Bangladesh J. Plant Taxon. **32**(2): 129-137, 2025 (December) DOI: https://doi.org/10.3329/bjpt.v32i2.86663

FIRST MOLECULAR CHARACTERIZATION AND TAXONOMIC ASSESSMENT OF *HUMARIA HEMISPHAERICA* FROM PAKISTAN

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Keywords: Anatomy; Oghi Forest*; Pyronemataceae;* Taxonomy.

Abstract

The present study marks the first molecular characterization of *Humaria hemisphaerica* (F.H. Wigg.) Fuckel from the temperate Oghi forest of Khyber Pakhtunkhwa, Pakistan, a region known for its ecological richness and underexplored fungal diversity. A comprehensive description of both the macro- and micro-morphological characteristics is included, supplemented with color photographs of fresh ascocarp in natural habitat and microscopic images with main anatomical features. Phylogenetic analysis of the studied sample was conducted using internal transcribed spacer (ITS) of nrDNA. This analysis supports the taxonomic identification of *H. hemisphaerica* and confirms its phylogenetic position. Additionally, comparisons are made with closely related taxa that exhibit phenotypic and molecular similarities. This is the first molecular description of this taxon occurring in Pakistan.

Introduction

Early taxonomic systems for operculate cup fungi emphasized morphological simplicity. Fries (1823) classified hairy *Peziza* species under the tribe Lachneae, relying primarily on the presence of hairs on the apothecial margin and receptacle. This approach neglected key anatomical traits such as apothecial shape, hymenial coloration, hair origin, and spore morphology. A major shift occurred in the mid-20th century with the development of more comprehensive, phylogenetically informed classification systems (Eckblad, 1968; Rifai, 1968; Dennis, 1978; Eriksson and Hawksworth, 1998), which significantly improved the accuracy of fungal taxonomy. Recently, molecular phylogenetic studies have further refined the taxonomy of Pezizomycetes, including the introduction of new families and clades within Pezizales (Ekanayaka *et al*., 2018).

Peziza hemisphaerica Fr. was originally placed in Lachneae due to its hairy fruiting body, but Fuckel (1870) reassigned it to the genus *Humaria* based on distinct morphological features. Boudier (1885) later elevated *Peziza* to family status and divided it into tribes, one being Lachneae (as "Lachnés") and suborders such as Cupules, Lenticles, and Mitres. Within this refined framework, *Humaria hemisphaerica* was placed in Lachnes under Cupules, reflecting its cupulate apothecial structure. In Pakistan, early records of Discomycetes were documented by Ahmad *et al*. (1997), and more recent studies have expanded the known diversity of cup fungi in the region. Notably, *Humaria laevispora*, a cryptic species closely related to *H. hemisphaerica*, was described from Pakistan using morpho-anatomical and molecular data (Niazi *et al*., 2021).

Globally, sixteen species of *Humaria* are known from temperate regions (Fuckel, 1870; Clements, 1909). In Pakistan, only three species viz., *H. gregaria*, *H. woolhopeia*, and now *H. hemisphaerica* have been reported (Ahmad *et al*., 1997; Niazi *et al*., 2021; Aman *et al*., 2022). The latter was collected during the 2022 monsoon season in the Oghi Forest of Khyber Pakhtunkhwa.

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This study presents the first molecular characterization of *Humaria hemisphaerica* from Pakistan, contributing to its taxonomic resolution and expanding the understanding of the genus's biogeographical range. It also highlights the importance of integrating molecular tools with classical taxonomy to uncover hidden fungal diversity in underexplored regions (Ekanayaka *et al*., 2018; Niazi *et al*., 2021).

Materials and Methods

Sampling and morpho-anatomical characterization

Sampling was carried out during the summer rainy season from Oghi Forest in Khyber Pakhtunkhwa, Pakistan, a region renowned for its ecological richness and diverse microhabitats shaped by complex topography. Field notes were prepared of fresh specimens and colors were designated following color charts of Munsell (1975). Specimens were dried using fan heater and stored in polythene bags. For further proceedings ascomata were brought back to laboratory. Free hand sections of dried specimens were made, placed in rectified spirit for 10 minutes and then rehydrated in water. The sections were mounted in 5% KOH & Melzer's reagent to see color reactions. Anatomical features were observed using compound microscope and photographed using microscope camera HDCE X5 5.0MP. Measurements were recorded in 5% KOH using Carl Zeiss Jena ocular micrometer. The dimensions of Ascospores are given in the form of (a–) b–c (–d), where *b* and *c* represent the range encompassing 90% of the observed values, and *a* and *d* denote the extreme minimum and maximum values, respectively. The average length and width of spores are indicated as av. L and av. W, while the quotient of length to width, reflecting spore shape, is represented as av. Q. Measurements of other microscopic structures (Asci, paraphysis) include the range between the extreme values measured in length and width. Line drawings were made using Leitz wetzlar camera lucida.

