Hymenoscyphus crataegi (Helotiales), a new species from Spain and its phylogenetic position within the genus *Hymenoscyphus**

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Hymenoscyphus crataegi is described as new to science. The species was found growing on *Crataegus monogyna* leaves collected in Spain. It is characterized by homopolar, non-scutuloid ascospores, being acute at both ends, with a high lipid content of large guttules, in combination with asci arising from simple septa. The species is morphologically close to *H. macroguttatus* nom. nov. and *H. caudatus*, but molecular phylogenetic analysis of the ITS 1-5.8S-ITS 2 region of the rRNA operon suggested a higher affinity with *H. immutabilis*.

Key words: *Hymenoscyphus crataegi*, *Hymenoscyphus macroguttatus*, phylogeny, taxonomy.

The genus *Hymenoscyphus* S.F. Gray is one of the large genera within the Helotiaceae, considering that it comprises more than one hundred species (Kirk *et al.* 2001). The large diversity and variability of the morphological features used to delimitate the genus makes it difficult to clarify the circumscription and relationships among species (Dennis 1963, Dumont & Carpenter 1982, Lizoň 1992, Korf & Lizoň 1994). Studies on the structure and ultrastructure of the ascus apical apparatus (Baral 1987, 1994, Verkley 1993) have

* We dedicate this paper to the memory of the late Dr. Ain Raitviir (10 Jul 1938 – 17 Sep 2006).

shown that the genus can be defined by a special type of apical ring (*Hymenoscyphus*-type), while several previously included species with a very different ring type (*Calycina*-type) are to be excluded from the genus. Just a few preliminary studies have been carried out to clarify its taxonomical position and the relationships among its species from a molecular phylogenetics perspective (Collado *et al.* 2002, Zhang & Zhuang 2004). The only exception is the mycorrhizal group of species ascribed to *H. ericae* (D.J. Read) Korf & Kernan, recently transferred to a separate genus as *Rhizoscyphus ericae* (D.J. Read) W.Y. Zhuang & Korf (Zhang & Zhuang, l.c.), which has been the subject of more extensive molecular studies (e.g. Vrålstad, Myhre & Schumacher 2002, Hambleton & Sigler 2005, Zijlstra *et al.* 2005). This species has been transfered to the genus *Pezoloma* Clem. as *P. ericae* (D.J. Read) Baral based on, e.g., apical rings of the *Calycina*-type and eguttulate paraphyses (Baral & Krieglsteiner 2006).

During a project concerning the molecular phylogeny of the order Helotiales based on the sequence analysis of the ITS 1-5.8S-ITS 2 region of the rRNA (Collado *et al.* 2002), several apothecia of an unknown species of the genus *Hymenoscyphus* were found growing on fallen leaves of *Crataegus monogyna*, collected in Western Spain. The new taxon resembles *H. caudatus* (P. Karst.) Dennis at a first glance, but its micromorphological features, together with phylogenetic results derived from ITS sequences of several related species, prompted us to describe a new species and to present a preliminary assessment of the phylogenetic relationships among selected species within the genus *Hymenoscyphus*. Taxonomic implications within the genus, including the new taxon, are discussed from both morphological and molecular points of view.

Material and Methods

Fungal isolation and growth conditions

Ascospores were released from fresh apothecia and spread on CMA (Corn Meal Agar-Difco plus streptomycin 5 µg mL^{-1} and terramycin 50 µg mL^{-1} Sigma) plates using a micromanipulator device. The isolates were preserved in the CIBE Culture Collection as frozen agar plugs in glycerol 10 %. The description of colonies was based on potato dextrose agar (PDA, Difco) and oatmeal agar (OTM, Difco) in 90 mm Petri dishes. Plates were incubated at 22 °C and 80 % relative humidity for three weeks. Colour names and codes are from Kornerup & Wanscher (1978).

Morphological observations

Light-microscopic observations were made by R. Galán and H.-O. Baral on living material in tap water (cf. Baral 1992). However, the fresh specimen collected in Western Spain, sent to Germany immediately after collection, arrived with all asci having lost their turgescence. An unexpected new, although poor finding of the same fungus in the North of Spain was studied by Carlos E. Hermosilla, allowing us to get an idea of the variability of the species.

Measurements of living cells were made in tap water; those of dead cells in tap water, Melzer's reagent (MLZ), Lugol's solution (IKI), and KOH 2 – 5 %. The light microscopes used were a Nikon Labophot-2 phase-contrast microscope, with magnification up to 1250X and a Zeiss Standard 20 using Euromex oculars, magnification 1500X. Freehand drawings were made by H.-O. Baral. Photographs 1 A and 1 B were made *in situ* by F. Prieto using an EOS 300 camera equipped with a 50 mm macro lens and approach rings. The remaining pictures were taken at laboratory by R. Galán under a binocular Nikon SMZ-2T using a Volpi AG Intralux 5000-1 fiber optics source.

The holotype is deposited in AH (University of Alcalá), an isotype in the private herbarium of H.B. (H.-O. Baral, Tübingen), and a subculture of the type (AH 7596) is deposited in the CIBE Culture Collection (Centro de Investigación Básica. Merck, Sharp & Dohme de España S. A., Madrid).

