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**Research Article**

# Morphological and molecular identification of *Dissingia confusa* based on the first record of the species in Türkiye

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*Dissingia*
*Helvella*
*Molecular taxonomy*
*New record*
*Phylogeny*

**Abstract:** In the present study, specimens kept in Fungarium of Van Yüzüncü Yıl University - Türkiye (VANF) were evaluated based on morphological and molecular characters, and identified as *Dissingia confusa* as a new record from Eastern Türkiye. Ascocarp, hymenium, stipe, ascospores, ascus and paraphyses were considered as diagnostic morphological characters and the nucleotide sequences of the large subunit (LSU) ribosomal DNA and the translation elongation factor 1-alpha (TEF1-α) were referred as molecular data. A phylogenetic analysis was performed with Maximum parsimony and Bayesian inference methods. The DNA sequences of 15 (7 for TEF1-α) *Dissingia* and 27 (14 for TEF1-α) *Helvella* specimens downloaded from the GenBank database were included in the analysis to estimate phylogeny between the two close genera. Morphological evaluations and genetic evidences confirmed that *Dissingia* is phylogenetically separated from *Helvella* at genus level and helped to identify the studied specimens as *D. confusa* as a new record in Türkiye. Detailed description, colour images of fresh and dried ascomata, along with photographs of microscopic characters and the obtained phylogenetic trees are given.

**Özet:** Van Yüzüncü Yıl Üniversitesi Fungaryumu'nda (VANF) muhafaza edilen *Dissingia confusa* türü morfolojik ve moleküler karakterler ile Türkiye'nin doğusundan yeni bir kayıt olarak tanımlanmıştır. Şapka, himenyum, sap, spor, askus ve parafiz yapıları morfolojik veri olarak, büyük alt ünite (LSU) ve translasyon uzama faktörü 1-alfa (TEF1-α) bölgelerinin nükleotid dizileri moleküler veri olarak kullanılmıştır. Filogenetik analiz Maksimum parsimoni ve Bayesian çıkarım yöntemi ile gerçekleştirilmiştir. Analizlere, iki cins arasındaki filogeniyi göstermek için GenBank veritabanından indirilen 15 (TEF1-α için 7) *Dissingia* ve 27 (TEF1-α için 14) *Helvella* örnekleri de eklenmiştir. Morfolojik çalışmalar ve genetik kanıtlar, *Dissingia* cinsinin cins düzeyinde *Helvella* cinsinden filogenetik olarak ayrıldığını ve *Dissingia confusa* türünün Türkiye'de ilk kez tanımlandığını doğrulamıştır. Detaylı deskripsiyon, taze ve kuru örneklerin renkli fotoğrafları ile mikroskobik karakterlerin görüntüleri ve filogenetik ağaçlar verilmiştir.

## Introduction

The genus *Dissingia* K. Hansen, X.H. Wang & T. Schumach. (Helvellaceae) is characterized by cupulate apothecia, being stipeless, or with a distinct short stipe with ribs gradually expanding and branching throughout the cup attachment. Hymenium is greyish to yellowish-brown, brown or darker close to black, and the external surface is covered with fine short, whitish to dark greyish brown hairs. Asci are operculate, 8-spored, cylindrical and typically originate from simple septa (i.e. aporhynchous). Ascospores are elongated ovoid, with a large guttule in the center. Paraphyses are septate, filiform, clavate or subcapitate at the tips (Hansen *et al.* 2019).

The species of *Dissingia* grow especially on calcareous soil, often in coniferous forests (Harmaja 1979, Skrede *et al.* 2017). The representatives of the genus were previously identified as a part of the *Leucomelaena* lineage within the broader concept of *Helvella* L. (Landeros *et al.* 2015, Skrede *et al.* 2017), which were then segregated in the genus *Dissingia* by Hansen *et al.* (2019) based on morphological and molecular characters. *Dissingia* species are separated from relatives mainly by asci that develop from simple septa (aporhynchous asci). The other genera in the *Helvellaceae* family have asci that originate from croziers

(i.e. pleurorhynchous) except the species belonging to the *corium-alpina* lineage of the genus *Helvella*. This situation proves that the ascus development from croziers is the ancestral trait for the family, and the development of asci from simple septa has evolved independently within the family (Hansen *et al*. 2019).