DNA extraction and PCR amplification

Genomic DNA was extracted from dried lamellar tissue using a modified 2% CTAB protocol following Bruns (1995). DNA quality was verified by 1% agarose gel electrophoresis. PCR amplification targeted the nrITS region using ITS1F forward primer (5′ CTTGGTCATTT AGAGGAAGTAA 3′) (Gardes and Bruns 1993) and ITS4 reverse primer (5′ TCCTCCGCTT ATTGATATGC 3′) (White *et al*. 1990). PCR reactions (50 µl) included EconoTaq buffer, dNTPs, primers, DNA polymerase, and template DNA. Thermal cycling comprised initial denaturation (94 °C, 2 min), 35 cycles of denaturation (94 °C, 30 s), annealing (54 °C, 1 min), extension (71 °C, 2 min), and a final extension (71 °C, 5 min). PCR products were cleaned and sent for sequencing to Tsingke, China.

Phylogenetic analysis

The ITS region was amplified and sequenced for all specimens. Forward and reverse sequences were reassembled using the BioEdit Sequence Alignment Editor (Hall, 2004). Initial identity verification was performed via BLAST searches at NCBI (https://www.ncbi.nlm.nih.gov/guide). ITS datasets were constructed by retrieving relevant sequences from the GenBank database. Alignment and manual editing were completed using BioEdit (Hall, 2004). Phylogenetic analyses were conducted on the CIPRES Science Gateway (https://www.phylo.org/) using GTR+GAMMA substitution model with 1,000 bootstrap replicates in RAxMLHPC2 v8.2.10. One thousand rapid bootstrap replicates were run to infer the evolutionary history of each species. The resulting Maximum Likelihood phylogenetic trees were visualized using FigTree v. 1.4.2 (Rambaut, 2014) and subsequently edited in Adobe Illustrator CC 2021. Appropriate species were selected as outgroups to root each phylogenetic tree.

Results and Discussion

Taxonomy

Humaria hemisphaerica (F.H. Wigg.) Fuckel, Jb. nassau. Ver. Naturk. 23−24: 322 (1870)

Macroscopic and Microscopic Characterization (**Figs 1-3**)

Apothecia 2 cm in diameter, cup shaped, involute margin, light gray (Hue 7.5YR 7/2), bright reddish (Hue 5YR 5/6) patch on surface, smooth, wavy and hairy cap margin, white interior. Hymenium smooth. Stipe absents. Taste and Odor are not checked. Ascospores [25/1/1] (10.1−)10.2−15.3 (−15.5) × (4.8−)4.9−6.7(−7.1) µm, av. L × av. W. 11.7 × 5.7 µm, av. Q. 2.1, ellipsoid to oblong, hilar appendage absent, have two oil droplets, guttulate, smooth, hyaline in 5% KOH. Asci (111−)112−138.9(−142.3) × (6.5−)6.9−8.5(−8.6) µm, av. L × av. W. 126.7 × 7.8 µm, 8−spored, operculate, eccentric operculum, unitunicate, uniseriate in the ascus, cylindrical, forked from base. Paraphysis 2.1– 4.2 µm, slender, filiform, septate, longer than asci, paraphyses tips variable, mostly broadened at the apices having prominent cellular contents, some knob shaped, hyaline in 5% KOH. Excipulum (12.4−)13.7−22.2(−26.5) × (10.8−)11.1−19.4(−20.8) µm, av. L × av. W. 18.6 × 15.3 µm, clavate to ovoid to narrowly cylindrical in shape. Excipulum hair 6.7−10.1 apices are pointed and slightly obtuse, septate, thick−walled, brown in 5% KOH.

Fig. 1. Morphological feature of *Humaria hemisphaerica*. A. Apothecia; B. Cap surface; C. Apothecia margin. Scale Bars A. 1.2cm, B. 0.6cm. C. 0.5cm.

