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Full Length Article

Phylogeny and Taxonomy of *Hebeloma theobrominum* and *H. mesophaeum* from Western Himalaya

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Abstract

This study deals with the molecular systematics of Asian collections of *Hebeloma theobrominum* and *H. mesophaeum* which have not been previously reported from any Himalayan part of the world, and are new to Pakistan. Both were identified using morpho-anatomical and molecular characterization. The target rDNA of both species was amplified by using universal fungal primers. In phylogenetic analysis, both species distinctly clustered with their respective groups. These both species have been described first time from Asia using ITS-rDNA barcoding. The sequences of both species were compared with those of same European species from the GenBank. These species clustered with European species in phylogenetic tree proving their cosmopolitan distribution. © 2017 Friends Science Publishers

Keywords: Euagarics, Internal transcribed spacers; Macrofungi; Pakistan

Introduction

Himalaya, literally means snow dwelling starting from Pakistan to Bhutan eastwards, is 25th hot spot of biodiversity of the world (Myers *et al.,* 2000). Western Himalaya, part of Himalaya in Pakistan, is also floristically diversified with more than 750 plant species and more than 300 macrofungi (Ahmad *et al.,* 1997; Samina and Salman, 2012; Razaq *et al.,* 2016; Hussain *et al.,* 2017). Mostly these Himalayan areas are distinguished by the luxurious growth of coniferous mixed with deciduous vegetation and eye catching mushrooms especially during rainy season. During summer temperature varies from 10.7–18°C, rainfall average is 59.3 cm, and humidity ranges up to 57% inducing a large number of macrofungi of different groups especially euagrics (Champion *et al.,* 1968). Although, the fungal diversity is very rich in this area and many new agarics and European species have been reported from these forests especially from moist temperate forests yet knowledge about *Hebeloma* (Fr.) P. Kumm is still lacking (Ahmad *et al.,* 1997; Tulloss *et al.,* 2001; Niazi *et al.,* 2006; Razaq *et al.,* 2012; Razaq *et al.,* 2013, 2016a).

Hebeloma is a genus comprising of 150 worldwide species especially in north temperate regions of the world (Kirk *et al.,* 2008). So far in Pakistan, only three species of *Hebeloma* have been reported (Ahmad *et al.,* 1997). This paper contributes to the documentation of two new European agarics from this area which have not been reported so far from any Himalayan part of the world. During field work in July 2010, two species of *Hebeloma* were collected which showed different reactions in Melzer's reagent. These species were identified as *H. mesophaeum* and *H. theobrominum* using morpho-anatomical and molecular characters. The ribosomal internal transcribed spacers (ITS) sequences were also used to determine the phylogenetic perspective of both these local species with respective to the European collections.

Materials and Methods

Basidiocarps were dug out from the soil with sharp knife and photographed in the field. Collected material was characterized morphologically and microscopically. For anatomical characterization, sections of fruitbodies were made by hand and mounted in 5% KOH, Congo red, and Melzer's reagent. Measurements for basidia, basidiospores and cheilocystidia were made using an ocular micrometer. At least 20 basidiospores, basidia and cheilocystidia were measured from each basidiome. Abbreviations Q is used for length/width of basidiospore. Line drawings were made using a camera lucida attached to compound microscope. Dried specimens were deposited in the LAH Herbarium, Department of Botany, University of the Punjab, Lahore.

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A sample from a dried specimen of each species was ground in liquid nitrogen and placed in 2% CTAB buffer and DNA was extracted (Porebski *et al.,* 1997). ITS regions of rDNA were amplified using universal primer pair ITS1F and ITS4 (White *et al.,* 1990). PCR was performed in 25 µL reaction volume following the protocol given by Gardes and Bruns (1993). The PCR product of the ITS-amplified region containing ITS-1, 5.8 and ITS-2 was directly sequenced in both directions using the same pair of amplification primers (Macrogen, Korea). For initial comparison and alignment of the sequence, BLAST (Basic Local Alignment Search Tool) analysis was performed using the National Center for Biotechnology Information (NCBI), USA database. For further phylogenetic analysis and alignment of sequence, closely related sequences were retrieved from GenBank. The sequence alignments and phylogenetic analysis were performed using Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura *et al.,* 2011). Maximum Likelihood (ML) method was based on the Jukes-Cantor model of nrITS sequences using Nearest-Neighbor-Interchange (NNI) as ML heuristic search method. Phylogeny was tested by bootsrap value of 1000 replicates. Consensus nucleotide sequences of *H. theobrominum* and *H. mesophaeum* were submitted to European Molecular Biology Laboratory (EMBL) database under accession numbers HE649368 and HE649369, respectively.

