Article

Phylogenetic analyses of new aquatic hyphomycetes provide molecular evidence for *Microthyriaceae* (Dothideomycetes, Ascomycota) anamorph

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Abstract: The fungal family *Microthyriaceae* is represented by relatively few mycelial cultures and DNA sequences. As a result, the taxonomy and classification of this group of organisms remain poorly understood. Here, based on DNA sequences at four gene fragments (nu*LSU* rDNA, nu*SSU* rDNA, *TEF*1 and *RPB*2) in our analyses of aquatic hyphomycetes from southern China, we identify and report four new genera (*Antidactylaria*, *Isthmomyces*, *Keqinzhangia*, *Pseudocoronospora*) and thirteen new species (*Antidactylaria minifimbriata*, *Pseudocoronospora hainanensis*, *Isthmomyces oxysporus*, *I. dissimilis, I. macrosporus, I. relanceatus, Keqinzhangia aquatica, Triscelophorus anakonajensis, T. anisopterioides, T. guizhouensis, T. mugecuoensis, T. multibrachiatus, T. neoseptatus;* new combinations *Isthmomyces asymmetrica, I. basitruncata, I. geniculata, I. lanceata, I. minima, I. rotundata*) belonging to *Microthyriaceae*. Our results provided the first molecular evidence of asexual morph of this family and strengthened the phylogenetic placement of the family in class Dothideomycetes. The addition of these new taxa made *Microthyriaceae* the largest family comprising freshwater asexual genera in Pleosporomycetidae. In addition, we confirmed the monophyly of the genus *Triscelophorus*, the paraphyly of the genus *Isthmolongispora*, and revised 6 new combinations in *Isthmolongispora*. ITS barcoding of 13 species were also provided to help identify aquatic hyphomycetes in the future. Our results suggest that the asexual genera and sexual genera identified so far within this family have completely different ecological niches.

Keywords: *Microthyriaceae*; aquatic hyphomycetes; asexual genera; phylogeny

1. Introduction

The family *Microthyriaceae* (Microthyriales, Dothideomycetes) was established by Saccardo [1], containing foliar epiphytes and saprobes on dead leaves and stems [2]. This family is characterized by having superficial, flattened thyriothecia, with cells of the upper wall radiating in a parallel arrangement from the central opening; the opening may or may not be surrounded by setae. Asci are fusiform or obclavate to cylindroclavate, bitunicate, fissitunicate, and ascospores are two-celled, hyaline to brown often with ciliate appendages [2-4]. Asthton et al. [3] estimated that there were 54 genera and 278 species in the family. In a subsequent series of papers, Wu et al. [2, 5-8] revised *Microthyriaceae* by examining the generic type species, and restricted *Microthyriaceae* to the species with morphological characteristics similar to *Microthyrium* Desm.. Based on morphological characteristics, 11 genera and about 230 species were proposed [9], but in a subsequent outline of Ascomycota, only 9 genera were listed in this family [10].

Microthyriaceae have been poorly studied, there are very few DNA sequences in public databases for this group of fungi. In the expanded multigene phylogeny of the Dothideomycetes, *Microthyriaceae* was not included because of the paucity of DNA sequence [11]. In the class-wide phylogenetic assessment of Dothideomycetes, Schoch et al. [12] included *Microthyriaceae* based on one strain of *Microthyrium microscopicum* Desm. (the type species of *Microthyriaceae*). So far, among the accepted 9 genera of the family, sequences of only five

species (out of more than 200 species) are available from public databases, representing *Chaetothyriothecium* Hongsannan & K.D. Hyde, *Microthyrium* Desm., *Palawania* Syd. & P. Syd., and *Tumidispora* Hongsannan & K.D. Hyde. One major contributing reason for the absence of DNA sequences is that few living cultures are available. As a result, researchers might have assumed that many of these species were obligate parasites and could not be cultured [2]. Later, Hongsannan et al.[13] isolated cultures of *Chaetothyriothecium elegans* Hongsanan & K.D. Hyde and *Tumidispora shoreae* Hongsanan & K.D. Hyde [14], but failed to observe anamorphs of the two species. Wu et al. [7] tried to isolate fresh cultures from *Microthyrium* propagulensis H.X. Wu & K.D. Hyde, but did not observe the germination of ascospores. Based on these situation, asexual genera of *Microthyriaceae* were recorded only from the literature. Before Wu revised *Microthyriaceae*, *Asterostomula* Theiss. and seven other genera were listed as asexual [15, 16]. With the exclusion of many genera from *Microthyriaceae* [2, 5, 6, 8], only *Hansfordiella* S. Hughes was retained as an asexual genus in *Microthyriaceae* [10], but this connection was not confirmed by molecular data because sequences of *Hansfordiella* were unavailable. Moreover, Hansfordiella was recorded as asexual state of Trichothyrium Speg., which belongs to *Trichothyriaceae* [3, 4, 15-17]. So strictly speaking, no asexual genus has been reported within the modern circumscription of *Microthyriaceae*.

In the early 1990s, molecular methods, in particular DNA sequence data, provided opportunities for phylogenetic inference, and have made a significant impact on the taxonomy and classification of fungi [18]. More importantly, sequence analysis can potentially place an asexual-state taxon within an order or even link it with a teleomorph genus without having to observe the latter (e.g., in [19]). The linkages between asexual and sexual genera have been accumulated during implementation of the "One fungus: One name" concept, allowing the asexual genera to be placed in a natural biological framework of fungi [9, 10, 20]. However, the phylogenetic position of about 1530 genera in Ascomycota still remain *incertae sedis* [10].

Aquatic hyphomycetes colonize allochthonous organic matter in fresh waters and are closely involved in the decomposition and conversion of biopolymers in aquatic habitats [21]. They are a polyphyletic group of fungi, mainly consisting of asexual morphs of Ascomycota and Basidiomycota, which have been identified based on conidium morphology and conidiogenesis [22]. Molecular approaches applied to phylogeny of aquatic hyphomycetes place some genera in a defined class and found multiple origins of aquatic hyphomycetes. Specifically, 7 strains (5 species) of *Tetracladium* De Wild. showed close relationships to the Ascomycete orders Onygenales, Erysiphales and Leotiales [23], but subsequently, *Tetracladium* was placed in Leotiomycetes based on combined ITS and 28S analyses [24]. Studies of 31 species of aquatic hyphomycetes placed the majority (74 %) within the Leotiomycetes [22, 25]. Duarte et al. [26] constructed and ITS phylogenetic tree for 79 aquatic hyphomycetes, and found *Tricladium* Ingold and *Triscelophorus* Ingold, are not monophyletic. Of course, with the availability of more and more reference sequences and the establishment of backbone trees of some classes, new aquatic hyphomycetes have been published with confirmed phylogenetic positions [10, 27-30]. Although these studies promoted phylogenetic development of aquatic hyphomycetes, the phylogenetic positions of most aquatic hyphomycetes have not been determined at the family level [10].

In recent years, we have studied the diversity and phylogeny of aquatic hyphomycetes from southern China in Yunnan, Sichuan, Guizhou and Hainan Provinces, a hot spot of world biodiversity. Previously we have reported some new species from these regions [29, 31-48]. During this process, we found some isolates similar to those in *Microthyriaceae*. After studying in detail, we described and illustrated these new taxa, placed them in *Microthyriaceae* based on analyses of four gene regions: nuclear large subunit (nu*LSU* rDNA), nuclear small subunit (nu*SSU* rDNA), translational elongation factor 1α (*TEF*1), and RNA polymerase II subunit 2 (*RPB*2), and discussed difference between each new taxon and its most similar genera or species. In addition, the internal transcribed spacers including the 5.8s subunit rDNA (ITS) were provided for each of the new species as DNA barcodes.

2. Materials and Methods

2.1 Collection of fresh samples, fungal isolation and characterization

Submerged dicotyledonous leaves were collected from streams in Yunnan, Guizhou, Sichuan, Hainan Provinces and Tibet. Samples were preserved in zip-locked plastic bags, labeled and transported to the laboratory. Each rotted leaf was cut into several 3–4 × 4–5 cm sized fragments, which were incubated on CMA (20 g cornmeal, 18 g agar, 40 mg streptomycin, 30 mg ampicillin, 1000 ml distilled water) for 5 days at room temperature. Conidia were isolated using a sterilized toothpick under a BX51 microscope and cultivated on

CMA plates. Morphological characteristics were observed from cultures growing on CMA after incubation at 25°C for a week. Pure cultures have been deposited in the Herbarium of the Laboratory for Conservation and Utilization of Bio-Resources, Yunnan University, Kunming, Yunnan, P.R. China (YMF, formerly Key Laboratory of Industrial Microbiology and Fermentation Technology of Yunnan). Ex-holotype living cultures were deposited in the China General Microbiological Culture Collection Center (CGMCC).

2.2 DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from fresh mycelia grown on potato dextrose agar (PDA) at 25°C as described by Turner et al. [49]. The air-dried precipitate was dissolved in 50 µl of sterilized distilled water and stored at -20°C until use for amplification reactions. The primer pairs NS1/NS4, LROR/LR7 [50], EF1-728F [51] and TEF1LLErev [52], and tRPB2-5F and tRPB2-7R [53] were, respectively, used for the amplification of the small subunit nuclear ribosomal RNA gene (*SSU* rRNA), the large subunit nuclear ribosomal RNA gene (*LSU* rRNA), translation elongation factor 1 alpha gene (*TEF*1), and the second largest subunit of the DNA-directed RNA polymerase II (*RPB*2). The PCR thermal cycle programs for the amplifications of these three DNA fragments followed those described in Su et al. [30]. PCR products were visualized on 1% agarose gel stained with Goldview (Geneshun Biotech, China) with D2000 DNA ladder (Realtimes Biotech, Beijing, China) and were then purified using a commercial Kit (Bioteke Biotechnology Co., Ltd., China). DNA forward and reverse sequencing was performed with a LI-COR 4000L automatic sequencer, using a Thermo Sequenase-kit as described by Kindermann et al. [54].

2.3 Sequence alignment and phylogenetic analysis

Preliminary BLAST searches with the *LSU* gene sequences of the new isolates against GenBank nucleotide databases determined species closely related to our isolates. Based on this information, sequences at the four marker loci were downloaded from *Microthyriaceae* and five sister families belonging to Dothideomycetes, including 44 strains representing 30 species (Supplementary Table 1). *Schismatomma decolorans* (Erichsen) Clauzade & Vězda was used as outgroup. For phylogenetic analyses of *Isthmolongispora*, *LSU* sequences of 32 strains representing 16 species of 7 genera, belonging to three families *Hyaloscyphaceae*, *Mollisiaceae* and *Loramycetaceae* were downloaded following BLAST searching of *LSU*. *Endocronartium harknessii* (J.P. Moore) Y. Hirats. belonging to *Cronartiaceae* was used as outgroup (Supplementary Table 2).

For *Microthyriaceae* the sequences of these representative strains were combined with the ones from our own cultures (Table 1). Four alignment files were generated, one for each gene, and there were then converted to NEXUS files with ClustalX 1.83 [55]. The four aligned were then concatenated with BioEdit 7.1.9.0 [56]. All characters were weighted equally and gaps were treated as missing characters. Maximum likelihood (ML) analysis was computed by RAxML [57] with the PHY files generated with ClustalX 1.83 [55], using the GTR-GAMMA model. Maximum likelihood bootstrap proportions (MLBP) were computed with 1000 replicates. Bayesian inference (BI) analysis was conducted with MrBayes v3.2.2 [58]. The Akaike information criterion (AIC) implemented in jModelTest 2.0 [59] was used to select the best fit models after likelihood score calculations were done. The base tree for likelihood calculations was ML-optimized. HKY+I+G was estimated as the best-fit model under the output strategy of AIC, Metropolis-coupled Markov chain Monte Carlo (MCMCMC) searches were run for 2,000,000 generations, sampling every 1000th generation. Two independent analyses with four chains each (one cold and three heated) were run until the average standard deviation of the split frequencies dropped below 0.01. The initial 25% of the generations of MCMC sampling were discarded as burn-in. The refinement of the phylogenetic tree was used for estimating Bayesian inference posterior probability (BIPP) values. The Tree was viewed in FigureTree v1.4. The values of maximum likelihood bootstrap proportions (MLBP) greater than 70% and Bayesian inference posterior probabilities (BIPP) greater than 90% at the nodes are shown along branches. For *Isthmolongispora* and Leotiomycetes, only Bayesian inference analysis based on LSU was used.

3. Results

3.1 Phylogenetic analyses

In the phylogenetic analyses of *Microthyriaceae*, several major clades were found, consistent with results of earlier multi-gene phylogenetic analyses [4, 9]. Our 13 species along with four known species of *Microthyriaceae* formed a clade with 98 % Maximum Likelihood Bootstrap (MLB) and 100% Bayesian Posterior Probability supports (BPP) (Figure 1), which is still as a sister clade of *Phaeotirchaceae* as previously indicated. Our

analyses revealed four distinct new clades which we describe as four new genera *Antidactylaria*, *Isthmomyces*, *Keqinzhangia*, *Pseudocoronospora*. In addition, *Triscelophorus* was found to form a distinct clade with 95 % MLB support and 100% BPP, while both the MLB and BPP supports of *Isthmomyces* were 100%. Of the four new genera, only *Isthmomyces* was closely related to the known genus *Microthyrium*, and these two genera formed a sister clade with a high support value.

In the phylogenetic analyses of *Isthmolongispora* and members of Leotiomycetes, three main clades were present, representing three families, *Hyaloscyphaceae*, *Mollisiaceae* and Loramycetaceae. Four strains of *I. quadricellularia* fell into the clade of *Hyaloscyphaceae*, as the closest sister clade to *Hyaloscypha* Boud (Figure 2).