Material Examined: Solitary on soil, Agror valley at 16000m. Mansehra district, Khyber Pakhtunkhwa, Pakistan. 20 August, 2022. NG20.20.8.22.

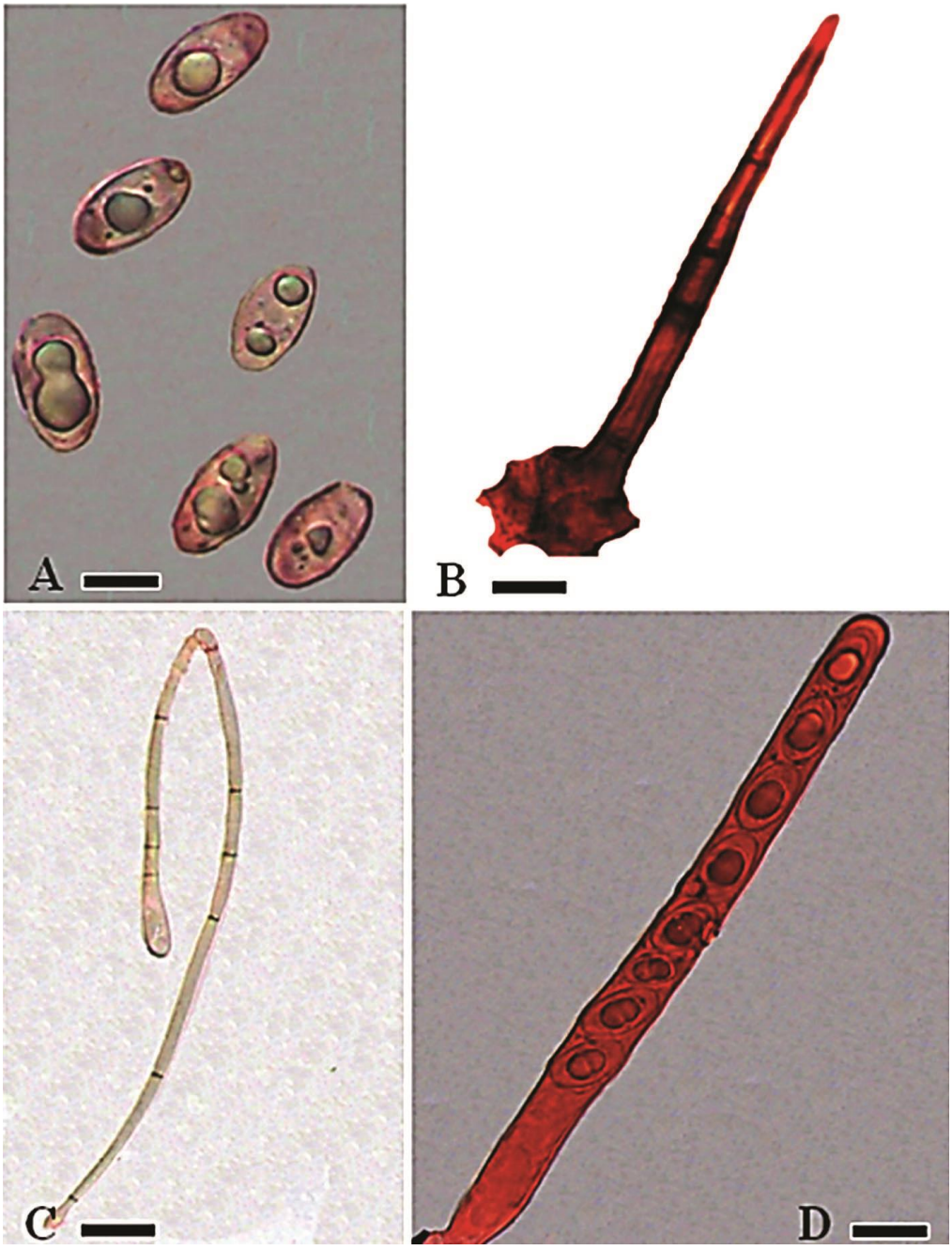

Fig. 2. Microscopic features of *Humaria hemisphaerica*. A. Ascospore; B. Hair; C. Paraphysis; D. Ascus. Scale Bars A. 5.7 µm, B. 28 µm, C. 15 µm, D. 7.8 µm.

