Molecular Phylogeny of Ascomycetes of the Helotiales

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Abstract

This work presents a preliminary assessment of the phylogenetic relationships among species in the Helotiales based on rDNA sequence analysis. We have analyzed the sequences of the ITS1-5.8s-ITS2 region from more than 100 strains corresponding at least to 32 species in the *Hyaloscyphaceae* and 37 in the *Helotiaceae*, with a special emphasis on the genera *Lachnum* and *Hymenoscyphus*. In addition, a subset of the species has been subjected to sequence analysis of the D1-D2 region of the 28s rRNA gene. The result of the cladistic analysis confirmed the heterogeneity of the taxa defined within the two families. The topology did not support the current morphology-based classification schemes of some of the taxa examined. In particular, a significant degree of incongruity was evident for several species in the genus *Hymenoscyphus* and less notably for *Lachnum* spp. The results are discussed in comparison with the morphological characters used to define taxa within the two families studied. The suitability of the ITS region for phylogenetic analysis of this heterogeneous group of fungi is also discussed.

Introduction

The Helotiales is the largest of the orders of inoperculate discomycetes, comprising 15 families, 372 genera, and 2022 species. (Dict. of the fungi, Kirk *et al*., 2001). The Helotiales spp. occur with a significant abundance across the world and are common in temperate regions. In particular, the Iberian Peninsula constitutes a reservoir of new species that remains partially unexplored. The subtropical Canary Islands, have been also reported to harbour a high variety of taxa (Iturriaga & Korf, 1997; Lizon *et al*., 1998). In other respect, some species in the Helotiales have been described as a source for secondary metabolites with nematicidal, antimicrobial and biochemical activities, with potential applications in a variety of therapeutic areas (Bills *et al*., 1999; Hosoya 1998).
The taxonomy of the Helotiales still remains unsettled, with at least 88 genera referred to the order level that are not assigned to any of the 8-15 recognised families. Except for the *Sclerotiniaceae* (Carbone & Kohn, 1993) and a few members of the *Hyaloscyphaceae* (Cantrell & Hanlin, 1997), these fungi have not been extensively subjected to molecular phylogenetic studies.
We have undertaken a study on the molecular phylogeny of two families, *Hyaloscyphaceae* and *Helotiaceae*, based on the analysis of sequences of the ITS-region and 28S rRNA-gene (D1-D2 region), with the goal of expanding the knowledge on the phylogenetic relationships within this relevant group of fungi. Most of strains in this study derive from apothecia collected in Spain (Iberian Peninsula, Majorca and Canary Islands) and Germany, in addition to some collection isolates from other geographic origins. The results are compared to classical morphology-based classification schemes.