Abbreviations: (*) = living cells, (†) = dead cells

DNA extraction

The extraction was carried out following a modification of a procedure previously described by Boysen *et al.* (1996): living mycelium was resuspended in 500 mL extraction buffer [50 mM Tris/HCl (pH 8.0), 100 mM EDTA and 1 % SDS] and heat-shocked. Afterwards it was extracted twice with an equal volume of phenol: chloroform: isoamylalcohol 25:24:1, followed by an extraction with chloroform: isoamylalcohol 24:1. The aqueous phase was then incubated at 37 °C for 30 min in the presence of 5 mg RNase A mL^{-1} and the DNA was precipitated with isopropanol, washed with 70 % ethanol and resuspended in 50 mL sterile water. Genomic DNA was visualized in 1 % agarose gels.

DNA amplification and PCR product purification

The entire ITS 1-5.8S-ITS 2 region was amplified using primers ITS 1 F and ITS 4 (White *et al.* 1990), as described (González del Val *et al.* 2003). All the resulting PCR products were purified before

sequencing using GFX™ PCR Gel Band Purification Kit (Amersham Pharmacia Biotech Inc, USA).

Sequencing and phylogenetic analysis

The amplified and purified products were sequenced with an ABI PRISM™ Dye Terminator Cycle Sequencing Kit (Perkin Elmer). The samples were sequenced in both directions as described for the ITS region (Sánchez-Ballesteros *et al.* 2000). Sequences were edited and assembled using the SeqEd v1.0.3 software. All sequences were deposited in GenBank (Table 1). DNA sequences were aligned and visualized using the multiple alignment program Clustal X 1.81 (Thompson *et al.* 1997) and the ends of the alignment were trimmed after with Se-Al v2.0a11 Carbon software (http://evolve.zoo.ox.ac.uk/software.html?id=seal). TuneClustalX software (Hall 2004) was used to obtain the mean Q-score of the entire alignment to estimate the accuracy of the alignment.

The phylogenetic analysis was carried out on a G4 Macintosh computer, using neighbor-joining (NJ) and maximum parsimony (MP) methods, using PAUP* ver. 4.0 b10 (Swofford 2001). NJ analysis was carried out using the Kimura 2-parameter (K2) to obtain a measure for the genetic distances. MP was conducted by heuristic search and performed with simple addition of sequences and TBR branch swapping, with MaxTrees set to 1000. All characters were unordered and equally weighted, with gaps treated as missing data. The trees were rooted with *Ciboria americana* Durand (Sclerotiniaceae) (F115887) as the outgroup. The confidence of the branches was measured in both methods by bootstrap analysis re-sampled with 1000 bootstrap replicates (Felsenstein 1985). Decay indexes (Bremer 1994) were also calculated for the MP analysis, using the TreeRoot ver. 2 software (Sorenson 1999). The trees were visualized with the application Treeview X.

Taxonomic Results

Based on morphological observations and results of the molecular phylogenetic analysis we describe *Hymenoscyphus crataegi* as a new species as follows:

Hymenoscyphus crataegi Baral & R. Galán **sp. nov.** – Figs. 1, 2.

Apothecia 0.4 – 0.6 mm in diam., dispersa, tota lacteo-alba, glabra, cum stipite tenui. Asci in statu emortuo 60 – 80 × 6 – 7.5 µm, octospori, cum apice conico, haud amyloideo seu saepius leviter caerulescens, e septis simplicibus excrescentes. Ascosporae in statu vivo (10.5) 12 – 16 × (3.8) 4 – 4.3 (4.8) µm, naviculiformes cum

ambis apicibus subacutis vel acutis, hyalinae, aseptatae, globulis aliquot magnis atque parvis continentur, setulis absentibus. Paraphyses cylindraceae, cellulis terminalibus in statu vivo cum vacuolis globulosis, haud seu parum refractivis. Excipulum ectale e textura prismatica (-porrecta) e cellulis tenuiter seu subcrasse tunicatis. Medulla e textura intricata.

Habitat: Ad folia Crataegi monogynae, in Jaraiz de la Vega, provincia Cáceres, Hispania; legit F. Prieto et E. Santiago (21 Sep 2002); holotypus in AH 7596, isotypus in H.B. 7211.

Apothecia moist 0.4 – 0.6 mm diam., round, non-gelatinous, scattered, disc pure milky-white, slightly concave, margin smooth, stipe slender, 0.4 – 0.8 × (0.1) 1.5 – 0.2 (0.25) mm, white, erumpent from small cracks in epidermis; receptacle turning yellowish-cream with age. Asci (†) 60 – 80 × 6 – 7.5 µm, 8-spored, spores biseriate; apex (†) conical to submammiform, apical thickening immature † 2 – 3 µm thick, IKI- or more often feebly blue (euamyloid = type bb, apical ring visible only in lower 1/3 – 1/4 as two thin lines in optical section, *Hymenoscyphus*-type); base gradually narrowed in a medium-long stalk, consistently arising from simple septa. Paraphyses apically cylindrical, not inflated, terminal cells (*) 14 – 33.5 × 2.2 – 2.7 (3) µm, containing some roundish, non- or very slightly refractive vacuoles in the upper 10 – 15 µm; lower cells (*) 14.5 – 22 × 1.7 – 2.5 µm. Ascospores (*) (10.5) 12 – 16 (18) × (3.8) 4 – 4.3 (4.8) µm, (†) 14 – 17.5 × (3) 3.5 – 4 (4.5) µm, naviculiform, both ends subacute to acute, hyaline, aseptate, straight or very slightly inequilateral, containing 1 – 3 large LBs c. 1.5 – 2.5 µm diam. in each half and several to many smaller ones, no sheath observed, without setulae; postmature spores not observed. Medullary excipulum of textura intricata, hyphae (†) 2.4 – 3.2 µm wide. Ectal excipulum hyaline, of thin-walled to slightly gelatinized textura prismatica/porrecta oriented at a low angle to the surface, individual cells (*) 12 – 43 × 10 – 16 µm, at margin of somewhat more gelatinized textura porrecta forming a palisade, in stalk of textura prismatica with cells measuring (†) 18 – 24 × 5 – 8 µm, externally covered on flanks by slightly undulating hyphae (*) 3 – 4 µm wide. No crystals observed, no staining of cell walls in IKI. Vacuoles in paraphyses and covering hyphae of ectal excipulum turn red-brown in IKI (multiguttulate) and extrude red-brown granules.