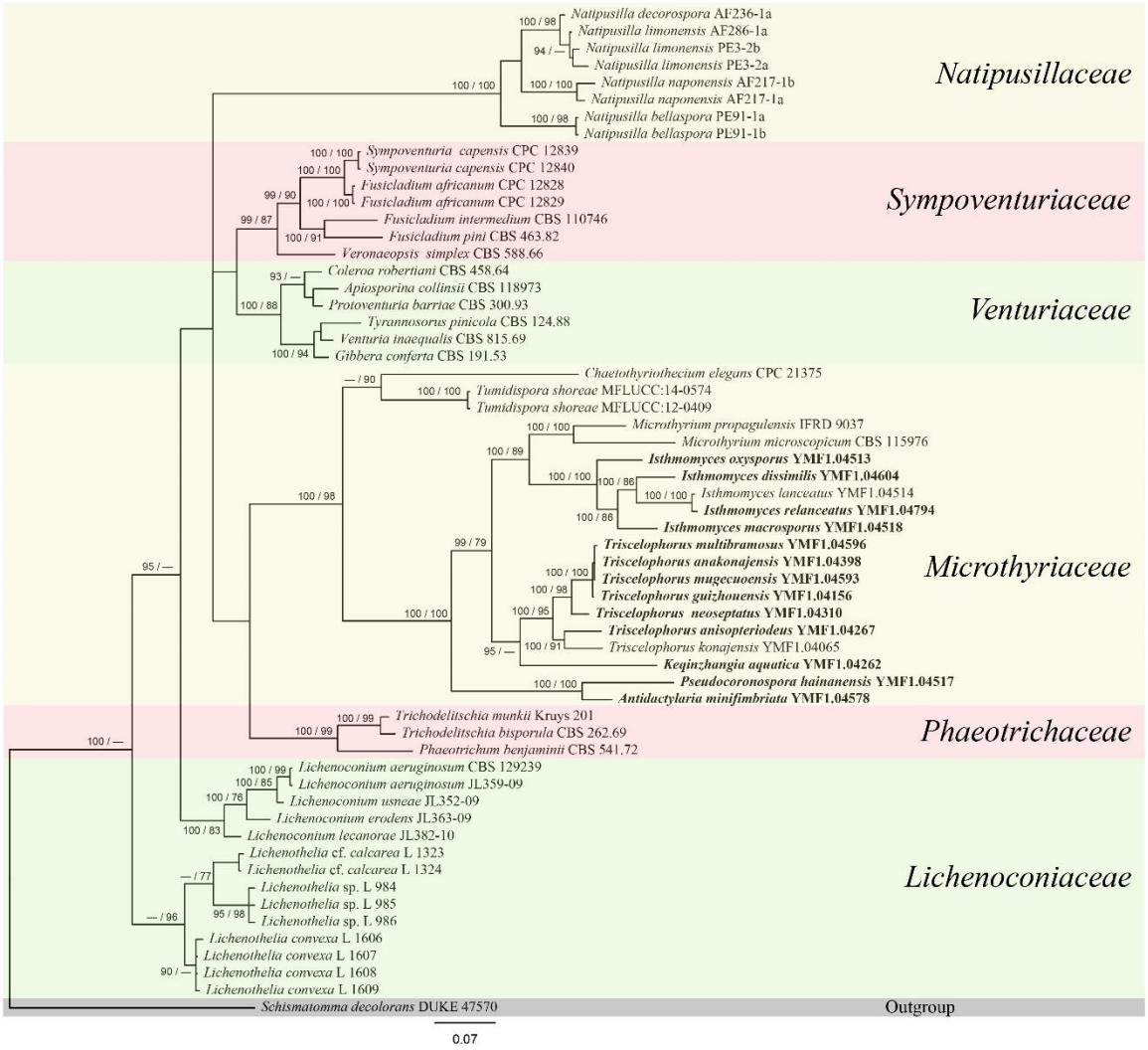

Figure 1. The best scoring RAxML Dothideomycetes tree from 59 taxa based on a combined dataset of LSU, SSU, TEF1 and RPB2 sequences. Bootstrap support values for maximum likelihood (ML) greater than 50% are given above the nodes; Bayesian posterior probabilities (BYPP, green) above 0.90 are given below the nodes. The original strain numbers are given after the species names. Type and ex-type strains are emphasized in bold. The tree was rooted with *Schismatomma decolorans* (DUKE 47570).

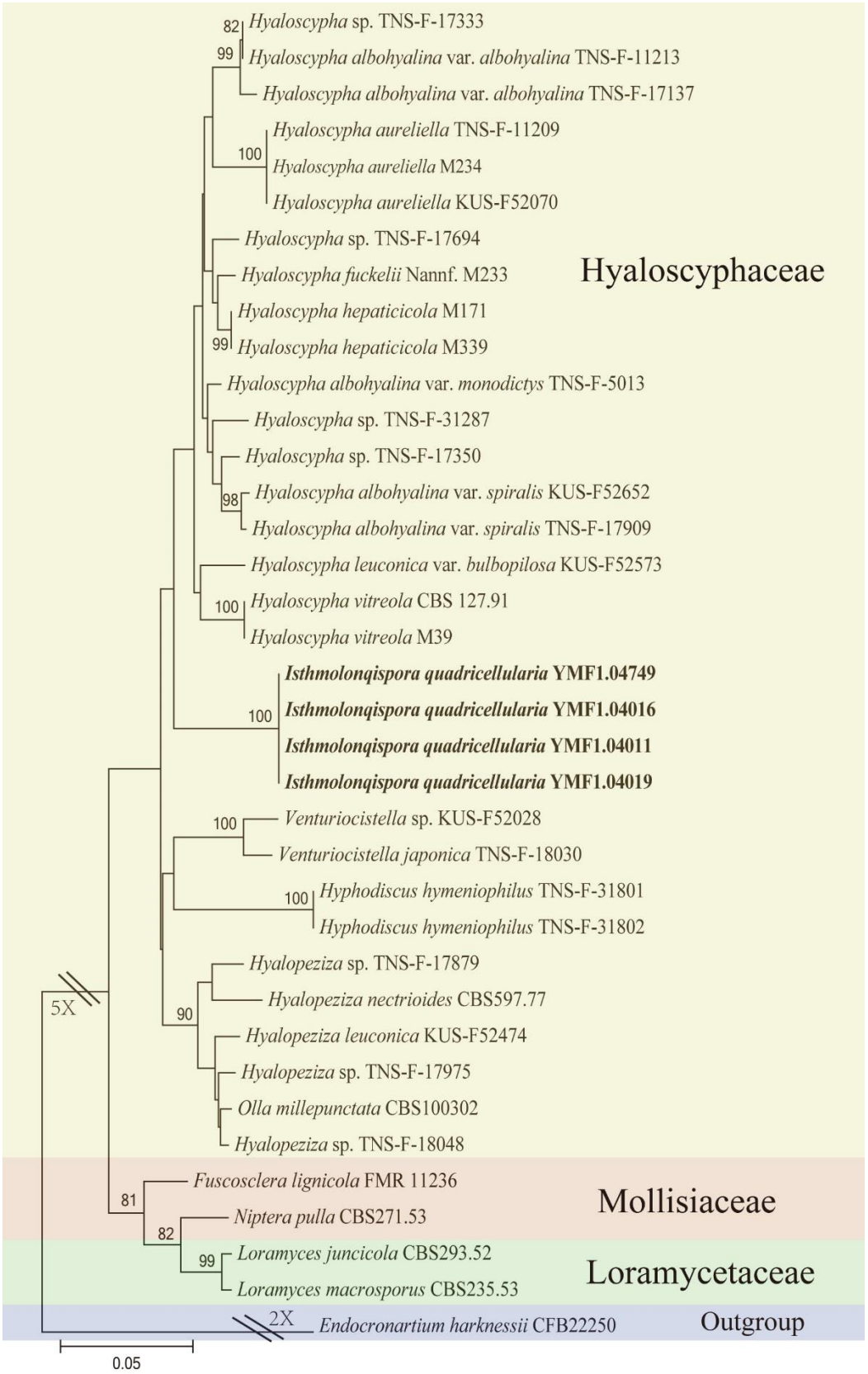

Figure 2. Phylogenetic tree from 37 taxa based on LSU sequences of three families of Leotiomycetes. Bayesian posterior probabilities (BYPP) above 0.80 were given above the nodes. The original strain numbers are given after the species names. The tree was rooted with *Endocronartium harknessii*.

3.2 Taxonomy

Microthyriaceae Sacc., Syll. fung. (Abellini) 2: 658 (1883). MycoBank 81008.

Type genus: Microthyrium Desm., Annls Sci. Nat., Bot., sér. 2 15: 137 (1841). MycoBank 3206.

Description: Hyde et al. 2013.

Notes: Microthyriaceae have been poorly studied, therefore, there are very few DNA sequences in public databases for this group of fungi. Our results provided the first molecular evidence of asexual morph of this family. We erected four new genera (*Antidactylaria, Isthmomyces, Keqinzhangia, Pseudocoronospora*) and recognized thirteen new species in *Microthyriaceae* based on DNA sequences at four gene fragments. In addition, six new combinations are proposed for *Isthmolongispora* species composed of two cellular isthmic segments.

Antidactylaria Z.F. Yu, M. Qiao & R.F. Castañeda, **gen. nov.**

Index Fungorum number: IF555876, Facesoffungi number: FoF 05734

Etymology. Greek, Anti- meaning against, + Latin, dactylaria, referring to the genus *Dactylaria*.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* effuse, white to rosy buff. *Mycelium* superficial and immersed. *Conidiophores* macronematous, erect, unbranched, septate, hyaline, sometimes reduced to conidiogenous. *Conidiogenous cells* polyblastic, sympodial elongated, integrated, terminal determinate or indeterminate, hyaline. Conidial secession rhexolytic. *Conidia* solitary, acrogenous, narrow obclavate, cylindrical to fusiform, navicular, attenuate towards the apex, rostrate, unicellular or septate, hyaline or subhyaline, smooth-walled, with a minute basal frill.

Type species: *Antidactylaria minifimbriata* Z.F. Yu, M. Qiao & R.F. Castañeda.

Notes: The genus *Dactylaria* Sacc., typified with *D. purpurella* (Sacc.) Sacc., is characterized by unbranched, septate, hyaline or pigmented conidiophores and denticulate, integrated, mostly terminal, sympodially extending conidiogenous cells and cylindrical, fusiform, filiform, ellipsoid, clavate, obclavate, unicellular or septate, hyaline or pale pigmented conidia that are liberated after schizolytic secession [60-62]. The rhexolytic conidial secession in *Antidactylaria* separates it from *Dactylaria* morphologically as conidiogenous event and an important criterion for generic delimitation, discussed by Paulus et al. [61] and supported by the molecular phylogeny analysis obtained from *Antidactylaria minifimbriata*.

Antidactylaria minifimbriata Z.F.Yu, M. Qiao & R.F. Castañeda, **sp. nov.** (Figure 3, 16E)

Index Fungorum number: IF556121, Facesoffungi number: FoF 05735

Etymology. Latin, mini- meaning very small, minutely, + Latin, fimbriata-, referring to edged, delicately toothed, fringe or frill that remained on the conidial base after rhexolytic secession.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* on CMA attaining 2.7 cm diam. after 20 days at 25°C, white to rosy buff, reverse buff. *Mycelium* partly superficial, partly immersed in the substrate, composed of branched, slender, spaced septate, hyaline, smooth-walled hyphae. *Conidiophores* semi-macronematous, mononematous, cylindrical, straight or slightly flexuous, unbranched, 0–1(–2)-septate, hyaline or pale brown, smooth, sometimes reduced to conidiogenous cells. *Conidiogenous cells* polyblastic, sympodial elongated, terminal, denticulate, denticles cylindrical, minute fringed. *Conidia* solitary, acrogenous, narrow obclavate, cylindrical to fusiform, attenuate, rostrate or caudate toward the apex, 27.7–40 × 2.5–3.3 μm, rostrum 10–19 × 1–1.8 μm, 2-septate, hyaline to subhyaline, smooth-walled, with a minute basal frill.

Material examined. CHINA, Hainan province, Diaoluoshan National Forest Park, on decaying leaves, 24 April 2014, Z.F Yu, holotype YMFT 1.04578, ex-type living culture YMF 1.04578 = CGMCC 3.18825.

Notes: Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits using the ITS sequence (GenBank MK569506) of *Antidactylaria minifimbriata* are *Isthmolongispora ampulliformis* (GenBank MH857845; Identities = 442/548 (81 %), Gaps = 37/548 (6 %)) and *Dactylaria ampulliformis* (GenBank AY265336; Identities = 431/535 (81%), Gaps = 35/535(6%)).

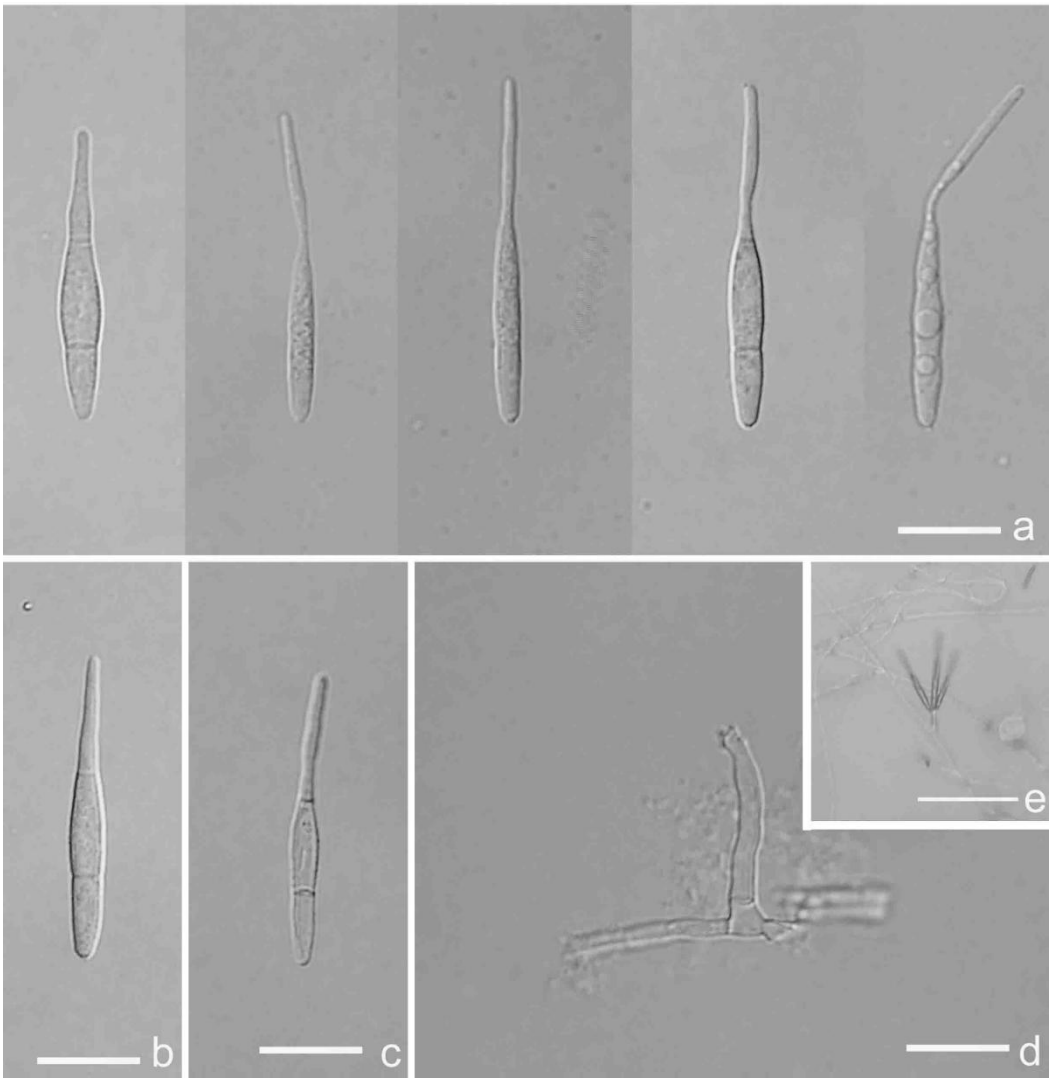

Figure 3. *Antidactylaria minifimbriata* (YMFT1.04578, **holotype**). **a–c** conidia. **d** conidiophore. **e** conidia on conidiophore under low objective. Scale bars: **a–d** = 10 μm, **e** = 50 μm.

