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Four new species of the genus *Hymenoscyphus* (fungi) based on morphology and molecular data

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Four new species of *Hymenoscyphus* (*H. brevicellulus*, *H. hyaloexcipulus*, *H. microcaudatus*, and *H. subsymmetricus*) and a new Chinese record (*H. subpallescens*) are described. These five species share common characteristics: small apothecia (<3 mm in diameter); hymenium whitish, pale yellow, to yellow in color; ectal excipulum of textura prismatica; asci arising from simple septa; ascospores scutuloid and guttulate; saprophytic nutrition; and leaf habitats, except for *H. subsymmetricus*, which grows on herbaceous stems. Phylogenetic analyses of internal transcribed spacer nuclear ribosomal DNA sequences, the universal DNA barcode for fungi, for 16 species in the genus indicated that these taxa were closely related to *H. microserotinus*, in accordance with their morphological features, but represented independent species. The distinguishing features of each new species from its relatives are discussed, and their phylogenetic relationships explored.

Helotiaceae, morphology, sequence analyses, taxonomy

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Hymenoscyphus Gray is a cosmopolitan genus in Helotiaceae (Helotiales, Leotiomycetes, Ascomycota) that has an estimated 155 species [1]. The genus was established in 1821 [2] and lectotypified by *H. fructigenus* (Bull.) Gray [3]. The known species are mainly reported from Europe [2,4–6], America [7–10] and Asia [11–14]. Species of the genus are normally saprophytic on plant debris, such as wood, twigs, fruits, leaves, and herbaceous stems. Morphologically, they are characterized by stipitate to sessile discoid apothecia; white to yellowish hymenium surface; ectal excipulum typically of textura prismatica or sometimes mixed with textura angularis; and subellipsoid, fusoid, or scutuloid ascospores [3, 6,12,15].

Published information on the economic importance of the genus is scattered. Extracellular degradative enzymes are produced by *H. scutula* (Pers.) W. Phillips [16], and useful compounds with antimicrobial and cytotoxic activities were found from *H. epiphyllus* (Pers.) Rehm ex Kauffman [17]. *Chalara fraxinea* T. Kowalski, the anamorphic stage of *H. pseudoalbidus* Queloz, Grünig, Berndt, T. Kowalski, T.N. Sieber & Holdenr [18], is reported to cause ash dieback disease in forest nurseries and has emerged in many European countries [19].

Studies on *Hymenoscyphus* have mostly concentrated on species diversity and morphology [3,6,8,9,12]. Species identification has been mainly based on morphological characters, such as apothecia size and color, ectal excipulum structure, and shape and size of asci and ascospores. Some of these characters may be influenced by environmental factors and developmental stages, making identification difficult. Recently, sequence data of the nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) have been used to aid species delimitation [18,20,21] and rapid identification [22]. Population structure and genetic variability of

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a few economically important species have also been studied [23,24].

In China, the first record of *Hymenoscyphus* was reported by S. C. Teng in 1934 [25]. Thus far, a total of 27 species have been identified in the country [15,26–31], most of which are saprophytic. As part of our ongoing study of the Chinese fungal flora, new species of the genus have been described and new Chinese records reported [13–15,30,31]. Here, based on the examination of additional collections, we describe four species new to science that morphologically resemble and are closely related to *H. microserotinus* and report one species new to China. In addition to morphological observations, ITS, the universal DNA barcode for fungi [32], was also analyzed to aid species identification.

1 Materials and methods

1.1 Materials studied

The specimens examined were collected from Anhui, Hainan, and Yunnan Provinces and deposited in the Mycological Herbarium, Institute of Microbiology, Chinese Academy of Sciences (HMAS), Beijing. A total of 35 ITS sequences representing 16 species of *Hymenoscyphus* and two outgroup taxa were analyzed (Table 1).

1.2 Morphological observations

Colors of fresh apothecia were recorded in field notes. Dried apothecia were rehydrated with distilled water and sectioned at a thickness of 10–20 μm with a freezing mi-

Table 1 Internal transcribed spacer sequences used in this study[a)]

Species	Collection number or source	GenBank accession number
Hymenoscyphus brevicellulus H.D. Zheng & W.Y. Zhuang	HMAS 264015	**JX977149**[a)]
	HMAS 264016	**JX977155**
	HMAS 264017	**JX977158**
	HMAS 264018	**JX977162**
	HMAS 264019	**JX977163**
H. caudatus (P. Karst.) Dennis	HMAS 82057	AY348576
	HMAS 82060	AY348577
H. crataegi Baral & R. Galán	F156966	DQ431177
H. fructigenus (Bull.) Gray	HMAS 75893	**JX977144**
	ARON3264.H	AJ430396
H. fucatus (W. Phillips) Baral & Hengstm.	HMAS 264028	**JX977147**
	HMAS 264029	**JX977148**
H. ginkgonis J.G. Han & H.D. Shin	KUS-F51352	EU096525
	KUS-F51854	EU219982
H. globus W.Y. Zhuang & Yan H. Zhang	HMAS 82107	AY348593
H. hyaloexcipulus H.D. Zheng & W.Y. Zhuang	HMAS 188542	**JX977145**
	HMAS 188538	**JX977146**
H. immutabilis (Fuckel) Dennis	HMAS 71809	AY348584
H. macroguttatus Baral, Declercq & Hengstm.	HB703	DQ431179
H. microcaudatus H.D. Zheng & W.Y. Zhuang	HMAS 264020	**JX977156**
H. microserotinus (W.Y. Zhuang) W.Y. Zhuang	HMAS 68520	DQ986481
	HMAS 264030	**JX977150**
	HMAS 264031	**JX977151**
	HMAS 264032	**JX977152**
H. scutula (Pers.) W. Phillips	HMAS 82092	AY348589
	HMAS 82093	AY348590
H. serotinus (Pers.) W. Phillips	F093261	DQ431168
H. subpallescens Dennis	HMAS 264022	**JX977154**
	HMAS 264024	**JX977157**
	HMAS 264025	**JX977159**
	HMAS 264026	**JX977160**
	HMAS 264027	**JX977161**
H. subsymmetricus H.D. Zheng & W.Y. Zhuang	HMAS 264021	**JX977153**
Hyaloscypha aureliella (Nyl.) Huhtinen	M234	EU940228
Hya. fuckelii Nannf.	M233	EU940230

a) Numbers in bold indicate newly submitted sequences.

crotome (YD-1508A, Yidi Medical Instrument Co., Jinhua, China). Measurements were taken from longitudinal sections and from squash mounts in lacto-phenol cotton blue solution using an Olympus BH-2 microscope (Tokyo, Japan). Iodine reactions of ascus apparatuses were tested in Melzer's reagent. Photographs were taken using a Canon G5 digital camera (Tokyo, Japan) connected to a Zeiss Axioskop 2 Plus microscope (Göttingen, Germany) for anatomical structure and to a Zeiss Stemi 2000C stereomicroscope for gross morphology.

