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Morphological and Molecular Identification of Cytotoxic Fungus Species; *Hebeloma crustuliniforme* and *Hebeloma sinapizans*

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Abstract

Taxonomic positions and phylogenetic relations of Hebeloma species have not yet been figured out molecularly in Turkey. Most species of the genus are mainly poisonous and a few of which are edible. Hebeloma crustuliniforme and *H. sinapizans* are two important species that contain deadly toxins so only morphological characters may not be enough for reliable identification. The DNA sequence of the nuclear ribosomal Internal Transcribed spacer (nrITS) region is used in addition to morphological characters in the present study. Phylogenetic analysis is conducted on the sequence dataset by using the Maximum Likelihood (ML) principle to see the taxonomic placement of two poisonous species. Studying morphological traits and molecular markers together is very useful to distinguish these two poisonous species from close relatives. The first sequence data of these species were added to the literature on behalf of our country with this study.

Keywords: DNA, fungal taxonomy, nrITS, poisonous fungi, phylogeny

INTRODUCTION

Hebeloma (Fr.) P. Kumm. is an ectomycorrhizal genus of family *Hymenogastraceae* widespread in alpine and arctic habitats (Vesterholt, 2005). Most species of the genus are mainly poisonous except a few of which are reported as edible (Montoya et al., 2004, 2008; Pérez-Moreno et al., 2008; Carrasco-Hernández et al., 2015; Eberhardt et al., 2020). Cytotoxic compounds may be implicated in *Hebeloma* toxicity (De Bernardi et al., 1983; Shao et al., 2005; Carrasco-Hernández et al., 2015) so the species are generally regarded as poisonous (Bresinsky and Besl, 1990; Benjamin, 1995). Identification of species within the genus is very difficult; only morphological characters may not be enough for correct and reliable discrimination of poisonous and edible specimens.

Hebeloma crustuliniforme (Bull.) Quél. and *Hebeloma sinapizans* (Paulet) Gillet are most often recorded poisonous species and widely confused with edible relatives (Vesterholt et al., 2014; O'Reilly, 2016). It is reported that both of them contain cytotoxic compounds named lanostane triterpene, named 3-β-acetyl-2-α (3′-hydroxy-3′-methyl) glutarylcrustulinol (De Bernardi et al., 1983). The patients who consume mushrooms are predominantly mycetismus gastrointestinal and this may be due to consumption of *H. crustuliniforme* and *H. sinapizans* (McDougall, 1925). Severe gastrointestinal nature, namely vomiting, diarrhea and colicky abdominal pain are common symptoms (Pamela, 1967; Roger, 2010).

In Turkey, most fungi species have been identified by only usage of anatomical/morphological characters that are considered flexible due to environmental factors. Therefore, many fungi are misidentified due to phenotypic changes. Molecular techniques are proved to be reliable approaches along with traditional methods for accurate identification and reclassification. The sequence of nrITS

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region is a superior molecular DNA barcode for molecular identification of Basidiomycetes (Schoch et al., 2012) so the region is used in the current study.

The present study aims to identify toxic fungus species, *H. crustuliniforme* and *H. sinapizans,* based on microscopic/macroscopic and molecular analyses and prove the utility of nrITS region to discriminate poisonous *Hebeloma* specimens from edible ones.

MATERIAL and METHOD

Two collections of *Hebeloma crustuliniforme* from Hakkari and one collection of *H. crustuliniforme* from Bingöl region of Turkey were collected in 2014-2018. Moreover, one collection of *Hebeloma sinapizans* was collected from Hakkari province in 2014. Samples were photographed with a Canon (EOS 60D) camera equipped using Tokina 100 mm macro lens in the fieldwork. Macroscopic features (pileus, lamellae, stipe, and cortina) were noted using fresh materials.

Descriptions of the samples were performed based on measures of spore ornamentation (O1–O4), spore dextrinoidity (D0–D4), and perispore loosening (P0–P4). At least 30 spores, 20 basidia, and cheilocystidia were measured under a Leica DM500 research microscope by using distilled water and Melzer's reagent solution. Measurements were made with Leica Application Suite (version 3.4.0) program and diagnosed based on the terminology of Beker et al. (2016). Dried samples were deposited in the Fungarium of Van Yüzüncü Yıl University (VANF).

