

Phylogenetic relationships of some members in the genus *Hymenoscyphus* (Ascomycetes, Helotiales)

by

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With 6 figures

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Abstract: The relationships among some species of *Hymenoscyphus* and a few genera in other families of the Helotiales with somewhat similar excipular structure were investigated based on sequence analyses of the rDNA ITS1-5.8S-ITS2 region. The phylogenetic trees were established with *Lachnum spartinae* and *L. cf. pygmaeum* as outgroup. Two clades were recognized within *Hymenoscyphus*. *Hymenoscyphus fructigenus*, the lectotype of the genus, together with *H. caudatus*, *H. cf. fructigenus*, *H. fucatus*, *H. cf. menthae*, and *H. scutululus*, as well as *H. epiphyllus*, *H. immutabilis*, *H. lasiopodius*, *H. serotinus*, and *H. globus*, represent a monophyletic group. *Hymenoscyphus ericae*, *H. sp. G1*, *H. rhodoleucus*, and *Calycina herbarum* form the other clade. *Hymenoscyphus caudatus* is possibly a species complex. *Hymenoscyphus globus* is described as a new species. The Ericaceae-associated species and *Calycina herbarum* treated formerly as members of *Hymenoscyphus* should be excluded from the genus and the family Helotiaceae. The new genus *Rhizoscyphus* is established to accommodate *H. ericae* and a related species.

Key words: Phylogeny, rDNA ITS1-5.8S-ITS2 region, DNA sequencing, *Hymenoscyphus globus*, *Rhizoscyphus*, *R. ericae*, *R. monotropae*

Introduction

Hymenoscyphus Gray is a large genus in the Helotiaceae containing about 100 species in the world (Kirk et al. 2001). Many taxonomic problems of the genus remain to be solved. The circumscription of the genus is not well-defined and relationships among species are not clear. Molecular approaches to phylogeny of the genus were mainly focused on *H. ericae* (D.J. Read) Korf & Kernan and its relatives (Egger & Sigler

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1993, Chambers et al. 1999, Vrålstad et al. 2002). Very limited work has been done with species other than *H. ericae* (Gernandt et al. 2001, Collado et al. 2002). In this study we use ITS1-5.8S-ITS2 rDNA sequences of several *Hymenoscyphus* species and taxa of a few other genera of the Helotiales to investigate the phylogenetic relationships among species of the genus.

Materials and methods

Material studied

Material used in this study is shown in Table I. Fungal genomic DNA was extracted from the dried herbarium specimens, except for *Lambertella* sp. 3176 which was available as a living culture. Specimens used in this study were deposited in the Herbarium Mycologicum Instituti Microbiologici Academiae Sinicae (HMAS). In order to explore the relationships among species of *Hymenoscyphus* and those between *Hymenoscyphus* spp. and other taxa of the Rutstroemiaceae and Sclerotiniaceae possessing somewhat similar ectal excipular structures, sequences of some species of *Ciboria*, *Lambertella*, and *Rutstroemia* were also introduced. *Lachnum* spp. of the Hyaloscyphaceae were used as the outgroup. Thirty-one sequences obtained for 22 selected taxa in the Helotiaceae, Rutstroemiaceae, Sclerotiniaceae, and Hyaloscyphaceae were analyzed. Among them, 20 sequences were newly obtained and 11 were retrieved from GenBank.

DNA extraction

The CTAB method was used for DNA extraction. The protocols of Doyle (1991), White et al. (1990) and Ausubel et al. (1995) were followed.

PCR amplification

GeneAmp PCR System 2400 (Perkin Elmer) was used for amplification. The primers for PCR amplification were ITS5 and ITS4 (White et al. 1990). The primers, Taq polymerase and dNTPs were supplied by Sino-America Biotechnology Company.

DNA sequencing

ABI Prism 377-96, BigDye terminator v. 2.0 was used for sequencing. Both strands were sequenced. The primers for sequencing were ITS5 and ITS4 (White et al. 1990). PCR product purification and DNA sequencing were performed by Songon Biotechnology Company and Genecore Biotechnology Company in Shanghai.

Alignment and sequence analyses

Sequences were aligned with ClustalX (Thompson et al. 1997). PAUP version 4.0b8 (Swofford 2001) was used for the phylogenetic analyses. The trees were rooted with *Lachnum spartinae* and *L. cf. pygmaeum* as outgroup. The phylogenetic trees were created after heuristic search and bootstrap (Felsenstein 1985) and viewed by TreeView (Page 1996).