1.3 Molecular methods

Genomic DNA was extracted from dried apothecia using the CTAB procedure with some modification [33]. Sequences of ITS nrDNA were amplified and sequenced with primers ITS1 and ITS4 [33]. DNA sequencing was performed on an ABI 3730 XL DNA Sequencer (Applied Biosciences, Foster City, CA, USA) at the Shanghai Majorbio Bio-pharm Technology Co., Ltd, Beijing Branch, China.

New sequences were assembled and manually edited using the program BioEdit 7.0.5.3 [34]. Alignment was generated with MUSCLE v. 3.6 [34], manually adjusted using BioEdit 7.0.5.3, and converted to nexus files in ClustalX 1.83 [35].

Neighbor-joining (NJ) analysis was performed in PAUP*4.0b10 [36] with 1000 bootstrap replicates. Unweighted maximum parsimony (MP) heuristic searches were performed with 1000 of random-addition replicates in PAUP*4.0b10 (TBR branch-swapping algorithm; MaxTrees=1000 and auto-increased by 100; MulTrees not in effect; 1000 bootstrap replicates, each with 10 random-addition replicates). Trees were examined in TreeView 1.6.6 [37], with neighbor-joining bootstrap proportions (NJBP) and maximum parsimony bootstrap proportions (MPBP) greater than 50% shown at the nodes. Sequence similarities were calculated using [38].

2 Results

2.1 Taxonomy

2.1.1 New species

(i) ***Hymenoscyphus brevicellulus*** H.D. Zheng & W.Y. Zhuang, **sp. nov.** (Figures 1A and 2)

Fungal name FN570045

Differing from *H. ginkgonis* in smaller asci and ascospores, habit, and ITS sequence; and from *H. microserotinus* in narrower asci and ascospores and in ITS sequence.

Apothecia flat to discoid, 0.3–1.4 mm in diam., stipitate; stipe 0.4–1 mm long. Hymenium surface whitish, cream to pale beige, drying whitish to pale yellowish, receptacle surface lighter. Outer covering layer present, of 1–2 hyphal layers, hyphae 3–4 μm wide. Ectal excipulum of textura prismatica, 20–50 μm thick; outer 2–3 layers of short rectangular to isodiametric cells, inner layer of long rectangular cells, cells and hyphae hyaline, walls about 1 μm thick, 6–25(–40)×4–10 μm. Medullary excipulum of two layers; outer layer of textura porrecta, 35–80 μm thick; inner layer of textura intricata, 55–180 μm thick; hyphae hyaline, 2.5–5(–7) μm wide. Stipe surface glabrous to having scarce hairs below; hairs septate, ends finger-shaped, about 10 μm long; basal hyphae hyaline to pale brown. Subhymenium not distinguishable. Hymenium 60–75 μm thick. Asci arising from simple septa, cylindrical-clavate, apex papillate, 8-spored, J+ in Melzer's reagent, pore walls bluing as two lines, 52–77×5.5–7.7 μm. Ascospores biseriate to biseriate above and uniseriate below, hyaline, scutuloid, slightly flattened on one side, with anterior ends round and slightly curved and posterior ends pointed, with (1–)2(–4) large oil drops, 12–19.8×2.5–3.5(–3.9) μm. Paraphyses filiform, 1.5–2.5 μm wide.

Etymology: The specific epithet refers to the short cells in the ectal excipulum.

Holotype: CHINA, Anhui, Jinzhai, Tiantangzhai, alt. 900–1000 m, on rotten leaf veins of unidentified trees, 24 Aug. 2011, S.L. Chen, W.Y. Zhuang, H.D. Zheng & Z.Q. Zeng 7841, HMAS 264018.

Additional material examined: CHINA, Anhui, Jinzhai, Tiantangzhai, alt. 700–900 m, on rotten leaf veins of trees, 22 Aug. 2011, S.L. Chen, W.Y. Zhuang, H.D. Zheng & Z.Q. Zeng 7728, HMAS 264015; *ibid*., alt. 900–1000 m, on rotten leaf veins of trees, 24 Aug. 2011, S.L. Chen, W.Y. Zhuang, H.D. Zheng & Z.Q. Zeng 7823, 7830, 7843, HMAS 264016, 264017, 264019.

Notes: Morphologically, *H. brevicellulus* is very similar

Figure 1 Dry apothecia of *Hymenoscyphus* spp. under a dissecting microscope showing habit and gross morphology. A, *H. brevicellulus* (HMAS 264018). B, *H. hyaloexcipulus* (HMAS 188542). C, *H. microcaudatus* (HMAS 264020). D, *H. subsymmetricus* (HMAS 264021); E, *H. subpallescens* (HMAS 264022). Scale bar, 0.5 mm.

