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Morphology and Phylogeny Reveal a New Record *Gyromitra* for Turkish Mycobiota

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Abstract: In the current study, *Gyromitra brunnea* Underw. belonging to the family *Discinaceae* was recorded for the first time from Turkey. Two candidate samples were described by using both morphological and molecular data. Short description of the newly reported species was given together with its photographs related to macro and micromorphologies and discussed briefly. Additionally, phylogenetic position of *Gyromitra brunnea* was indicated in the phylogenetic tree constructed based on the sequence of 28S (LSU) rRNA gene.

Key words: *Gyromitra brunnea*, fungal taxonomy, new record, LSU, phylogeny

Türkiye Mikobiyotası İçin Yeni Kayıt *Gyromitra*'nın Morfolojik ve Filogenetik Olarak Ortaya Çıkarılması

Öz: Bu çalışmada, *Discinaceae* ailesine ait olan *Gyromitra brunnea* Underw. Türkiye için ilk kez kaydedilmiştir. Hem morfolojik hem de moleküler veriler kullanılarak iki örnek tanımlanmıştır. Rapor edilen yeni kayıt türün kısa açıklaması, makro ve mikromorfolojiyle ilgili fotoğraflar ile birlikte verilmiş ve kısaca tartışılmıştır. Ek olarak, *Gyromitra brunnea*'nın filogenetik konumu, 28S (LSU) rRNA gen dizisine dayalı olarak oluşturulan filogenetik ağaçta gösterilmiştir.

Anahtar kelimeler: *Gyromitra brunnea*, fungal taksonomi, yeni kayıt, LSU, filogeni

Introduction

Gyromitra Fr. (*Discinaceae*) is a widespread macrofungus genus of Ascomycetes and is represented by 76 species worldwide (www.MycoBank.org; Robert et al., 1999). The genus has brain shaped ascocarps with generally reddish brown to yellowish brown and well developed stems. Most of *Gyromitra* species are poisonous and causes gastrointestinal syndrome. There is limited number of reliably identified *Gyromitra* species due to many taxonomic problems between *Discina* and *Gyromitra* genera. In regards to mutual generic boundaries among them, reliable and exact taxonomical studies have not been established (Van Vooren and Moreau, 2009). For instance, Fries (1849) considered that *Discina* and *Gyromitra* were different genera. However, Harmaja (1969) suggested calling *Discina perlata* Fr. and *D. leucoxantha* Bres. species as *Gyromitra perlata* and *G. leucoxantha*, respectively stating that the name *Gyromitra* is older than the name *Discina*. He also transferred *Discina macrospora* Bubak to *Gyromitra* genus (Harmaja, 1973). Eventually, *Discina* was accepted as a subgenus of *Gyromitra* by Abbott and Currah (1997).

Taxonomic ambiguities between these taxa have been originated from traditional classification which is unclear due to similar morphological and ecological features. Therefore, not only morphological characters but also molecular data are needed to identify macrofungus, correctly (Undan et al., 2016). All eukaryotic and prokaryotic cells contain rRNA genes which are widely conserved during evolutionary time and they are used for phylogenetic classification. The LSU (28S) region has been used extensively for fungal phylogeny and taxonomic

placement (Asemaninejad et al. 2016). This region contains the D1 and D2 hypervariable domains which are valuable for species identification in various fungal groups (Raja et al. 2017). Methven and his colleagues (2013) resolved nomenclatural problems between *Discina* and *Gyromitra* taxa by using sequences of 28S rRNA gene region. They show that *Discina macrospora* nested with *Gyromitra perlata* with high bootstrap value and produced *Discina* subgenus and at the end of the study they proposed five subgenera for the genus as *Gyromitra*, *Discina*, *Caroliniana*, *Pseudorhizina* and *Melaleucoides*. In their study, *G. brunnea* was also studied and characterized by an apical hymenophore that has 2-5 distinct lobes with thick margins, seams joining the lobes and a whitish undersurface that is partially exposed. This sample was also characterized by Kuo (2012) and he distinguished the species from other *Gyromitra* species by reddish brown cap, which is decidedly lobed and often gathered into two or three points, creating a saddle-shaped appearance.