Molecular Studies

Genomic DNA was extracted from dried basidiomata using the CTAB method (Doyle & Doyle 1987). The purity and quantity of extracted DNA were determined by using NanoDrop2000c UV–Vis Spectrophotometer (Thermo Scientific) and 0.8% agarose gel electrophoresis. DNA amplification was performed in a 25 µl volume mixture containing genomic DNA (10 ng/µl), 10X PCR Buffer, MgCl2 (25 mM), dNTP mixture (10 mM), selected primer pair (10 µM), Taq polymerase (5u/µl) and sterile water. Amplification of ITS (ITS1-5.8S-ITS2) region was performed using primer pairs N-nc18S10 5'AGGAGAAGTCGTAACAAG3'/C26A 5'GTTTCTTTTCCTCCGCT3' (Wen & al. 1996). After amplification, PCR products were run in a 1 % agarose gel and visualized by staining with Gelred dye. Positive reactions were sequenced with forward and reverse PCR primers using ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA).

The sequences were taken by using forward and reverse primer were evaluated and edited via Alibee Multiple Alignment 3.0 software (www.genebee.msu.su/genebee.html). Ambiguous sites were checked and corrected. Sequence data of nrITS region were deposited in GenBank and accession numbers were given in the text. Three sequences of *H. crustuliniforme* and two sequences of *H. sinapizans* generated from the present study and additional sequences retrieved from the GenBank database were combined and analyzed together to see the phylogenetic relation and position of the studied species within the genus. *Galerina pruinatipes* (AJ585510) was chosen as an outgroup for rooting topology. All sequences were aligned with the ClustalW program (Thompson et al., 1994) and adjusted manually where it was necessary.

The sequence alignments were performed using MEGA 6.0 software (Tamura et al., 2013). Phylogram was constructed using Maximum Likelihood (ML) principle by using PHYML v.3.1 software (Guindon et al., 2010). GTR model was implemented with six rate classes and invariable sites. Across site variations were fixed with parameters achieved from Modeltest and 1000 bootstrap

replicates were carried out from a BioNJ starting tree running the best of nearest neighbor interchange (NNI) and subtree pruning and regrafting (SPR) branch swapping.

RESULTS and DISCUSSION

Morphological Identifications

Hebeloma crustuliniforme (Bull.) Quél. (Figure 1).

Description:

Pileus; 25–80 mm broad, convex, umbonate and smooth margin, involute, white to cream, cream-brown, with a slightly brown pale center. Lamellae; emarginate, L=60–100, cream coloured, droplets present. Stipe; 30–100 mm, cylindrical, with clavate-bulbose base, floccose, whitish.

Basidiospores; 9.0–12 × 5.0–7.0(7.2) µm, amygdaloid, guttulate, (n=30 and Q=1.8), light yellow-yellow brown, verrucose (O2-O3), indistinctly dextrinoid (D0-D1-D2), perispore loosening (P0-P1-P2). Basidia; 26–40 × 6–10 µm, 4- rarely 2-spored. Cheilocystidia; 30–80(100) × 4.0–9.0(12) × 2-6(7) × 2-7 µm, clavate, spathulate, occasionally lageniform. Pleurocystidia; none. Pileipellis; an ixocutis, up to 6 µm wide hyphae. Caulocystidia; resemble to cheilocystidia, up to 80 µm long.

Specimens examined: TURKEY, Bingöl, Genç forest, under Conifer trees, 38° 41'52.78"N, 40° 29'25.70"E, 1165 m, 29.11.2018. Acar. 1091 (VANF). TURKEY, Hakkâri, under *Populus* sp. trees, 37° 22'18.63"N, 44° 28'34.16"E, 1484 m, 01.11.2014. Acar. 778 (VANF). TURKEY, Hakkâri, under *Populus* sp. trees, 37° 22'18.63"N, 44° 28'34.16"E, 1484 m, 15.05.2014. 29.11.2018. Acar. 483 (VANF).