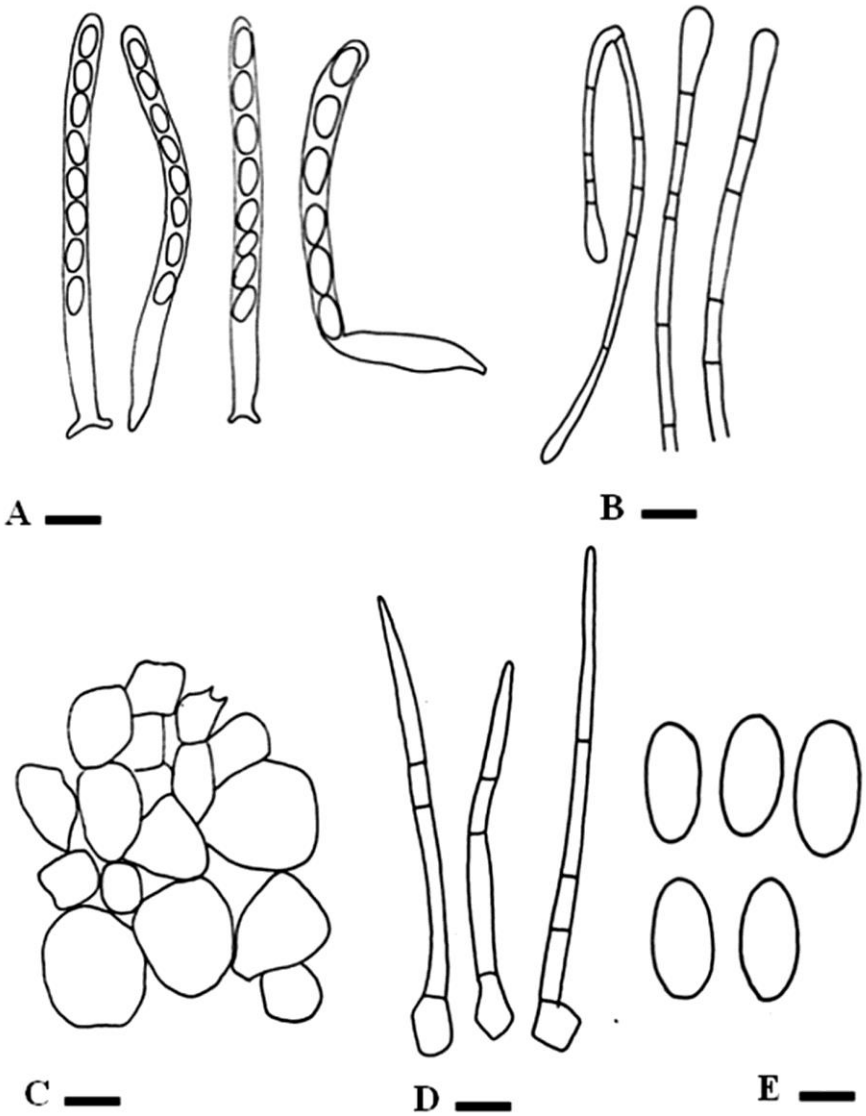

Fig. 3. Line drawing of microscopic features of *Humaria hemisphaerica*. Ascus; B. Paraphysis; C. Excipulum cells; D. Hair; E. Ascospore. Scale Bars A. 15.6 µm, B. 7.8 µm, C. 10.2 µm, D. 14 µm, E. 5.7 µm.

Molecular and Phylogenetic analysis

Humaria hemisphaerica species collected from Pakistan is described on molecular basis using base sequence of rDNA gene. The Internal transcribed spacer (ITS) of rDNA is amplified and sequenced. The sequence was analyzed by comparing with the data in the GenBank using BLAST (Basic local alignment search tool) searches and phylogenetic analysis of sequence as well. For phylogenetic analysis, sequences of the genus *Humaria* were retrieved from GenBank and from the most recent published literature (based on their similarity to our sequences. Our newly generated sequence showed 99.57% similarity with *Vouchered mycorrhizae Humaria* (EU024878)

with 98% query coverage. *Helvella crispa* (KT254551) was selected as the outgroup. All the sequences in Blast analysis of Pakistani sequence belong to the same genus or different genus of the same family. Other closely related sequences in the Blast below the closest sequence showed 76%, with *Humaria cazaresii* (Table 1; Fig. 4).