Isthmomyces Z. F. Yu, M. Qiao & R. F. Castañeda, **gen. nov.**

Index Fungorum number: IF556126, Facesoffungi number: FoF 05740

Etymology. Latin, isthmus, Greek (isthmós, "neck") meaning a narrow cellular structure that connecting two larger bodies or cells, +Greek, myces referring to fungus.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* effuse, pale mouse grey to dark mouse grey. *Mycelium* superficial and immersed. *Conidiophores* macronematous, mononematous, erect, unbranched, smooth, pale brown or hyaline, septate, sometimes reduced to conidiogenous cells. *Conidiogenous cells* polyblastic, denticulate, integrated, terminal, sympodial extended. Conidial secession schizolytic. *Conidia* acrogenous, isthmospore, composed two cellular isthmic-segment obclavate, clavate, pyriform, obpyriform, lageniform, subulate fusiform to navicular to lanceolate, unicellular or septate, smooth, hyaline, connected by a very narrow, distinct or inconspicuous isthmus.

Type species: *Isthmomyces oxysporus* Z.F. Yu, M. Qiao & R.F. Castañeda.

Notes: *Isthmolongispora* Matsush. was established with *I. intermedia* Matsush. as type species [63]. The genus is characterized denticulate, sympodially extending conidiogenous cells and conidia composed by two or several cellular structures, which are connected by very narrow isthmuses. In present study, isthmospore with two and more cellular isthmic-segment specimens were collected respectively. Phylogenetic analysis inferred from four loci showed that the species with isthmospore composed by two cellular isthmic-segment (hemi-

isthmospore) belong to *Microthyriaceae* (Figure 1), while species composed by more than two cellular isthmic-segments belong to Leotiomycetes based on phylogenetic analysis inferred from LSU (Figure 2). We retained species with 3(–4) cellular isthmic-segments which were similar to type species *Isthmolongisp*ora intermedia in *Isthmolongispora*, and established *Isthmomyces* to comprise two cellular isthmic-segments (hemi-isthmospore).

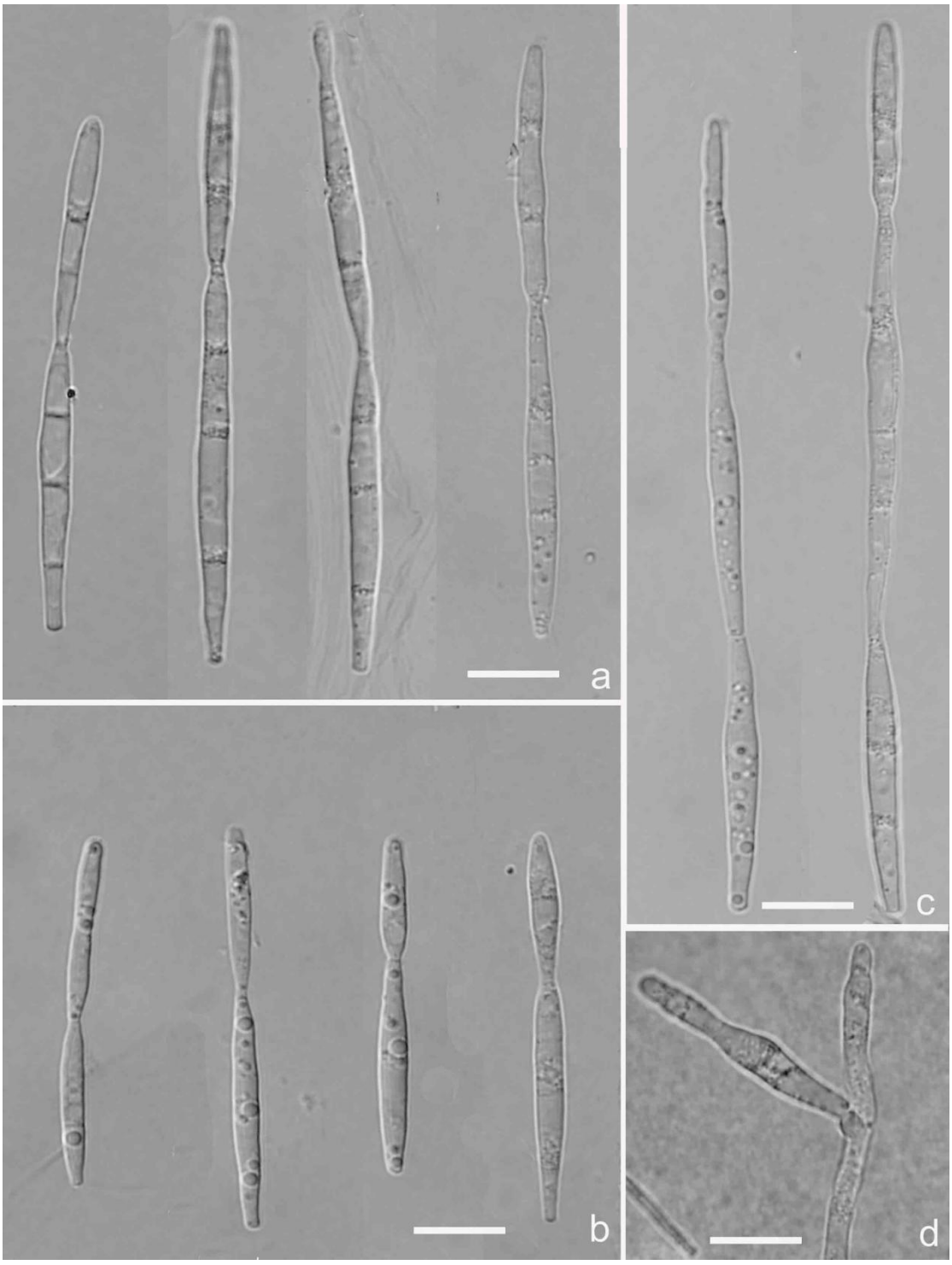

Figure 4. ***Isthmomyces dissimilis*** (YMFT1.04604, **holotype**). **a** the larger isthmospore with 2-cellular isthmic-segmentsi. **b** the smaller isthmospore with 2-cellular isthmic-segmentsi. **c** isthmospore with 3-cellular isthmic-segmentsi. **d** conidiogenous cell and developing conidia. Scale bars: **a–d** = 10 μm.

Isthmomyces dissimilis Z. F. Yu, M. Qiao & R. F. Castañeda **sp. nov.** (Figure 4, 16B)

Index Fungorum number: IF556129, Facesoffungi number: FoF 05743

Etymology. Latin, dissimilis, variation of the conidial shaped related with generic concept of the genus.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* on CMA after 20 days at 25°C, attaining 2.5 cm diam., white to dark salmon, reverse pale yellow. *Mycelium* superficial or immersed, composed of branched, septate, brown, hyphae. *Conidiophores* macronematous, mononematous, erect, straight, unbranched or slightly branched, 0–1- septate, smooth, subhyaline13.8–51 × 2.3–3.2 μm. *Conidiogenous cells* polyblastic, ampulliform to cylindrical, sympodial extended, integrated, terminal, subhyaline. *Conidia* acrogenous, isthmospore, with inconspicuous isthmus, (isthmus mostly reduced to a constricted at the septa) subhyaline, guttulate, smooth, composed of 2–3-cellular isthmic-segments, more or less symmetrical: A) the larger isthmospore with 2-cellular isthmic-segments: i) basal isthmic-segment cylindrical-fusiform, truncate below, 1–3 septate, 35–60 × 4–4.5 μm, ii) apical isthmic-segment fusiform, rounded at the tip, 0–2 septate, 17–36.5 × 4–4.5 μm; total long 70–95 μm. B) the smaller isthmospore with 2-cellular isthmic-segments: i) basal isthmic-segment cylindrical-fusiform, truncate below, 0–1 septate, 23–33 × 3.5–4.5 μm; ii) apical isthmic-segment fusiform, rounded at the tip, 0–1 septate, 17–22 × 3.5–4.5μm; total long 47–57μm. C) isthmospore with 3-cellular isthmic-segments: i) basal isthmic-segment fusiform, truncate below, 2–3-septate, 18.5–38.5 × 2.8–5.0 μm; ii) central isthmic-segment cylindrical-fusiform, 2–3-septate, 20.1–44.5 × 3.0–6.2 μm; iii) apical isthmic-segment fusiform, rounded or obtuse at the tip, 0–2-septate, 17.4–31.6 × 2.3–4.8 μm.

Material examined. CHINA, Hainan Province, Diaoluo Mountain Nature Reserve, on decaying leaves, 17 Aug. 2015, J. Peng, holotype YMFT 1.04604, ex-type living culture YMF 1.04604 = CGMCC 3.18826.

Notes. In this genus, the conidia of *Isthmomyces variabilis* are the longest ones, 36–136 μm. *I. dissimilis* varies in conidia shape. Although it has 3-cellular isthmic-segment conidia, its isthmic is not distinct as *Isthmolongispora* species. However, cells of *Isthmolongispora* are bead, while those of *I. dissimilis* are cylindrical-fusiform.

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits using the ITS sequence (GenBank MF740794) of *I. dissimilis* are *Isthmolongispora lanceata* (= *Isthmomyces lanceatus*, GenBank MH858897; Identities =312/339 (92 %), Gaps = 8/339 (2 %)). In addition, the similarity of ITS sequence of this species with other close *Isthmomyces* species, *I. relanceatus*, is 92 % (312/339, Gaps = 8/339 (2 %)).

Isthmomyces macrosporus Z. F. Yu, M. Qiao & R. F. Castañeda **sp. nov.** (Figure 5, 16D)

Index Fungorum number: IF556128, Facesoffungi number: FoF 05742

Etymology. Greek, macrosporus referred to the large, great conidia.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* on PDA attaining 2 cm diam. after 20 days at 25°C, amber to fawn, reverse fawn *Mycelium* mostly immersed, composed of branched, septate, slender, colorless hyphae. *Conidiophores* macronematous, mononematous, cylindrical, erect, straight, unbranched, 0–1- septate, smooth, pale brown, 25–35 × 3–3.5 μm. *Conidiogenous cells* polyblastic, cylindrical, denticulate, sympodial extended, integrated, terminal, pale brown or subhyaline. *Conidia* acrogenous, isthmospore, long fusiform, hyaline, smooth, 36.5–73.0 μm long, strongly constricted at the conspicuous. narrow, tiny central isthmus, sometime not differentiated, composed of two cellular isthmic-segments: i) basal isthmic-segment clavate, truncated at the base, 1(–2)-septate, hyaline or subhyaline, smooth, 19.2–31.1 × 4.5–6.7 μm; ii) apical isthmic-segment narrow obclavate, sometimes sub-obspathulate, rounded at the tip, unicellular, guttulate, hyaline or subhyaline, smooth, 21.1–42.0 × 3.3–5.4 μm.

Material examined. CHINA, Hainan Province, Limu Mountain National Conservation Area, on decaying leaves, Apr. 24 2015, J. Peng, holotype YMFT 1.04518, ex-type living culture YMF 1.04518 = CGMCC 3.18824.

Notes: *Isthmomyces macrosporus* is different from all species within this genus by having larger conidia, and the conidiophores are obviously brown, few denticulate conidiogenous cells also distinguish it from other species [64].

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits using the ITS sequence (GenBank MF740796) of *I. macrosporus* are *Isthmolongispora lanceata* (= *Isthmomyces lanceatus*, GenBank MH858897; Identities = 250/273 (92 %), Gaps = 12/273 (4 %)) and *Stenocladiella neglecta* (GenBank KX858624; Identities = 254/278 (91 %), Gaps = 14/278 (5 %)).

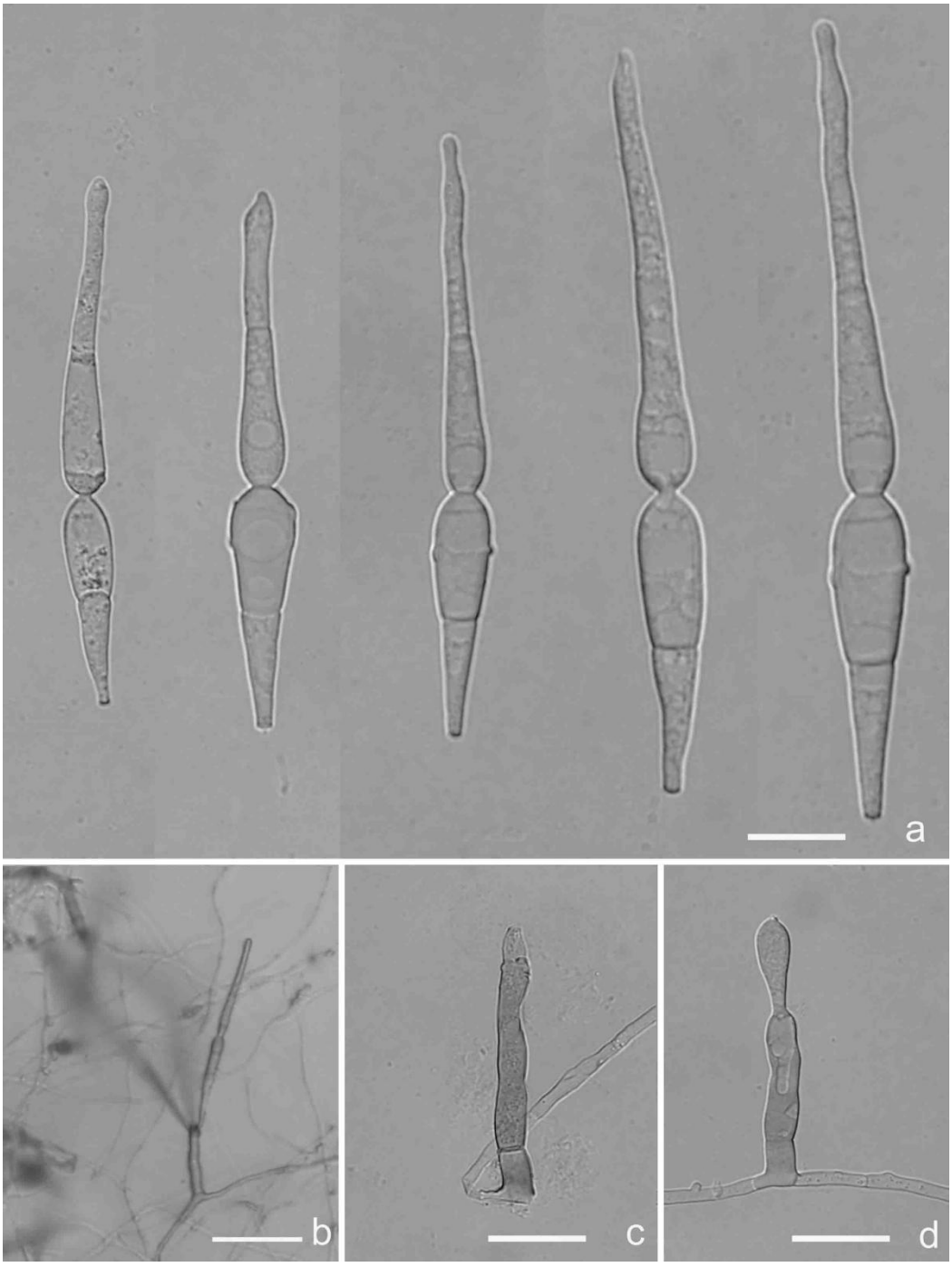

Figure 5. ***Isthmomyces macroporus*** (YMFT1.04518 , **holotype**).**a** conidia. **b** conidiophore with conidia under low objective. **c** conidiophore and conidiogenous. **d** conidiophore and developing conidia. Scale bars: **a,c,d** = 10 μm, **b** = 50 μm.