Table 1.Strains used in this study

Name	Strain code*	Original substrate	Origin
Amicodisca svrcekii	HB7022	Rotten stump of *Quercus sp.*	Not reported
Arachnopeziza aurata	F119682	Rotten wood of *Laurus azorica*	M. Mercedes. Tenerife. SPAIN
Arachnopeziza aurata	F119695	Rotten wood of *Myrica faya*	P. Inglés. Tenerife. SPAIN
Arachnopeziza aurata	F122082	*Eucalyptus globulus*	P.N. Garajonay. Tenerife. SPAIN
Arachnopeziza aurata	F146760	*Populus alba*	Casavieja. Ávila. SPAIN
Arachnopeziza aurata	HB6921b	Rotten branch of *Carpinus betulus*	Ellergronn. LUXEMBOURG
Arachnopeziza aurata	CBS11654	Not reported	FRANCE
Arachnopeziza aff. aurata	HB6961	Rotten branch of *Ulex europaeus*	Ruffiac. FRANCE
Arachnopeziza aurelia	F118039	*Eucalyptus globulus*	Maqueda. Toledo. SPAIN
Arachnopeziza aurelia	CBS11754	Not reported	FRANCE
Ascocoryne cylichnium	F115894	Unbarked branch of *Fagus sylvatica*	Pto. Quesera. Segovia. SPAIN
Ascocoryne cylichnium	HB6768	rotten stump of deciduous tree	Reutlingen-Gönningen. GERMANY
Ascocoryne sarcoides	F092788	Branches of *Fagus sylvatica*	Pto. Quesera. Segovia. SPAIN
Ascocoryne sarcoides	F115896	Unbarked branch of *Fagus sylvatica*	Pto. Quesera. Segovia. SPAIN
Ascocoryne sarcoides	HB6789	Rotten branch of *Fagus sylvatica*	Tübingen-Pfrondorf. GERMANY
Bisporella citrina	F115889	Unbarked branch of *Fagus sylvatica*	Pto. Quesera. Segovia. SPAIN
Bisporella citrina	F118000	*Quercus robur*. Mixed forest	O. Sajambre. León. SPAIN
Bisporella subpallida	HB6849a	Stump of *Fraxinus excelsior*	Tübingen. GERMANY
Calycellina albida	F119880	*Quercus ilex* leaves	Tamajón. Guadalajara. SPAIN
Calycellina albida	F132989	*Quercus ilex* leaves	Menut, Escorca. Mallorca. SPAIN
Calycellina aff. guttulifera	HB6787	Rotten branch of *Tilia*	Tübingen-Pfrondorf. GERMANY
Calycina subherbarum	HB7052	Stem of *Solidago canadensis*	Tübingen. GERMANY
Capitotricha bicolor	HB6923a	Rotten branch of *Crataegus laevigata*	Ellergronn. LUXEMBOURG
Cyathicula cyathoidea	F124806	*Urtica* dead stems	P. de Babia. León. SPAIN
Cyathicula cyathoidea	F144616	*Atropa belladona*	Peñacerrada, Álava. SPAIN
Cyathicula tomentosa	F144620	Grasses	Izarra. Vizcaya. SPAIN
Dasyscyphella angustipila	F132987	*Erica multiflora* stems	Menut, Escorca. Mallorca. SPAIN
Dasyscyphella angustipila	HB6874	Rotten stump of *Fagus sylvatica*	Tübingen. GERMANY
Dasyscyphella calongei	F116601	*Fagus sylvatica* rotten wood	Pto. Quesera. Segovia. SPAIN
Dasyscyphella nivea	F119674	*Erica arborea* wood	M. Mercedes. Tenerife. SPAIN
Dasyscyphella pulverulenta	F119704	*Pinus canariensis* leaves	La Esperanza. Tenerife. SPAIN
Dasyscyphella pulverulenta	F119705	*Pinus canariensis* leaves	La Esperanza. Tenerife. SPAIN
Dematiosccyphe dematiicola	F119694	Decorticated branches of *Erica arborea*	P. Inglés. Tenerife. SPAIN
Discinella terrestris var. inamyloidea	Uncultured	Humus under *Eucalyptus globulus*	Verdicio . Asturias. SPAIN
Discinella terrestris var. terrestris	Uncultured	Humus under *Eucalyptus globulus*	Puerto de Mirabete. Cáceres. SPAIN
Durella macrospora	HB6871c	Stem of *Fagus sylvatica*	Tübingen. GERMANY
Durella melanochlora	HB6922	Rotten branch of *Fagus sylvatica*	Ellergronn. LUXEMBOURG