Culture. Colonies on PDA attaining 11 mm in 21 days; wrinkled, velvety, with radial grooves, copper green (26B6) at the center, becoming light green (30A5) outwards and white at the edge of the colony, margin irregular; reverse dark green (30F3). Dark green (30F6) exudates produced predominantly at the center. Diffusible pigment olive brown (4F7), visible around the colony. Colonies on OTM 26 mm in diameter, flat, velvety, dull green (27D4) at the center, becoming light green (30A4) in outer parts, margin regular; reverse

dark green (28F5). Exudates absent. Diffusible pigment reddish brown (8E5). Anamorph not observed.

Etymology: Referring to the substrate (leaves of *Crataegus*).

Holotype: SPAIN, Cáceres, Jaraiz de la Vera, 570 m a.s.l.; *Quercus pyrenaica* forest over acid soil, on *Crataegus monogyna* leaves of the previous year, on lower, sometimes also upper face, on both main- and net-veins, 21 Sep 2002, F. Prieto & E. Santiago. AH 7596 (holotype), H.B. 7211 (isotype).

Further specimens examined: SPAIN, Alava, Opakua mountain pass, Opakua, 1000 m a.s.l.; mixed forest (*Pinus spp., Pseudotsuga menziesii, Fagus sylvatica* etc.), on fallen leaves of *Crataegus monogyna*, 13 Oct 2002, C.E. Hermosilla. Drawing performed by C.E. Hermosilla, material not preserved.

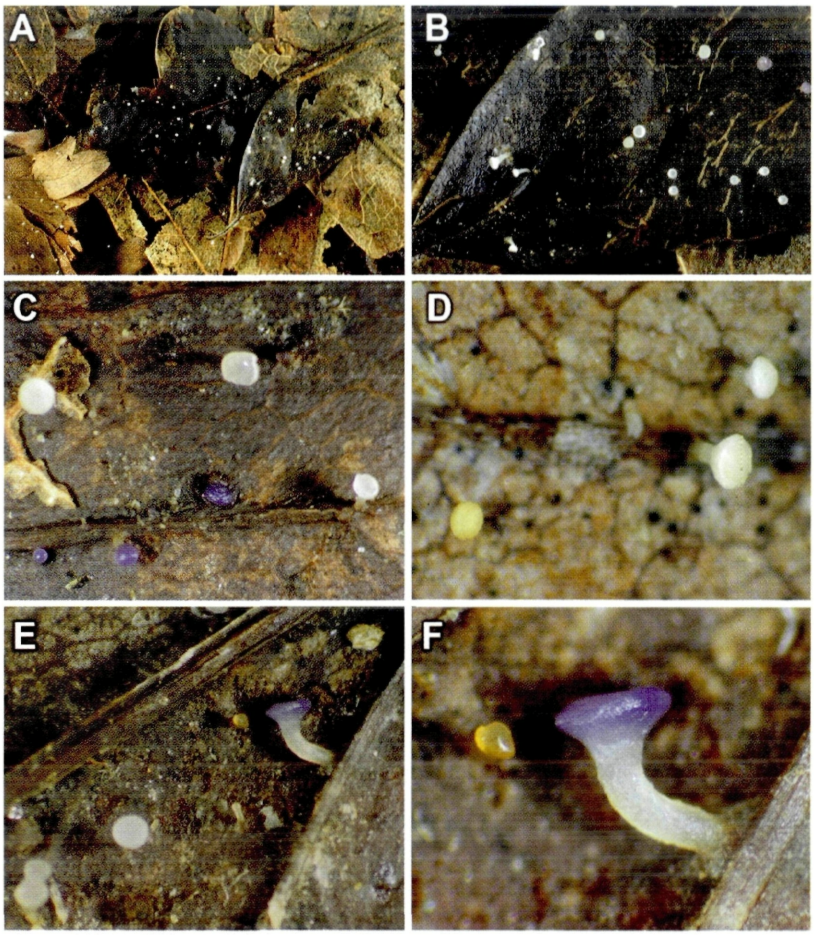

Fig. 1. – *Hymenoscyphus crataegi* (holotype): **A, B.** Apothecia in their natural habitat (Phot. F. Prieto, *in situ*). **C – F.** Enlarged view of fresh apothecia under dissecting microscope. Some apothecia show a violet pigment, supposedly due to some bacterial infection (Phot. R. Galán, *ex situ*).