Results

Molecular Description and Phylogenetic Analysis

The target region of fungal genomic DNA was amplified generating a fragment ca. 750bp consisting of internal transcribed spacers (ITS) regions and the 5.8S region of rDNA. Initial BLAST analysis in both cases revealed that *H. theobrominum* (Pakistani collection K-74) matched with sequences of different *Hebeloma* species. In the Blast, the top most sequences were of *H. theobrominum* showing maximum percentage similarity (GenBank accession # FJ816623.1, FJ816621.1, FJ816619.1). In the second case, our other *Hebeloma* species (*H. mesophaeum*, SR-108) in Blast analysis showed its maximum base percentage similarity with *H. mesophaeum* (GenBank accession # EF091826.1, EF451057.1, EF644106.1).

The phylogenetic analysis of *Hebeloma* (Fr.) P. Kumm species collected from Pakistan was carried out using maximum likelihood method. The sequences included in this analysis had 1254 characters, from which 690 characters were used in final analysis after trimming the alignment from both 5' and 3' sides. In phylogenetic analysis gaps are given as weight age as the other letters. A total 24 sequences of *Hebeloma* species were included in phylogenetic analysis out of which two belonged to Pakistan. To clarify the phylogenetic position of each Pakistani collection, sequences of different sections of the genus were retrieved from the GenBank database. Two major clades formed in the phylogenetic tree (Fig. 1), which have been labeled by clade names. Out of two, each Pakistani collection lied separately in separate clade (Fig. 1).

In Clade I, only the species of *Hebeloma* section *Theobromina* Vesterholt are present (HE649368.1, FJ816619.1, FJ816621.1, FJ816622.1, FJ816623.1, FJ816624.1, FJ816625.1, EU570180.1, EU570181.1, EU570182.1, FJ816629.1, FJ816631.1, FJ816638.1), but our specimen (HE649368.1) distinctly separated with the *H. theobrominum* Quadr. sub-clade in clade I under a notable bootstrap value (64%) with a supportive branch length. This means that Pakistani collection is also a real member of this sub-clade and it is *H. theobrominum* from different geographical area. This sub-clade formed a sister sub-clade with its closely related *H. vesterholtii* Beker and U. Eberh.

In the Clade II of the phylogenetic tree; all the sequences of *H. mesophaeum* (Pers.) Quél. (HE649369.1, EF091826.1, EF451056.1, EF451057.1, EF644106.1, HQ453395.1, AB211272.1, AY312980.1, AY311521.1) are present among which our specimen (HE649369.1) clustered under a significant bootstrap value (87%). This clade formed a sister clade to another closely related species *H. cistophilum* R. Maire (EU570177.1, EU570178.1).

In the second case, *H. mesophaeum* takes its position in the opposite Clade II (Fig. 1) in which members belong to *Hebeloma* sect. *Mesophaeum*. The author's species separated with *H. mesophaeum.*

Taxonomy

Hebeloma theobrominum Quadr. Mycotaxon 30: 311, 1987. Fig. 2, 3 A–D, bold please Fig. 2, 3 A–D.

Pileus 30–55 mm diam., plano-convex, broadly umbonate, undulating surface, deep cinnamon to red–brown to dark brick, central disc darker, in mature specimens surface zonate, surface smooth, viscid, shiny, cap margin clearly white to cream with dentate processes, involute. Context moderately thick, white, remaining unchanged on bruising. Lamellae broad, adnate to adnexed, white, margin entire, crowded in younger specimens, at maturity two zones, outer crowded with several tiers of lamellulae while inner has distant to sub-distant arrangement without lamellulae. Stipe 30–50 × 7–10 mm, cylindrical, centrally attached, dry, silky, fibrillose texture, light brown with overall white pruina.

Basidiospores 8.55–11 × 4.5–6 µm, Q= 1.8–1.84 or ≥ 1.8, elliptical with prominent apiculus, rounded at the distal end, smooth to poorly ornamented; yellowish brown to brown, with oily drops, highly dextrinoid (deep reddish brown) in Melzer's reagent. Basidia 4–sterigmate, 24–30 × 7–8.5 µm, cylindrical to clavate, pale yellow with oil drops, basal septa or clamps rarely present. Cheilocystidia 30–36.5 × 8–9.5 µm, hyaline to pale yellow in 5% KOH, polymorphic with the following forms observed: subcylindrical, clavate, fusoid-ventricose and clavate with flexuous sides.