Haplographium sp./Dematiosccyphe sp.	F119719	P. and *Eucalyptus camaldulensis*	La Esperanza. Tenerife. SPAIN
Hyaloscypha albohyalina	CBS12491	Coniferous wood	NORWAY
Hyaloscypha aureliella	F119712	*Pinus canariensis* wood	La Esperanza. Tenerife. SPAIN
Hyaloscypha aureliella	F115881	Wood of *Pinus pinaster*	S. Aracena. Huelva. SPAIN
Hyaloscypha aureliella	F119710	*Pinus canariensis* wood	La Esperanza. Tenerife. SPAIN
Hymenoscyphus calyculus	F093262	Rotten wood of *Corylus avellana*	Somosierra. Madrid. SPAIN
Hymenoscyphus caudatus	F130793	Leaves of *Salix*	P.N. Cabañeros. Ciudad Real. SPAIN
Hymenoscyphus epiphyllus	HB7054	Stem of *Quercus*	Tübingen. GERMANY
Hymenoscyphus grex epiphyllus	F115879	Leaves of *Castanea sativa*+*Quercus suber*	S. Aracena. Huelva. SPAIN
Hymenoscyphus fructigenus	F065166	Not reported	S.Villuercas. Cáceres. SPAIN
Hymenoscyphus fructigenus	F093275	*Quercus suber*	S. Aracena. Huelva. SPAIN
Hymenoscyphus fructigenus	F115882	Fruits *Quercus suber*	S. Aracena. Huelva. SPAIN
Hymenoscyphus fructigenus	F127542	*Betula alba*+*Quercus petrea* forest	Somosierra. Madrid. SPAIN
Hymenoscyphus imberbis	HB6797	Rotten branch of *Salix sp.*	Tübingen-Pfrondorf. GERMANY
Hymenoscyphus cf. immutabilis	F137632	*Rubus sp.* leaves	Colmenarejo. Madrid. SPAIN
Hymenoscyphus kathiae	HB6924	Rotten branch of *Salix sp.*	Ellergronn. LUXEMBOURG
Hymenoscyphus macroguttatus	HB7034	Leaves of *Acer pseudoplatanus*	Tann. Hessen. GERMANY
Hymenoscyphus monotropae		*Monotropa uniflora* roots	New York. USA
Hymenoscyphus paradoxus (Helicodendron paradoxum)	F060490	Fresh water	JORDAN
Hymenoscyphus salmanovicensis	HB6977	Leaves of *Eriophorum vaginatum*	Lac de Lispach. FRANCE
Hymenoscyphus scutula	F109077	Not reported	S.M. Cogolla. Logroño. SPAIN
Hymenoscyphus serotinus	F093261	Branches of *Fagus sylvatica*	Pto. Quesera. Segovia. SPAIN
Hymenoscyphus serotinus	F115891	Branches of *Fagus sylvatica*	Pto. Quesera. Segovia. SPAIN
Hymenoscyphus suspectus	F115899	Grasses	Pto. Quesera. Segovia. SPAIN
Hymenoscyphus tamaricis	F110125	Not reported	Not reported
Hymenoscyphus vernus	HB6915	Rotten branch of *Alnus*	Grosbous. LUXEMBOURG
Lachnellula calycina	F119707	*Pinus canariensis* trunk	La Esperanza. Tenerife. SPAIN
Lachnellula calycina	F119708	*Pinus canariensis* trunk	La Esperanza. Tenerife. SPAIN
Lachnellula willkommii	CBS17035	*Larix decidua*	Massachusetts. USA
Lachnum balearicum sp. nov. ad int.	F132995	*Ampelodesma mauritanica*, shells	Valldemossa. Mallorca. SPAIN
Lachnum balearicum sp. nov. ad int.	F136257	*Ampelodesma mauritanica*, shells	Valldemossa. Mallorca. SPAIN
Lachnum balearicum sp. nov. ad int.	F142279	Not reported	Escorca. Mallorca. SPAIN
Lachnum cf. balearicum sp. nov. ad int.	F132986	*Ampelodesma mauritanica*, shells	Menut, Escorca. Mallorca. SPAIN
Lachnum brevipilosum	HB6885	Rotten stump of *Fraxinus excelsior*	Tübingen. GERMANY
"Lachnum" cerinum	F115892	Unbarked branch of *Fagus sylvatica*	Pto. Quesera. Segovia. SPAIN
"Lachnum" cerinum	F119685	Lauroid forest.	M. Mercedes. Tenerife. SPAIN
"Lachnum" cerinum	HB6871b	Stem of *Fagus sylvatica*	Tübingen. GERMANY
Lachnum impudicum	HB6871a	Rotten stem of *Fagus sylvatica*	Tübingen. GERMANY
Lachnum cf. pudibundum	F119698	*Rubus* branches	P. Inglés. Tenerife. SPAIN
