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A new species of *Microthyrium* from Yunnan, China

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Abstract

A new species, *Microthyrium propagulensis,* collected in Yunnan Province, southwestern China is introduced. The species is typical of Microthyriaceae (Microthyriales) in having superficial thyriothecia with a poorly developed basal layer and a prominent darker central ostiole, bitunicate asci and 1-septate ascospores. It is similar to the generic type, *M. microscopicum*, but differs in having relatively small ascospores, with two apical cilia, which lie downloads from the ascospore apex at a 45° angle. Phylogenetic analysis based on combined LSU and SSU gene sequence data clearly place this species in *Microthyrium*, but distinguishes it from *Microthyrium microscopicum*. This second sequence from a *Microthyrium* species indicates that the putative sequence of the type of this genus is CBS has been correctly named and supports the distinctiveness of Microthyriales and Microthyriaceae.

Key words: Microthyriaceae, phylogeny, taxonomy

Introduction

Microthyrium has been poorly studied, although species of the genus are common in China (Wu *et al*. 2011a). The important morphological characters of *Microthyrium* are superficial thyriothecia, with a poorly developed basal layer, a prominent darker central ostiole, bitunicate asci and 1-septate ascospores, usually with fine appendages.

Microthyrium is the type genus of Microthyriaceae in Microthyriales. Wu *et al.* (2010, 2011a,b,c, 2013) reappraised the family Microthyriaceae and Hyde *et al.* (2013) treated Microthyriaceae and Micropeltidaceae as separate families in Microthyriales. However, there are no reliable sequences in GenBank and it is not sure that the one sequenced culture of the generic type, *Microthyrium microscopicum*, from CBS in GenBank is correctly named. Sequences from this strain have been used in several higher level phylogenetic studies, but confirmation that the strain is correctly named would be reassuring.

In this paper, we collected a new species of *Microthyrium* from Yunnan Province, which is introduced as *M. propagulensis*. The new species is described and illustrated and compared with similar *Microthyrium* species. We also sequenced the SSU and LSU genes of this taxon and provide welcome verified sequence data for a species of Microthyriaceae/Microthyriales. A phylogenetic tree is presented to support previous data on the placement and relationships of Microthyriaceae/Microthyriales with other members of Dothideomycetes.

Materials and methods

Morphology

The new species was collected from Gaoligong Mountain, Bao Shan County, Yunnan Province, China. Leaf samples were placed in paper bags and returned to the laboratory for further study. Microscopic study and

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sectioning follow the method outlined in Wu *et al*. (2011a) and used Leica MZ16A and KEYENCEVHX-1000 stereomicroscopes, Nikon E800 and 80i compound microscopes and a Leica CM1100 freezing microtome. Although we tried several times to obtain fresh cultures from this collection following the single spore isolation method detailed in Chomnunti *et al*. (2011), ascospores did not germinate. Herbarium material is deposited in IFRD (International Fungal Research & Development Centre Research Institute of Resource Insects, Kunming).

DNA sequencing alignment and phylogenetic analyses

Total genomic DNA was extracted from fresh collections (ascomata) using a modified protocol of E.Z.N.A. Forensic DNA Kit (www.omegabiotek.com). The two markers (SSU and LSU rDNA) were used in this study. Primer pairs NS1 and NS4 (White *et al*. 1990) were used to amplify a region spanning the SSU rDNA gene; LROR and LR5 primer pairs (Vilgalys & Hester 1990) were used to amplify a segment of the LSU rDNA gene. DNA amplification was performed by PCR (polymerase chain reaction). The 28S (LSU) and 18S (SSU) gene data were combined in the analysis. Sequences were downloaded from GenBank using data from the publications of Schoch *et al*. (2009), Hofmann *et al*. (2010), Wu *et al*. (2011a), Hyde *et al*. (2013) and aligned using MUSCLE (Edgar 2004) and CLUSTALX (v. 1.83) (Thompson *et al*. 1997) and then manually refined and adjusted using BioEdit (Hall 1999). MrModeltest v. 2.2 (Nylander 2004) was used to estimate the best-fitting model for the combined LSU and SSU genes. Maximum parsimony (MP) analyses were performed using PAUP v. 4.0b10 (Swofford 2002); using the heuristic search option with 1,000 random sequence additions and TRB as branch swapping algorithm. All characters were unordered and of equal weight and gaps were treated as missing data. Maxtrees were set up to 5,000, branches of zero length were collapsed and all multiple parsimonious trees were saved. Tree length [TL], consistency index [CI], retention index [RI], relative consistency index [RC] and homoplasy index [HI] were determined. Clade stability was assessed using bootstrap (BT) analysis with 1000 replicates, each with ten replicates of random stepwise addition of taxa (Hillis & Bull 1993). Bayesian analyses were performed by using MrBayes v. 3.0b4 (Ronquist & Huelsenbeck 2003). Posterior probabilities (PP) were implemented with Markov Chain Monte Carlo sampling (MCMC) (Rannala & Yang 1996, Zhaxybayeva & Gogarten 2002). Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100 generation, in total saved 10,000 trees. The first 2,000 trees submitted to the burn-in phase and were discarded; the remaining 8,000 trees were used for calculating posterior probabilities (PP) in the majority rule consensus tree (Cai *et al*. 2006, 2008, Liu *et al*. 2012). Maximum likelihood analysis was performed in RAxML (Stamatakis 2006) by using raxmlGUIv.0.9b2 (Silvestro & Michalak 2010). The search strategy was set to rapid bootstrapping and the analysis implemented in the GTR (general time reversible) model of nucleotide substitution. The best scoring RAxML tree had a final value of -12401.299512.