Table 1. Taxa used for constructing the phylogenetic tree, along with their voucher numbers, geographical localities and GenBank accession numbers.

Taxa	Voucher/ Isolate	Origin	GenBank Accession number (ITS)	References
Genea gardnerii	src867	USA	DQ206851	Niazi *et al*. (2021)
Genea gardnerii	src831	USA	DQ206850	Niazi *et al*. (2021)
Genea gardnerii	SOC 690	USA	AY830857	Niazi *et al*. (2021)
Genea verrucosa	BP104856	Spain	KJ938936	Niazi *et al*. (2021)
Genea verrucosa	AH44208	Spain	KJ938935	Niazi *et al*. (2021)
Genea harknessii	bg3c_c8	USA	DQ218290	Niazi *et al*. (2021)
Genea harknessii	sm16b_a3 clone	USA	DQ218298	Niazi *et al*. (2021)
Wilcoxina rehmii	NS211	Sweden	DQ069001	Niazi *et al*. (2021)
Wilcoxina rehmii	87-ITS-1F	USA	KT800250	Niazi *et al*. (2021)
Humaria cazaresii	Trappe18044	USA	DQ206863	Sánchez-Flores *et al*. (2023)
Humaria cazaresii	OSC:111670	USA	NR_182778.1	Sánchez-Flores *et al*. (2023)
Humaria hemisphaerica	SUB15569834	Pakistan	PX238431	Sequence generated during this study
Humaria hemisphaerica	JMP0104	USA	EU819470	Niazi *et al*. (2021)
Humaria hemisphaerica	RT00017	USA	EU819506	Palmer *et al*. (2008)
Humaria hemisphaerica	388536	USA	OM987404	-
Vouchered mycorrhizae Humaria	BP 97494	Hungary	EUO24878	-
Humaria hemisphaerica	FLAS-F-68295	Pakistan	OR149264	-
Humaria umbrosa	PC:Le Gal s.n.	Switzerland	OL832168	Unpublished
Genea verrucosa	Trappe 11775 (FH, OSC)	USA	DQ220335	Niazi *et al*. (2021)
Genea harknessii	Trappe 13313 (FH, OSC)	USA	DQ220334	Niazi *et al*. (2021)
Trichophaea hybrida	KH.04.39 (FH)	USA	DQ220454	Niazi *et al*. (2021)
Trichophaea hybrida	AMNH-49682 (AMNH)	USA	DQ220455	Niazi *et al*. (2021)
Humaria setimarginata	Type ITCV Mexico	Mexico	OP521892	Sánchez-Flores *et al*. (2023)
Helvella crispa	HKAS:75434	China	KT254551	Niazi *et al*. (2021)

The phylogenetic analysis was carried out using maximum likelihood method. The sequences obtained from these sources, along with those generated during the study, were aligned using the online MUSCLE alignment tool. Manual editing of the aligned dataset was performed in BioEdit,

resulting in a final dataset containing ITS sequences, 1016 characters, categorized as follows: 407 conserved positions, 567 variable sites, 448 parsimony-informative characters, and 117 singleton sites. Subsequently, the finalized dataset underwent phylogenetic analysis using the Maximum Likelihood method in MEGA 12. The phylogenetic analysis revealed that the sequence under investigation was positioned with *Humaria hemispherica* (OR149264), supported by strong bootstrap value.