According to the checklists of Turkish macrofungi, six *Gyromitra* species are found in Turkey and *Gyromitra brunnea* has not been previously reported (Sesli and Denchev, 2014; Solak et al., 2015). Therefore, the main goal of the present study is to identify the collected specimens by using both morphological and molecular data and add newly reported species, *Gyromitra brunnea,* to the macrofungi of Turkey.

Material and Method

Taxon sampling and morphological studies

The macrofungus samples were collected from Şemdinli district of Hakkari, Turkey. Two samples, representatives of *Gyromitra brunnea*, were collected and used for morphological and molecular studies. Collected samples were deposited in the Fungarium of Van Yüzüncü Yıl University (VANF). Specimens were photographed in situ, using with a Canon (EOS 60D) camera equipped with Tokina 100 mm macro lens during field work. Macroscopic characters (cap and stipe) were recorded using fresh materials. Microscopic structures (ascus, paraphysis and ascospores) were observed in distilled water under a Leica EZ4 stereo microscope and sections were examined under a Leica DM500 research microscope. Microscopic structures were measured with the Leica Application Suite (version 3.2.0) programme and described based on different studies [Murrill (1913), Bresinsky and Stangl (1977), Bon (1991), Breitenbach and Kränzlin (1991), Dähncke (2004), Jordan (2004), Gerault (2005), Clémençon (2009), Vizzini et al. (2011), Buczacki (2012), Garcia et al. (2013), Kuo and Methven (2014)].

DNA extraction, PCR amplification and Sequencing

Total DNA was extracted from dried ascomata using the CTAB method (Doyle and 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, $MgCl_2$ (25 mM), dNTP mixture (10 mM), selected primer pair (10 µM), Taq polymerase (5u/µl) and sterile water. Primer pair for LSU; LROR 5'ACCCGCTGAACTTAAGC3'/LR5 5'TCCTGAGGGAAACTTCG3' (Vilgalys and Hester, 1990) was used for amplification (Figure 1). PCR products were run in a 1.0 % agarose gel and visualized by staining with Gelred dye and positive reactions were sequenced with forward and reverse PCR primers using ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA).

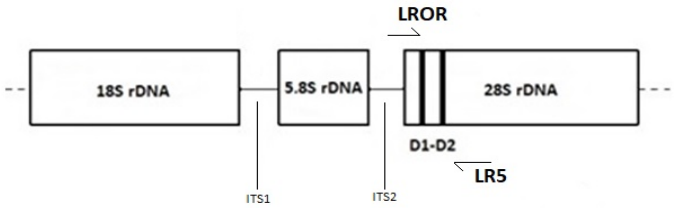

Figure 1. Primers [LROR (forward) and LRS (reverse)] used for amplification of D1 and D2 hypervariable domains of 28S large subunit (LSU). Length of amplified area was about 880 bp.

Sequence alignment and phylogenetic analysis

Sequences of *Gyromitra brunnea* generated from the current study and additional sequences retrieved from GenBank were combined and analyzed together to see phylogenetic relationships among species in the phylogenetic tree. Even though sequences downloaded from GenBank are named as *Gyromitra* species, some of them are accepted as *Discina* species or vice versa in the Index Fungorum. For instance, *Gyromitra brunnea, G. fastigiata, G. caroliniana, G. perlata* and *G. montana* are given as *Discina brunnea*, *D. fastigiata* and etc. in the Index Fungorum. Border of the region was decided using sequence downloaded from GenBank database (*Gyromitra brunnea* accession no; KC751521, Methven et al., 2013). All sequences were aligned with the aid of the program ClustalW (Thompson et al., 1994).

Prior to construction of phylogenetic tree, total nucleotide length (bp) and variable sites were calculated using Molecular Evolutionary Genetics Analysis software (MEGA 6.0; Tamura et al., 2013). Phylogenetic tree was constructed using two different methods; Maximum Likelihood (ML) and Maximum Parsimony (MP). To test branch support, bootstrap analysis was used with 1000 replicates. *Morchella esculenta* (KM485969) was utilized as an outgroup (Methven et al., 2013). In the ML method, initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ

algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then the topology with superior log likelihood value was selected. The Tree-Bisection-Reconnection (TBR) search method was employed with 100 random addition replications to construct the MP trees and the consensus tree inferred from 10 most parsimonious trees was used. All positions containing gaps and missing data were eliminated.