Results

Phylogenetic analysis

The combined 18S (SSU) and 28S (LSU) nrDNA consists of 31 taxa, with *Schismatomma decolorans* as the outgroup taxon. The maximum parsimony dataset comprised 2580 characters, including 1687 constant characters, 496 variable parsimony-uninformative characters and 397 parsimony-informative characters. Kishino-Hasegawa (KH) test showed length= 1876 steps, CI= 0.638, RI= 0.690, RC= 0.440 and HI= 0.362. A heuristic search with random addition of taxa (1000 replicates) and treating gaps as missing characters generated six equally parsimonious trees. All trees were similar in topology and not significantly different (data not shown). The first of 1,000 equally most parsimonious trees is shown (Fig. 1). Bootstrap support (BS) values of MP and ML (equal to or above 50% based on 1,000 replicates) are shown on the upper branches. Values of the Bayesian posterior probabilities (PP) (equal to or above 90% based on 1,000 replicates) are shown under the branches.

In the phylogenetic tree, *Trichothyrium peristommalis* is the only representative in Trichothyriaceae, and clusters alone in a clade with low bootstrap support (BS). Description and illustration of *T. peristommalis*, with the appropriate placement of the family Trichothyriaceae will be provided in a later paper.

Microthyrium propagulensis sp. nov., clustered in the family Microthyriaceae with *M. microscopicum* (the generic type) in a clade with high bootstrap value (100%, 100% BS and 1.00 PP) (Fig. 1). The Microthyriales forms a sister order to Venturiales.