Isthmomyces oxysporus Z. F. Yu, M. Qiao & R. F. Castañeda **sp. nov.** (Figure 6, 16A)

Index Fungorum number: IF556127, Facesoffungi number: FoF 05741

Etymology. Greek, oxys-, meaning sharp, keen + -sporum referring to the conidia.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* on CMA, attaining about 2 cm diam. after 20 days at 25°C. Pale mouse grey to dark mouse grey, reverse olivaceous grey. *Mycelium* mostly immersed, composed of branched, septate, subhyaline to hyaline hyphae. *Conidiophores* macronematous, mononematous, cylindrical, erect, smooth, 0–1-septate, subhyaline to hyaline, mostly reduced to conidiogenous

cells, up to 30 μm long, 2.5–3 μm wide, arising from the creeping hyphae. *Conidiogenous cells* polyblastic, cylindrical, denticulate, integrated, terminal, sympodial extended, hyaline. *Conidia* isthmospore, fusiform, hyaline, smooth, 20.5 –25.5 μm long, strongly constricted at the narrow, tiny central isthmus, composed of two cellular isthmic-segments: i) basal isthmic-segment broadly clavate to clavate, unicellular, hyaline 9.7–13× 2.0–4.0 μm; ii) apical isthmic-segment narrow obclavate to obclavate, obpyriform or rarely lecythiform, unicellular, hyaline, 9.0–13.0 × 2.0–3.0 μm.

Material examined. CHINA, Hainan Province, Diaoluo Mountain Natural Reserve, on decaying leaves, 24 Aug. 2015, J. Peng, holotype YMFT 1.04513, ex-type living culture YMF 1.04513 = CGMCC 3.18821.

Notes: *Isthmomyces oxysporus* resembles *I. asymmetricus* in having both isthmic-segment ends tapering, but *I. asymmetricus* has asymmetrical conidia with the basal isthmic-segment longer (*I. asymmetricus* 17–20 μm long). Besides, *I. oxysporusis* somewhat similar to *I. rodundatus* in conidial sizes, but the apical isthmic-segment in *I. rotundatus* are rounded at the tip.

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits using the ITS sequence of *I. oxysporusis* are *Isthmolongispora lanceata* (= *Isthmomyces lanceatus*, GenBank MH858897; Identities = 201/206 (98 %), Gaps = 2/206 (0 %)) and *Stenocladiella neglecta* (GenBank KX858624; Identities = 238/260 (92 %), Gaps = 9/260 (3 %)). In addition, the similarity of ITS sequence of this species with other close *Isthmomyces* species, *I. dissimilis*, is 89 % (292/327, Gaps = 25/327 (7 %)).

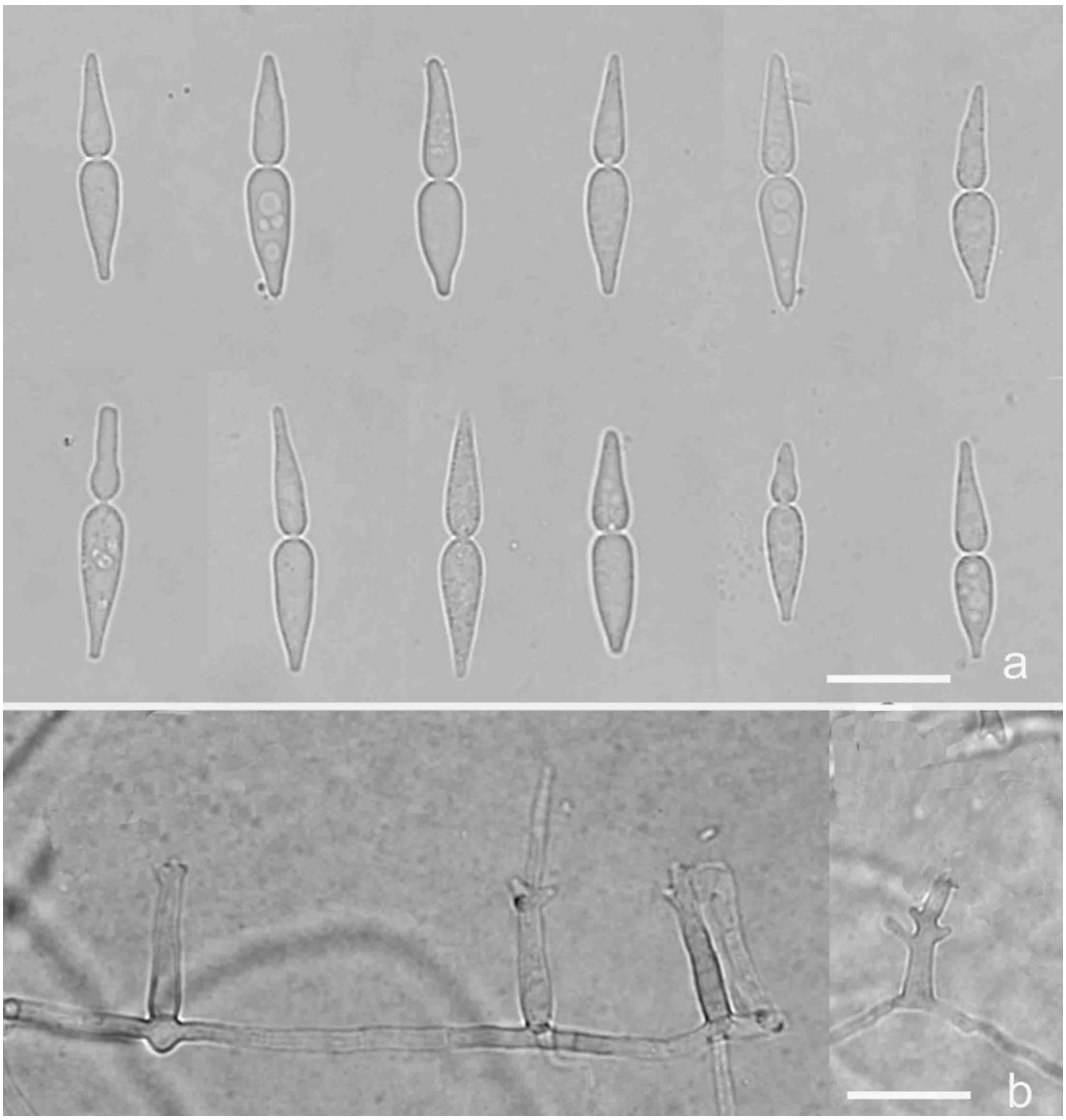

Figure 6. ***Isthmomyces oxysporus*** (YMFT1.04513, **holotype**). **a** conidia. **b** conidiophore. Scale bars: **a–b** = 10 μm.

Isthmomyces relanceatus Z. F. Yu, M. Qiao & R. F. Castañeda **sp. nov.** (Figure 7, 16E)

Index Fungorum number: IF556130, Facesoffungi number: FoF 05744

Etymology. Latin, re- meaning back, against, again + -lanceatus, referred to the conidia resemblance with *Isthmomyces lanceatus*.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* on CMA attaining about 2 cm diam. after 20 days at 25°C, white to dark salmon, reverse pale brown. *Mycelium* partly superficial, partly immersed in the substrate, composed of branched, septate, slender, hyaline hyphae. *Conidiophores* macronematous, mononematous, cylindrical, erect, straight, unbranched, 0–1- septate, smooth, hyaline, up to 30 μm long, 3–3.5 μm wide. *Conidiogenous cells* polyblastic, cylindrical, denticulate, sympodial extended, integrated, terminal, hyaline. *Conidia* acrogenous, isthmospore, somewhat fusiform, hyaline, smooth, 21.3–39.7 μm long, strongly constricted at the conspicuous, narrow, tiny, distinct or inconspicuous central isthmus, composed of two cellular isthmic-segments: i) basal isthmic-segment narrow clavate, sometimes cylindrical-clavate, truncated at the base, unicellular, hyaline or subhyaline, smooth, 12.5–18.5 × 3.0–4.8 μm; ii) apical isthmic-segment broadly obclavate, obspathulate, rounded at the tip, unicellular, hyaline, smooth, 13.0–30.0 × 2.3–3.8 μm.

Material examined. CHINA, Tibet, Nanyigou Scenic Area, on decaying leaves, 1 Oct. 2016, Z.F. Yu, holotype YMFT 1.04794, ex-type living culture YMF 1.04794 = CGMCC 3.18827.

Notes: Isthmomyces relanceatus is similar to *I. lanceatus* in the shape of conidia, but both the basal cell and distal cellular isthmic-segments of *I. relanceatus* are longer and wider than those of *I. lanceatus* (basal isthmic segment: 10–15 × 2.5–3.8 μm; apical isthmic segment 14–22 × 2.0–3.4 μm) as described by Hoog and Hennebert [64]. Morphologically, *I. relanceatus* is also similar to *I. oxysporus* in the conidial shape. However, conidia of *I. relanceatus* are longer than those of *I. oxysporus* (20.5 – 25.5 μm long).

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits using the ITS sequence (GenBank MK577896) of *I. relanceatus* are *Isthmolongispora lanceata* (= *Isthmomyces lanceatus*, GenBank MH858897; Identities = 541/584 (93 %), Gaps = 22/584(3%)) and *Stenocladiella neglecta* (GenBank KX858624; Identities = 348/375 (93 %), Gaps = 12/375 (3 %)).

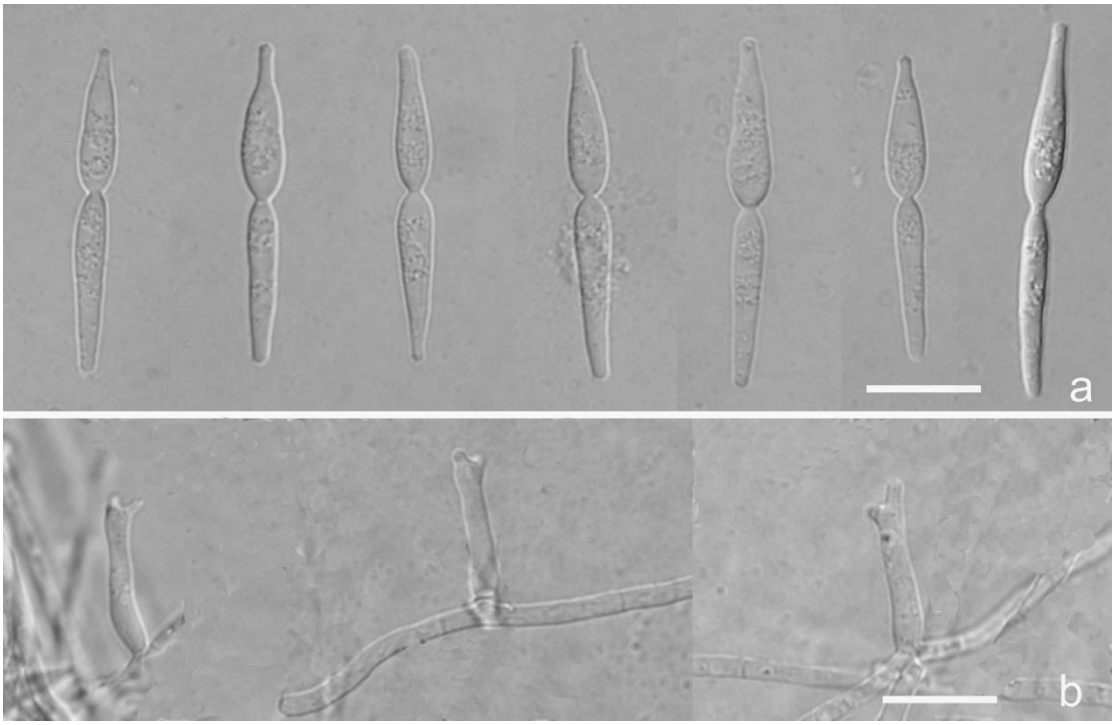

Figure 7. ***Isthmomyces relanceatus*** (YMFT1.04794 , **holotype**). **a** conidia. **b** conidiophores. Scale bars: **a, b** = 10 μm.

Isthmomyces asymmetricus (Aramb. & Cabello) Z. F. Yu & R. F. Castañeda, **comb. nov.**

Index Fungorum number: IF566155, Facesoffungi number: FoF 05756

Basionym: Isthmolongispora asymmetrica Aramb. & Cabello, in Arambarri, Cabello & Mengascini, Mycotaxon 29: 30 (1987).

Description: Arambarri et al. 1987.

Isthmomyces basitruncatus (Matsush.) Z. F. Yu & R. F. Castañeda, **comb. nov.**

Index Fungorum number: IF556156, Facesoffungi number: FoF 05755

Basionym: *Isthmolongispora basitruncata* Matsush., Icon. microfung. Matsush. lect. (Kobe): 89 (1975).

Description: Matsush 1975.

Isthmomyces geniculatus (Nawawi & Kuthub.) Z. F. Yu & R. F. Castañeda, **comb. nov.**

Index Fungorum number: IF556157, Facesoffungi number: FoF 05758.

Basionym: Isthmolongispora geniculata Nawawi & Kuthub., Mycotaxon 31(2): 339 (1988).

Description: Nawawi & Kuthub 1975.

Isthmomyces lanceatus (de Hoog & Hennebert) Z. F. Yu & R. F. Castañeda, **comb. nov.**

Index Fungorum number: IF556158, Facesoffungi number: FoF 05757

Basionym: Isthmolongispora lanceata de Hoog & Hennebert, Proc. K. Ned. Akad.Wet., Ser. C, Biol. Med. Sci. 86(3): 343 (1983).

Description: Nawawi & Kuthub Matsush 1975.