Genbank accession number: OK356899-OK356900-OK356901

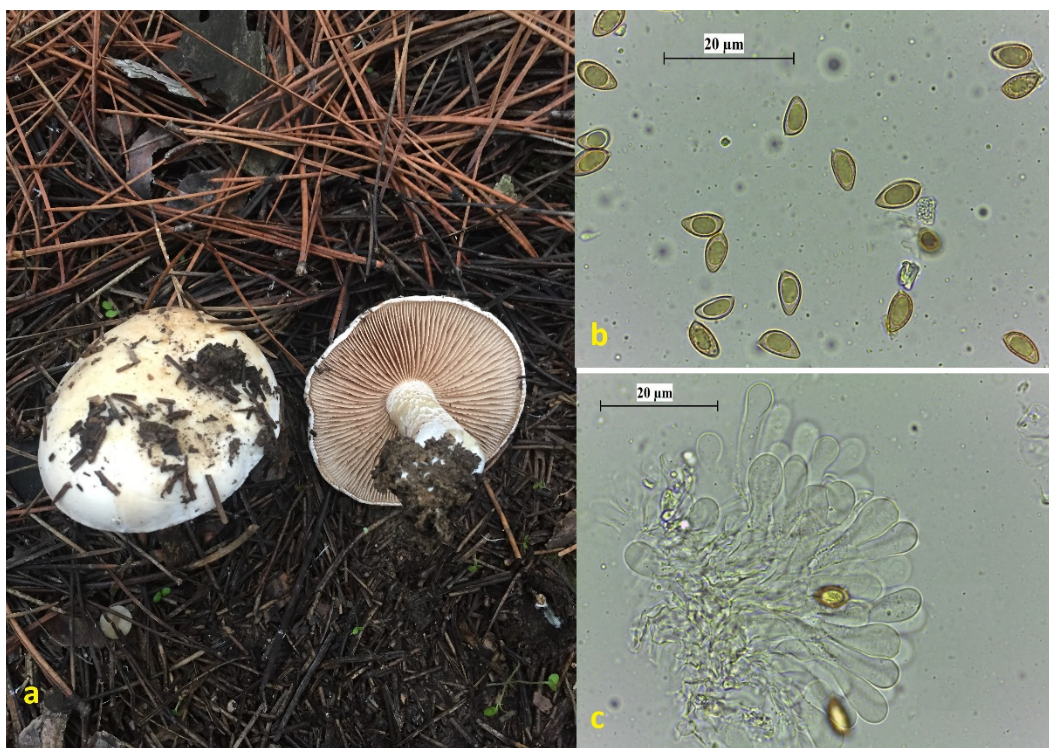

Figure 1. *Hebeloma crustuliniforme* a. Basidiomata b. Spores in distilled water c. Cheilocystidia (Scale bar=20 µm).

Hebeloma sinapizans (Paulet) Gillet (Figure 2).

Description:

Pileus; 30–100 mm; convex with umbonate, margin often involute, smooth, unicolor, cream, yellowish, yellow-brown. Lamellae; adnate to emarginate, crowded (L= 80–120). Stipe; 30–120 mm, clavate to bulbous, pruinose floccose, white, mycelial cords mostly present. Smell usually raphanoid.

Basidiospores; (8)9.5–12(14) × (5.2)6–8(9.5) µm (n=30 and Q=1.7), amygdaloid, limoniform, yellow-brown, guttulate, ornamentation distinct (O3, O4), perispore loosening (P1, P2, P3), dextrinoidity strong (D3, D4). Basidia; 25–35 × 7–9 µm, four-spored, rarely two-spored. Cheilocystidia; 30–70(92) × (3)3.7-9.0× 4–7(7.5) × 4-15 µm, mostly ventricose, sometimes cylindrical, clavate. Pleurocystidia; similar to cheilocystidia. Caulocystidia; ventricose, septate, similar to cheilocystidia, up to 150 µm long. Pileipellis; thick, hyphae 8 µm wide.

Specimens examined: TURKEY, Hakkâri, under *Quercus* sp. trees, 37° 22'18.63"N, 44° 28'34.16"E, 1484 m, 24.10.2014. Acar. 476 (VANF).

Genbank accession number: OK356902- OK356903

Figure 2. *Hebeloma sinapizans* a. Basidiomata b. Spores in distilled water c. Cheilocystidia (Scale bar=20 µm).

Molecular Identifications

The amplified DNA part of the nrITS region was approximately 650 bp in length encompassing complete ITS1, 5.8S, and ITS2 subregions. Basic Local Alignment Search Tool (BLAST) analysis was performed using the GenBank database. For each genotype, the sequence similarity search in GenBank used the ‘blastn’ (Megablast) option excepting ‘uncultured/environmental sample sequences. The sequences of putative *Hebeloma crustuliniforme* samples showed 100% homology with the sequences of *H. crustuliniforme* found in the database. The homology value was 99% for *Hebeloma sinapizans*.