With the improvement of techniques used in the field of molecular phylogeny, the taxonomy of *Helvellacae* has been studied in detail and *Dissingia* was proposed as a new genus. The phylogenetic inference of *Helvella* and its related genera has been examined by using DNA sequences of numerous DNA regions (Hansen & Pfister 2006, Tedersoo *et al*. 2006, Laessoe & Hansen 2007, Bonito *et al*. 2013, Hansen *et al*. 2013, Nguyen *et al*. 2013, Landeros *et al*. 2015, Skrede *et al*. 2017, Hansen *et al*. 2019, Wang *et al*. 2019, Skrede *et al*. 2023, Wang *et al*. 2023). Even if the internal transcribed spacer (ITS) region is suggested as the potential barcode for fungi (Schoch *et al*. 2012), amplification of the region using universal primers generally failed because of the primer and DNA mismatch (Skrede *et al*. 2017). Therefore, the amplification achievement rate of ITS sequence is low, especially in herbarium materials (Skrede *et al*. 2017, Wang *et al*. 2019). In the present study, the nuclear large subunit ribosomal (nrLSU) DNA and translation elongation factor alpha (TEF1-α) were preferred since these regions were proved to be useful for assessing the species boundaries within the genus *Helvella* in Europe (Landeros *et al*. 2015, Skrede *et al*. 2017, Mao *et al*. 2023, Wang *et al*. 2023).

Four species, *Dissingia confusa*, *D. crassitunicata* (N.S. Weber) T. Schumach. & Skrede, *D. leucomelaena* (Pers.) K. Hansen & X.H. Wang and *D. oblongispora* (Harmaja) T. Schumach. & Skrede have been ascribed to the genus (mycobank.org, 2023) and only *D. leucomelaena* has been reported from Türkiye so far (Uzun 2023). The aim of the study is to identify *D. confusa* as a new record from Türkiye and figure out taxonomic status with *Helvella pedunculata* Harmaja by using molecular and morphological data.

## Materials and Methods

*Morphological studies*

The fungi specimens collected from Erzurum (Türkiye) in 2014 and deposited in the Van Yüzüncü Yıl University Fungarium – Türkiye (VANF) were included in the study. Morphological investigations and measurements of the specimens were based on ten apothecia. To determine the dimensions of ascospores, thirty ejected mature ascospores from one individual were measured. Morphological evaluations and measurements were performed on material mounted either in distilled water, 5% KOH (potassium hydroxide) or Congo red solution prepared with water. Microscopic investigations were made using a light microscope (Leica DM500) and measurements with the Leica Application Suite (version 3.2.0) software. The obtained morphological data were compared with the relevant literature (Hansen *et al*. 2019, Skrede *et al*. 2020, Carbone *et al*. 2021).

*DNA extraction, PCR and Sequencing*

Isolation of DNA was performed from a dried specimen based on the slightly modified procedure of Doyle & Doyle (1987). Primer pairs LR0R / LR5 (Vilgalys & Hester 1990) and EF1-983F / EF1-1567R (Rehner & Buckley 2005) were used to amplify LSU and TEF1-α regions, respectively. PCR reaction was done in a 25 µl reaction mixture containing 10X Buffer, $MgCl_2$ (25 mM), genomic DNA (10 ng/µl), selected primer pair (10 µM), dNTP mixture (10 mM), Taq polymerase (5u/µl) and sterile water. The Tm values were as follows; initial denaturation at 94°C for 4 min; 30 cycles at 94°C for 1 min, 53°C (54°C for TEF1-α) for 45 s, 72°C for 1 min; and a final extension at 72°C for 5 min. PCR products were separated in a 1.0% agarose gel stained with Gelred dye. The PCR products were sequenced by using appropriate primer pairs for amplification reactions by BM Labosis, Ankara. In addition to the sequences (two regions from one sample) produced in the current study, several representative sequences of *Dissingia* and *Helvella* species were downloaded from the GenBank database and included in the analyses. The accession numbers of the used GenBank sequences are provided in Supplementary Material Table S1.