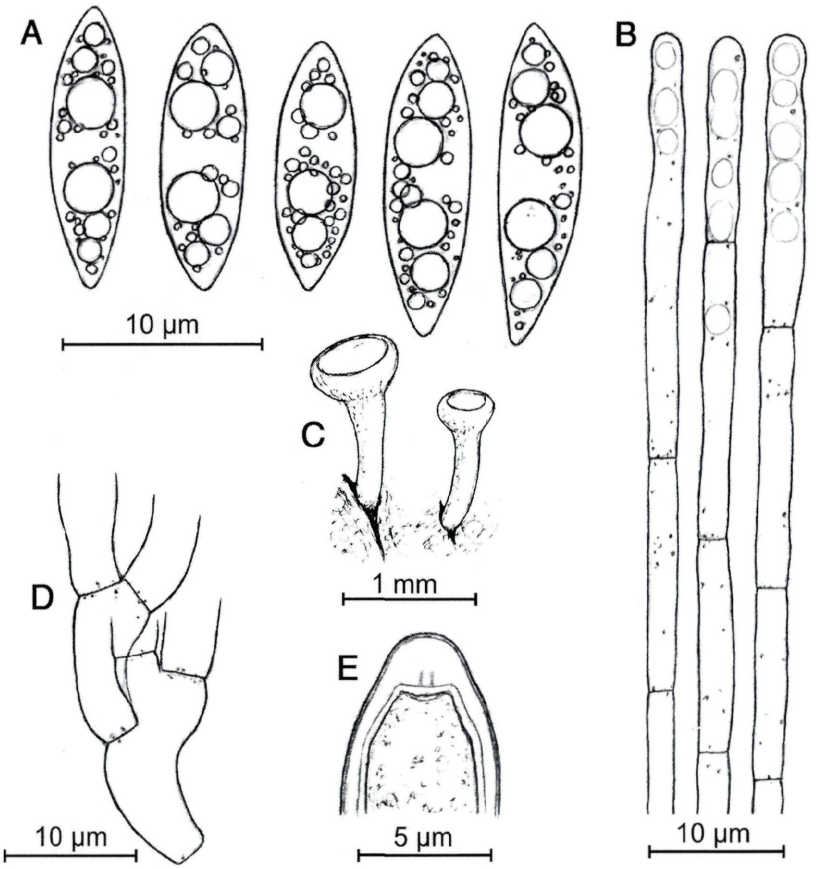

Fig. 2. – *Hymenoscyphus crataegi* (isotype): **A.** Ascospores. **B.** Paraphyses. **C.** Fresh apothecia. **D.** Base of the asci showing simple septa. **E.** Apex of an immature ascus, in Lugol (Drawings H. O. Baral; living state except Fig. E).

Phylogenetic Results

To clarify the relationships of the new species with other *Hymenoscyphus* taxa, we analyzed the sequences of the ITS 1-5.8S-ITS 2 region of 31 specimens (Table 1). A total of 13 new sequences were generated to complete the study. Amplifications of the ITS regions resulted in single DNA fragments about 450 – 900 bp. PCR products longer than approximately 500 bp were sometimes obtained, due to the presence of different group I introns inserted at the end of the 18S-rRNA gene. These introns were subsequently removed from the alignment prior to the phylogentic analysis. The resulting alignment data matrix consisted of 31 sequences and 504 characters.

Tab. 1. – Material used in this study. The new sequences produced for this study are indicated in bold type, including the newly described species. Strain codes: F, BR, CIBE-MSD Culture Collection; H.B. – Hans-Otto Baral Herbarium; ARON – Ascomycete Research group of Oslo, Norway; HMAS – Herbarium Mycologicum Instituti Microbiologici Academiae Sinicae.

Species	Strain code	GenBank Accession No	Substrate	Geographic origin
H. cf. *macroguttatus*	HMAS 82089	AY348589	On herbaceous stem	Jilin (China)
H. macroguttatus	HMAS 82093	AY348590	On fern	Jilin (China)
H. macroguttatus	HMAS 82098	AY348591	On herbaceous stem	Beijing (China)
H. macroguttatus	**H.B.7034**	**DQ431179**	On leaves of *Acer pseudoplatanus*	Hessen (Germany)
H. fructigenus	ARON3264.H	AJ430396	From decaying nut of *Quercus robur*	Norway
H. fructigenus	**F115882**	**DQ431171**	On fruits of *Quercus suber*	Aracena, Huelva (Spain)
H. fructigenus	**F156965**	**DQ431176**	On *Abies* twigs	Boi Taul, Lérida (Spain)
"H. fructigenus"	**F109077**	**DQ431169**	Unknown	San Millán de la Cogolla, Logroño (Spain)
H. serotinus	**F093261**	**DQ431168**	On rotten wood of *Fagus sylvatica.*	Pto. de la Quesera, Segovia (Spain)
H. serotinus	**F115891**	**DQ431173**	On branches of *Fagus sylvatica.*	Riaza, Segovia (Spain)
H. serotinus	**F159526**	**DQ431178**	On *Fagus sylvatica*	Pto. de la Quesera, Segovia (Spain)
H. caudatus	HMAS 82057	AY348576	On herbaceous stem	Anhui (China)
H. caudatus	HMAS 82063	AY348578	On herbaceous stem	Jiangxi (China)
H. caudatus	HMAS 82073	AY348579	On herbaceous stem	Jiangxi (China)
H. cf. *menthae*	HMAS 75934	AY348588	Dead wood	Sichuan (China)
H. globus		AY348593	Wet hard wood	Jiangxi (China)
H. crataegi sp. nov.	**F156966**	**DQ431177**	On leaves of *Crataegus monogyna*	Jaraiz de la Vera, Cáceres (Spain)
H. immutabilis	**F137632**	**DQ431174**	On leaves of *Ulmus* sp.	Colmenarejo, Madrid (Spain)

Tab. 1 continued.