The names obtained from GenBank for each collection were also verified for nomenclatural and taxonomic synonyms by using Mycobank (http://www.mycobank.org/) and Index Fungorum (http://www.indexfungorum.org/names/names.asp) and valid names were surely given. The sequences having the highest homologies loaded by researchers specialized in *Hebeloma* were retrieved from the

database and analyzed with our sequences. The dataset including 72 nrITS sequences was analyzed and observed that the region had 461 conserved and 137 variable (74 in ITS1 and 63 in ITS2) sites. The identification of the samples was also supported by establishing a phylogenetic tree. Phylogenetic tree constructed based on nrITS region separated the sequences at the species and section levels (Figure 3). Molecular results supported the monophyly of the *Hebeloma crustuliniforme* with 78% bootstrap support value grouped within the section *Denudata* and close relationships with *H. pallidolabiatum* and *H. salicicola*. *Hebeloma sinapizans* were grouped within the section *Sinapizantia* with its representative (97%) and it was closely related with *H. bulbiferum.*

Morphological and Molecular Assessment

Several specific characters are distinguished *Hebeloma crustuliniforme* from other species such as floccose stem; the presence of tears on lamellae; large number of lamellae (60-100); voluminous cheilocystidia and amygdaloid, indextrinoid large (<12 µm long and <7 µm wide) spores. In the field, *Hebeloma crustuliniforme* may be confused with *H. alpinum* which is an edible species of the genus. At first glance, they show macromorphological similarities such as light color basidiomes and shape of pileus. However, both species have their distinctive characteristics examined in detail by mycologists. *Hebeloma alpinum* is characterized by a robust pileus; mostly equal and shorter stipe; dark brown gills; elliptical, most weakly dextrinoid spores (Beker et al. 2016). Moreover, *H. crustuliniforme* is molecularly differentiated from *H. alpinum* based on 4 bp observed in the DNA sequences of the nrITS region. *Hebeloma crustuliniforme* grouped within the section *Denudata* and close relationships with *H. pallidolabiatum* and *H. salicicola* species. Four nucleotide substitutions detected at 439th (C-T), 493th (T-C), 534th (A-T) and 576th (C-T) bases caused the separation of *H. crustuliniforme* from *H. pallidolabiatum*. Moreover, three variations at bases 493th (T-C), 534th (A-T) and 590th (G-A) differentiated *H. crustuliniforme* from *H. salicicola*.

Hebeloma sinapizans is sometimes confused with *H. crustuliniforme* by the reason of sharing the beaded lamellae. However, basidiomata of *H. sinapizans* seems larger compared to *H. crustuliniforme* and it often has a darker pileus colour. Key characters of *Hebeloma sinapizans* are rather large basidiomes with a more bulbous stem base; a persistent incurved cap margin, and dark brown spots (not watery) on the gills. Furthermore, this species has some morphological similarities with *H. alpinum* because of the pale coloration and shape of pileus. However, *H. sinapizans* produces relatively large basidiomes and robust floccose white stipe. *Hebeloma sinapizans* is similar to *H. bulbiferum* both molecularly and morphologically. Basidiomes of these taxa are almost similar in size and both have floccose stipe and crowded lamellae. However, *H. bulbiferum* can be separated by more distinct droplets on the lamellae (Grilli et al., 2016). The shape of cheilocystidia is the other important feature to identify species; *H. bulbiferum* has capitate to clavate to stipitate or clavate-lageniform or gently clavate cheilocystidia and *H. sinapizans* has mostly ventricose or lageniform cheilocystidia (Beker et al., 2016; Grilli et al., 2016). Studied *Hebeloma sinapizans* samples were grouped within the section *Sinapizantia* with 97% bootstrap support value and it was closely related with *H. bulbiferum.* Many variations were observed between *H. sinapizans* and *H. bulbiferum* [15th (T-G), 21th (C-T), 32th (C-T), 55th (T-C), 119th (A-G), 129th (T-G), 203th (A-G), 207th (C-A), 416th (G-A), 441th (C-T), 595th (G-A)].