*Phylogenetic analysis*

The DNA sequences were edited and assembled using the Mesquite 3.7 software (Maddison & Maddison 2009) using Mafft 7.311 (Katoh & Standley 2013). The sequences obtained were deposited in NCBI database with accession numbers OR133577 (LSU) and OR192540 (TEF1-α). For initial comparison, Basic Local Alignment Search Tool (BLAST) (Altschul *et al*. 1990) analysis was performed and related sequences were added in the input data to clarify the phylogenetic positions of the studied samples within the genus. Separate alignments of the two regions and concatenated (LSU+TEF1-α) data were prepared for phylogenetic analyses. Our sequences in addition to 43 other sequences (16 *Dissingia* and 27 *Helvella*) for LSU data, 22 sequences (8 *Dissingia* and 14 *Helvella*) for TEF1-α data and 20 sequences (5 *Dissingia* and 15 *Helvella*) for the concatenated data were analyzed. The sequences of *Wynnella subalpina* Q. Zhao, Zhu L. Yang & K.D. Hyde (KX034104, MK113895 and UPS-F-005992) were chosen as outgroups for LSU, TEF1-α and concatenated data, respectively.

Phylogenetic analyses of the sequence data were performed using the maximum parsimony (MP) in PAUP version 4.0b10 (Swofford, 2003). For MP analysis, all characters were equally weighted, gaps were treated as missing, and character states were treated as unordered. A heuristic search was implemented with 100 random additional sequence replicates, treebisection-reconnection (TBR) branch swapping, MULPARS option, and ACCTRAN optimisation. Clade robustness was assessed using the bootstrap (BT) analysis with 1,000 replicates.

MrBayes v.3.2.6 (Ronquist & Huelsenbeck 2003) was employed to conduct the Bayesian inference (BI) analysis. Markov Chain Monte Carlo (MCMC) method was used with all the remaining settings set as default (incremental heating scheme of chains, unconstrained branch length, and uninformative topology priors). MCMC run for 3 million generations when the average standard deviations of split frequencies were <0.01 (the first 25% of generations were treated as burn-in). A majority rule consensus tree of the remaining trees was calculated. Branch support was determined by Bayesian Posterior Probabilities (BPP). Trees were visualized using Figtree 1.4.3 (Rambaut 2010).

## Results

### *Taxonomy*

*Dissingia confusa* (Harmaja) K. Hansen & X.H. Wang. Persoonia 42: 197. (Figs 1, 2).
≡ *Helvella confusa* Harmaja, Karstenia, 17 (1): 43 (1977).
= *Helvella pedunculata* Harmaja, Karstenia, 18 (2): 57 (1978), *fide* Carbone *et al*. (2021).

### *Description*

Apothecium, 1-3.5 cm wide, more or less cup-shaped, remaining concave, hymenium dry, mostly dark brown, rarely blackish-brown; outer surface gray to dark brown at the edge, whitish below, rarely yellowish, unribbed or with ribs extending slightly inward at the base, finely hairy under lens, appearing smooth or nearly smooth to the naked eye. Stipe, well-developed, fairly short, more or less cylindric; ribs blunt, always simple (Fig. 1a, b). Medullary excipulum composed of a *textura intricata*, hyphal width 3-5 (7) µm, uniformly thick. Ectal excipulum quite irregular, composed of a *textura angularis*, medium to pale brown due to intercellular and frequently distinctly encrusted, even dark brown pigment, noticeably darker than the medullary excipulum, length of elongated terminal cells of the *textura angularis* 6-20 µm. Asci subcylindrical, 285-350 × 12-15 µm, 8-spored (Fig. 2a), with aporhynchous base and lacking an apical apparatus (Fig. 2c). Ascospores 19-23 (24) × 12-15 µm; mostly elongate ellipsoid, immature spores slightly larger than matures and subfusiform (Fig. 2b). Paraphyses filiform to slightly clavate, club-shaped at tips, 5-9 µm in width, thin-walled, without encrustations, contents pale brown, homogeneous; terminal cells (50) 70-95 µm (Fig. 2d).

### *Specimens examined*

Türkiye, Erzurum province, Narman, Telli Village, under *Picea* trees, 40°12'37.0"N, 41°47'47.8"E, 2045 m a.s.l., 07.06.2018, CS 405. GenBank accession numbers: LSU, OR133577 and TEF1-α, OR192540.