Results and discussion

Three most parsimonious trees were obtained from 31 sequences with 519 characters, all characters equally weighted, 275 constant characters, 48 characters parsimony-uninformative, and 196 characters parsimony-informative. Gaps were treated as “missing” data. Fig. 1 shows one of the three most parsimonious trees after heuristic search. Fig. 2 shows the parsimony consensus tree after bootstrap with 1000 replicates. The two phylogenetic trees are of very similar topology (Figs 1, 2).

Table I. Material used

Species	Source of the Chinese material	GenBank accession numbers
<i>Hymenoscyphus caudatus</i> (P. Karst.) Dennis	1105, HMAS 82057 Anhui	AY348576
	1354, HMAS 82060 Beijing	AY348577
	1459, HMAS 82063 Jiangxi	AY348578
	1578, HMAS 82073 Jiangxi	AY348579
<i>H. epiphyllus</i> (Pers. : Fr.) Rehm	1489, HMAS 82075 Jiangxi	AY348580
	1491, HMAS 82076 Jiangxi	AY348581
<i>H. ericae</i> (D.J. Read) Korf & Kernan		AF069440
<i>H. fructigenus</i> (Bull. : Fr.) Gray		AF430396
<i>H. cf. fructigenus</i>	2247, HMAS 75877 Sichuan	AY348582
<i>H. fucatus</i> (W. Phillips) Baral & Hengstm.	2178, HMAS 75902 Sichuan	AY348583
<i>H. immutabilis</i> (Fuckel) Dennis	1274, HMAS 71809 Beijing	AY348584
<i>H. lasiopodius</i> (Pat.) Dennis	1221, HMAS 71820 Beijing	AY348585
	1255, HMAS 71821 Beijing	AY348586
	0242, HMAS 75878 Beijing	AY348587
	0015, HMAS 75934 Sichuan	AY348588
<i>H. cf. menthae</i> (W. Phillips) Baral		AJ430395
<i>H. rhodoleucus</i> (Fr.) W. Phillips		AY348589
<i>H. scutululus</i> (Pers. : Fr.) W. Phillips	3546, HMAS 82092 Jilin	AY348590
	3548, HMAS 82093 Jilin	AY348591
	1278, HMAS 82098 Beijing	AY348591
	1544, HMAS 82122 Jiangxi	AY348592
<i>H. serotinus</i> (Pers. : Fr.) W. Phillips		AY348593
<i>H. globus</i> W.Y. Zhuang & Y.H. Zhang	1558, HMAS 82107 Jiangxi	AY348593
<i>H. sp. G1</i>		AJ292199
<i>Calycina herbarum</i> (Pers. : Fr.) Gray	1549, HMAS 82102 Jiangxi	AY348594
<i>Ciboria betulae</i> (Woronin ex Navashin) W.L. White		Z81427
<i>Ciboria caucus</i> (Rebent.) Fuckel		Z73766
<i>Lachnum cf. pygmaeum</i> (Fr.) Bres.		AJ430215
<i>Lachnum spartinae</i> S.A. Cantrell		AF422970
<i>Lambertella</i> sp.	3176, Yunnan	AY348595
<i>Rutstroemia bolaris</i> (Batsch) Rehm		Z80894
<i>Rutstroemia firma</i> (Pers.) P. Karst.		Z80893

Core species of *Hymenoscyphus* and the *H. caudatus* complex

Among the investigated species of *Hymenoscyphus* and a few genera of the Rutstroemiaceae and Sclerotiniaceae, two clades are recognized and corroborated with 100% bootstrap support (Fig 2). *Hymenoscyphus caudatus*, *H. cf. menthae*, *H. fucatus*, *H. fructigenus*, *H. cf. fructigenus*, and *H. scutululus* form a homogeneous clade; *H. serotinus*, *H. immutabilis*, and *H. globus* 1558 (described as a new species below) are associated with the same group with relatively low bootstrap values (less than 50%). *Hymenoscyphus epiphyllus* and *H. lasiopodius* form a closely related