Lachnum cf. pudibundum	F119730	Branches of *Laurus azorica*	M. Mercedes. Tenerife. SPAIN
Lachnum pygmaeum	HB6903	Rotten twigs of *Ulex minor*	M.de Cojoux. Ille-et-Vilaine. FRANCE
Lachnum sp.	F119696	Decorticated branches of *Erica arborea*	P. Inglés. Tenerife. SPAIN
Lachnum virgineum	F119679	Lauroid forest	M. Mercedes. Tenerife. SPAIN
Lachnum virgineum	F119683	Decorticated branches of *Erica arborea*	M. Mercedes. Tenerife. SPAIN
Lachnum virgineum	F119684	Decorticated branches of *Erica arborea*	M. Mercedes. Tenerife. SPAIN
Lachnum virgineum	F119686	Decorticated branches of *Erica arborea*	M. Mercedes. Tenerife. SPAIN
Lachnum virgineum	F119699	Decorticated branches of *Erica arborea*	P. Inglés. Tenerife. SPAIN
Lachnum virgineum	F119703	Lauroid forest	Anaga. Tenerife. SPAIN
Lachnum virgineum	F119722	*Erica arborea* branches	La Esperanza. Tenerife. SPAIN
Lachnum virgineum	F122062	*Erica arborea*	P.N. Garajonay. Tenerife. SPAIN
Lachnum virgineum	F122063	*Erica arborea*	P.N. Garajonay. Tenerife. SPAIN
Lachnum virgineum	F119687	Decorticated branches of *Erica arborea*	M. Mercedes. Tenerife. SPAIN
Mitrula paludosa	F124810	Peat bog under *Pinus sylvestris*	Navacerrada. Madrid. SPAIN
Mollisia cinerea	CBS41281	*Juniperus comunis*	SWITZERLAND
Mollisia fusca	CBS23471	*Fagus sylvatica*	SWITZERLAND
Mollisia hydrophila	CBS55663	*Phragmites australis*	FRANCE
Mollisia melaleuca	F115897	*Fagus sylvatica*	Pto. Quesera. Segovia. SPAIN
Neobulgaria pura	HB6880	Stem of *Fagus sylvatica*	Tübingen-Pfrondorf. GERMANY
"Ombrophila" hemiamyloidea	F115893	Submerged branch of *Fagus sylvatica*	Pto. Quesera. Segovia. SPAIN
Ombrophila janthina	HB7044	Cones of *Picea abies*	Tübingen. GERMANY
Ombrophila lilacina	HB6921a	Rotten branch of *Carpinus betulus*	Ellergronn. LUXEMBOURG
"Pezizella" amenti	F144619	*Salix atrocinerea*	Montoria. Álava. SPAIN
"Pezizella" amenti	HB6884	catkins of *Salix caprea*	Tübingen. GERMANY
"Pezizella" gemmarum	F144622	*Populus sp.*	M. Herrera. Burgos. SPAIN
"Peizzela" junipericola	HB6910	Rotten twigs of *Juniperus sp.*	Maasheggen. Boxmeer. THE NETHERLANDS
"Pezizella" languida	F116599	*Fagus sylvatica*	Pto. Quesera. Segovia. SPAIN
"Pezizella" languida	F116600	*Fagus sylvatica*	Pto. Quesera. Segovia. SPAIN
Perrotia flammea	F115890	Unbarked branch of *Fagus sylvatica*	Pto. Quesera. Segovia. SPAIN
Pezoloma sp./Anguillospora furtiva	F057390	Fresh water	SPAIN
Phaeohelotium trabinellum	HB6810b	Rotten branches of *Fagus sylvatica*	Tübingen-Pfrondorf. GERMANY
Proliferodiscus alboviridis	HB6922a	Rotten branch of *Fagus sylvatica*	Ellergronn. LUXEMBOURG
Proliferodiscus pulveraceus	F119675	Branches of *Erica arborea*	M. Mercedes. Tenerife. SPAIN
Proliferodiscus pulveraceus	F122075	*Laurus azorica*	P.N. Garajonay. Tenerife. SPAIN
Tatraea dumbirensis	F115898	Unbarked branch of *Fagus sylvatica*	Pto. Quesera. Segovia. SPAIN
Trichopeziza barbata	HB6947	Branch of *Lonicera*	Graubünden. SWITZERLAND
Trichopeziza leucophaea	F146748	Stems of *Epibolium sp.*	Montoria. Álava. SPAIN
Trichopeziza leucophaea	HB6967	stem of *Aconitum napellus*	Tübingen. GERMANY
Trichopeziza mollissima	HB6968	Stem of *Scrophularia nodosa*	Tübingen. GERMANY