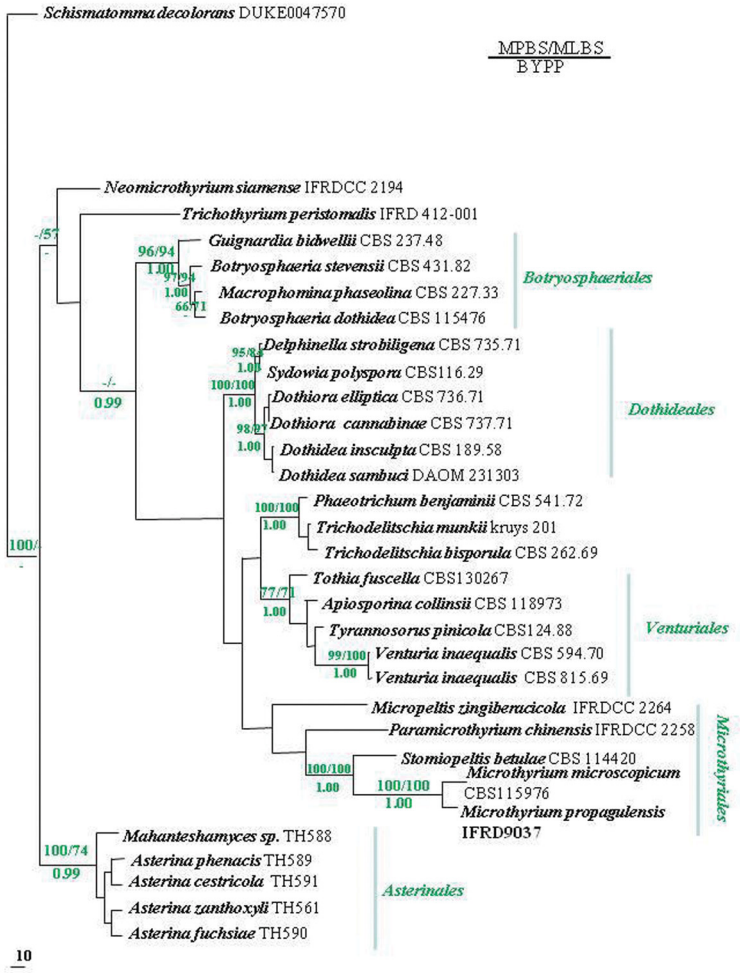

FIGURE 1. Topology showing the most parsimonious tree obtained from a heuristic search with 1,000 random taxon additions of the combined dataset of SSU and LSU sequences alignment using PAUP v. 4.0b10. The scale bar shows 10 changes. Bootstrap support values for maximum parsimony (MP) and maximum likelihood (ML) greater than 50% above the nodes. The values below the nodes are Bayesian posterior probabilities above 0.95. Hyphen ("-") indicates a value lower than 50% (BS) or 0.90 (PP). The original isolate numbers are noted after the species names. The tree is rooted to *Schismatomma decolorans*.

Taxonomy

The new taxon *Microthyrium propagulensis* is well differentiated from *Microthyrium microscopicum* based on molecular phylogeny and morphology, as discussed below.

Microthyrium propagulensis H.X. Wu & K.D. Hyde, *sp. nov.* MycoBank: MB 805473 (Fig. 2)

Type:—CHINA. Yunnan Province: Mount Gaoligong, on decaying leaves of *Castanopsis histrix* Miq. (*Fagaceae*) 18 August 2011, *Y.M. Li* (IFRD 9037, holotype).

Etymology:—from the Latin *propagulum,* in reference to the appearance of the ascospores as propagules for dispersal.

Differs from *M. microscopicum* based on saprobic on decaying leaves. Mycelium superficial, abundant, almost colourless. Sexual state: Thyriothecia, on the upper and lower of leaves, orbicular, up to 270 µm diam, solitary or often gregarious, superficial, membranaceous, brown to light brown, with a poorly developed basal layer, with a prominent darker central ostiole, in section lenticular. Upper wall comprising cells of *textura epidermoidea*, radiating outwards in parallel rows from the ostiole. Hamathecium comprising asci inclined from the base and rim

towards the central, pseudoparaphyses absent, possibly deliquescing early. *Asci* 41–56 × 5–8 µm ($\overline{x}$ = 49.8 × 6.7 µm, n = 15), 8-spored, numerous, bitunicate, clavate to cylindrical, with a 3–4 × 2–3 µm pedicel. Ascospores 10–11.5 × 3–4 µm ($\overline{x}$ = 10.9 × 3.4 µm, n= 15), 2–3-seriate, ellipsoidal to ovate, with rounded ends, hyaline, 1-septate, septum in lower part, not constricted at the septum, upper cell larger with 2 distinct guttules, apex with 2 cilia, which lie downloads at an 45° angle to the ascospore, cilia 5.4–7.3 µm long. Asexual state: Unknown.