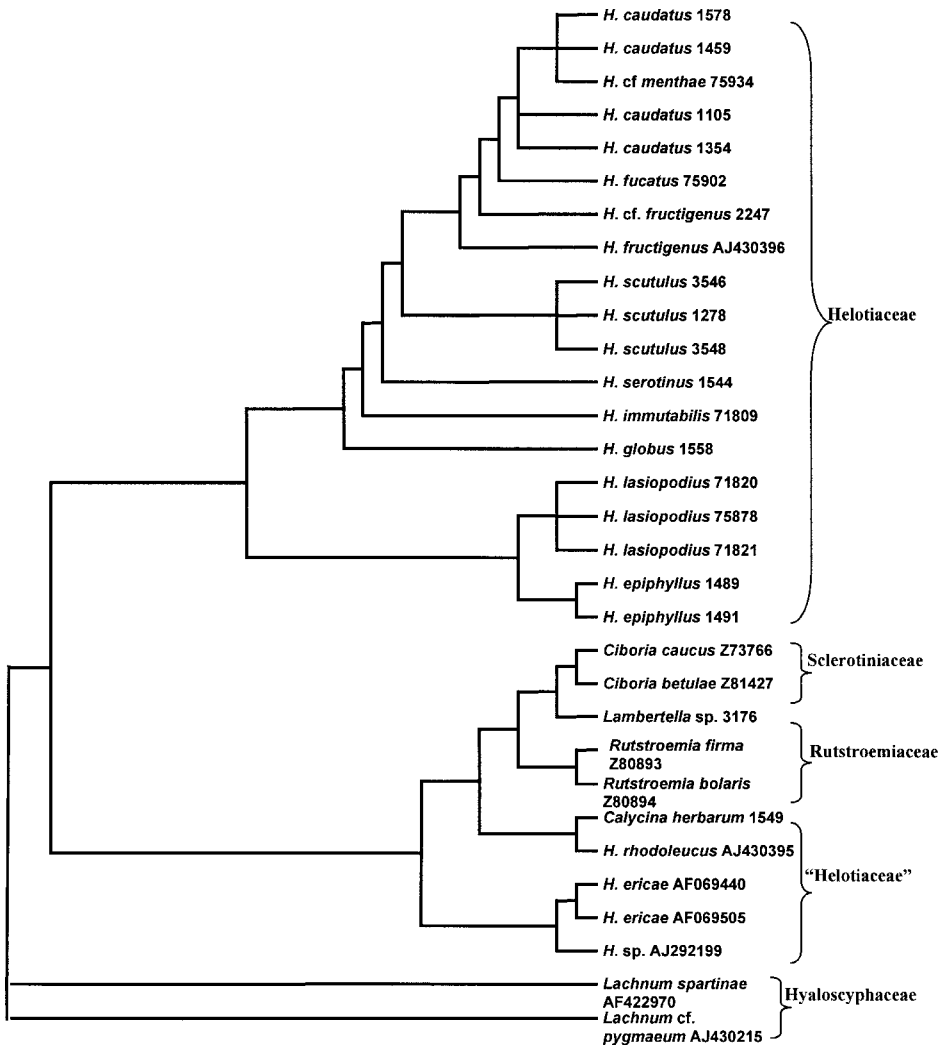


Fig. 1. Phylogenetic relationships of *Hymenoscyphus* species inferred from the ITS1-5.8S-ITS2 region, showing one of three most parsimonious trees obtained after heuristic search. Tree length (TL) = 712 steps, consistency index (CI) = 0.5562, homoplasy index (HI) = 0.4438, retention index (RI) = 0.7380, rescaled consistency index (RC) = 0.4104; outgroup *Lachnum spartinae* and *L. cf. pygmaeum*, "H." is the abbreviation of *Hymenoscyphus*. Numbers preceded by letters are from GenBank.

sister group with 74% bootstrap support. The remaining taxa investigated, including the *H. ericae*, *H. sp.* G1 and *H. rhodoleucus*, together with *Calycina herbarum* and members of the Rutstroemiaceae and Sclerotiniaceae (*Ciboria* spp., *Lambertella* sp., and *Rutstroemia* spp.), form a second clade.