Results

Macroscopic and Microscopic Characters

Ascocarp; 50-110 × 50-80 mm, 2-5 lobes in different shapes but usually saddle-shaped, pinkish brown, reddish brown or tan color, loose, wrinkled, often as if planted with lobed lines, hairless, whitish and often associated with the stipe. **Stipe**; 30-80 × 20-45 mm, irregularly but usually slightly widening to the base, pale pinkish color or white color, hairless, generally near to base is protruding. **Asci**; 17-25 × 220-250 µm, hyaline, 8 spored. **Ascospores**; 22-29 × 10-13.5 µm, ellipsoid, 1-3 drops, firstly smooth then slightly warty. As the spore matures, ornaments grow in the length of 1-2 µm, these ornaments can then extend to 2-5 µm. **Paraphysis**; 5-11 µm wide, clavate or slightly lobed, with several septate and orangish-reddish brown (Figure 2).

Hakkari, Şemdinli, Bozyamaç village, under *Quercus* sp., 37° 22'084"N - 44° 26'384"E, 1371 m, 10.04.2015, Acar 850.

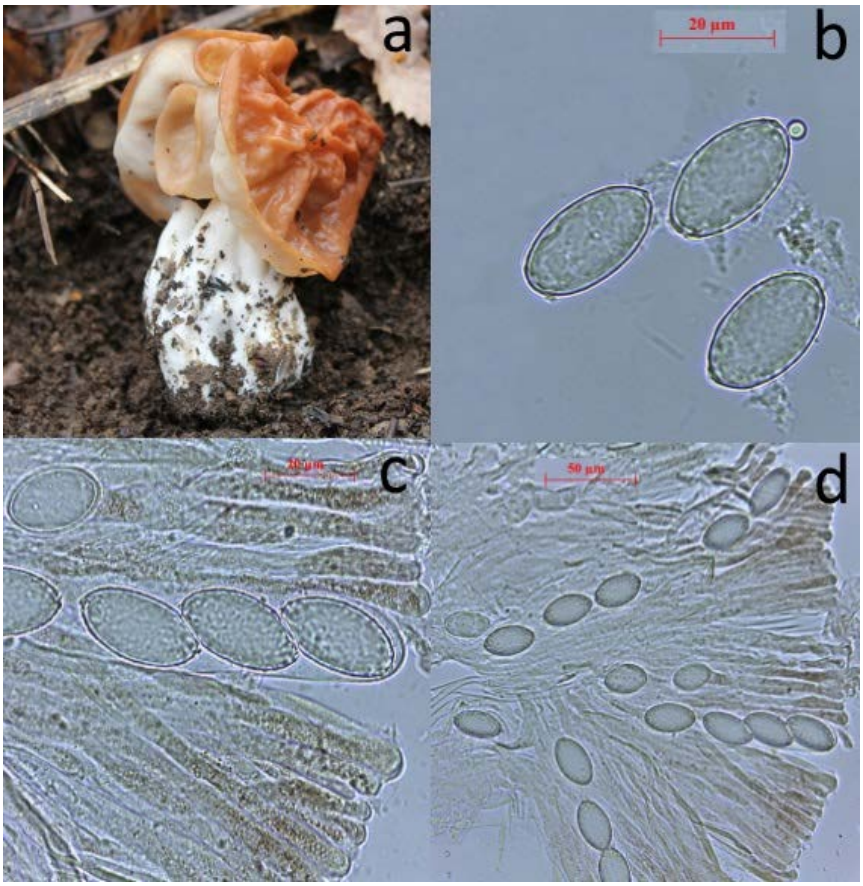

Figure 2. Macroscopic and Microscopic characters of *Gyromitra brunnea*. a. ascocarp, b. ascospores, c-d. asci and paraphysis.

Molecular phylogeny

LSU data matrix consists of 27 sequences and the aligned data was about 881 bp length. The final alignment included 126 polymorphic nucleotides with 65 parsimony informative sites. Sequences of the studied region were submitted to GenBank and the accession numbers were assigned as MH376402 and MH698933 for two different specimens. The Maximum Likelihood analysis resulted in similar phylogenetic topologies with Maximum Parsimony and Neighbour Joining analyses so only ML tree was given and discussed. Bootstrap values of the all used algorithms were also given in the tree by separating slash sign (Figure 3). The phylogenetic tree composed from four clades and each of them corresponded one subgenus as *Caroliniana*, *Discina*, *Pseudorhizina*, *Gyromitra*. Determination of subgenus was done according to the study of Methven et al. (2013). The *Caroliniana* clade consisted of all *G. brunnea* samples (including studied specimens) with a

bootstrap value of 98 % (Figure 3). *Gyromitra fastigiata* and *G. caroliniana* samples located closely to *G. brunnea* in this clade. Although these three species are morphologically similar they can be distinguished from each other based on some macroscopic and microscopic characters (Table 1).