* F numbers correspond to monosporic isolates obtained from ascospores or conidia. HB numbers are isolates from H.O. Baral's collections (cultured by Evi Weber and Guy Marson). ATCC and CBS are strains purchased from the American Type Culture Collection (Rockville, Maryland, USA) and Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, respectively.

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Materials and Methods

Fungal strains A total of 119 strains were analyzed in this study. The information on these strains is in Table 1.

DNA extraction and PCR amplification The procedures followed are in Sánchez-Ballesteros *et al*.(2000). The primers used for amplification of the ITS region were ITS4 (White *et al*., 1990), ITS1F (Gardes & Bruns, 1993), and ITS4A (Larena *et al*., 1999). Primers LR1 (Acero, 2000) and LR4 (O'Donnell, 1992) were used for amplification of the 28S D1-D2 region.

DNA sequencing ABI PRISM™ Dye Terminator Cycle sequencing Kit (Perkin Elmer) was used. The primers used for sequencing were ITS3 and ITS5, when ITS4/ITS1F were used for amplification, and ITS2 and ITS4, when ITS1F/4A were used for amplification (White *et al*., 1990). For 28S, the primers were LR2 and LR3 (Acero, 2000).

Alignment and analyses ITS and 28S sequences were assembled using the GCG Fragment Assembly System (Program Manual for the Wisconsin Package, Version 8, Wisconsin). Alignments were performed with CLUSTALW (Thompson *et al*., 1994) (IntelliGenetics, Inc., Mountain View, California). PAUP 4.0 (Swofford, 1998) was used for the phylogenetic analysis of the aligned sequences. Bootstrap (Felsenstein, 1985) was calculated to estimate the robustness and reliability of the branches.

Results and Discussion

We analyzed the molecular data obtained from a total of 74 species, 69 in the Helotiaceae and Hyaloscyphaceae, and 5 belonging to the Dermateaceae and Leotiaceae. The amplification products were within the range of 432-588 bp for ITS, and 565-612 bp for the 28S. The phylogenetic trees based on the sequences of the ITS and 28S are in Figs. 1 and 2 respectively.

According to Fig. 1 (I), species of *Lachnum*, *Proliferodiscus*, *Perrotia*, *Dasyscyphella*, *Capitotricha*, *Lachnellula*, and *Trichopeziza* consistently group in the same cluster of Hyaloscyphaceae, although in some cases the branches are poorly supported by the bootstrap values. Fig. 1 (II) contains all the analysed species of *Hymenoscyphus*, except for *Helicodendron paradoxum* (F060490)- teleomorph *Hymenoscyphus paradoxus* (Fisher & Webster, 1983)-, *Cyathicula*, *Discinella* and *Tatraea*. The species of *Hymenoscyphus* in this clade are heterogeneous. In particular, *H. monotropae* (ATCC52305), *H. salmanovicensis* (HB6977), *H. tamaricis* (F110125), *H. epiphyllus* (HB7054, F115879), *H. imberbis* (HB6797), *H. vernus* (HB6915) and *H. kathiae* (HB6924), cluster apart from the bulk of *Hymenoscypus* spp., which is supported by moderate to high bootstrap values. This segregation might support the value of the genus *Cudoniella*. Its type species, *C. acicularis*, which is under study in this project, is probably very close to *Hymenoscyphus vernus*, *H. imberbis* and *H. kathiae*.