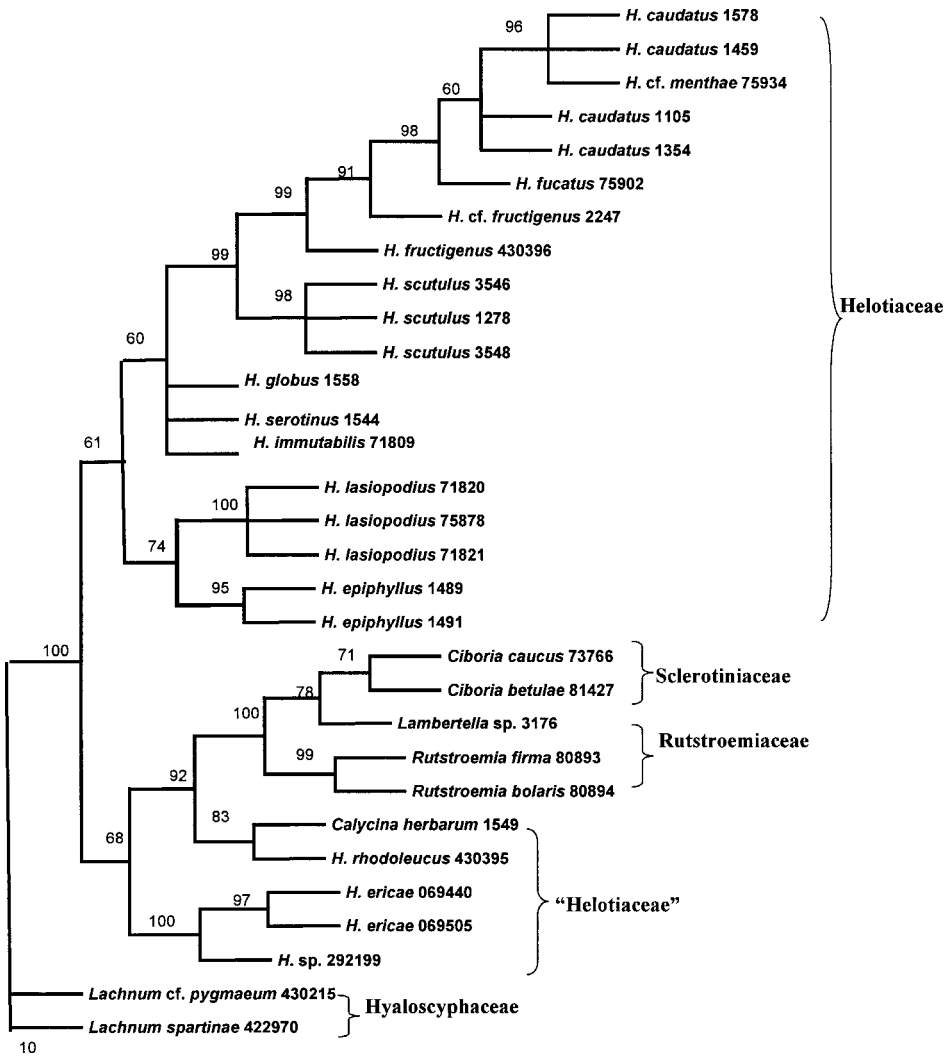


Fig. 2. Parsimony consensus tree of *Hymenoscyphus* species inferred from the ITS1-5.8S-ITS2 region, after bootstrap, 50% majority rule; outgroup *Lachnum spartinae* and *L. cf. pygmaeum*. "H." is the abbreviation of *Hymenoscyphus*. Numbers as in Fig. 1.

The collections of *Hymenoscyphus caudatus* (1105, 1354, 1459 and 1578) are divided into three small terminal branches with a bootstrap value of 60%, correlating with morphological variations observed among the collections. We suspect that *H. caudatus* is possibly a species complex. HMAS 75934, previously reported as *Hymenoscyphus cf. consobrinus* (Boud.) Hengstm. (Zhang & Zhuang 2002) and later assigned to *H. cf. menthae* (H.-O. Baral, pers. comm.), is grouped within these collections of *H.*

caudatus. Because HMAS 75934 is distinct from *H. caudatus* (Dennis 1956, Dumont & Carpenter 1982) in its ascospore shape, size, and guttulation, as well as the substrates (Zhang & Zhuang 2002), we think that it should stand as a separate taxon. Further studies on the *H. caudatus* complex are certainly required.

Different collections of *Hymenoscyphus lasiopodius* (HMAS 71820, 71821 and 75878) are grouped together with 100% bootstrap support. Both morphology (Zhang & Zhuang 2002, Zhuang 2003) and our sequence analyses indicate that HMAS 75878, the holotype of *H. adlasiopodius* Zheng Wang (Wang & Pei 2001), is identical with *H. lasiopodius* except that some of the apothecia in that material are somewhat young and their ascospores have not yet fully developed 3 septa.

