.** MycoBank MB500077 (gen.), MB500164 (sp.). Figs 1–4.

Fungus ascomycetum Amphisphaerialium, ascosporis fuscis, inaequaliter biseptatis, in parte media et basilari biappendiculatis, hyalinis.



Figs 1–4. *Funiliomyces biseptatus.* 1, 2. Culture on MEA after 3 wk at 20 ºC (surface and reverse). 3, 4. Ascospores. Scale bars: 1, 2 = 1 mm; 3, 4 = 10 µm.

Ascomata black, nearly globose, immersed to erumpent, *ca.* 0.1–0.2 mm diam.; wall consisting of irregular layers of regularly melanized, flattened cells, unchanged in KOH. *Asci* cylindrical, tip thickend with a central refractive, IKI-negative apical apparatus, 8-spored. *Physes* absent, but asci surrounded by a parenchymatous tissue with *ca.* 10 µm wide cells. *Ascospores* (Figs 3, 4), pale brown, torpedo-shaped, 2-septate with both septa in close proximity near the middle of the ascospore, 12–15 × 4–5 µm, upper cell pointed, lower cell conspicuously rounded, upper two cells characteristically with numerous hyaline granules or oil droplets, with two hyaline mucilaginous appendages of 15–27 × 2–4 (base up to 10) µm, one at the lower pole, and one median, sticking out sideways.

Type: **Brazil**, Minas Gerais, Catas Altas, Serro do Caraça, Parque Natural do Caraça, near Funil, 1 km NW of monastery Santuário do Caraça, 20º 06' S, 43º 29' W, on dead leaf of *Bromeliaceae* in rock field, 18 Sept. 1997, A. Aptroot, **holotype** herb. CBS H-10505, **isotypes** herb. SP, living culture ex-type CBS 100373, also dried culture CBS H-10506.

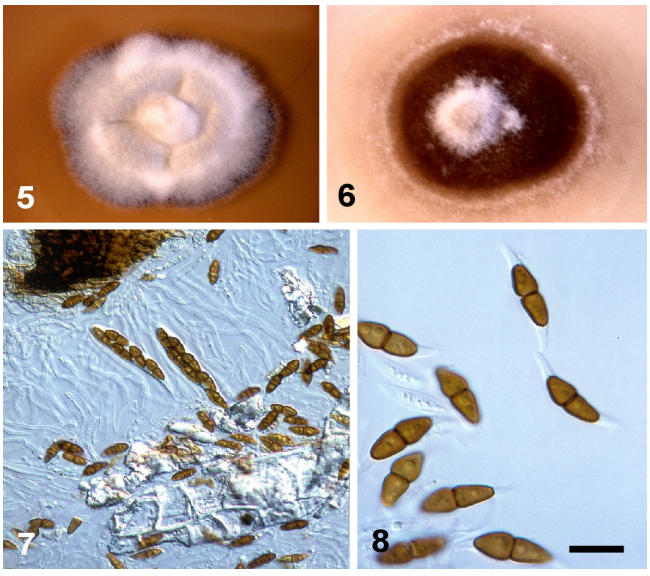
Cultural characteristics: Cultures slow growing, attaining a diameter of 4 cm after 3 wk at 20 ºC on OA or MEA (Figs 1, 2). Growth was obtained between 4 ºC and 24 ºC. Aerial mycelium dense, cottony, creamy white. Reverse mottled pale brownish with darker reddish brown sectors and paler radiate streaks.

Notes: The new genus belongs probably to the *Amphisphaeriales* because of the ascus type, but no known genus seems to fit this species. It is strongly characterised by the torpedo-shaped ascospores with two nearly central septa and one polar and one median appendage.

Munkovalsaria appendiculata Aptroot, **sp. nov.** MycoBank MB500078. Figs 5–8.

Munkovalsaria ascosporis fuscis, uniseptatis, biappendiculatis, hyalinis, 4–7 µm longis.

Ascomata black, nearly globose or elongated due to interaction with the substratum, immersed to erumpent, *ca.* 0.1–0.2 mm diam; wall consisting of two layers of regularly melanized, flattened cells, unchanged in KOH. *Asci* (Fig. 8) cylindrical to clavate, tip thickend with central ocular chamber, IKI-negative, 8-spored. *Pseudoparaphyses* septate, little branched, 1.5–2.5 µm wide. *Ascospores* (Figs 7, 8), reddish brown, broadly fusiform, 1-septate, 12–15 × 4–5 µm, upper cell wider and shorter than lower cell, both cells with 2–5 greenish oil droplets, both ends rather pointed, with two polar hyaline appendages of 4–7 × 1.5–2.5 µm.



Figs 5–8. *Munkovalsaria appendiculata.* 5, 6. Culture on MEA after 3 wk at 20 ºC (surface and reverse). 7. Asci with ascospores and hamathecium. 8. Ascospores. Scale bars: 5, 6 = 1 mm; 7, 8 = 10 µm.