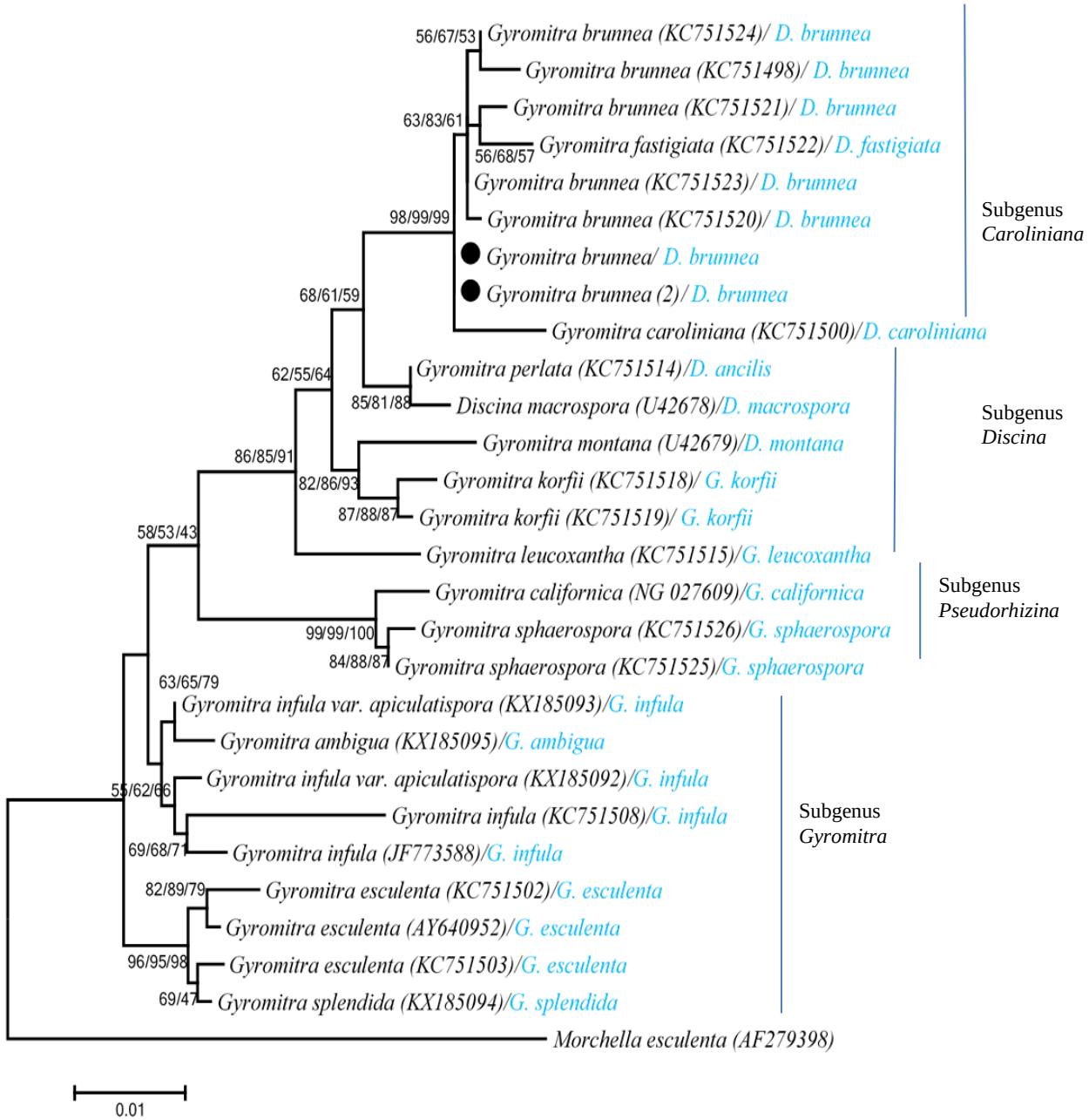

Figure 3. Phylogenetic tree of *Gyromitra* species based on ML analysis of the LSU rRNA gene region. Black circles indicate studied specimens. *Morchella esculenta* was used as outgroup. Bootstrap analyses of ML, MP, NJ were based on 1000 replicates and values higher than 50% were indicated on branches, respectively. The names written before and after the slash are the names accepted by GenBank and Index Fungorum, respectively.