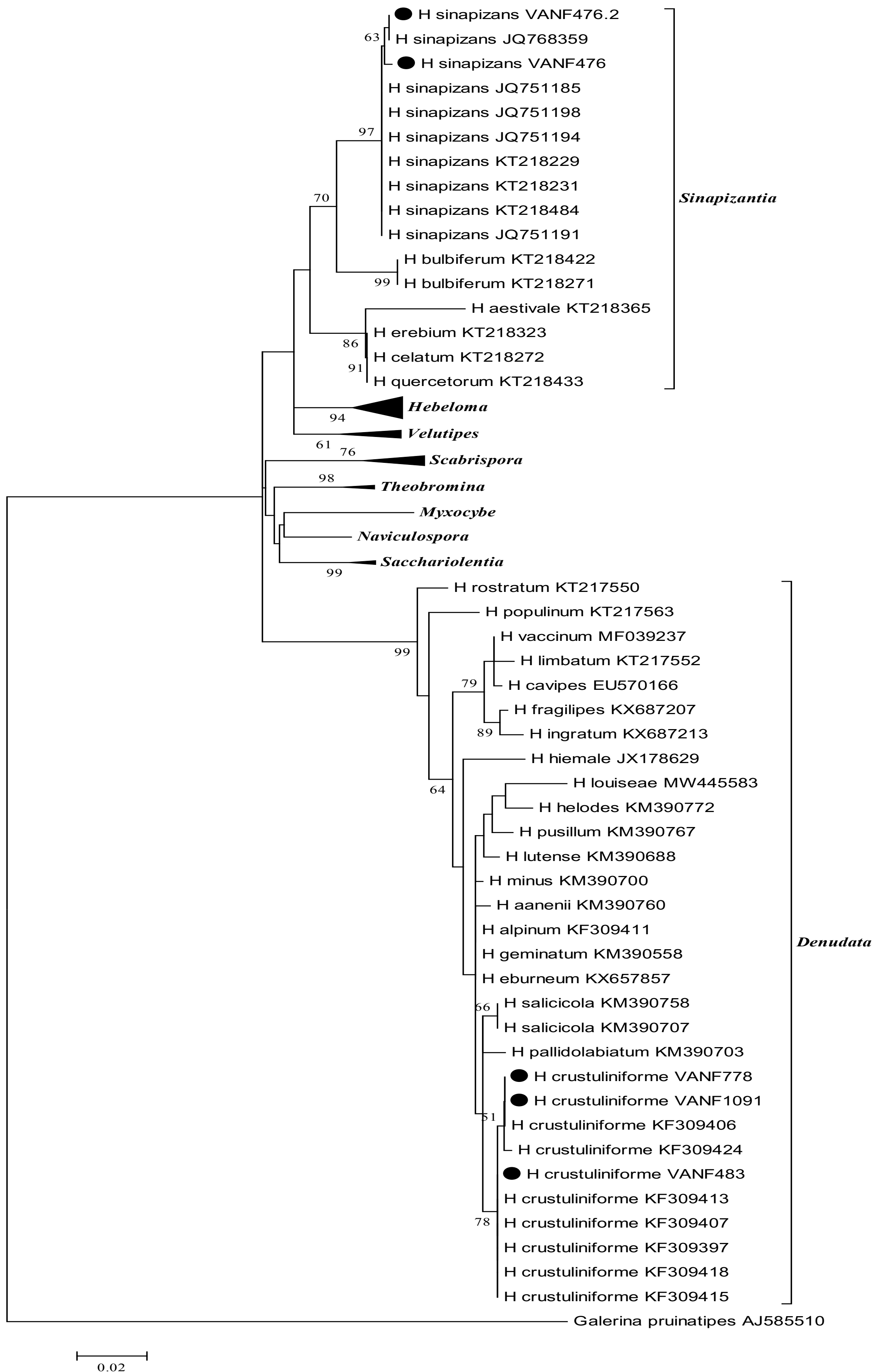

Figure 3. Phylogram based on ML analysis of the nrITS region. The black circle shows studied specimens. *Galerina pruinatipes* are used as an outgroup. Bootstrap values higher than 50% were showed on branches.

CONCLUSIONS

Most species of the genus *Hebeloma* are mainly poisonous and a few of which are edible. Some cases of poisoning by previously unidentified fungi have been reported. Therefore, the fungus must be identified based on multi-discipline studies and offered to the public. For this reason, we focused on making reliable identifications for fungi that are common in nature. *Hebeloma crustuliniforme* and *H. sinapizans* are the most poisonous species within the genus so Turkey collections of these species were identified in the study. Both morphological characters and the DNA sequence of a barcode region are used for correct identification.

Morphological and molecular approaches play important roles to identify *Hebeloma* species correctly. Each approach has its limitations and strengths so the combination of two techniques is needed to describe *Hebeloma* species successfully.

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AUTHOR CONTRIBUTIONS

The authors have contributed equally to this study.

CONFLICT of INTEREST

The authors declare no conflict of interest

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