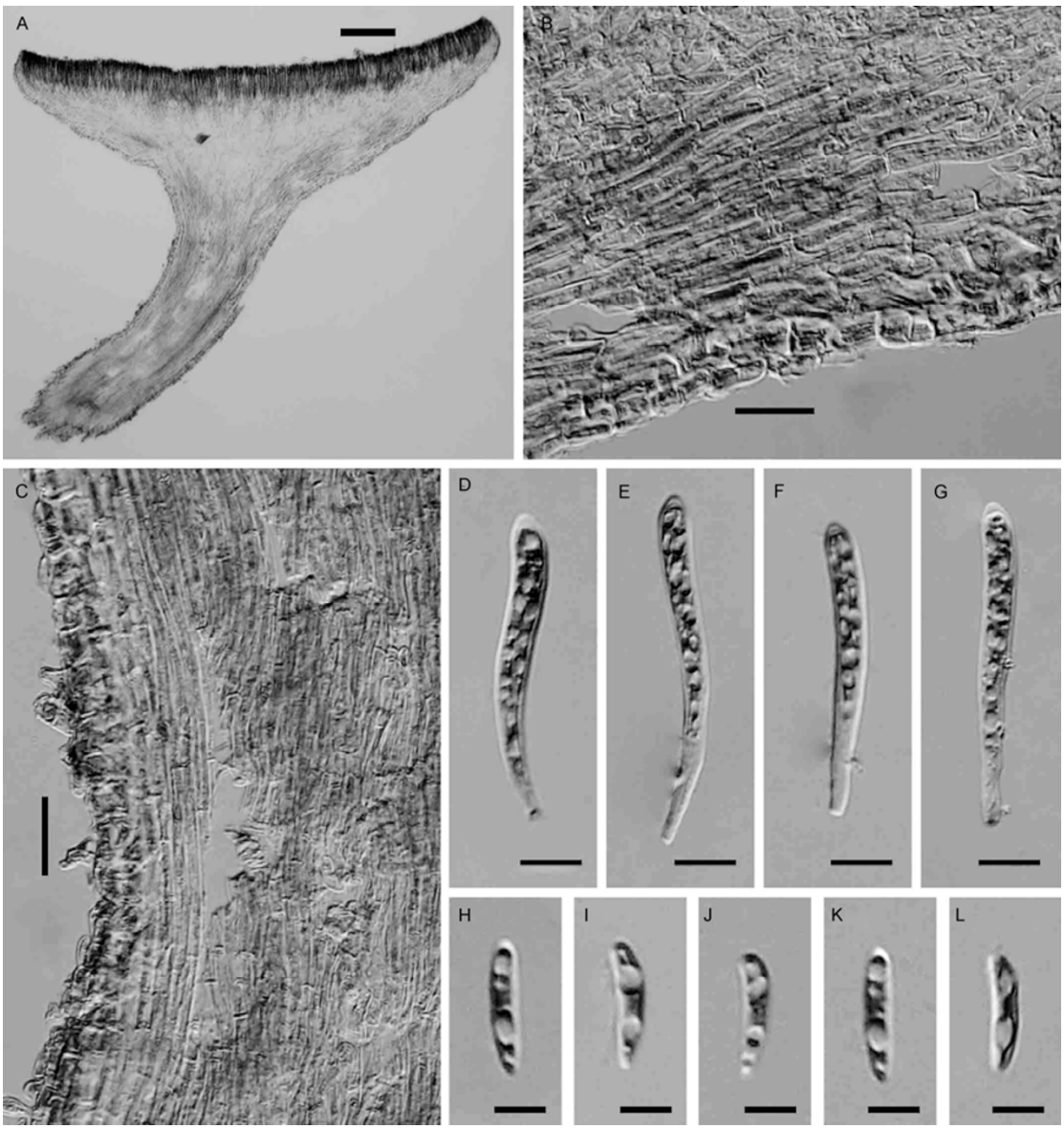

Figure 2 *Hymenoscyphus brevicellulus* (HMAS 264018). A, Longitudinal section of apothecium. B, Excipular structure at flank. C, Structure near stipe base. D–G, Ascus with ascospores. H–L, Ascospore. Scale bars: A, 100 μm; B–C, 20 μm; D–G, 10 μm; H–L, 5 μm.

to *H. ginkgonis* and *H. microserotinus* in ectal excipular structure and ascospore shape but these are obviously different fungi. *Hymenoscyphus ginkgonis* can be easily separated from *H. brevicellulus* by its larger asci (72–98×8–11 μm) and ascospores (17–22×3–4 μm), and it grows on fallen leaves of *Ginkgo biloba* [41]. The holotype of *H. microserotinus* (HMAS 68520) has yellow apothecia, broader asci (58–67×7.5–8.8 μm), and relatively broader ascospores (length/width ratio 3.8–4.4 vs. 4.0–6.0).

Sequence analyses of ITS supported the morphological distinctions between the new species and its two allies. The bootstrap values among the three species varied from less than 50% to 63% NJBP and less than 50% MPBP, whereas those within an individual species were 88%–100% NJBP and 82%–100% MPBP (Figure 7). The ITS sequence similarities of *H. brevicellulus* to *H. ginkgonis* and *H. microserotinus* were 95.5% and 94.6%, respectively.

(ii) ***Hymenoscyphus hyaloexcipulus*** H.D. Zheng & W.Y. Zhuang, **sp. nov.** (Figures 1B and 3).

Fungal name FN570046

Differing from *H. caudatus* in much broader ectal excipular cells, broader ascospores, and ITS sequence.

Apothecia flat to discoid, 0.3–1 mm in diam., stipitate; stipe 0.2–1 mm long. Hymenium surface whitish to pale yellow, drying whitish to pale orange, receptacle surface lighter. Outer covering layer absent. Ectal excipulum of textura prismatica, 20–55 μm thick, composed of 3–4 cell layers, cells hyaline, thin- to slightly thick-walled, walls 1–2 μm thick, short rectangular to isodiametric or nearly so, 6–30(–60)×6–25 μm. Medullary excipulum of two layers; outer layer of textura porrecta, 20–50 μm thick; inner layer