Table 1. Comparisons of *G. brunnea*, *G. caroliniana* and *G. fastigiata* based on macroscopic and microscopic characters.

Species	Pileus	Stipe	Asci	Spores	Paraphysis	Ecology
G. brunnea	50-110 ×50-80 mm	30-80 × 20-45 mm	220-250 × 17-25 µm	22-29 × 10-13.5 µm	5- 11 µm, clavate to subcapitate	Under hardwoods
G. caroliniana	50-130 × 60-120 mm	40-100 × 20-100 mm	320–420 × 18.5–23 µm	22-35 × 10-16 µm	5-7 µm wide, clavate to subcapitate	Under hardwoods
G. fastigiata	60-170 ×60-130 mm	40-100 × 20-50 mm	220-260 × 15-18 µm, apex flattened.	28–33.5 × 13–15.5 µm	5-9 µm, clavate to subcapitate	Under hardwoods

Discussion

Gyromitra genus has many taxonomic problems because of morphological similarities among the specimens. Especially, taxonomic position of the genus with *Discina* taxon is very problematic. According to Fries (1849) *Discina* and *Gyromitra* are two different genera. However, Harmaja (1973) reduced *Discina* to a subgenus in *Gyromitra* based on ascospores features. Several recent studies (Abbott and Currah 1997, Van Vooren and Moreau 2009, and Methven et al 2013) and data taken from current study confirmed this arrangement by using additional specimens and phylogenetic analyses. At this point, we used both morphological and molecular data to get correct and reliable results for identification of *Gyromitra brunnea.*

Methven and his colleagues (2013) showed that *Discina* samples nested in *Gyromitra* species and they propose five subgenera for the genus (*Gyromitra*, *Discina*, *Caroliniana* and *Pseudorhizina*) by using sequences of 28S rRNA gene region. The data taken from present study supported their results; *Discina* samples retrieved from GenBank (*Discina macrospora*, U42678) located within *Gyromitra* species. This sample is called as *Gyromitra macrospora* in the Index Fungorum. Same situation was also observed in *G. brunnea*, *G. fastigiata, G. caroliniana, G. perlata* and *G. montana* species that are named under *Discina* in Index Fungorum. This circumstance indicates complexities between boundaries of *Gyromitra* and *Discina* taxa.

Studied sample (*G. brunnea)* grouped closely with *G. fastigiata* and *G. caroliniana* samples in *Caroliniana* clade (Figure 3). Actually, these species not only molecularly but also morphologically can be separated from each other. For instance, *G. brunnea* is characterized by an apical hymenophore with thick margins, lobes usually joined in seam-like lines, a whitish undersurface partially exposed in places while *G. caroliniana* has a brain-like apical hymenophore that is irregularly wrinkled, lacks the seams and fused to the stipe so that the undersurface is not exposed. *Gyromitra fastigiata* and *G. brunnea* resemble each other macroscopically but structure of paraphysis can be used to separate them (Table 1). In addition to macroscopic and microscopic features these three species can also be separated based on nucleotide variations of D1-D2 regions of LSU. McKnight et al. (1987) indicated that *G. brunnea* may be the same species (or variety) known in some recent European books as *G. fastigiata*. However, in the current study *G. brunnea* was separated from this sample based on not only macroscopic features but also molecular data so we named our sample as *G. brunnea* to avoid uncertainty which originated from different comments in Europe.

The present study is valuable because *Gyromitra brunnea* from Turkey has never been studied by using morphological and molecular characters. At the end of the study, *Gyromitra brunnea* was firstly reported for mycobiota of Turkey and the total number of the sepecies was increased from six to seven for *Gyromitra* genus. However, taxonomic positions of *Gyromitra* and *Discina* are still problematic and need exact boundaries for correct identification and nomenclature. Therefore, further studies covering detailed morphological analyses and additional

DNA markers are necessary for realiable delimitations of mentioned taxa.

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