Species	Strain code	GenBank Accession No	Substrate	Geographic origin
H. immutabilis	**F155012**	**DQ431175**	On decayed leaves of *Salix caprea*	Lanuza, Huesca (Spain)
H. immutabilis	HMAS 71809	AY348584	Rotten leaves of *Populus* sp.	Beijing (China)
H. tamaricis	**BR020**	**DQ431167**	On *Tamarix gallica* debris	Alcalá de Henares, Madrid (Spain)
H. epiphyllus	HMAS 82075	AY348580	On fallen leaves	Jiangxi (China)
H. epiphyllus	**H.B.7054**	**DQ431180**	On bark of *Quercus* sp.	Tübingen (Germany)
H. epiphyllus	**F115879**	**DQ431170**	On leaves of *Castanea sativa* and *Quercus suber*	Aracena, Huelva (Spain)
H. lasiopodius	HMAS 71820	AY348585	Dead root of *Carex* sp.	Beijing (China)
H. lasiopodius	HMAS 71821	AY348586	Dead root of *Carex* sp.	Beijing (China)
H. lasiopodius	HMAS 75878	AY348587	Dead root of *Carex* sp.	Beijing (China)
Rhizoscyphus ericae		AY394685	Unknown	Ecuador
R. ericae		AY394686	Unknown	Ecuador
R. ericae		AY394687	Unknown	Ecuador
Ciboria americana	**F115887**	**DQ431172**	On fruits of *Quercus suber*	Aracena, Huelva (Spain)

The NJ analysis of the aligned sequences resulted in the phylogenetic tree shown in Fig. 3. The *Hymenoscyphus* species were distributed across six main clades, grouped together in a branch with high bootstrap support (98 %), and separated from a basal clade representing the recently erected genus *Rhizoscyphus* (formerly *H. ericae*). Four of the main six clades (groups I to IV) would represent the genus *Hymenoscyphus s. str.* (*H. caudatus* species complex, *H. serotinus, H. fructigenus,* and *H. macroguttatus* complex). These four clades were supported by high bootstrap values (98 – 100 %), and grouped together within a branch with bootstrap support of 100 %. Group V contained the new species *H. crataegi,* clustered together with *H. immutabilis* and *H. globus* in a clade with high bootstrap support (99 %). Group VI included *H. lasiopodius, H. tamaricis,* and *H. epiphyllus,* and this clade was supported by just a moderate bootstrap index (78 %). Groups V and VI would account for the species considered as *Hymenoscyphus s. lat.*

For the MP analysis, 504 characters were considered of which 320 were constant, 46 were variable but parsimony-uninformative, and 138 were parsimony-informative. A single most parsimonious tree was obtained (Fig. 4). The tree length was 341 steps, with consistency index CI = 0.557, homoplasy index HI = 0.443, retention index RI =