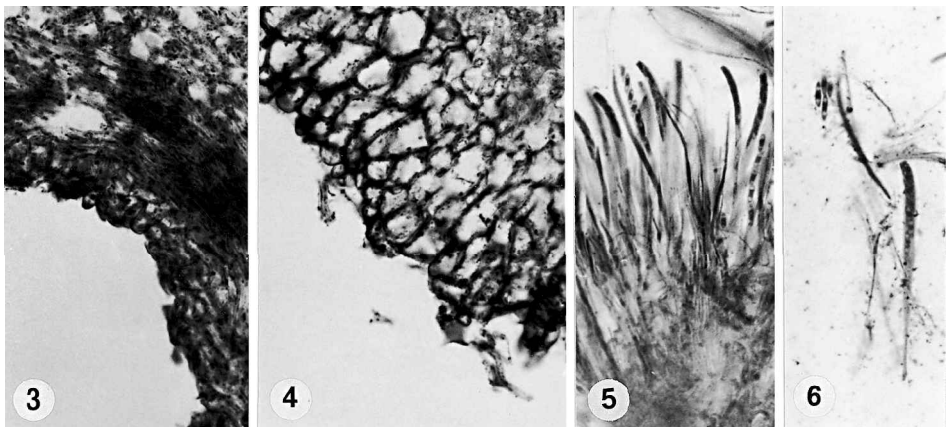
A new species of *Hymenoscyphus*

Hymenoscyphus globus W.Y. Zhuang & Y.H. Zhang, sp. nov.

Figs 3-6

Ab *Hymenoscypho epiphylo* differt apotheciis turbinatis vel disciformibus, 1-4 mm diam.; ascis J-, 86-95 × 5.5-6.5 μm; ascosporis subellipsoideis, 7.5-11.5 × 2.5-3.5(-4) μm.

Apothecia turbinate to discoid, sessile to short-stipitate, 1-4 mm diam, hymenium surface yellow to light yellow when fresh, receptacle surface paler than hymenium surface, stipe base dark when dry; ectal excipulum of textura prismatica at the margin and of textura angularis at the flanks, 33-75 μm thick, cells more or less isodiametric, 9-25 μm diam.; medullary excipulum of textura intricata, 90-650 μm thick or thicker, hyphae hyaline, 1.5-2.5 μm wide; hymenium ca 130-135 μm thick; asci subcylindrical to cylindric-clavate, 8-spored, with repeating croziers, J- in Melzer's reagent, 86-95 × 5.5-6.5 μm; ascospores subellipsoid to elongate-ellipsoid, with 2(-3) guttules, hyaline, unicellular, occasionally with a septum, irregularly uniseriate to irregularly biseriata, 7.5-11.5 × 2.5-3.5(-4) μm; paraphyses filiform, ca 2 μm wide.



Figs 3-6. *Hymenoscyphus globus* (HMAS 82107): 3. Ectal excipular structure near apothecial margin, 4. Excipular structure near apothecial base, 5-6. Asci and ascospores; all × 320.

HOLOTYPE: China. Jiangxi, Jinggangshan, Ciping, 860 m alt., on wet hard wood, 26-X-1996, Z. Wang & W.Y. Zhuang 1558, HMAS 82107. Other specimens examined: Jiangxi, Jinggangshan, Ciping, 860 m alt., on wet hard wood, 26-X-1996, Z. Wang & W.Y. Zhuang 1551, 1559, HMAS 82106, 82108.

ETYMOLOGY: The specific epithet refers to the globose shape of the ectal excipular cells of the fungus.

Anatomically, this fungus is very similar to *Hymenoscyphus epiphyllus* and *H. immutabilis* in excipular structure, i.e. presence of angular to somewhat isodiametric cells in the ectal excipulum, and shape of ascospores (Dennis 1956, 1964). It is distinct from the latter two fungi by much larger apothecia, smaller asci with a negative iodine reaction, smaller and narrower ascospores, and occurrence on wood instead of on leaves. Our phylogenetic trees (Figs 1, 2) reveal the relationship between *H. globus* and the above two closely related species in the genus. In the ITS1-5.8S-ITS2 sequences, 15 nucleotides were different between *H. globus* and *H. immutabilis* and 32 between *H. globus* and *H. epiphyllus*.