Type: **Hong Kong**, Lantau Island, Cheung Sha Hang near Discovery Bay, 22º 16' N, 114º 00' E, on dead culms of *Zea mays* in secondary forest, 7 Jul. 2000, A. Aptroot, **holotype** herb. CBS H-10503, **isotypes** herb. HKU(M), living culture ex-type CBS 109027, also dried culture CBS H-10504.

Cultural characteristics: Cultures relatively slow growing, attaining 5 cm diam after 3 wk at 20 ºC on OA or MEA (Figs 5, 6). Growth was obtained between 4 ºC and 24 ºC. Aerial mycelium rather loose, cottony, off-white. Reverse mottled, pale brownish, with darker reddish brown concentric bands.

Notes: The new species belongs undoubtedly to the genus *Munkovalsaria* (Aptroot 1995b), but is immediately characterised by the brown, 1-septate ascospores with long hyaline appendages. These appendages were observed to form already when the ascospores are still immature (and hyaline). This firmly excludes a possible confusion with germ tubes. *Munkovalsaria appendiculata* is close to *Phaeodothis winteri* (Niessl) Aptroot and *Montagnula opulenta* (De Not.) Aptroot, two taxa with brown, 1-septate ascospores that were excluded from the genus *Didymosphaeria* by Aptroot (1995b).

DISCUSSION

Most ascomycetes with ascospores with long appendages are aquatic or even marine. Surprisingly, the two species described above are truly terrestrial. They are also among the first to be described with the combination of brown ascospores and hyaline appendages: most appendaged ascomycetes have entirely hyaline ascospores.

The partial (approx. 850 bp) sequences of the LSU 28S rDNA confirmed the taxonomic uniqueness of the new taxa, as no close relatives were found. A phylogenetic analysis (Fig. 9) provided additional suggestions as to their phylogenetic position. Unfortunately, no LSU sequences are available from the other species of *Munkovalsaria,* but it clusters with high bootstrap support in the *Pleosporales. Funiliomyces biseptatus* shows only up to 94 % similarity with some anamorphs in the *Amphisphaeriales*. Various parsimony analyses were performed, which yielded mostly identical results. Most branches were supported by high bootstrap values, but the ordering within the *Amphisphaeriales* remains unresolved.

The ITS sequences of the two new species described above were vastly different from any fungus known to date. Therefore no conclusions could be drawn from them. The obvious reason is that thus far too few related ascomycetes have been studied in culture, and subjected to DNA sequence analysis.

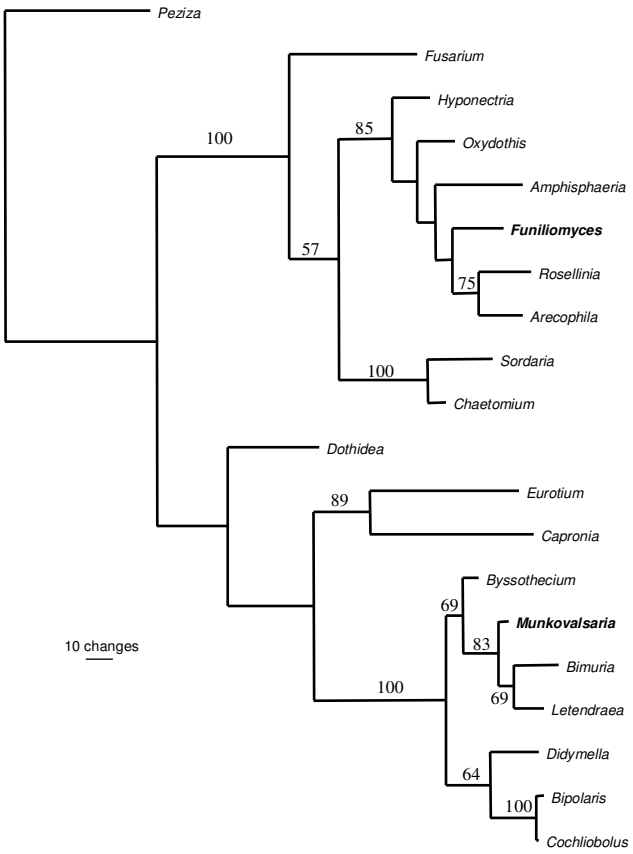


Fig. 9. One of two equally most parsimonious cladograms from phylogenetic analysis with characters unordered with equal weight, *Peziza* as outgroup, alignment gaps treated as fifth state, after deletion of the most variable regions of which the alignment was ambiguous. Parsimony bootstrap values are added on branches with over 50 % support. Tree statistics: CI = 0.586, RI = 0.707, RC = 0.414, HI = 0.414.

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