Isthmomyces minimus (Matsush.) Z. F. Yu, M. Qiao & R. F. Castañeda, **comb. nov.**

Index Fungorum number: IF556159, Facesoffungi number: FoF 05759

Basionym: *Isthmolongispora minima* Matsush., Microfungi of the Solomon Islands and Papua-New Guinea (Osaka): 32 (1971).

Description: Nawawi & Kuthub Matsush 1975.

Isthmomyces rotundatus (Matsush.) Z. F. Yu, M. Qiao & R. F. Castañeda, **comb. nov.**

Index Fungorum number: IF556160, Facesoffungi number: FoF 05760

Basionym: *Isthmolongispora rotundata* Matsush., Matsush. Mycol. Mem. 5: 17 (1987)

Description: Nawawi & Kuthub Matsush 1975.

Keqinzhangia Z. F. Yu, M. Qiao & R. F. Castañeda, **gen. nov.**

Index Fungorum number: IF556124, Facesoffungi number: FoF 05738

Etymology. Latin, name in honors to Prof. Keqing Zhang of the Yunnan University for his contribution to the biological sciences.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* effuse, mouse grey. *Mycelium* superficial and immersed. *Hyphae* branched, septate, hyaline, smooth-walled. *Conidiophores* prostrate, not differentiated. *Conidiogenous hyphae* holothallic, branched, septate, discrete, determinate, forming conidia by random thallic-arthric conidial ontogeny. Conidial secession schizolytic. *Conidia* thallic-arthric, solitary or in chains, obclavate, bacilliform, cylindrical, fusiform, sub-oblecythiform or cuneiform, unicellular to septate, hyaline. *Chlamydospores* globose, terminal, solitary or short catenulate, subhyaline.

Type species: *Keqinzhangia aquatica* Z.F. Yu, M. Qiao & R.F. Castañeda.

Keqinzhangia aquatica Z.F. Yu, M. Qiao & R. F. Castañeda, **sp. nov.** (Figure 8, 16F)

Index Fungorum number: IF556125, Facesoffungi number: FoF 05739

Etymology. Latin, aquatic, referring to it growing in water.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* flat, growing slowly on CMA, attaining about 2.4 cm diam. after 20 days at 25°C. Pale mouse grey, reverse mouse grey. *Mycelium* mostly immersed, composed of branched, septate, subhyaline to hyaline hyphae. *Conidiophores* prostrate, undifferentiated. *Conidiogenous hyphae* holothallic, branched, septate, hyaline, forming conidia by random thallic-arthric

disarticulation. *Conidia* thallic-arthric, solitary or in chains, obclavate, bacilliform, cylindrical, fusiform, sub-oblecythiform or cuneiform, truncate at the ends or truncate at the base and obtuse or rounded at the apex, 0–6(–7)-septate, slightly or strongly constricted at the septa, sinuate, guttulate, smooth, hyaline, 12–76.5 × 3–6.2 μm. *Clamydospores* solitary or catenate, broad globose, subglobose to ellipsoidal, terminal, slightly or densely guttulate, smooth, subhyaline, 8–12.6 × 4.1–5.4 μm.

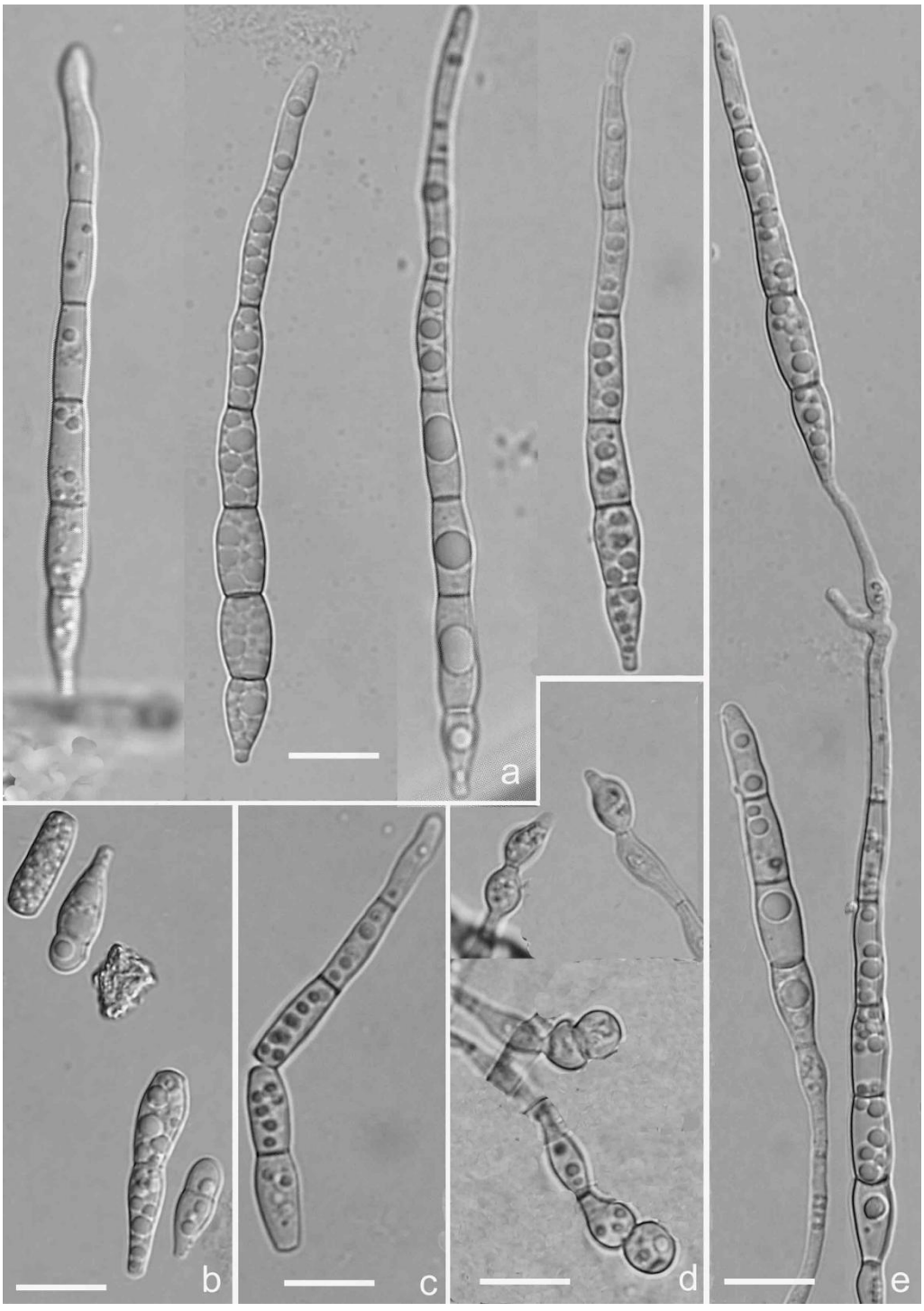

Figure 8. ***Keqinzhangia aquatica*** (YMFT1.04262, **holotype**). **a** conidia. **b–c** broken conidia. **d** clamydospores. **e** conidiophores and conidia. Scale: **a–e** = 10 μm.

Material examined. CHINA, Sichuan province, E'mei National Conservation Area, on decaying leaves, Z.F Yu, 24 June 2014, J. Peng, holotype YMFT 1.04262, ex-type living culture YMF 1.04262.

Notes: Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits using the ITS sequence (GenBank MK569507) of *Keqinzhangia aquatica* are *Microthyrium macrosporum* (GenBank MG844147;

Identities = 188/200 (94 %), Gaps = 3/200 (1 %)) and *Triscelophorus monosporus* (GenBank KF730840; Identities = 188/204 (92%), Gaps = 2/204 (0%)).

Pseudocoronospora Z.F.Yu, M. Qiao & R.F. Castañeda, **gen. nov.**

Index Fungorum number: IF556122, Facesoffungi number: FoF 05736

Etymology. Latin: hainanensis, referred to the region where type strain isolated.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* effuse, hairy, white to pale flesh. Mycelium superficial and immersed. *Conidiophores* macronematous, mononematous, erect, septate, unbranched, brown. *Conidiogenous cells* polyblastic, denticulate, integrated, sympodial extended, terminal, indeterminate. Conidial secession rhexolytic. *Conidia* solitary, acropleurogenous obclavate, crowned, with mammiform protuberances arranged near the apex; septate, smooth or verruculose, hyaline, fringed at the base.

Type species: *Pseudocoronospora hainanensis* Z.F. Yu, M. Qiao & R.F. Castañeda.

Notes: The genus *Coronospora* was established by Ellis with *C. dendrocalami* M. B. Ellis as type species, in which after the conidiogenous events the cicatrized loci are produced following sympodial extensions of the polyblastic conidiogenous cells disposed in geniculate conidiophores and the conidia are liberated via schizolytic conidial secession [62, 65, 66], but in *Pseudocoronospora hainanensis* the conidiogenous loci are tiny or conspicuous denticles and the conidial basal cells are fringed after the rhexolytic conidial secession. Matsushima [67] observed the *Coronospora* in culture of *Ascoronospora*, so he thought that *Coronospora* is asexual state of *Ascoronospora*. Then Wijayawardene et al. [9] and Asthton et al. [3] accepted the link between two genera. So far, molecular sequences of two genera were not obtainable, so the connection between two genera was not confirmed by molecular data. Ascoronospora Matsush. was treated as Pleosporales genera *incertae sedis* [17], morphologically which is different from sexual members of *Microthyriaceae*.

Pseudocoronospora hainanensis Z.F.Yu, M. Qiao & R.F. Castañeda, **sp. nov.** (Figure 9, 16G)

Index Fungorum number: IF556123, Facesoffungi number: FoF 05737;

Etymology. Latin hainanensis, referred to the region where type strain isolated.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* on CMA attaining 3 cm diam. after 20 days at 25°C, effuse, white to pale flesh, reverse buff. *Hyphae* thin-walled, septate, hyaline, smooth. *Conidiophores* macronematous, mononematous, straight or slightly flexuous, somewhat geniculate toward the apex, septate, unbranched, mid brown or pale brown below, pale brown to subhyaline towards the apex, 16.5–49 μm long, 3.5–5.0 μm wide. *Conidiogenous cells* polyblastic, denticulate, denticles conspicuous, narrowly cylindrical, integrated, sympodial extended, terminal, sometimes intercalary, indeterminate, pale brown to subhyaline. Conidial secession rhexolytic. *Conidia* solitary, acropleurogenous, obclavate, crowned with 2–3 broadly mammiform protuberances, radially arranged near the rounded to obtuse apex; 2 septate, smooth or slightly verruculose at the basal and central cells, hyaline, 27.2–33 × 3.7–8.0 μm, with a minute basal frill.

Material examined. CHINA, Hainan province, Diaoluoshan National Forest Park, on decaying leaves, 24 April 2014, Z.F Yu, holotype YMFT 1.04517, ex-type living culture YMF 1.04517 = CGMCC 3.18823.

Notes: Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits using the ITS sequence (GenBank MK569505) of *Pseudocoronospora hainanensis* are *Neoscolecobasidium agapanthi* (GenBank NR_152546; Identities = 317/393 (81%), Gaps = 27/393 (6 %)). Phylogenetically, the genus *Pseudocoronospora* near to the genus *Antidactylaria*, and the similarity of *P. hainanensis* with *A. minifimbriata* is 93 % (179/193, Gaps = 3/193(1%)).

Triscelophorus Ingold, Trans. Br. mycol. Soc. 26(3-4): 151 (1943).

Description: Ingold 1943.

Type species: *Triscelophorus monosporus* Ingold, Trans. Br. mycol. Soc. 26(3-4): 152 (1943).

Notes: *Triscelophorus* was established by Ingold, with *T. monosporus* as type species [68]. The genus is characterized by macronematous, mononematous, erect, straight or flexuous, sometimes sinuate, septate, unbranched or sparingly branched, hyaline, smooth conidiophores. The conidiogenous cells are monoblastic,

sometimes sympodially extended, integrated, hyalinethat produced a solitary, acrogenous, septate, staurospore composed by a main axis and 3 or more branches verticillate arranged from the basal cell of the main axis [62, 68]. Duarte et al. [26] found that *Triscelophorus* was polyphyly based on ITS analysis, but our phylogenetic based on four-loci and ITS showed the genus should be monophylogenetic, more details refer discussion.

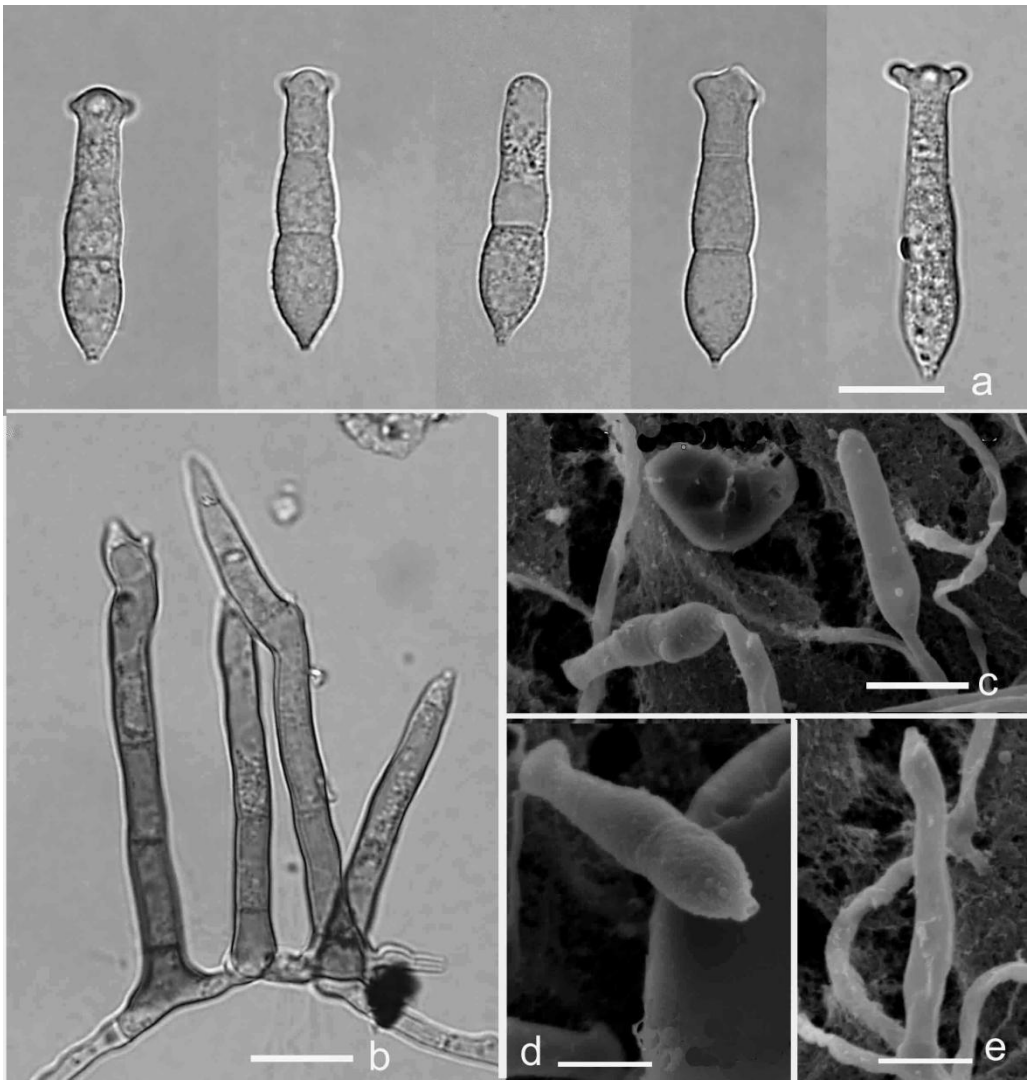

Figure 9. ***Pseudocoronospora hainanensis*** (YMFT1.04517, **holotype**). **a,d** conidia. **b,e** conidiophores and conidiogenous cells. **c** conidia with conidiophores. **c, d, e** were taken with SEM. Scale bars: **a–e** = 10 μm.