The two "*Pezizella*" *amenti* strains (F144616 and HB 6884) clustered together in the same clade (Fig. 1, II) as *Cyathicula cyathoidea* and *C. tomentosa*. This supports the previous classification of Carpenter (1981) based on morphological criteria, who recognised all these species as members of *Crocicreas*, a generic name used by him in a rather wide sense including *Cyathicula*. In the absence of recent collections of the type species of *Crocicreas*, *C. gramineum*, its position can not be clarified, neither by molecular data nor by morphology.

According to Fig. 1 (IV), *Arachnopeziza aurata* (CBS11654, F146760, HB6921b, F122082, F119682, F119695) and *A. aurelia* (CBS11754, F118039) are closer to species in the Helotiaceae rather than to those in the Hyaloscyphaceae. This is inconsistent with the morphological data, that suggest affinities with *Trichopeziza*, especially for *A. aurelia*, but confirms the uncertain taxonomic position of the genus *Arachnopeziza*, considered occasionally as a doubtful member of the Hyaloscyphaceae (Raitvir, 1970).

There is a close relationship between the ITS sequences from *Ombrophila lilacina* (HB6921A) (=*Neobulgaria lilacina* (Wulf.:Fr.) Dennis) and the *Ascocoryne* spp., which is clearly supported by bootstrapping Fig. 1 (IV). Therefore, the new combination "*Ascocoryne lilacina*" might be proposed for the species. In addition, *Ombrophila janthina* (HB7044) and *Neobulgaria pura* (HB6880) (=*Ombrophila pura* (Pers.:Fr.) Fr), the type species for *Neobulgaria* (Fig. 3), appear as somewhat related to species in the Helotiaceae (Fig. 1, IV and Fig. 2), but the branch length and the bootstrap values hardly support this relationship. Further analysis is needed to assess the taxonomic position of *Neobulgaria* (Baral, 1999; Lizon *et al*.., 1998; Korf & Lizon, 2001).

As for *Pezoloma* (Fig. 1, II), another genus recently segregated from the Helotiales (Lizon *et al*.., 1998; Korf & Lizon, 2001), *Pezoloma* sp. (F057390) and *Pezoloma kathiae* Korf (2000) (in the tree as *Hymenoscyphus kathiae* (Korf) Baral comb. nov. in press) (HB 6924), are related to species in the Helotiaceae. Further analysis of other representative species of *Pezoloma* is needed.

The position in the tree of *Discinella terrestris* var. *terrestris* (CFB1861) *and D. terrestris var. inamyloidea* Galán & Baral (unpublished) (Fig. 1, II; Figs. 4,5) seems reasonable according to the morphological affinities existing between *D. terrestris*, *Tatraea* and *Phaeohelotium*. Nevertheless, analysis of the type species, *Discinella boudieri*, is required to assess the taxonomic relationships of *Discinella*.

Ombrophila hemiamyloidea (F115893) is distant from other *Ombrophila* spp. (Fig. 1, IV; Fig. 6), and apparently is not related to any of the species in the tree. This unrelated position fits with the morphological characters of *O. hemiamyloidea*, which somewhat resemble to those of the Dermateaceae spp. (Baral, 1999). The results from the analysis of the 28S sequences also indicate that there is a significant relationship between the sequences of *O. hemiamyloidea* and some *Mollisia* spp (Fig. 2).