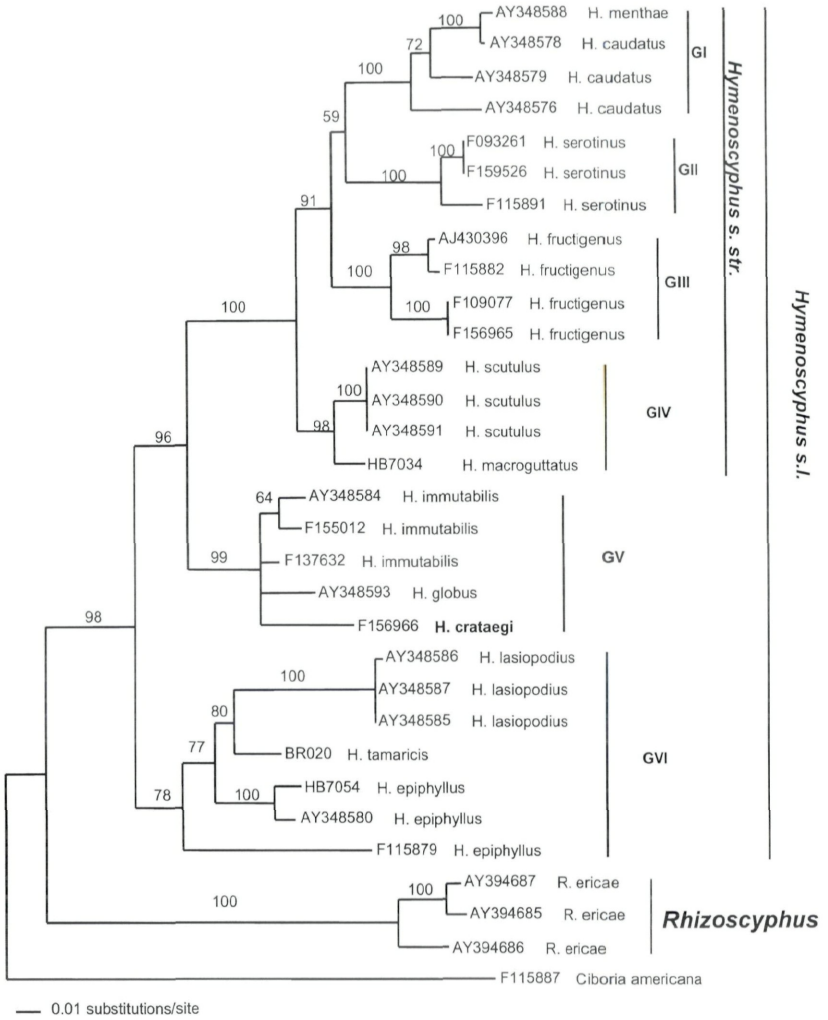

Fig. 3. – Phylogenetic tree based on neighbor-joining (NJ) method using Kimura 2-parameter as distance measure for the ITS 1-5.8S-ITS 2 region of *Hymenoscyphus* species. The newly described species is shown in bold type. Bootstrap support values are indicated (when higher than 50%) at the base of the corresponding clade (above the line). The tree was rooted with the sequence of *Ciboria americana* (Sclerotiniaceae) (F115887) as outgroup.

0.802 and rescaled consistency index RC = 0.447. The MP analysis resulted in a tree with very similar topology to the one obtained

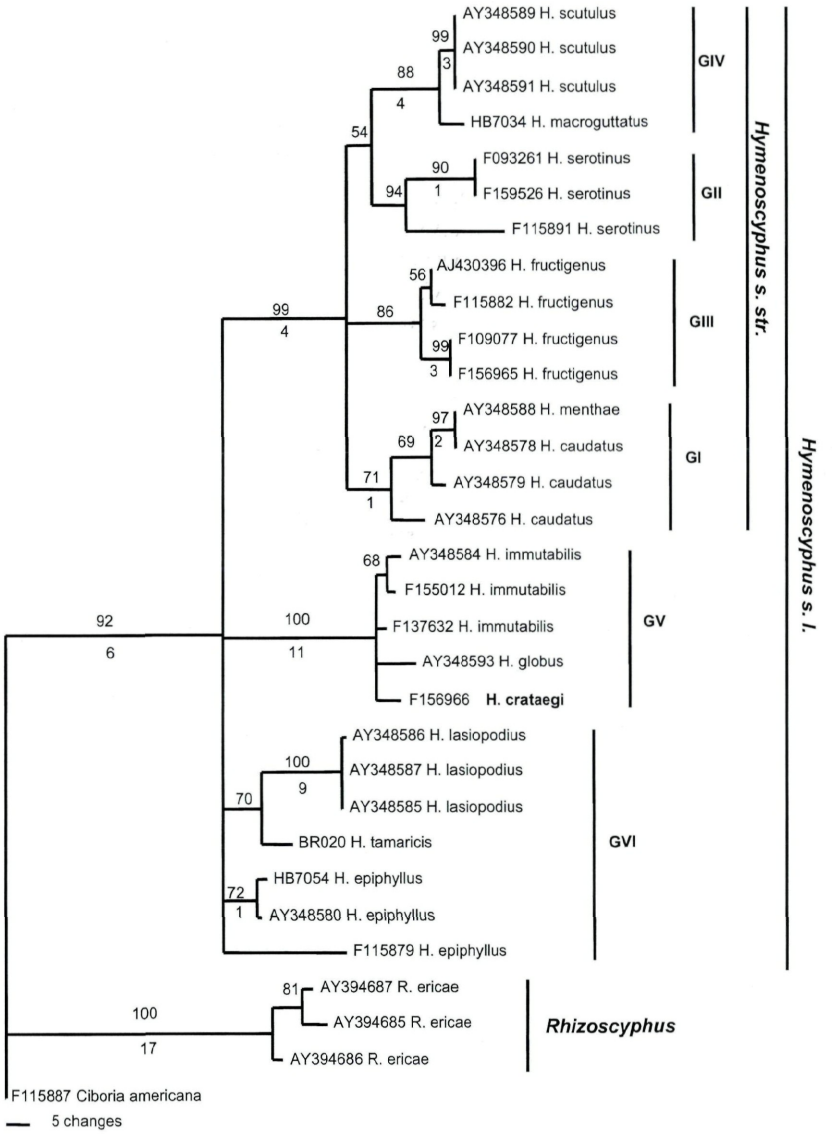

Fig. 4. – Phylogenetic tree generated by maximum parsimony (MP) analysis using the ITS 1-5.8S.ITS 2 region of *Hymenoscyphus* species. Bootstrap support values are indicated (when higher than 50 %) at the base of the corresponding clade (above the line) and decay indexes are indicated below. The newly described species is shown in bold type. The tree was rooted with the sequence of *Ciboria americana* (Sclerotiniaceae) (F115887) as outgroup.

by NJ method. The tree (Fig. 4) also grouped together all the *Hymenoscyphus* species within a single clade with high bootstrap support (92 %), segregated from a basal branch containing the *Rhizoscyphus* strains. The tree contained the same four monophyletic groups (I to IV) representing *Hymenoscyphus s. str.,* supported by moderate to high bootstrap values (71 – 94 %), and clustered together within a branch with high statistical support (99 %). The group V, including the new species *H. crataegi,* was also conserved in this tree, with a bootstrap support of 100 %. The main difference with the NJ dendrogram was that *H. lasiopodius, H. tamaricis,* and *H. epiphyllus* were not grouped into a single clade (group VI). Instead, the strains of *H. lasiopodius* were grouped together with *H. tamaricis* in a cluster with weak support (70 %), whereas the strains of *H. epiphyllus* were divided into two groups and rooted at the same level in the tree.