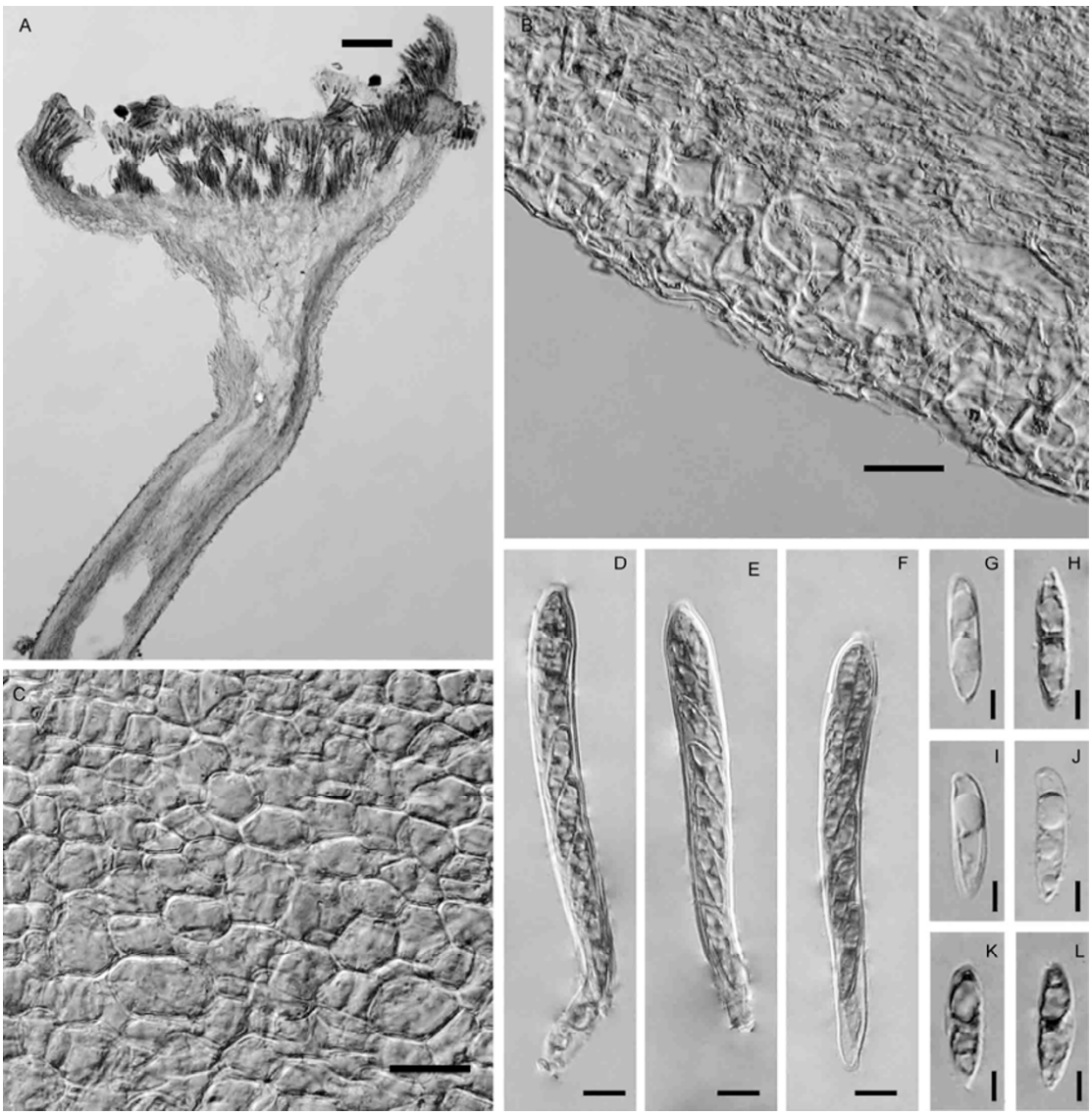

Figure 3 *Hymenoscyphus hyaloexcipulus* (HMAS 188542). A, Longitudinal section of apothecium. B, Excipular structure at flank. C, Surface view of receptacle. D–F, Ascus with ascospores. G–L, Ascospore. Scale bars: A, 100 μm; B–C, 20 μm; D–F, 10 μm; G–L, 5 μm.

of textura intricata, 30–150 μm thick; hyphae hyaline, 3–4 μm wide. Stipe surface glabrous or nearly so, base not dark, hyphae hyaline. Subhymenium present or not distinguishable, 0–40 μm thick. Hymenium 120–165 μm thick. Asci arising from simple septa, cylindrical-clavate, apex papillate, 8-spored, J+ in Melzer's reagent, pore walls bluing as two lines, 110–128×9–12.5 μm. Ascospores uniseriate, biseriate above and uniseriate below to biseriate, hyaline, elliptical-fusoid, anterior ends round and posterior ends pointed, slightly flattened on one side, with (1–)2(–4) large oil drops and smaller ones, 17.5–25.5×5.0–6.5 μm. Paraphyses filiform, 1.5–2.5 μm wide.

Etymology: The specific epithet refers to the refractive cell wall in the ectal excipulum.

Holotype: CHINA, Yunnan, Pingbian, Daweishan, alt. 1900 m, on rotten leaf veins of trees, 4 Nov. 1999, W.Y. Zhuang & Z.H. Yu 3290, HMAS 188542.

Additional material examined: CHINA, Hainan, Changjiang, Bawangling, alt. 1150 m, on rotten leaf veins of trees, 7 Dec. 2000, Y.H. Zhang, Z.H. Yu & W.Y. Zhuang 3682, HMAS 188538.

Notes: Morphologically, *H. hyaloexcipulus* is easily confused with *H. caudatus* because of their similarities in color and size of apothecia and shape and size of asci and ascospores. The two Chinese collections (HMAS 188538, 188542) were previously identified as *H. caudatus* [31]. *Hymenoscyphus caudatus* differs in having much narrower ectal excipular cells [4–22×3–8 μm], and narrower ascospores [(14–)16–22(–26)×4–5(–6) μm] [10].

Although *H. hyaloexcipulus* and *H. caudatus* are morphologically similar, sequence analyses indicated that they are distantly related and belong to different phylogenetic