**Fig. 1. a.** Photograph of the fresh ascocarp of *Dissingia confusa* in the field, **b.** dried samples.

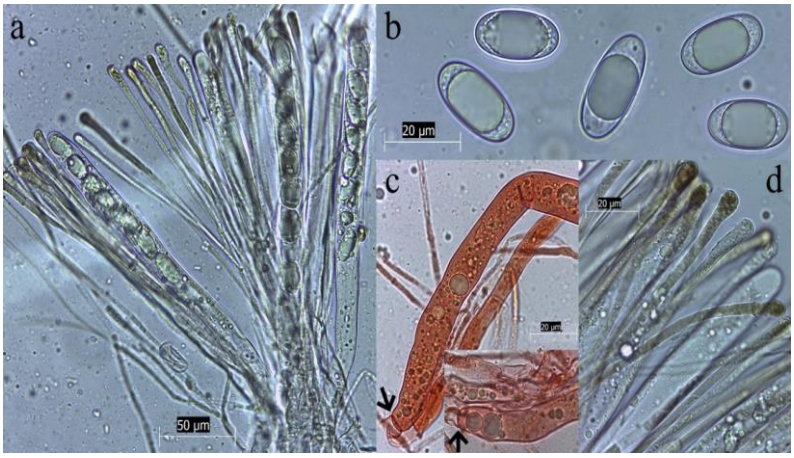


**Fig. 2.** Microscopic views of some parts of *Dissingia confusa*. **a.** Asci and paraphyses (in KOH), **b.** ascospores (in distilled water), **c.** aporhynchous ascus bases (stained with congo red), **d.** apices of paraphyses (in distilled water).

*Molecular phylogeny*

The LSU data matrix consisted of 44 sequences representing 30 taxa; 27 of the genus *Helvella*, 16 of the genus *Dissingia*, and one outgroup from the genus *Wynnella* (Schaeff.) Boud. The aligned dataset was 878 bp in length after removing poorly aligned sites with 185 variable and 142 parsimony informative sites. The TEF1-α data consisted of 18 taxa with 23 sequences; 14 of the genus *Helvella*, 8 of the genus *Dissingia*, and one outgroup from the genus *Wynnella*. The aligned dataset was 553 bp in length with 200 variable and 151 parsimony informative sites. The concatenated dataset contained 20 taxa with 21 sequences and the aligned length of the data was 1444 bp with 345 variable and 232 parsimony-informative sites. The analysis revealed two distinct clades corresponding to the genera *Helvella* and *Dissingia* in the LSU phylogeny (Fig. 3). The studied specimen grouped with its representatives downloaded from database (BPP ≥ 0.9, BT ≥ 90). The analyses showed 6 variable bases between the studied *D. confusa* sample and the downloaded sequences of *D. confusa* and *Helvella pedunculata* (including the holotype). The external position of the studied sample was driven mainly by these variations (Fig. 3). Similar phylogenetic relationships were also observed when TEF1-α and concatenated data were analyzed (Figs 4, 5). Phylogenetic relation between *D. confusa* and *H. pedunculata* could not be shown in TEF1-α tree due to missing data in the database.