Ericaceae-associated species of “*Hymenoscyphus*”

The Ericaceae-associated species, *Hymenoscyphus ericae* aggregate and *Hymenoscyphus* sp. G1, grouped together with a few members of the Sclerotiniaceae and Rutstroemiaceae (*Ciboria* spp., *Lambertella* sp. and *Rutstroemia* spp.) instead of in the *H. fructigenus* clade (Figs 1, 2). They represent a group of mycorrhizal inoperculate discomycetes living in symbiotic relationship with plant roots; they should be excluded from *Hymenoscyphus*, as also proposed by Vrålstad et al. (2002). A new genus is here described on the basis of morphological, ecological, and molecular characteristics to accommodate *H. ericae* and a related fungus associated with *Monotropa* (Read 1974, Kernan & Finocchio 1983). Further study is required to investigate whether the new genus merits a separate family.

Rhizoscyphus W.Y. Zhuang & Korf, gen. nov.

Genus mycorrhizale. Apothecii calyciformibus, stipitatis; excipulo ectali textura prismatica, excipulo medullari textura intricata; asci inoperculatis, 8-sporis, oblongo-clavatis, J+; ascosporis ellipsoideis, unicellularibus; paraphysibus filiformibus.

TYPE: *Pezizella ericae* D.J. Read.

ETYMOLOGY: The generic name refers to association of the fruitbodies with roots.

Associated with plant roots, mycorrhizal; apothecia discoid, stipitate; ectal excipulum of textura prismatica, with or without short hyphal protrusions on receptacle surface; medullary excipulum of textura intricata; asci inoperculate, 8-spored, cylindrical-clavate, J+ in Melzer's reagent; ascospores ellipsoid to elongate-ellipsoid, unicellular; paraphyses filiform.

Rhizoscyphus ericae (D.J. Read) W.Y. Zhuang & Korf, comb. nov.

≡ *Pezizella ericae* D.J. Read, Trans. Brit. Mycol. Soc. 63: 381, 1974.

≡ *Hymenoscyphus ericae* (D.J. Read) Korf & Kernan, Mycologia 75: 919, 1983.

Rhizoscyphus monotropae (Kernan & Finocchio) W.Y. Zhuang, comb. nov.

≡ *Hymenoscyphus monotropae* Kernan & Finocchio, *Mycologia* 75: 916, 1983.

The taxonomic position of *Calycina herbarum*

Calycina herbarum is not closely related to *Hymenoscyphus fructigenus* as shown in our phylogenetic trees (Figs 1, 2) and is not a member of *Hymenoscyphus*, as was also repeatedly noted in morphological studies (Baral & Krieglsteiner 1985, Verkley 1993). The earlier classification as *Hymenoscyphus herbarum* (Pers. : Fr.) Dennis (Dennis 1964, Lizon 1992) should be reconsidered. Morphological and molecular differences suggest that *Calycina* is distinct from *Hymenoscyphus*, stands for a separate genus, and might even belong to a different family than the Helotiaceae.

Weight of the excipular structure in taxonomy

Our phylogenetic analysis includes some sequences of taxa of the Rutstroemiaceae and Sclerotiniaceae possessing ectal excipular structures similar to *Hymenoscyphus*. The results show that the presence of basal substratal stromata and sclerotia in members of the Rutstroemiaceae and Sclerotiniaceae is a stable characteristic that is diagnostic in separating the two families from the Helotiaceae. Although *textura prismatica* and *textura angularis* occur both in the ectal excipulum of species of *Hymenoscyphus* and in taxa of the rutstroemiaceous and sclerotiniaceous fungi, such as *Lambertella*, *Lanzia*, *Ciboria*, etc., this might be a newly derived character not reflecting phylogenetic affinities but it is certainly a useful morphological feature in the taxonomy of the Helotiales.

In conclusion, the current concept of “*Hymenoscyphus*” includes a group of polyphyletic taxa. *Hymenoscyphus fructigenus* (lectotype of the genus) together with *H. caudatus*, *H. cf. menthae*, *H. fucatus*, *H. cf. fructigenus*, and *H. scutulus* represent a monophyletic group; *H. globus*, *H. serotinus*, and *H. immutabilis* join the group with certain distinctions in both morphology and sequence composition; *H. lasiopodius* and *H. epiphyllus* are well-placed as the sister group within the above clade. *Hymenoscyphus ericae*, *Hymenoscyphus* sp. G1, and *H. rhodoleucus* should be excluded from the genus and might even merit their own family.

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