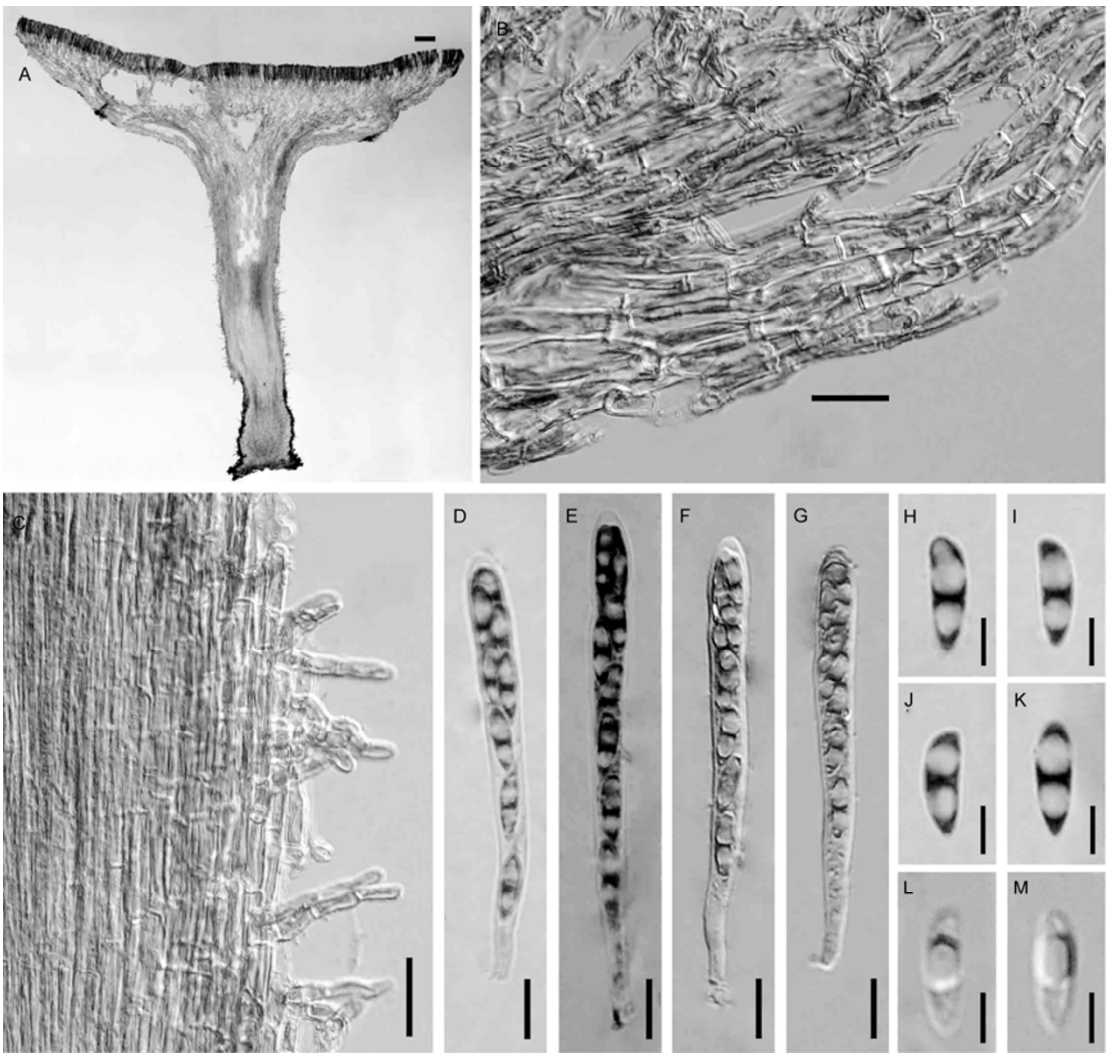

Figure 4 *Hymenoscyphus microcaudatus* (HMAS 264020). A, Longitudinal section of apothecium. B, Excipular structure at flank. C, Structure at middle of stipe. D–G, Ascus with ascospores. H–M, Ascospore. Scale bars: A, 100 μm; B–C, 20 μm; D–G, 10 μm; H–M, 5 μm.

clades (Figure 7). Their ITS sequence similarity was only 86.6%.

Very minor infraspecific variations were detected between the two collections of *H. hyaloexcipulus* from Hainan and Yunnan, such as apothecial size and ascospore guttulation, and the ITS sequences of these accessions differed by five base pairs. However, the differences were not sufficient to recognize them as separate species.

(iii) ***Hymenoscyphus microcaudatus*** H.D. Zheng & W.Y. Zhuang, **sp. nov.** (Figures 1C and 4).

Fungal name FN570047

Differing from *H. caudatus* in longer ectal excipular cells, smaller asci and ascospores, and ITS sequence; and from *H. subpallescens* in yellow apothecia, scutuloid ascospores, and ITS sequence.

Apothecia flat to discoid, 1–2.8 mm in diam., stipitate; stipe about 1.5 mm long. Hymenium surface yellow, drying orange, receptacle surface lighter. Outer covering layer absent. Ectal excipulum of textura prismatica, 30–55 μm thick, composed of elongated cells, cells hyaline, 5–45(–75)×5–8 μm. Medullary excipulum of two layers; outer layer of textura porrecta, 55–85 μm thick; inner layer of textura intricata, 80–220 μm thick; hyphae hyaline, 4–6(–8) μm wide. Stipe surface with dense, short to long hairs from the middle to the base; hairs septate, ends finger-shaped, 15–30 μm long; base dark, cells brownish. Subhymenium not distinguishable. Hymenium about 90 μm thick. Asci arising from simple septa, cylindrical-clavate, apex broadly conical, 8-spored, J+ in Melzer's reagent, pore walls bluing as two lines, 78–91.5×6.6–8.3 μm. Ascospores biseriate above and uniseriate below, hyaline, scutuloid, slightly flattened on one side, with anterior ends round and slightly curved and posterior ends pointed, with (1–)2 large oil drops, 12–14.3×3.9–4.4 μm. Paraphyses filiform, 2–2.5 μm wide.

Etymology: The specific epithet refers to the smaller ascospores compared with those of *H. caudatus*.

Holotype: CHINA, Anhui, Jinzhai, Tiantangzhai, alt.