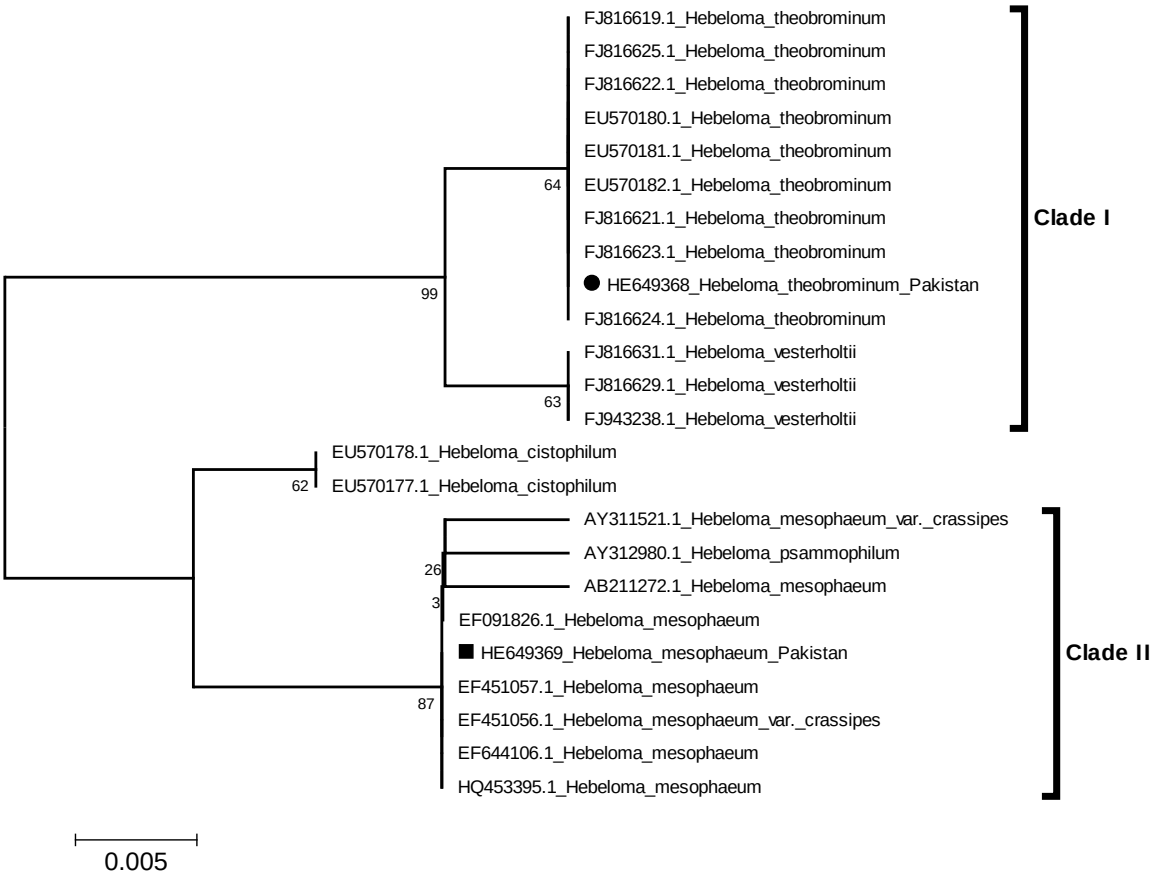

Fig. 1: Phylogenetic analysis of *Hebeloma* species collected from Pakistan using nrITS-rDNA regions data. This tree is based on maximum likelihood method using Jukes-Cantor model. The values given above the branches is bootstrap

Material Examined

Pakistan: Khyber Pakhtankhaw, Abbotabad, Ayubia-Khanspur (34° 4' 0" N, 73° 24' 0" E), on the ground humified soil under *Abies pindrow-Pinus wallichina* thick vegetation, submitted to herbarium, Department of Botany, University of the Punjab, Lahore. Abdul Razaq, 10-08-2010. LAH. 10081074.