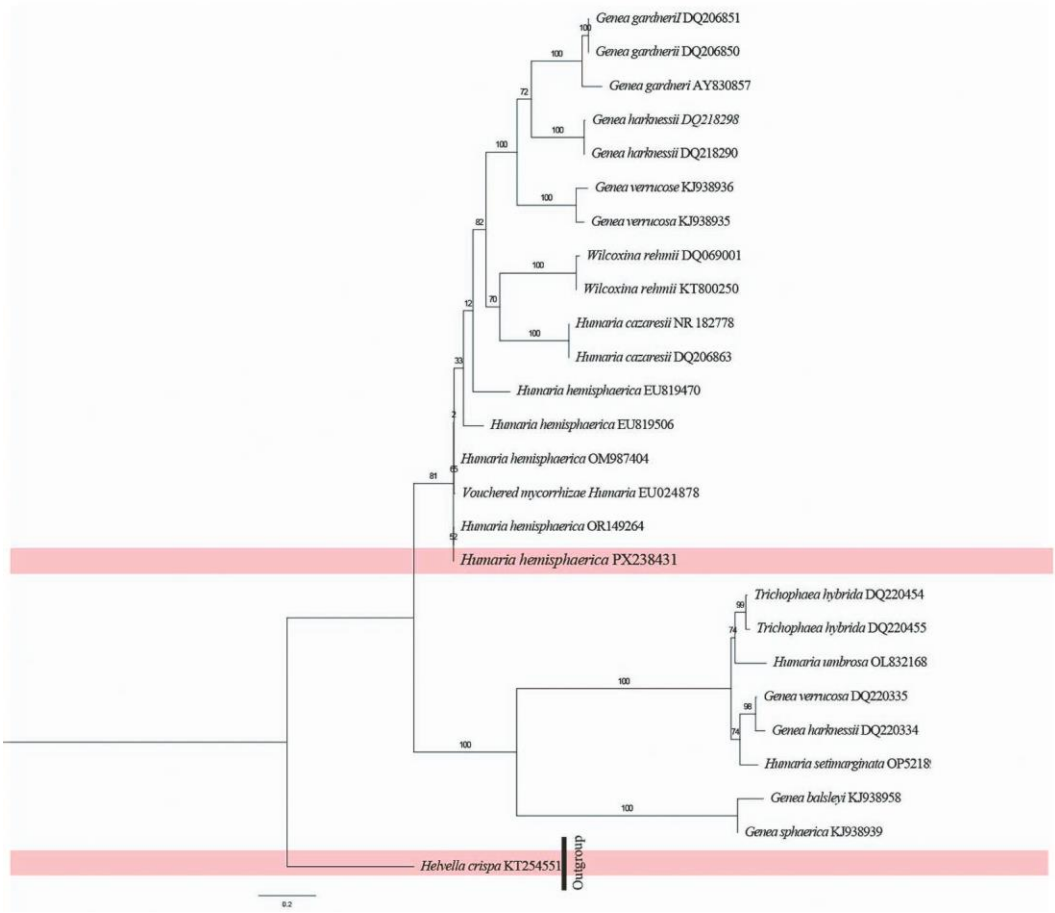

Fig. 4. Phylogenetic tree based on the ITS sequences of *Humaria hemisphaerica*. Values that are indicated at the nodes show bootstrap value. Sequence generated in our study is highlighted with pink colour. *Helvella crispa is* outgroup in this tree.

Discussion

Humaria hemisphaerica is distinguished by its cup-shaped apothecia with an involute margin and a bright reddish patch on the hymenial surface. The margin of the apothecium is smooth, wavy, and covered with hairs. Microscopic features include filiform paraphyses, ellipsoidal spores, and the presence of excipular hairs. This species has been previously documented in the United States, Hungary, and Central India.

Verma (2018) reported that *Humaria hemisphaerica* produces ellipsoidal, smooth-walled spores measuring 20–24 × 10–12 µm, while Fuckel (1870) described slightly smaller dimensions,

ranging from 19–22 × 11–14 µm. In contrast, specimens collected from Pakistan exhibit smaller spores, measuring 10–15 × 4–8 µm. This variation in spore size may be attributed to geographical differences. Both Fuckel (1870) and Verma (2018) noted that the spores are ellipsoidal and contain two oil droplets, which become separated upon maturation, a characteristic also observed in the Pakistani specimens. Additionally, *H. hemisphaerica* specimens from Central India and the United States have been reported to possess brown excipular hairs, which are visible in potassium hydroxide (KOH) preparations. Regarding macroscopic features, both Fuckel and Verma described the apothecia as cup-shaped, undulate, and covered with hairs. The presence of oil droplets within the spores, the presence of excipulum hairs, and the similar spore morphology suggest that the Pakistani collection of *H. hemisphaerica* is morphologically consistent with specimens reported from India and the United States. Some of the species that are closely related to our species can also be distinguished to our specie by having some different features. Phylogenetic analysis reveals *H. cazaresii* is closely related to our species yet possess distinct features. *H. cazaresii* reported from United State of America is characterized by 10 mm diameter of hypogeous apothecium, no marginal hair, 170-220 × 10-13 µm long asci, 2.5- 5 µm long paraphysis (Smith *et al*., 2006). All of these characters make this specie is different from present species examined in Pakistan.

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(Manuscript received on 26 July 2025; revised on 12 December 2025)