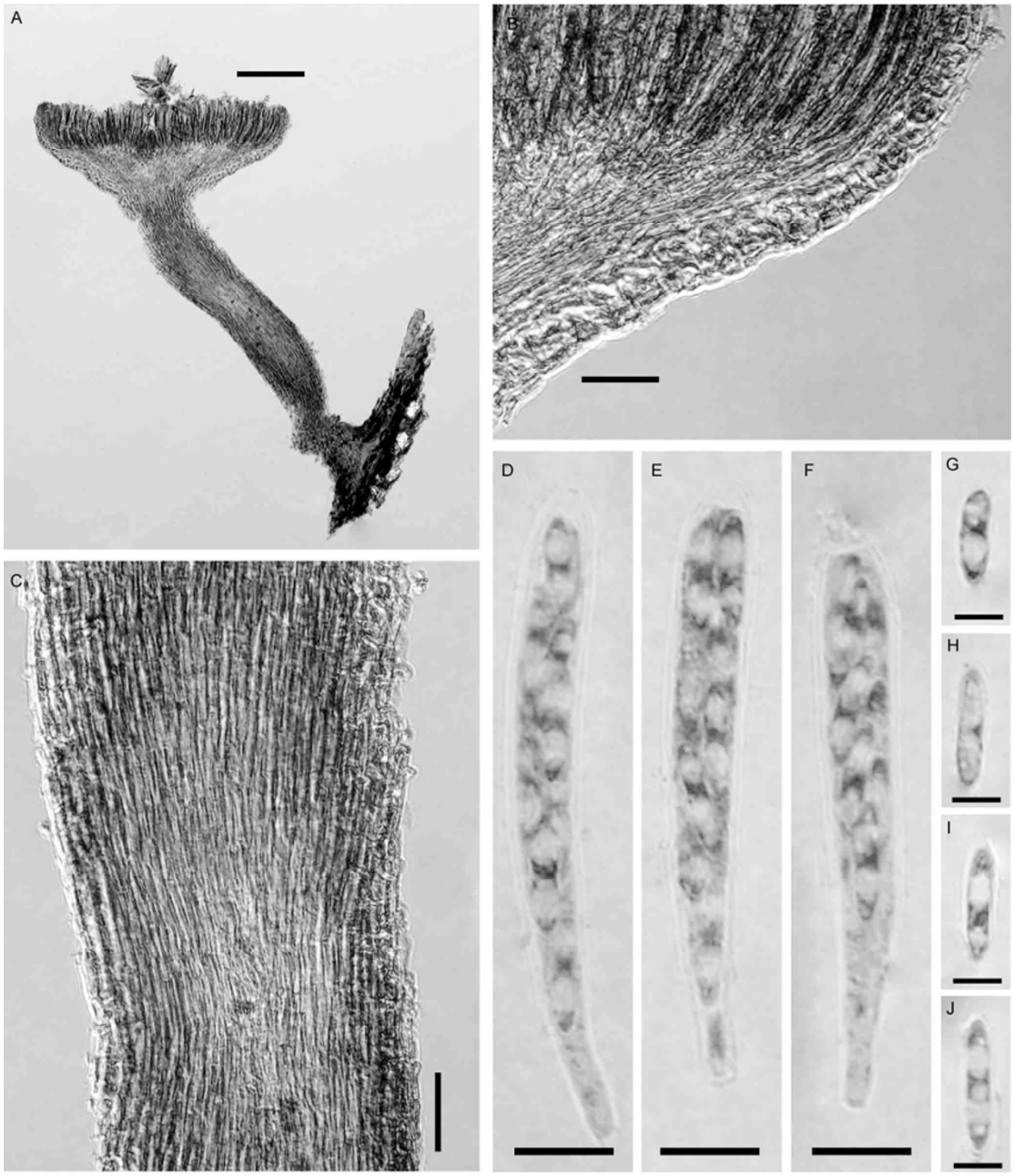

Figure 5 *Hymenoscyphus subsymmetricus* (HMAS 264021). A, Longitudinal section of apothecium. B, Excipular structure at flank. C, Structure in the middle of stipe. D–F, Ascus with ascospores. G–J, Ascospore. Scale bars: A, 100 μm; B–C, 20 μm; D–F, 10 μm; G–J, 5 μm.

900–1000 m, on petioles of rotten leaves, 24 Aug. 2011, S.L. Chen, W.Y. Zhuang, H.D. Zheng & Z.Q. Zeng 7824, HMAS 264020.

Notes: The ascospore shape of *H. microcaudatus* is very similar to that of *H. caudatus*. The latter is a common species in China and can be easily recognized by smaller (0.75–1.0 mm) white to pale yellow apothecia, shorter ectal excipular cells (4–22×3–8 μm), and larger asci [(90–)105–140(–150)×8–12(–15) μm] and ascospores [(14–)16–23(–26)×4–5(–6) μm] [10].

Hymenoscyphus subpallescens is also similar to the new species in excipular structure and size of asci and ascospores, but differs in having lighter apothecia (dirty white, pale beige to pale yellow vs. yellow) and symmetrically fusoid ascospores with pointed instead of scutuloid ends. *Hymenoscyphus phyllogenus* (Rehm) Kuntze is similar in size of ascospores and foliicolous habit but is distinguishable by smaller (0.3–0.5 mm in diam.) white apothecia, narrower ectal excipular cells (15–20×4–5 μm), and guttulate ascospores with more or less round ends [5,7].

Sequence analyses of ITS showed that *H. microcaudatus*, in Clade III, was distantly related to the morphologically

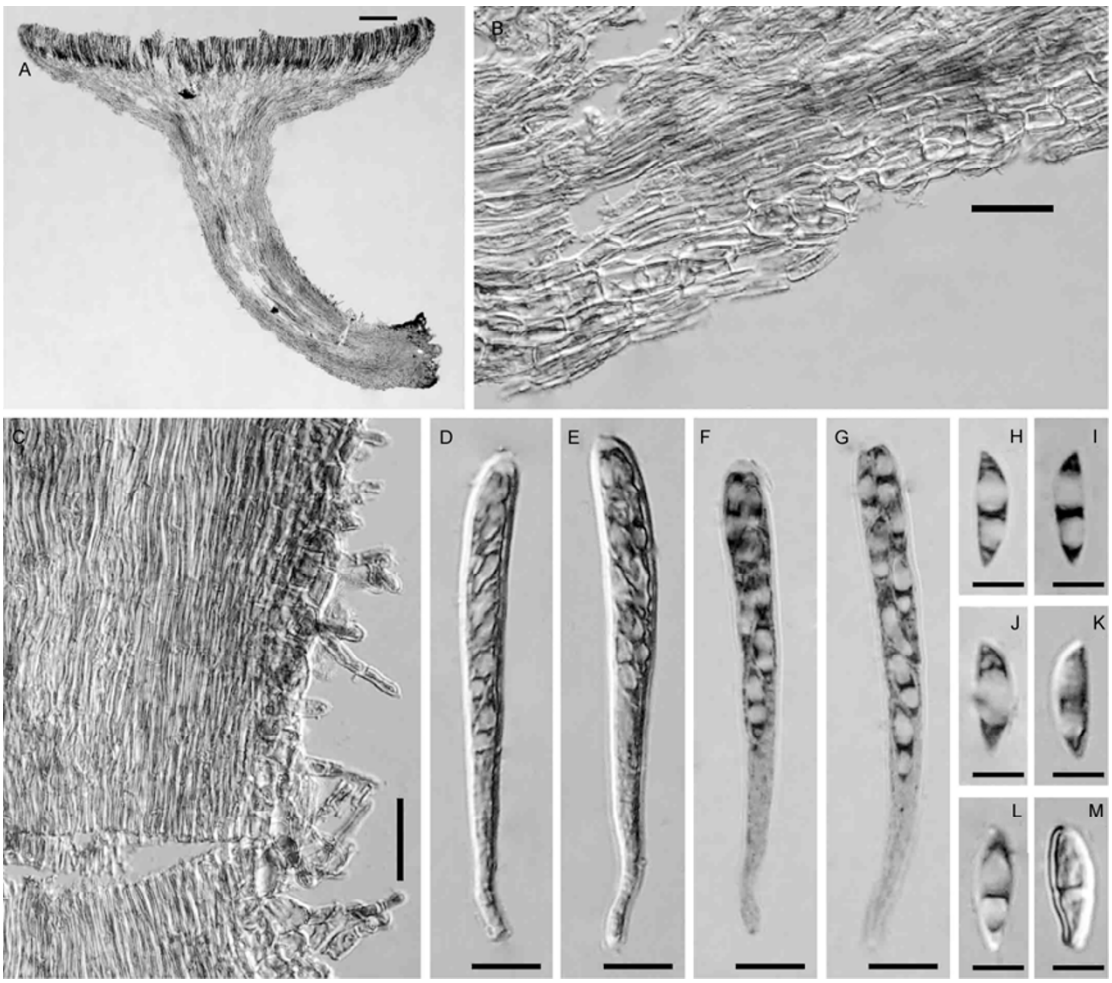

Figure 6 *Hymenoscyphus subpallescens* (HMAS 264022). A, Longitudinal section of apothecium. B, Excipular structure at flank. C, Structure near stipe base. D–G, Ascus with ascospores. H–M, Ascospore. Scale bars: A, 100 μm; B–C, 20 μm; D–G, 10 μm; H–M, 5 μm.

similar *H. caudatus* (Clade II) and *H. subpallescens* (Clade III) (Figure 7).