Triscelophorus anakonajensis Z. F. Yu, M. Qiao & R. F. Castañeda **sp. nov.** (Figure 10, 16K)

Index Fungorum number: IF556149, Facesoffungi number: FoF 05748

Etymology. Greek, ana-, means back, again, + -konajensis referred to *Triscelophorus konajensis*, the resemblance of conidial morphology of both species.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* on CMA, attaining about 2 cm diam. after 20 days at 25°C, white to dark salmon, reverse mouse grey. *Mycelium* superficial and immersed, composed of branched, septate, hyaline hyphae. *Conidiophores* macronematous, mononematous, cylindrical, erect, flexuous, unbranched, smooth, hyaline, up to 90 μm long. *Conidiogenous cells* monoblastic, cylindrical, terminal, integrated, determinate, smooth, hyaline. *Conidia* solitary, staurospore, acrogenous, septate, composed of a main axis and 2–3 lateral branches: i) main axis obclavate, 2–4-septate, slightly constricted at the septa, straight, smooth, hyaline, 30.3–50 × 3.2–5.3 μm; ii) lateral branches obclavate, broad ovoid, 0–2-septate, straight, smooth, hyaline, 5.8–32.4 × 2.7–4.7 μm, arising from the basal cell of the main axis in a regular or irregular verticillate arranged.

Material examined. CHINA, Sichuan Province, Mugecuo Nature Reserve, on decaying leaves, 10 Sep. 2015, J. Peng, holotype YMFT 1.04398, ex-type living culture YMF 1.04398 = CGMCC 3.18980.

Notes: *Triscelophorus anakonajensis* is similar to *T. konajensis* K.R. Sridhar & Kaver in the number of lateral branches and shape of conidia, but main axis of *T. konajensisare* 1–3-septate, 20–35 × 3.5–4 μm [69].

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits using the ITS sequence (GenBank MK569512) of *T. anakonajensis* are *T. konajensis* (GenBank MK569513; Identities = 202/210 (96 %), Gaps = 1/210 (0%)) and *T. monosporus* (GenBank KX858630; Identities = 286/313 (91 %), Gaps = 8/313 (2 %)). In addition, the similarity of ITS sequence of T. anakonajensis with phylogenetically close Triscelophorus species, *T. multibrachiatus*, *T. mugecuoensis*, and *T. guizhouensis*, are 96 % (620/646, Gaps = 15/646 (2 %)), 96 % (599/623, Gaps = 13/623 (2 %)), and 86 % (405/473, Gaps = 20/473 (4 %)), respectively.

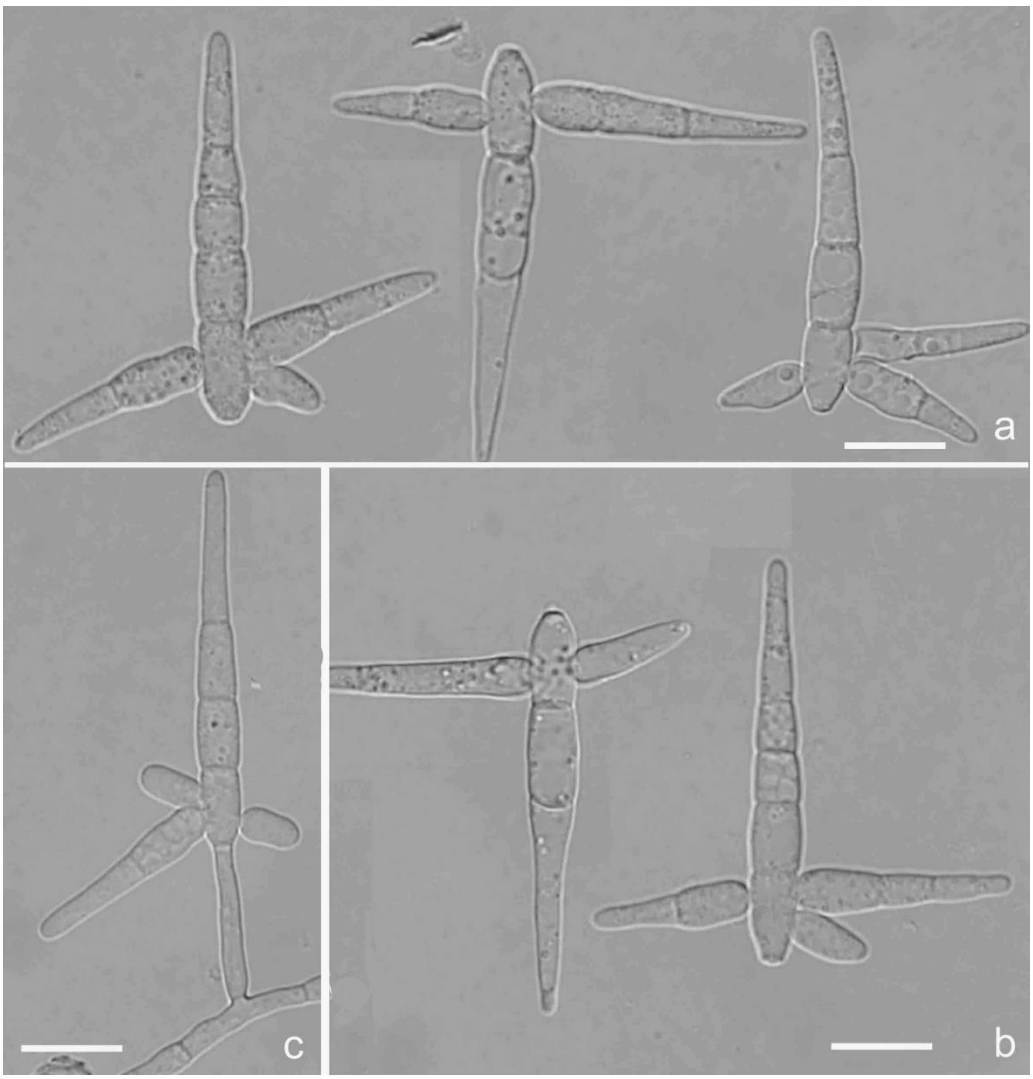

Figure 10. ***Triscelophorus anakonajensis*** (YMFT1.04398, **holotype**). **a, b** conidia. **c** conidiophone with conidia. Scale bars: **a–c** = 10 μm.

Triscelophorus anisopteriodeus Z. F. Yu, M. Qiao & R. F. Castañeda **sp. nov.** (Figure 11, 16H)

Index Fungorum number: IF556148, Facesoffungi number: FoF 05747

Etymology. Latin, anisopteriodeus, referred to the resemblance of conidial body with an adult of *Anisoptera* sp.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* on CMA, attaining about 1cm diam. after 20 days at 25°C, light smoke grey. Reverse smoke grey. *Mycelium* superficial and immersed, composed of branched, septate, hyaline hyphae. *Conidiophores* macronematous, mononematous, cylindrical, erect,

flexuous, unbranched, smooth, hyaline, up to 20–110 μm long. *Conidiogenous cells* monoblastic, cylindrical, terminal, integrated, determinate, smooth, hyaline. *Conidia* solitary, acrogenous, staurospore, septate, composed of a main axis and 2–4 lateral branches: i) the main axis elongate obclavate, 2–4-septate, straight, smooth, hyaline, 31.2–48 × 3–5.2 μm; ii) 2–4-lateral branches obclavate to broad obclavate, straight, smooth, hyaline, all arising divergent, unequal, from the basal cell of the main axis: ii a) 2 lateral branches, 2–3-septate, 8.2–38.7 ×2.5–4.8 μm, more or less opposite, just below the supra-basal septum arranged; ii b) (1–) 2-lateral branches, 0–1-septate, 14–20 × 5–5.5 μm, sequential opposite near the middle of the basal cell arranged.

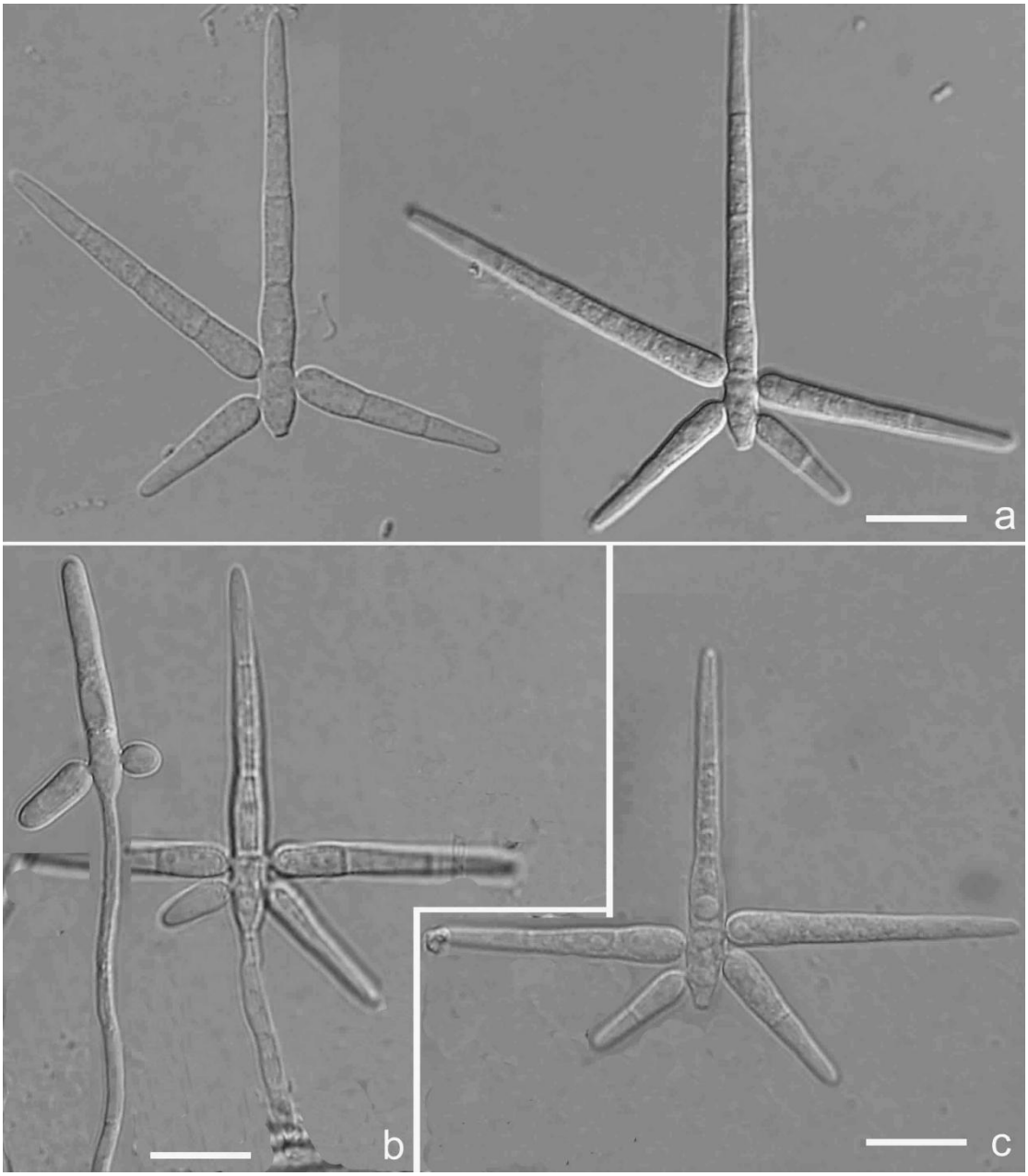

Figure 11. ***Triscelophorus anisopteriodeus*** (YMFT1.04267, **holotype**). **a, c** conidia. **b** conidiophore with conidia. Scale bars: **a-c** = 10 μm.

Material examined. CHINA, Hainan Province, Limu Mountain Nature Reserve, on decaying leaves, 26 Apr. 2015, J. Peng, holotype YMFT 1.04267, ex-type living culture YMF 1.04267 = CGMCC 3.18978.

Notes – *Triscelophorus anisopteriodeus* is differentiated from other known *Triscelophorus* species and new species in the present work by conidia with four laterals branches in pairs, which make conidia looks like a dragonfly-shape. Four lateral branches are not arising from the same level at the basal cell of main axis. Two shorter ones are lower, and two longer ones are upper. Among conidia of *Triscelophorum* spp., three lateral

branches are often growing in a whorl, while 2 lateral branches are in pairs. Four lateral branches in pairs in *T. anisopteriodeus* make it recognizable easily.

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits using the ITS sequence (GenBank MK569511) of *T. anisopteriodeus* are *T. monosporus* (GenBank KX858630; Identities = 199/210 (95 %), Gaps = 2/210 (0 %)) and *T. konajensis* (GenBank MK569513; Identities = 215/234 (92 %), Gaps = 9/234 (3 %)).