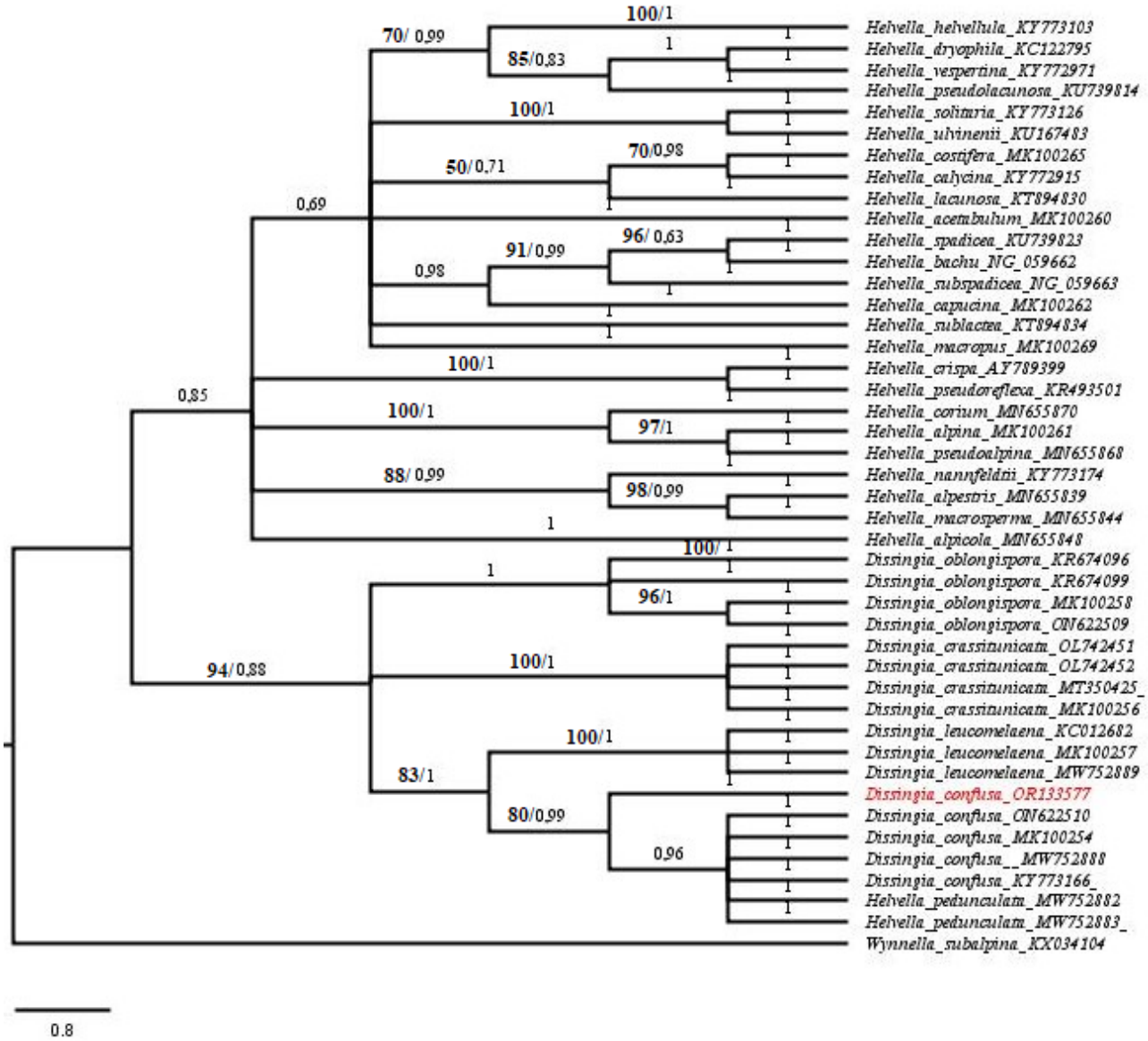


**Fig. 3.** Majority Rule Consensus tree of LSU region conducted by Bayesian analysis. Branches are labelled with Maximum parsimony bootstrap value and Bayesian posterior probabilities, respectively. The specimen of the current study, *Dissingia confusa*, is shown in red.