Discussion

Taxonomy: At first glance, *H. crataegi* resembles both macro- and microscopically *H. caudatus* (P. Karst.) Dennis. It differs from that species predominantly in the constantly homopolar spores, acute at both ends, in the asci and spores being narrower, and apparently also in the very faint iodine reaction of the ascus apical ring (strongly euamyloid in *H. caudatus*). Like *H. crataegi*, *H. caudatus* has occasionally been recorded on *Crataegus* leaves (Dennis 1986, Arendholz 1979: 84). Also, *H. caudatus*, in a restricted sense, has asci arising from simple septa and paraphyses with only faintly refractive vacuoles. *Hymenoscyphus caudatus* is a collective species in which different taxa have been included (Dennis 1956: 82), some of them having paraphyses with strongly refractive vacuoles (Baral & Krieglsteiner 1985: 123), and in a few cases asci arising from croziers (White 1943: 151). White (1943) examined the type material in FH, but the legend to his plate (showing for *H. caudatus* slightly scutuloid spores) lacks any remark on this species and therefore does not indicate from which specimen the drawing derives.

Hymenoscyphus phyllogenus (Rehm) O. Kuntze, a species possibly restricted to leaves of *Populus,* is similar to *H. crataegi* in the spores being homopolar, the paraphyses with scarcely refractive vacuoles staining deep red-brown in IKI, and the asci arising from simple septa, besides a similar macromorphology. It differs from the new species in the spores with rounded ends and very low lipid content, and in the asci with a more distinct amyloid ring (see Baral & Krieglsteiner 1985: 132).

Hymenoscyphus immutabilis (Fuckel) Dennis, reported on leaves of various trees, is very distinct in having larger apothecia with

comparatively short and wide stalks, and asci arising from croziers. The ectal excipulum towards the base of the receptacle and in the stipe is composed of a textura globulosa-angularis oriented at a high angle to the surface, much unlike *H. crataegi.* Similar to *H. crataegi* are the contents of the living paraphyses, which show a low refractivity and produce abundant extruding red-brown granules when treated with IKI (Baral & Krieglsteiner 1985: 131).

A species having spores very similar to *H. crataegi* in size, shape, and oil drops is *H. macroguttatus* Baral *et al.* (see below). This species is mainly caulicolous but rarely also lignicolous, foliicolous, and fructicolous. The two known "foliicolous" collections were on leaves of *Acer pseudoplatanus* and *Pteris vitatta* fronds (the collection from the latter host is the type of *H. pteridicola* Thind & Sharma). Despite the strong similarity in spore morphology and macromorphology between *H. crataegi* and *H. macroguttatus,* the latter sharply differs in the asci consistently arising from croziers, in a more or less strongly amyloid apical ring, and in the living paraphyses containing strongly refractive vacuolar guttules in their terminal cells.

Re-examination of the type material of *H. menthae* (W. Phillips) Baral proved that this taxon was misapplied previously, but is in fact an earlier synonym of *H. consobrinus* (Boud.) Hengstm., a species with multiguttulate homopolar spores and simple-septate ascogenous hyphae. When searching for an available name for *H. menthae s. auct.,* the type material of *Hymenoscyphus pteridicola* Thind & Sharma was found to obviously represent the same species. However, due to the homonymy with *Hymenoscyphus pteridicola* (P. & H. Crouan) O. Kuntze, a new name must be chosen.

Hymenoscyphus macroguttatus Baral, B. Declercq & Hengstm. **nom. nov.**

Basionym: *Hymenoscyphus pteridicola* Thind & Sharma, Nova Hedwigia 32: 125, Figs. 5 – 7 (1980) (non *Hymenoscyphus pteridicola* (P. & H. Crouan) O. Kuntze = *Crocicreas cyathoideum* var. *pteridicola* (P. & H. Crouan) S. E. Carp.)

= *H. menthae* (W. Phillips) Baral ss. Baral & Krieglsteiner 1985, Hengstmengel 1996.

= *H. scutula* var. *solani* ss. Korf & Zhuang 1985: 500.

Ecology: The new species was found in the litter of a *Quercus pyrenaica* forest with intermingled *Crataegus* shrubs. Although the fallen leaves of both *Quercus* and *Crataegus* lay tightly mixed on the ground, the apothecia of this species were only found on *Crataegus* (Fig. 1 A).

Some of the apothecia had a bright violet-blue hymenium and receptacle in overmature stages (Figs. 1 C, 1 E, 1 F). This was thought

to be due to some bacterial infection, but bacteria could not be seen under the LM, while the violet pigment was located in scattered particles outside the cells of paraphyses.