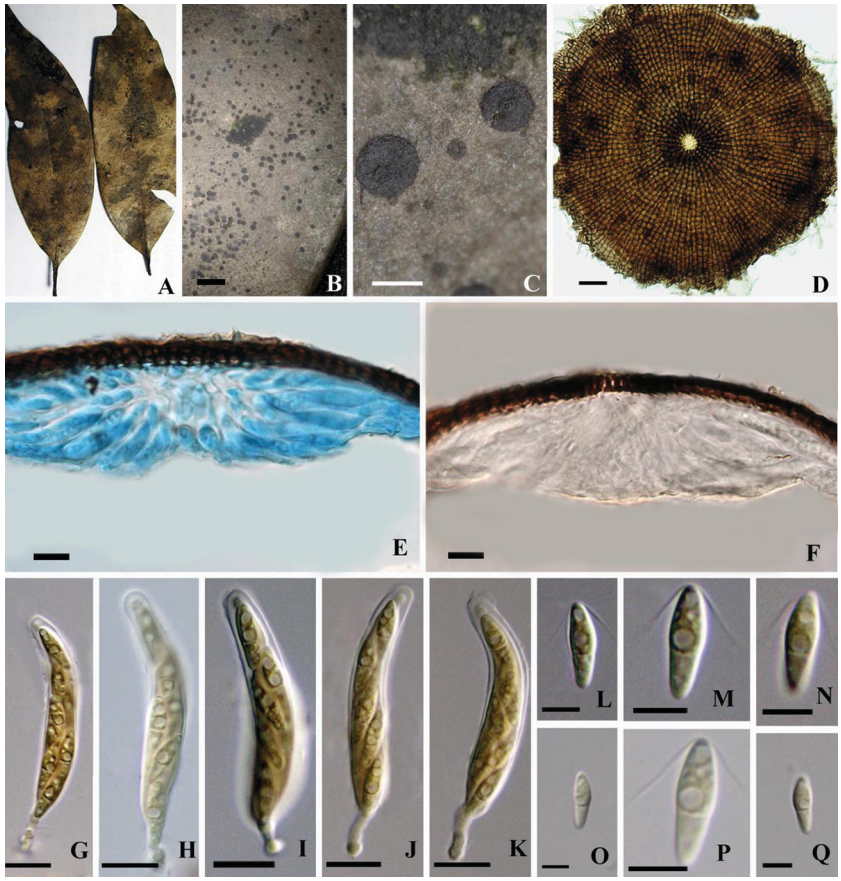

FIGURE 2. *Microthyrium propagulensis* (holotype) on leaf. A–C. Superficial thyriothecia on host leaves. D. Thyriothecium with ostiole. E, F. Section through the thyriothecium with thin peridium. G–K. Bitunicate asci. L–Q. Ascospores with apical appendages. Scale bars: B = 500 µm, C = 200 µm, D–F = 20 µm, G–K = 10 µm, L–Q = 5 µm.

Discussion

Microthyrium propagulensis is similar to *M. microscopicum*, but has smaller ascospores (10–11.5 × 3–4 µm ($\overline{x}$ = 10.9 × 3.4 µm, n = 15) versus 12–16 × 2–3 µm ($\overline{x}$ = 14.4 × 2.5 µm, n = 20) (Wu *et al.* 2011a). The shape of ascospores can also distinguish the taxa. *M. propagulensis* has ellipsoidal to ovate ascospores with rounded ends while *M. microscopicum* has fusiform to ellipsoidal ascospores. The ascospore cilia can also be used to distinguish the species. *Microthyrium propagulensis* with two equal length apical cilia, while *M. microscopicum* has apical or subapical tufts of 2–4 cilia. *Microthyrium propagulensis* should also be compared with the British *Microthyrium* species detailed in Ellis (1976). *Microthyrium propagulensis* (ascospores ellipsoidal to ovate, 10–11.5 × 3–4 µm) is most similar to *M. versicolor* (Desm.) Höhnel (1910: 453) (ascospores slipper-shaped, about 11–13.5 × 3.5–4.5 µm); but can be distinguished as *M. versicolor* has pseudoparaphyses and the cilia are often lost. During investigations of Microthyriaceae in southwestern China, the genus *Paramicrothyrium* was introduced by Wu & Hyde (2011a). The type species, *Paramicrothyrium chinensis*, has cylindrical to cylindro-clavate asci and ascospores in a uniseriate arrangement, with cilia at the ends. *Paramicrothyrium chinensis* is very different from *Microthyrium propagulensis* and the phylogenetic tree also confirmed this viewpoint.

Microthyriales is a well supported order, which includes two families (Microthyriaceae and Micropeltidaceae). Hyde *et al*. (2013) accepted this order and families based on morphological data and some phylogenetic support. Members of Trichothyriaceae are parasitic on other fungi and have a well-developed lower wall. These form a separate clade without close relatives and should probably be introduced as a new order. Hyde *et al*. (2013) considered Trichothyriaceae as a good family, and in this paper we confirm Trichothyriaceae is new family by molecular data. *M. propagulensis* clearly belongs to the genus *Microthyrium* with high bootstrap supports (100% MPBS, 100% MLBS, 1.00 BYPP, respectively); *M. propagulensis* and *M. microscopicum* are distinct based on the molecular data.

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