Hebeloma mesophaeum Quélet, L. 1872, *Mémoires de la Société d'Émulation de Montbéliard* 5: 128 Fig. 4A-D.

Pileus 2.5 cm diam, hemispherical to plano-convex, with obtuse umbo, dark brown in the centre fading towards camel brown at margin, fibrillose, margin entire; surface smooth, shiny, viscid. Lamellae creamy to brown, sub-crowded, adnate to adnexed, broad, margin entire to somewhat crenuate. Stipe 6.5×0.3 cm, cylindrical, centrally attached, woody in texture; reddish brown, veil remnants present, dry, stuffed.

Basidiospores 7.5–12.5 × 4–5.7 um, elliptical, obtuse, with prominent apiculus, hyaline to pale yellow, spore wall loses its colour in Melzer's regeant. Basidia 26–33 × 7–11 µm, subcylindrical to subclavate, hyaline to pale yellow in KOH. Cheilocystidia 35–61.5 × 9–15 µm fusoid-ventricose, subutriform, saccate, clavate and filamentous types present, hyaline to yellowish in KOH.

Material Examined

Pakistan Gilgit-Baltistan, Himalayan Moist Temperate Forests, Fairy Meadows forest, (34.400570°S 150.891738°E), at 3360 m a.s.l., solitary, on moist ground under coniferous vegetation, 27 July 2010, Abdul Razaq (SR-108) LAH. No. 270710108.' GenBank Accsession # HE649369.

Discussion

The collection of *Hebeloma theobrominum* collected from Pakistan is characterized macroscopically by the reddish brown basidiomata and microscopically, by the elliptical basidiospores, which are highly dextrinoid in Melzer's reagent. Pleurocystidia were missing, while cheilocystida were present forming clusters. The size and shape of the spores from our collection match with original description of *H. theobrominum* (Quadraccia, 1987).

Fig. 2: A-B *Hebeloma theobrominum* A-B. Basidiomata. Scale bar .A= 1.06, B=0.8 cm

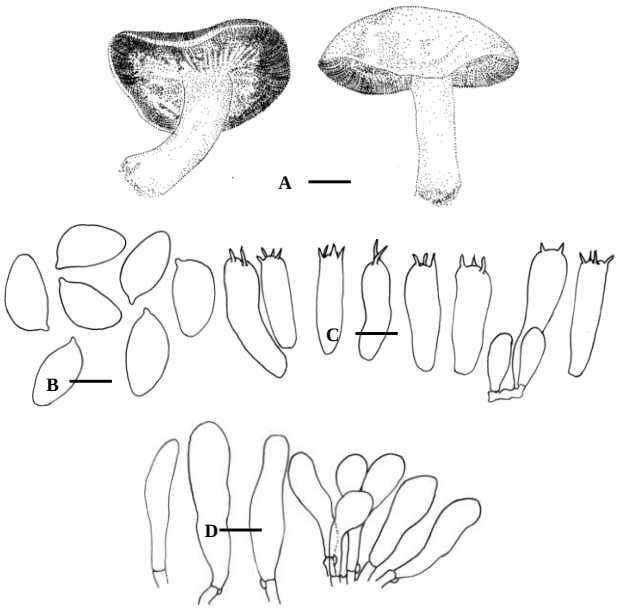

Fig. 3: A-D. *H. theobrominum.* A- line drawings of Basidiocarps B-Basidiospores C-Basidia D-Cheilocystidia. Scale Bar = A=1cm; B = 3.0 µm; C=3.45 µm; D= 4.5 µm

The spore Q value is more than 1.8 and the colour of the pileus is deep reddish brown as given for European *H. theobrominum* (Eberhardt and Beker, 2010). This species is distributed in different parts of Europe and has been collected from mixed or woodland forest but in our case this species is collected from a forest area purely dominated by *Abies pindrow* (Royle ex D. Don) Royle (Eberhardt and Beker, 2010). In molecular analysis of ITS regions of rDNA of our collection, nucleotide base matching of our collection is more than 97%. Furthermore, in the phylogenetic analysis based on nrITS-r DNA sequence, our specimen separated with European species of *H. theobrominum* in clade I. This clade forms a sister clade with *H. vesterholtii* Beker and U. Eberh., a closely related species.