Molecular phylogenetic analysis: The phylogenetic analysis derived from the ITS sequences using either NJ or MP methods grouped the selected *Hymenoscyphus* species into a strongly supported monophyletic clade separated from the basal branch of *Rhizoscyphus ericae*. The *Hymenoscyphus* sequences were distributed across six main groups. Groups I to IV contained species considered as *Hymenoscyphus* s. str., whereas groups V and VI included species within *Hymenoscyphus* s. lat. All these clades showed significant bootstrap support, except group VI, which had only modest bootstrap support in the NJ tree and did not appear as a monophyletic clade by MP. Interestingly, groups I to IV, representing the core of the genus, were grouped as a monophyletic higher level clade with strong bootstrap support by both methods, although the internal relationships among these four groups were not consistently resolved by the two methods of analysis. Overall, the clades resulting from the phylogenetic analysis of the ITS sequences were consistent with the known morphological relationships among the species included in the study, except for *H. immutabilis* which from its micromorphology would better fit to *H. imberbis* (Bull.: Fr.) Dennis (which belongs in a clade not included in this phylogenetic tree). Furthermore, *H. crataegi* would have been expected to cluster with *H. macroguttatus* whilst, on the other hand, the three Chinese strains filed under *H. scutula* in GenBank clustered with *H. macroguttatus*.

The first clade (group I) grouped together three strains of *H. caudatus* and one named "*H.* cf. *menthae*" in GenBank. Our molecular analysis suggests that the latter strain is conspecific with one of the *H. caudatus* strains. It further indicates that the name *H. menthae* was obviously not applied by Zhang & Zhuang (2004) in the sense of Hengstmengel (1996) who described a species which is here called *H. macroguttatus*. The strain "*H.* cf. *menthae*" derives from a collection on wood, with asci with croziers and heteropolar ascospores without setulae (W.-Y. Zhuang; pers. comm.).

Hymenoscyphus caudatus is currently used for foliicolous collections, but within this ecological group *H. caudatus* is a collective species being morphologically rather variable and heterogenous. Our molecular analysis supports the concept of a *H. caudatus* species complex, as previously suggested (Zhang & Zhuang 2004).

The sequenced strains of *H. serotinus* and *H. fructigenus* were grouped as two well supported monophyletic groups (II and III, respectively). *Hymenoscyphus fructigenus* is considered as a species complex comprising several fructicolous species, taxonomically

treated as varieties, being difficult to delimit at the species level (Baral 1996). The two subgroups within *H. fructigenus* differ in the substrate (fructicolous versus lignicolous) and therefore probably belong to two different species. A thorough study of fresh collections should help clarifying the taxonomical delimitation and phylogenetic relationships within this species complex.

Group IV including three sequences from GenBank identified as *H. scutula* (Zhang & Zhuang 2004) grouped together with our sequence of *H. macroguttatus*. Given that *H. scutula* and *H. macroguttatus* (= *H. menthae* s. auct.) were frequently confused in the past (*H. scutula* var. *solani* ss. Korf & Zhuang 1985 is *H. macroguttatus* according to a study of the herbarium specimen) it seems possible that the three Chinese strains labelled as *H. scutula* belong to *H. macroguttatus*. In fact, our strain of *H. scutula* from Spain clustered as a sister group of *H. caudatus* (results not shown here). However, two of the GenBank strains (AY348590, AY348591) had heteropolar ascospores with setulae (W.-Y. Zhuang; pers. comm.) what excludes identity with *H. macroguttatus*. Whether or not these two strains represent *H. scutula* remains unclear, but our phylogenetic data indicate that they are not conspecific with our personal collection we have referred to *H. scutula*.

Group V appeared consistently as a monophyletic group containing three strains of *H. immutabilis*, one strain of *H. globus*, and the strain of the new *H. crataegi*. Morphologically, *H. globus* resembles *H. immutabilis* but differs in having larger apothecia, smaller asci reacting negatively to iodine, narrower spores, and in the habitat (Zhang & Zhuang 2004). Group VI, containing *H. lasiopodius*, *H. tamaricis*, and *H. epiphyllus*, was the only group without a clear monophyletic origin. It appeared as a monophyletic clade with moderate bootstrap support in the NJ analysis, but as polyphyletic by MP. The nucleotide homology in the ITS region among the species in this clade was lower than within any of the clades discussed above (91.2 % between *H. epiphyllus* F115879 and AY348580), suggesting a large degree of variability within this group.

Our phylogenetic analysis confirms the consistency of the morphological differences observed between *H. crataegi* and the related species described above. Interestingly, the analysis suggests that *H. crataegi* is phylogenetically very distant from both the *H. caudatus* species complex and the *H. macroguttatus* clade, which are considered to be the closest species from the morphological perspective. *Hymenoscyphus crataegi* instead is clearly related with *H. globus* and *H. immutabilis* from the molecular perspective. Although the relationships among the three species were not resolved in the phylogenetic trees, nucleotide divergence in the ITS region between *H. crataegi* and *H. immutabilis* was lower than 4 %, whereas

it was about 8% with *H. globus*, suggesting a higher affinity with *H. immutabilis*. However, the latter species is morphologically very different from *H. crataegi*.

In conclusion, we have described the new species *H. crataegi*, based on morphological and molecular evidences, and we have discussed its taxonomic position among related species from both points of view. We have produced 13 new sequences of seven different *Hymenoscyphus* species, but further studies including additional taxa will be required for a better understanding of the taxonomy and phylogenetic affinities within the genus *Hymenoscyphus*.

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