Dasyscyphella pulverulenta (F119704, F119705) clusters near typical *Dasyscyphella* spp. and even nearer to *Capitotricha bicolor*., but separately from *Lachnum* spp. (Fig.1). This gives some molecular support to the transfer of this species from *Lachnum* to *Dasyscyphella* (Baral, 1993). The position of *Capitotricha bicolor* (HB6923A), distantly clustered from the *Lachnum* spp. group, is also in accordance with the segregation of *Capitotricha* from *Lachnum* (Baral & Krieglsteiner, 1985). *Lachnellula willkommii* (CBS17035) and *L. calycina* (F119707, F119708) are in the same cluster as *Dasyscyphella* spp. when analysing both ITS and 28S sequences (Figs. 1 and 2). That may suggest the artificial segregation of the two genera on the basis of morphological characters. *Lachnellula* is a xerotolerant genus confined to conifers, characterized by carotenoids and *Naemaspora*-anamorph, and *Dasyscyphella* might have evolved from it by loss of pigment, anamorph, and xerotolerance (Baral, ined.).

"*Lachnum*" *cerinum* (*Neodasyscypha cerina* nom. inval.) strains (F115892, HB6871B, F119685) are out of the cluster containing the representative species of *Lachnum* (Fig. 1, I; Fig. 7). It is generally accepted that the morphological features of *L. cerinum* do not match those of *Lachnum* spp., but are similar to those of *Perrotia* and *Proliferodiscus*. Our molecular data agree with this difference.

The position of *Bisporella subpallida* (HB6849a) in the ITS tree (Fig. 1, IV) is unexpected when considering its morphological features. This species and the type species, *B. pallescens*, exhibit apical rings of the *Hymenoscyphus*-type and multiguttulate paraphyses (Baral; 1987), and are assumed to be related to *Hymenoscyphus-Cyathicula*. The results from the 28S sequences are consistent with these affinities to *Hymenoscyphus* (Fig. 2). *B. citrina* (F115889, F118000) has similar morphological characteristics to *Calycina* spp.. It has long-cylindrical refractive vacuoles in the paraphyses, and an apical ring of the *Calycina-Allophylaria*-type (Baral, l.c.). These morphological similarities are well supported by our sequence data (Fig.1, III; Fig. 2).

An appropriate genus for *Phaeohelotium trabinellum* (HB 6810b) and the similar *Calycellina* aff. *guttulifera* (HB 6787) (Fig. 1, III; Figs. 8, 9) has not yet been found, but the morphological characteristics, in particular the presence of an apical ring of the *Calycina*-type (Baral, ined.) have suggested a relation to *Calycina* or *Calycellina*, which is supported by our molecular data.

Calycina gemmarum (Boud.) Baral (in the tree as "*Pezizella*" *gemmarum*, F144622) and "*Pezizella*" *junipericola* Svrcek (HB6910) are close one to each other and related to *Calycellina* and *Calycina* (Fig. 1, III). This result is supported by micromorphology (Baral & Krieglsteiner, 1985). *Pezizella gemmarum* was combined in *Hyphodiscus* by Raitviir & Galán (1994), a transfer which needs further molecular study to support.

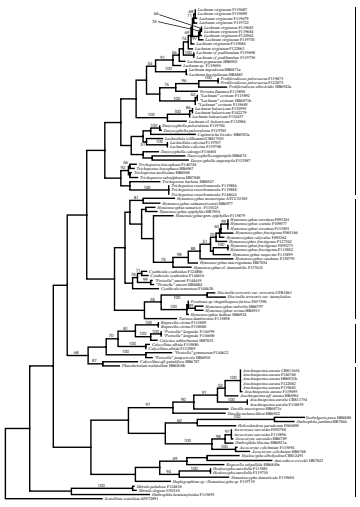


Fig. 1. Most parsimonious phylogenetic tree generated from a branch and bound algorithm in PAUP 4.0 after alignment of the ITS1-5.8S-ITS2 region. Tree length = 2398 steps, consistency index = 0.334 and retention index = 0.733. Bootstrap replicates values are indicated at the base of the corresponding clade when more than 50%. GenBank sequence from *Mitrula elegans* U92310 was used to confirm the identity of *M. paludosa* (F124810). GeneBank sequence from *Scutellinia scutellata* was the outgroup.