The collection of *Hebeloma mesophaeum* described from Pakistan is characterized by having dull brown basidiomes, brown velar remnants on stipe, and elliptical spores with prominent apiculus. Pleurocystidia are absent but the clavate, filamentous, subutriform, saccate and fusoid-ventricose cheilocystidia are striking features of

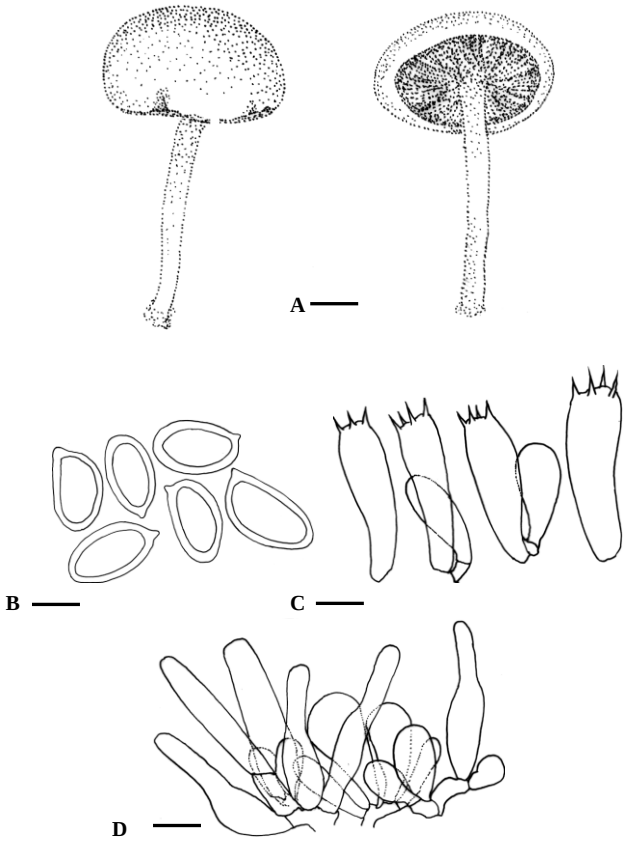

Fig. 4: A-D. *H. mesophaeum* A- Line drawings of basidiomata B- Basidiospores C-Basidia D- Cheilocystidia. Scale Bars = A = 1.0 cm; B= 3.5 µm; C=3.0 µm; D= 6.0 µm

this variety. In Melzer's reagent the spores lose their coloration or remain pale. Because of these features, *H. mesophaeum* falls clearly in subsect. *Mesophaeae* of sect. *Mesophaea* of *Hebeloma* (Smith *et al.,* 1983).

Phylogenetic analysis based on nrITS-rDNA of our collection very clearly clustered with sequences of *H. mesophaeum* (Fig. 2. Clade II). In the phylogenetic tree, Clade II purely consists of sequences of *H. mesophaeum* and its varieties. This clade also formed a sister clade with its closely related species *H. cistophilum* Maire R. The comparison of morphological, anatomical and molecular characters of our collection with what are already described in the literature, leads us to conclude that our collection is *H. mesophaeum*. Maximum sequences of this species are lacking in the GenBank, especially from Asian even single sequences was not available for comparison.

A lot of variation in this species has been found especially with respect to shape and type of cheilocystidia. Smith *et al.* (1983) described 10 varieties of *H. mesophaeum* in their book based on morphological and anatomical differences especially in the size and shape of the cheilocystidia. In *H. mesophaeum*, clavate, filamentous, cylindrical, sub-cylindrical and most

commonly fusoid-ventricose cheilocystidia are reported (Smith *et al*., 1983). But as far as shape and types of cheilocystidia are concerned the authors' variety is closely related to *H. mesophaeum var. fluviatile,* which has filamentous, clavate and fusoid-ventricose cheilocystidia however in that variety the clavate cystidia are elongate, while in case of *Hebeloma mesophaeum* these are saccate and subutriform. Bessette *et al.* (1997) also given a description for American collection of this species which has slightly smaller basidiospore size of Pakistani collection. This non-significant difference may be due to the geographical isolation of both populations. These morpho-anatomical variations in different varieties of this species are predictable on genetic level if all the molecular data is available for comparison and phylogeny construction.

Conclusion

In the phylogenetic perspective, both Pakistani collections of the same species clustered with European collections showing that the both species are well represented not only in European continent but also in the other continents. *H. mesophaeum* is commonly distributed in Asia, Europe and North America, while *H. thoebrominum* is endemic to Europe and Asia.

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