(iv) ***Hymenoscyphus subsymmetricus*** H.D. Zheng & W.Y. Zhuang, **sp. nov.** (Figures 1D and 5)

Fungal name FN570048

Differing from *H. brevicellulus* in smaller apothecia, cylindrical-fusoid ascospores, growing on herbaceous stems, and ITS sequence.

Apothecia flat to discoid, 0.15–0.4 mm in diam., stipitate; stipe about 0.5 mm long. Hymenium surface yellowish, drying grayish brown, receptacle surface lighter. Covering layer one hyphal layer thick, hyphae 2–4 μm wide. Ectal excipulum of textura prismatica, 14–25 μm thick, composed of 2–3 cell layers with short rectangular to isodiametric cells, hyaline, walls 1–2 μm thick, cells 6–15×5–11 μm. Medullary excipulum of two layers, 75 μm thick; outer layer of textura porrecta, 20–28 μm thick; inner layer of textura intricata, 15–60 μm thick; hyphae hyaline, 1.5–3.5 μm wide. Stipe surface glabrous, base not dark. Subhymenium not distinguishable. Hymenium about 70 μm thick. Asci arising from simple septa, cylindrical-clavate, apex round, 8-spored, J+ in Melzer's reagent, pore walls bluing as two lines, 60–66×6.5–8.5 μm. Ascospores biseriate or biseriate above and uniseriate below, hyaline, cylindrical-fusoid, with 2 large oil drops and some smaller ones, 13.2–14.3×3.3–3.6 μm. Paraphyses filiform, 1–2 μm wide.

Etymology: The specific epithet refers to the subsymmetric ascospores.

Holotype: CHINA, Anhui, Jinzhai, Tiantangzhai, alt. 800–900 m, on herbaceous stem, 23 Aug. 2011, S.L. Chen, W.Y. Zhuang, H.D. Zheng & Z.Q. Zeng 7802, HMAS 264021.

Notes: *Hymenoscyphus brevicellulus* is similar to *H. subsymmetricus* in excipular structure and size of asci and ascospores but differs in having larger apothecia (0.3–1.4 mm), scutuloid ascospores with anterior ends slightly curved and posterior ends pointed, and occurring on leaf veins. The new species is also similar to *H. albopunctus* (Peck) Kuntze in ascospore size and guttulation, but the latter differs in larger (up to 0.8 mm in diam.) and cream-white to yellowish-stained apothecia, broader asci (60–70×8–10 μm), scutuloid ascospores, and growing on leaf veins of *Fagus* [5,6].

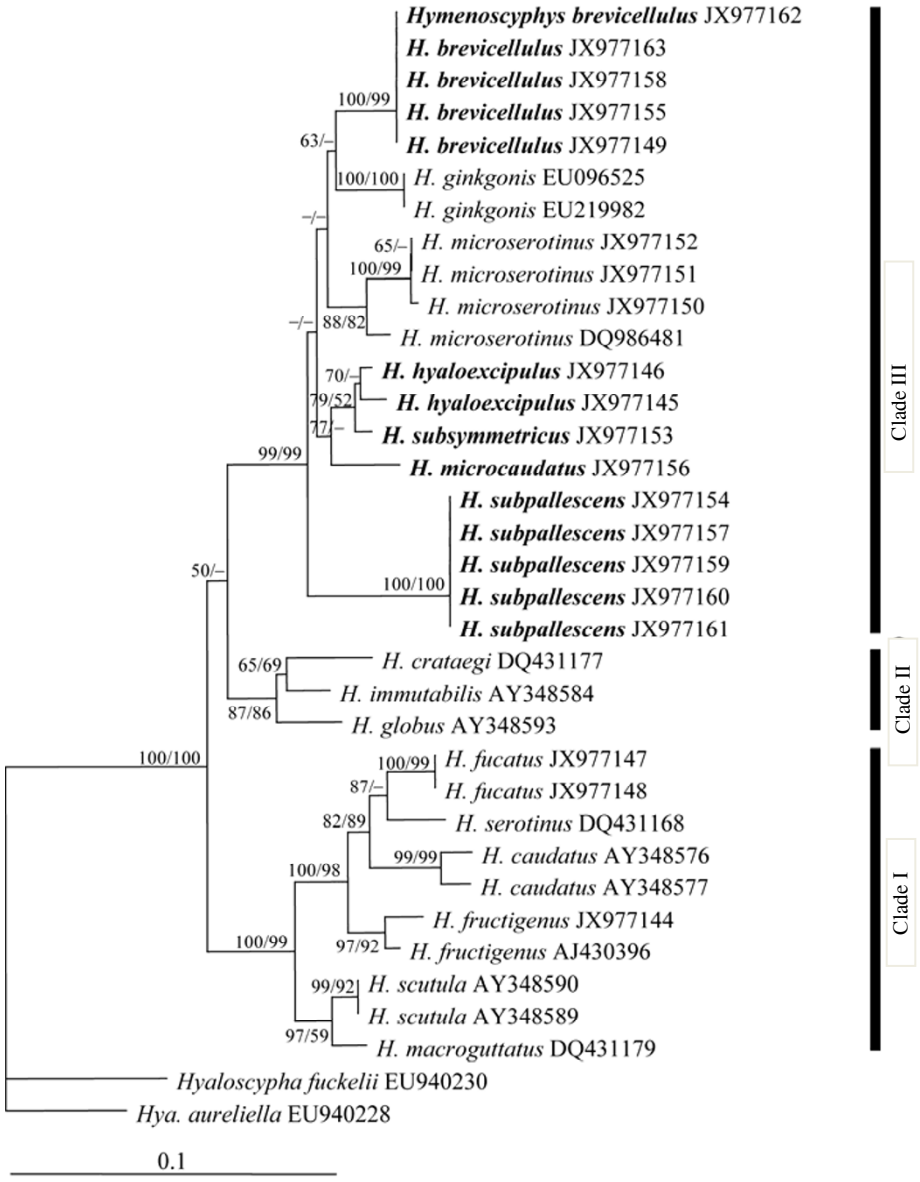

Figure 7 Neighbor-joining tree inferred from internal transcribed spacer nuclear ribosomal DNA sequences showing the relationships among some species of *Hymenoscyphus*. Statistical support values at nodes are neighbor-joining (left) and maximum parsimony (right) bootstrap values (⩾50%).