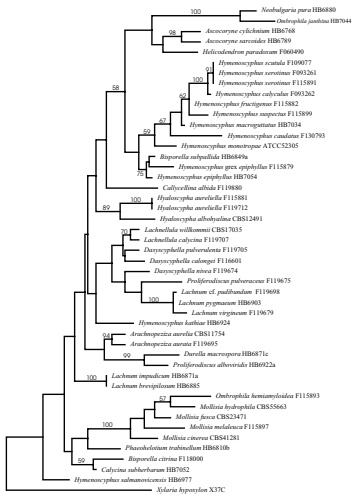


Fig. 2. Most parsimonious phylogenetic tree generated from a branch and bound algorithm in PAUP 4.0 after alignment of the 28S 5´ region. Tree length = 646 steps, consistency index = 0.450 and retention index = 0.629. Bootstrap replicates values are indicated at the base of the corresponding clade when more than 50%. *Xylaria hypoxylon* X37C was the outgroup.

Fig. 3. *Neobulgaria pura*

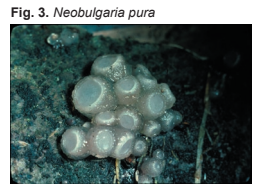


Fig. 4. *Discinella terrestris* var. *terrestris*

Fig. 5. *D. terrestris* var. *inamyloidea*

Fig. 6. *Ombrophila hemiamyloidea*

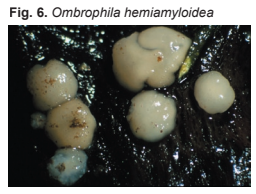


Fig. 7. "*Lachnum*" *cerinum*

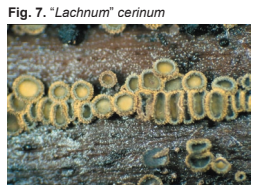
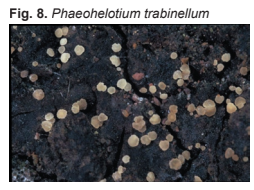


Fig. 8. *Phaeohelotium trabinellum*



Conclusions

- The ITS sequences are an acceptable tool for assessing the taxonomic relationships of the Hyaloscyphaceae and Helotiaceae. Nevertheless, a combination of characters, including more molecular markers and morphological data, must be considered, especially in some controversial cases.
- There is an overall consistency between the classical morphology-based classification and the molecular data in *Lachnum* and *Hymenoscyphus*. Some molecular divergences concur with the morphology, but others are in disagreement and need further analysis.
- The molecular results confirm that *Lachnum cerinum* is not *Lachnum*, and a new combination should be provided.
- The segregation of *Dasyscyphella pulverulenta* and *Capitotricha* from *Lachnum* is supported by molecular data.
- The taxonomic position of *Neobulgaria* and *Pezoloma* needs further molecular study.
- *Ombrophila lilacina* could be combined as "*Ascocoryne lilacina*". *O. hemiamyloidea* is unrelated to the other *Ombrophila* spp. and close to the Dermateaceae.

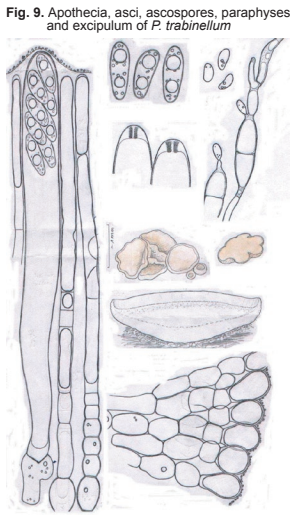



Fig. 9. Apothecia, asci, ascospores, paraphyses and excipulum of *P. trabinellum*