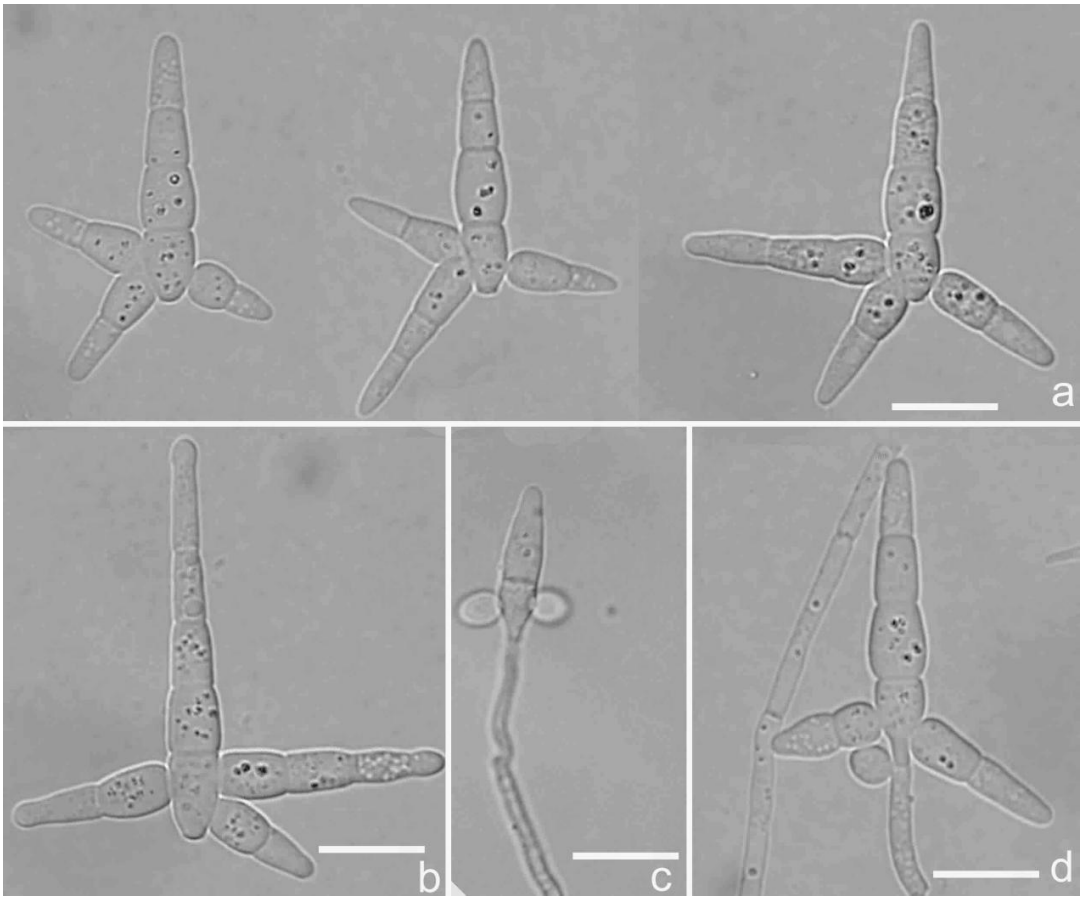

Figure 12. ***Triscelophorus guizhouensis*** (YMFT1.04156, **holotype**) **a,b,d** conidia. **c** developing conidium on conidiaphore. Scale bars: **a–d** = 10 μm.

Triscelophorus guizhouensis Z. F. Yu, M. Qiao & R. F. Castañeda **sp. nov (**Figure 12, 16I)

Index Fungorum number: IF556146, Facesoffungi number: FoF 05745

Etymology. Latin, guizhouensis, referred to the Guizhou Province.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* on CMA, attaining about 1.5 cm diam. after 20 days at 25°C, pale mouse grey to greyish sepia, reverse mouse grey. *Mycelium* most immersed, composed of branched, septate, subhyaline to hyaline hyphae. *Conidiophores* macronematous, mononematous, cylindrical, erect, straight or flexuous, mostly unbranched, rare sparingly branched, smooth, hyaline, up to 20–80 μm long. *Conidiogenous cells* mono- and polyblastic, cylindrical terminal, integrated, determinate or sympodial extended, smooth, hyaline. *Conidia* solitary, acrogenous, staurospore, septate, composed of a main axis and (2–)3 lateral branches: i) the main axis elongate obclavate, gradually tapering towards the rounded apex, 2–4-septate, sometimes constricted slightly at the septum, guttulate, smooth, hyaline, 23.5–38.8 ×4.7–5.7 μm; ii) the (2–)3-lateral branches broad obclavate, 0–3 septate, slightly constricted at the septa, guttulate, smooth, hyaline, the longer two sizes 24–27 × 6–6.5 μm the shorter and 14–19 × 6–7 μm, arising more or less in verticillate from main axis basal cell.

Material examined. CHINA, Guizhou Province, Leigong Mountain Nature Reserve, on decaying leaves, 4 Apr. 2012, Z. F. Yu, holotype YMFT 1.04156, ex-type living culture YMF 1.04156 = CGMCC 3.18919.

Notes: *Triscelophorus guizhouensis* is similar to *T. acuminatus* Nawawi and *T. monosporus* Ingold, they all have three lateral branches at the base, but *T. acuminatus* has larger and more septate conidia [70] and *T. monosporus* has shorter conidiophores and larger and much less septate conidia [68].

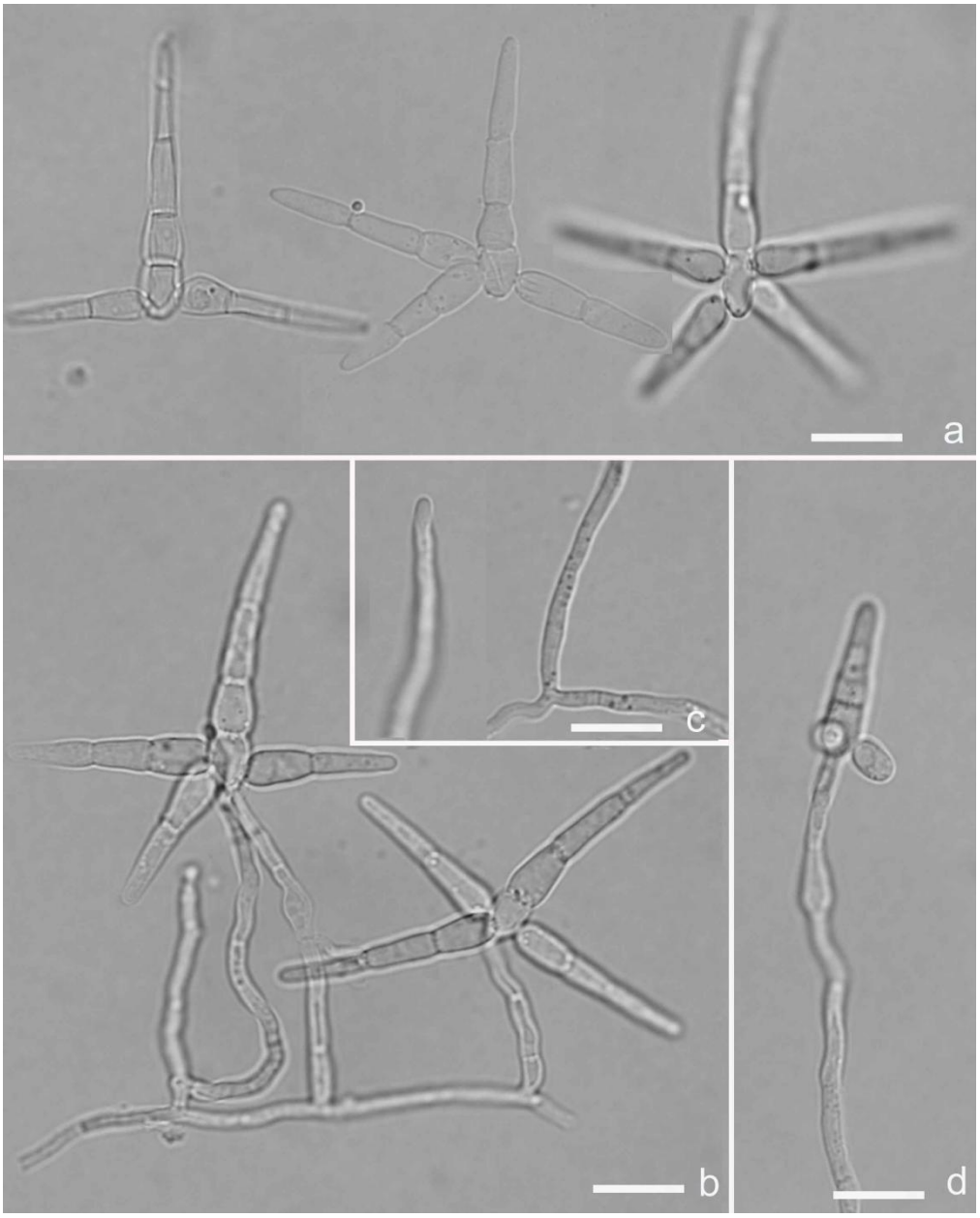

Figure 13. ***Triscelophorus mugecuoensis*** (YMFT1.04593, **holotype**). **a** detached conidia. **b** conidia on conidiophores. **c** the apex and the base of conidiophore. **d** developing conidia. Scale bars: **a–d** = 10 μm.

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits using the ITS sequence (GenBank MK569514) of *T. guizhouensis* are *T. monosporus* (GenBank KF730840; Identities = 191/194 (98 %), Gaps = 0/194 (0 %)) and *T. konajensis* (GenBank MK569513; Identities = 197/207 (95 %), Gaps = 2/207 (0 %)). In addition, the similarity of ITS sequence of *T. guizhouensis* with phylogenetically close *Triscelophorus* species, *T. multibrachiatus* and *T. mugecuoensis*, are 85 % (412/484, Gaps = 30/484 (6 %)) and 85 % (412/485, Gaps = 32/485 (6 %)), respectively.

Triscelophorus mugecuoensis Z. F. Yu, M. Qiao & R. F. Castañeda **sp. nov.** (Figure 13, 16L)

Index Fungorum number: IF556151, Facesoffungi number: FoF 05750

Etymology. Latin mugecuoensis, referred to the locality where the fungus was found.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* on CMA, attaining about 1.4 cm diam. after 20 days at 25°C, white to pale mouse grey, reverse mouse grey. *Mycelium* superficial and immersed, composed of branched, septate, hyaline hyphae. *Conidiophores* macronematous, mononematous, sinuate, unbranched, smooth, hyaline, up to 40 μm long. *Conidiogenous cells* monoblastic, cylindrical, terminal, integrated, determinate, smooth, hyaline. *Conidia* solitary, staurospore, acrogenous, septate, composed of a main axis and 2–4 (mostly3) lateral branches: i) main axis obclavate, 3-septate, slightly constricted at the septa, straight or slightly curved toward the apex, smooth, hyaline, 27–38 × 4–5 μm; ii) lateral branches obclavate, 1–2-septate, straight, smooth, hyaline, 14–24 × 2.7–4.3 μm, arising verticillate from basal cell of the main axis.

Material examined. CHINA, Chongqing, Dafengbao Nature Reserve, on decaying leaves, 17Aug. 2015, J. Peng, holotype YMFT 1.04593, ex-type living culture YMF1.04593.

Notes: *Triscelophorus mugecuoensis* resembles *T. konajensis* K.R. Sridhar & Kaver in the number of lateral branches and the size of conidia, but the latter has longer and wider main axis of conidia (20–35× 3.5–4 μm) and are 3–4-septate [69]. Morphologically, *T. mugecuoensis* also similar to *T. monosporus* in the lateral branches number of conidia. However, it can easily be distinguished from *T. monosporus* by having smaller and much less septatec onidia.

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits using the ITS sequence (GenBank MK569508) of *T. mugecuoensis* are *T. monosporus* (GenBank KF730840; Identities = 200/204 (98 %), Gaps = 1/204(0%)) and *T. konajensis* (GenBank MK569513; Identities = 200/207 (97 %), Gaps = 2/207 (0 %)).

Triscelophorus multibrachiatus Z. F. Yu, M. Qiao & R. F. Castañeda **sp. nov.** (Figure 14, 16M)

Index Fungorum number: IF556150, Facesoffungi number: FoF 05749

Etymology. Latin, multi-, means many, + -brachiatus, referred to the branches.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* on CMA, attaining about 1.7 cm diam. after 20 days at 25°C, white to dark salmon, reverse iron grey. *Mycelium* superficial and immersed, composed of branched, septate, hyaline hyphae. *Conidiophores* macronematous, mononematous, cylindrical, erect, straight or flexuous, unbranched, smooth, hyaline, up to 27–53 μm long. *Conidiogenous cells* monoblastic, cylindrical, terminal, integrated, determinate, smooth, hyaline. *Conidia* solitary, staurospore, acrogenous, septate, composed of a main axis and 2–5 lateral branches: i) main axis obclavate, long obclavate-subulate, 2–4-septate, sometimes slightly constricted at the septa, straight, somewhat curved toward the apex, smooth, hyaline, 29.3–48.8 ×3.5–5.5 μm; ii) lateral branches obclavate or long obclavate, 0–3-septate, straight, smooth, hyaline, 5.8–32.4 × 2.7–4.7 μm, arising from both side or unilateral from the basal and supra cells of the main axis, sequential, opposite, more or less parallel or divergent arranged.

Material examined. CHINA, Yunnan Province, Yulong Snow Mountain Nature Reserve, on decaying leaves, 17 Aug. 2015, Z. F. Yu, holotype YMFT 1.04596, ex-type living culture YMF 1.04596 = CGMCC 3.18981.

Notes: *Triscelophorus multibrachiatus* is characterized by conidia with 4–5 lateral branches not in a whorl. Among known *Triscelophorus* species, only conidia of *T. magnificus* R.H. Petersen and has over 4 lateral branches, but conidia of *T. magnificus* have longer and narrower main axis (55–75 × 2–2.9 μm), and lateral branches arise from the third cell of the base of main apex [71].

Triscelophorus neoseptatus Z. F. Yu, M. Qiao & R. F. Castañeda **sp. nov.** (Figure 15, 16J)

Index Fungorum number: IF556147, Facesoffungi number: FoF 05746

Etymology. Latin, neoseptatus, referred to the resemblance with T. septatus in the conidial shape.

Figure 14. ***Triscelophorus multibrachiatus*** (YMFT1.04596, **holotype**). a conidia. b conidia connecting conidiophore. Scale bars: **a, b** = 10 μm.

Sexual morph undetermined. Asexual morph hyphomycetous. *Colonies* on CMA, attaining about 1.8 cm diam. after 20 days at 25°C, white to pale olivaceous grey, reverse dark brown. *Mycelium* most immersed, composed of branched, septate, subhyaline to hyaline hyphae. *Conidiophores* macronematous, mononematous, cylindrical, erect, straight or flexuous, unbranched or sparingly branched, smooth, hyaline, up to 40 μm long. *Conidiogenous cells* monoblastic, cylindrical terminal, integrated, determinate, smooth, hyaline. *Conidia* solitary, acrogenous, staurospore, septate, composed of a main axis and 3 lateral branches: i) main axis elongate obclavate-subulate, 3–5-septate, flexuous, sometimes slightly curved, smooth, hyaline, 47.3–54.1 × 3.0–4.1 μm; ii) lateral branches obclavate-subulate, straight or curved, 1–4 septate, smooth, hyaline, 25–40.5 × 2.3–3.5 μm arising verticillate from main axis basal cell.

Material examined. CHINA, Sichuan Province, Le Mountain natural reserve, on decaying leaves, 24 Nov. 2015, Z. F. Yu, holotype YMFT 1.04310, ex-type living culture YMF 1.04310 = CGMCC 3.18979.