Although a high sequence similarity among the species tested was found between *H. brevicellulus* and *H. subsymmetricus* (96%), the latter fungus differs distinctly in apothecial size and ascospore shape. In the phylogenetic tree (Figure 7), *H. subsymmetricus* and *H. brevicellulus* belonged to different lineages of Clade III, indicating that they are different species. *Hymenoscyphus subsymmetricus* clustered with *H. hyaloexcipulus* and *H. microcaudatus* (77% NJBP). *Hymenoscyphus hyaloexcipulus* appeared to be a sister of *H. subsymmetricus* and their ITS sequence similarity was very high (98.8%), statistical support for this relationship was very low (79% NJBP, 52% MPBP), agreeing with the morphological differences between these species. The former fungus possesses larger ectal excipular cells (6–30(–60)×6–25 μm), asci (110–128×9–12.5 μm), and ascospores (16.7–25.5×5.5–6.5 μm) and occurs on rotten leaf veins of trees. *H. microcaudatus* has larger apothecia, narrower ectal excipular cells, and scutuloid ascospores.

2.1.2 A new record for China

Hymenoscyphus subpallescens Dennis, Kew Bull. 30(2): 349, 1975 (Figures 1E, 6).

Apothecia flat to discoid, 0.5–2 mm in diam., stipitate; stipe 0.8–1.3 mm long. Hymenium surface dirty white, pale beige to pale yellow, drying pale tan with grayish tints, receptacle surface lighter, drying cream to dirty white or concolorous with hymenium. Outer covering layer present, of 1–5 layers, hyphae about 3 μm wide. Ectal excipulum of

textura prismatica, 10–55 μm thick, cells hyaline, walls about 1 μm thick, 7–35×5–11 μm. Medullary excipulum of two layers; outer layer of textura porrecta, 15–55 μm thick; inner layer of textura intricata, 30–165 μm thick; hyphae hyaline, 2–6 μm wide. Stipe surface with scarce to dense short to long hairs on lower part, hairs septate, ends finger-shaped, 6–30 μm long; base light to dark in color, cells hyaline, light brown to brown. Subhymenium not distinguishable. Hymenium 80–90 μm thick. Asci arising from simple septate, cylindrical-clavate, apex broadly papillate or rounded, 8-spored, J+ in Melzer's reagent, pore walls bluing as two lines, 66–92.5×7–8.8 μm. Ascospores uniseriate to biseriate above and uniseriate below, hyaline, broadly fusoid, slightly flattened on one side, with 1–2(–4) large oil drops, occasionally 1-septate, 12–15×3.9–5 μm. Paraphyses filiform, 2–2.5 μm wide.

Specimens examined: CHINA, Anhui, Jinzhai, Tiantangzhai, alt. 900–1000 m, on rotten leaf veins of trees, 24 Aug. 2011, S.L. Chen, W.Y. Zhuang, H.D. Zheng & Z.Q. Zeng 7815, 7817, 7829, 7837, 7839, 7840, HMAS 264022, 264023, 264024, 264025, 264026, 264027.

Notes: *Hymenoscyphus subpallescens* was originally described from the United Kingdom and characterized by yellowish-white apothecia, ectal excipulum composed of rectangular cells, and fusoid ascospores uniseriate in asci [39]. The Anhui collections agree with the original description of the fungus in most aspects. This is a new record for China.

2.2 Sequence analyses

The ITS dataset included 35 sequences representing 16 *Hymenoscyphus* species and two outgroup taxa (Table 1). After deleting the unaligned and ambiguously aligned sites, 446 characters remained for analyses, including 307 invariant, 25 parsimony-uninformative, and 114 parsimony-informative characters. The NJ tree with bootstrap proportions is shown in Figure 7. The MP analysis resulted in the 12 most parsimonious trees (tree length=312; consistency index=0.635; homoplasy index=0.365; retention index=0.861; rescaled consistency index=0.546); MP bootstrap proportions are also shown in Figure 7.

The phylogenetic analyses indicate that all the investigated species formed a strongly supported (100% NJBP, 100% MPBP) monophyletic group, in agreement with previous studies [20,21,30]. These species were further divided into three clades with moderate to high bootstrap support. Clade I (100% NJBP, 99% MPBP) contained *H. fructigenus* (type species of the genus) and five other species. Clade II (87% NJBP, 86% MPBP) included *H. crataegi*, *H. globus*, and *H. immutabilis*. Clade III (99% NJBP, 99% MPBP) consisted of the four new species (*H. brevicellulus*, *H. hyaloexcipulus*, *H. microcaudatus*, and *H. subsymmetricus*), a new record for China (*H. subpallescens*), and two other known species (*H. ginkgonis* and *H. microserotinus*). Collections of the same species grouped together and formed well-supported terminal branches, indicating that they are tenable taxa.

3 Discussion

In this paper, the relationships among 16 *Hymenoscyphus* species, which produce scutuloid (or similarly shaped) ascospores, were explored. The phylogenetic trees hint at a possible connection between the molecular data and taxonomic morphological criteria. In the genus *Hymenoscyphus*, the shape and size of ascospores and the type of excipular structure seem to convey phylogenetic information. For example, most of the small-spored species (ascospore length <20 μm) clustered in Clades II & III, while those bearing somewhat larger ascospores (>20 μm) were in Clade I. Furthermore, species in Clade II had subfusoid ascospores and large-celled textura angularis in the ectal excipulum, while most species in Clades I & III produced scutuloid ascospores, except for *H. subsymmetricus* and *H. subpallescens*. Combining morphology and DNA sequence data proved helpful in recognizing fungal species diversity, as well as in establishing clear species concepts.

As indicated by Hawksworth [40,41], fungi have extremely high species diversity; there are around 1.5 million extant fungal species worldwide. However, only about 100000 species are currently known [1]. Discovering new species is a long-term task for mycologists. Clear species concept and accurate species identification are essential for the economic exploitation of useful species and control of harmful ones.

Species recognition in fungi is still a matter of intensive debate [42,43]. Limited morphological features have been used in taxonomy, and species concepts of some *Hymenoscyphus* taxa, as in some other fungal groups, have not been accurately resolved. Future work will improve our knowledge of fungal species diversity and firmly establish a logical generic circumscription. A multi-gene phylogeny of *Hymenoscyphus* that is based on broad sampling is certainly needed. With the aid of integrated studies, i.e., combining morphology, culture characterization, sequence data, etc., we believe that new fungal species will continue to be discovered.

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