Notes – *Triscelophorus neoseptatus* resembles *T. septatus* Wolfe in the shape and size of conidia, but in *T. septatus*, the number of septa of the main axis was not mentioned in the original publication, but illustrated conidia are up to 9-septate [72] which are more than those in *T. neoseptatus*. Also *T. acuminatus* superficially resembles *T. neoseptatus* in the shape and size of conidia. However, *T. acuminatus* is distinguished by longer

main axis and lateral branches (main axis 44–66 × 3.5–5 μm, lateral branches 21–54 × 3–4.5 μm). In the phylogenetic analysis inferred from ITS, *T. neoseptatus* formed a separate clade, as a sister clade of *T. acuminatus*.

Based on a megablast search of NCBIs GenBank nucleotide database, the closest hits using the ITS sequence (GenBank MK569510) of *T. neoseptatus* are *T. monosporus* (GenBank KX858630; Identities = 394/455 (87 %), Gaps = 26/455 (5 %)).

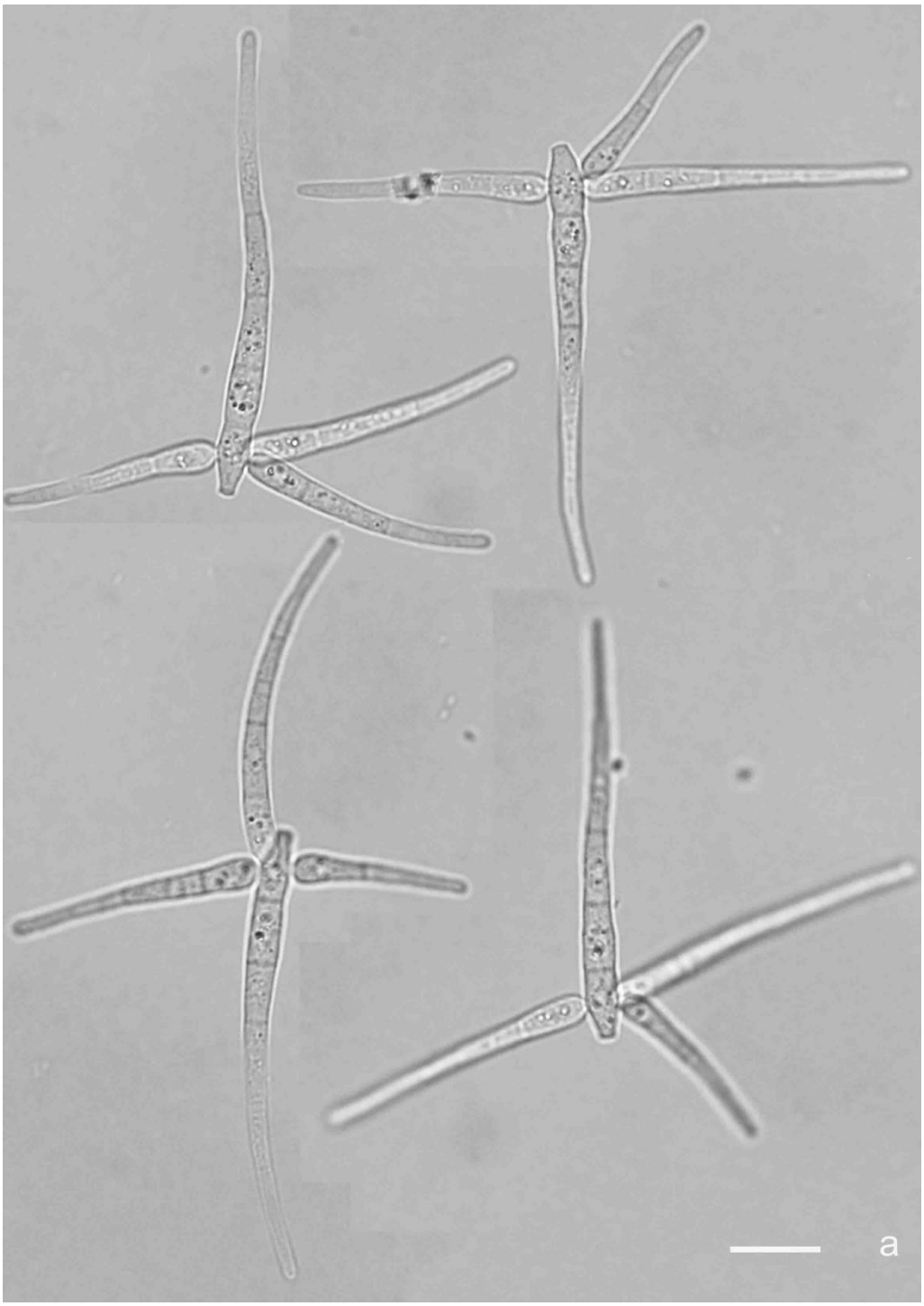

Figure 15. *Triscelophorus neoseptatus* (YMFT1.04310, holotype) **a** conidia. Scale bars: 10 μm.

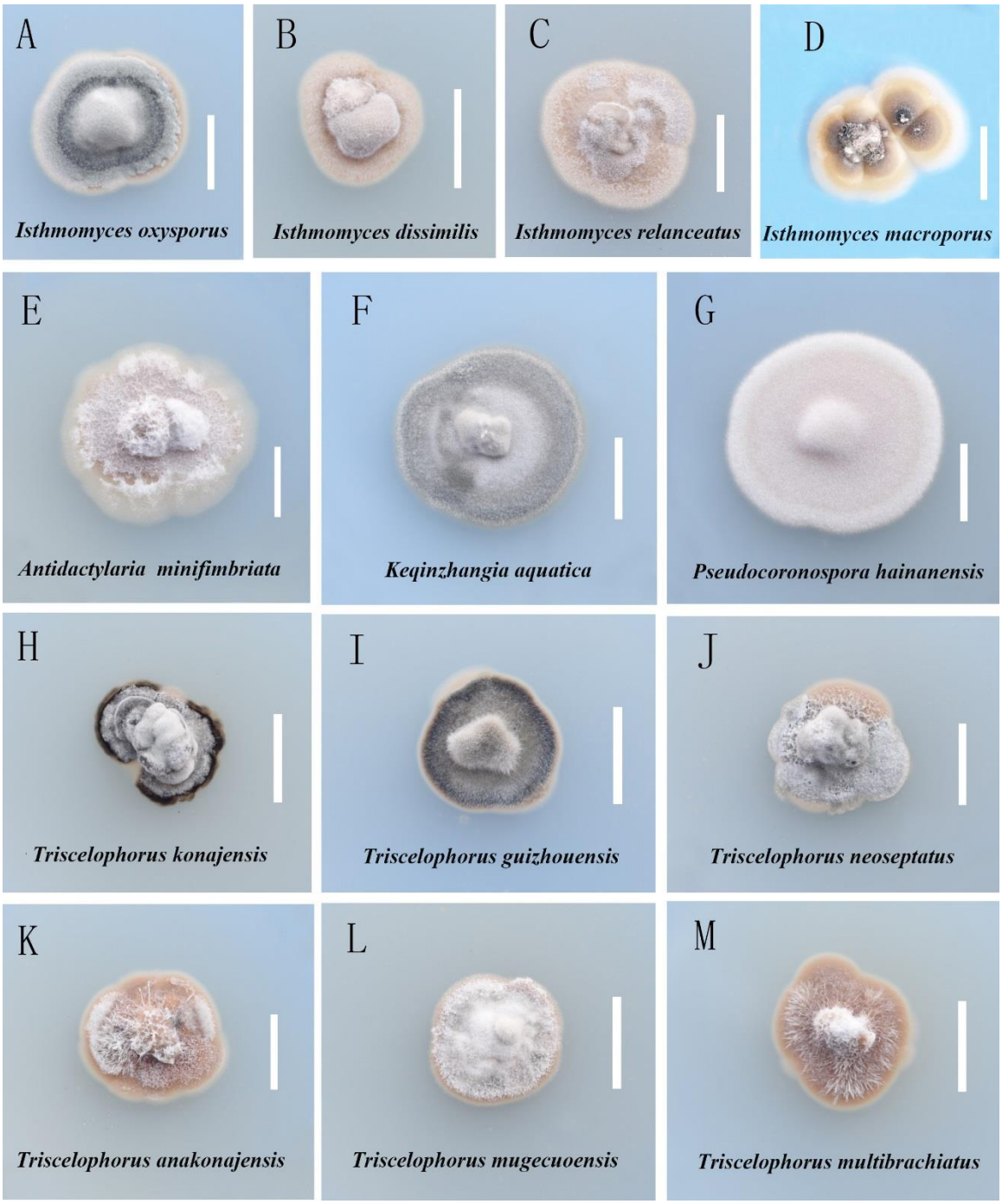

Figure 16. Colony after 20 days on CMA at 25°C. Scales bars: a–m = 2 cm.

4. Discussion

Molecular phylogeny of freshwater fungi in Dothideomycetes has been studied by Shearer et al. [73] using SSU and LSU for 84 isolates representing 29 genera. The results showed that the majority of freshwater Dothideomycetes belonged to Pleosporomycetidae, including four clades comprised of only freshwater taxa while the remaining freshwater taxa were distributed among other clades. In the largest phylogenetic assessment of Dothideomycetes by 2009, members of the class from various ecological niches were included, and freshwater were in different clades [12]. Unfortunately, like other studies, though representative, these two studies of Dothideomycetes and freshwater ascomycetes had very few aquatic asexual genera. In the paper of Shearer et al. [73], only 10 asexual genera were included, while in the paper of Schoch et al. [12], only four asexual genera were included (*Monotosporella* S. Hughes and *Beverwykella* Tubaki belonging to *Melanom-*

mataceae G. Winter, while *Helicomyces* Link and *Helicosporium* Nees belonging to *Tubeufiaceae*). Among the accepted genera of Dothideomycetes, only 11 aquatic or aero-aquatic asexual genera have been described as belonging to different families of the subclass Pleosporomycetidae [9]. By our addition of new aquatic hyphomycetes to *Microthyriaceae* makes this family the largest of Pleosporomycetidae comprising aquatic asexual genera.

With increasingly widespread use of molecular techniques, multigenes were concatenated to resolve phylogenetic affiliations and taxonomic placements at family or higher ranks. For example, nuc*SSU*, nuc*LSU* rDNA, *TEF*1, *RPB*1 and *RPB*2, were combined to assess phylogeny [9, 11, 12]. However, sequence data and cultures of many aquatic hyphomycetes were unavailable. By 2013, over 300 aquatic hyphomycete species had been described based on conidia morphology and conidiogenesis. However, fewer than 50 species had published ITS sequences in the International Nucleotide Sequence Database [74]. In addition, most of these species with ITS sequences were considered Ascomycota genera incertae sedis because of the limitations of ITS as a phylogenetic marker for these organisms. Duarte et al. [26] found that *Triscelophorus* spp. were polyphyly based on ITS analysis, but did not determine its phylogenetic position. We carried out phylogenetic analysis based on all ITS of *Triscelophorus* spp. available from GenBank and generated from our *Triscelophorus* strains, found four strains of *T. cf. acuminatus* (GenBank accession number: KF730836 – 838) and one unnamed *Triscelophorus* strain (KF730841) formed a clade, but other 6 unnamed *Triscelophorus* strains (Genbank accessible number: KF730842 – 847) fell into another clade, we suspected these six strains might be misidentified at genus level. Based on our four-loci phylogenetic analysis, in our opinion, *Triscelophorus* should be monophylogenetic. At present, the majority of ascomycetous aquatic hyphomycetes were only placed at the class level [75-77]. In order to bring more aquatic hyphomycetes into lower taxonomic levels, it is necessary to obtain sequences from more genes.

The most obvious result achieved on the phylogeny of aquatic hyphomycetes is that the multiple origins of aquatic hyphomycetes was found [22]. So far, at least 14 genera have shown to be polyphyletic using sequence information from a single or two genes [23-26, 78]. Likewise, polyphyly of *Isthmolongispora* was found for the first time here. Although there are 9 ITS sequences, one sequence is from *I. lanceate*, and another one from *I. ampulliformis* (Tubaki) de Hoog & Hennebert, while other 7 sequences were from unidentified species. Three LSU sequences were available from GenBank. However, *Isthmolongispora* was listed as Ascomycota genera *incertae sedis* [17]. These results show it is necessary to sequences more loci for confirm polyphyly or monophyly.

Difficulty in conidia development may be the reason why the asexual states of *Microthyriaceae* have not been found. According to previous studies, cultures were isolated but no sporulation [13, 14]. According to our experience from studying aquatic hyphomycetes and their relevant relationships in *Microthyriaceae*, in our opinion, it is necessary to induce conidia using various methods. Aquatic hyphomycetes often grow slowly, and do not produce generative structures easily. Based on our experiment, conidia can be induced after preserving the isolates at a low temperature of 4°C, sometimes water is needed. Anyhow, development of conidia takes about two weeks or longer.

Morphologically, five asexual genera of *Microthyriaceae* do not have any characteristics in common. Genetically there are large differences between each other, including between species within the same genus. For example, ITS of *Isthmomyces lanceatus* has 98.02 similarity with that of *I. relanceatus*, but only 59.76% similarity between *I. lanceatus* and *I. macroporus*. Within *Triscelophorus*, the largest ITS similarity of 95.83% is between *T. anakonajensis* and *T. mugecuoensis*, while the lowest ITS similarity of 73.96% is between *T. anakonajensis* and *T. mugecuoensis*. The larger genetic difference suggests that there are likely many more un-identified taxa in this genus and/or that the evolution of ITS in *Microthyriaceae* is very fast.

In this study, all taxa were described based on their asexual characteristics. Although we observed cultures for long time on CMA, we did not see any sexual reproductive structures. According to our phylogenetic analyses, only *Ishmomyces* is closely related to the genus *Microthyrium*, but their ITS sequence similarity is low, so we can't determine the connection between them.

In conclusion, this study described four new genera and 13 new species of aquatic hyphomycetes. Our phylogenetic analyses placed several other aquatic genera in family *Microthyriaceae*. Though we failed to connect teleomorphs and anamorphs at genus level, our results showed close phylogenetic relationships between

aquatic hyphomycetes and *Microthyriaceae* at the family rank. This study also revealed the importance of obtaining pure cultures of aquatic fungi and multiple gene sequences from them in order to identify the origins and phylogenetic positions of aquatic hyphomycetes and their relationships with their terrestrial relatives.

Author Contributions: KZ, and ZY conceived and designed the study. MQ, JG and HZ wrote the manuscript. JG and JP conducted the experiments. R.F.C contributed actively in the identification and the taxonomy of the fungal strains. ZY and JX revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financed by the National Natural Science Foundation Program of PR China (31770026, 31760012).

Acknowledgments: We are grateful to two reviewers for critically reviewing the manuscript and for providing helpful suggestions to improve this paper.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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