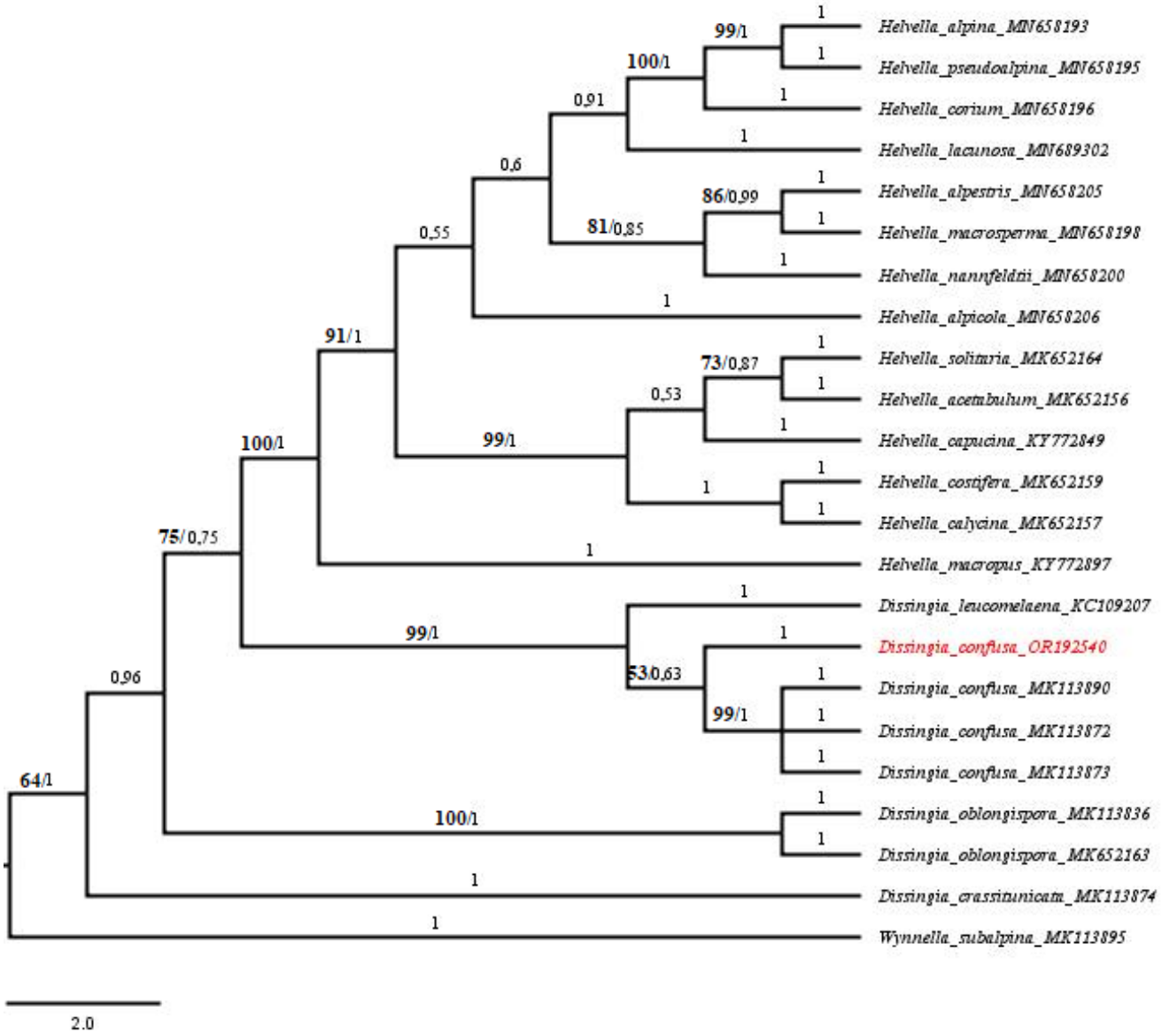


**Fig. 4.** Majority Rule Consensus tree of TEF1-α region conducted by Bayesian analysis. Branches are labelled with Maximum parsimony bootstrap value and Bayesian posterior probabilities, respectively. The specimen of the current study, *Dissingia confusa*, is shown in red.

## Discussion

The development of ascus was first studied in the genus *Helvella* by Weber (1972) and *Helvella leucomelaena* (= *Dissingia leucomelaena*) with its aporhynchous asci was separated from all other species (with pleurorhynchous asci) and located in the separate *Helvella* section *Leucomelaenae*. Later, three species (*Helvella crassitunicata*, *H. confusa* and *H. oblongispora*) were added to the section *Leucomelaenae* (Weber 1975, Harmaja 1977, 1979). Then, Häffner (1987) identified the aporhynchous ascus as a key character for the section *Leucomelaenae*. Abbott and Currah (1997) classified these species (*H. leucomelaena*, *H. crassitunicata* and *H. oblongispora*) within *Helvella* subg. *Leucomelaenae* without considering the feature of ascus development. However, they stated that this feature is a supportive character for determining the boundaries of *Leucomelaenae*, which is accepted as both a section and a subgenus.

The phylogenetic relationships of *Helvella* and its related genera have been investigated using DNA sequences from various genomic regions (Landeros *et al*. 2015, Skrede *et al*. 2017, Hansen *et al*. 2019, Loken *et al.* 2019, Wang *et al*. 2019, Skrede *et al*. 2023, Wang *et al*. 2023). With advancements in molecular phylogenetics, the taxonomy of *Helvellaceae* has been extensively studied, leading to the proposal of *Dissingia* as a new genus (Hansen et al. 2019).

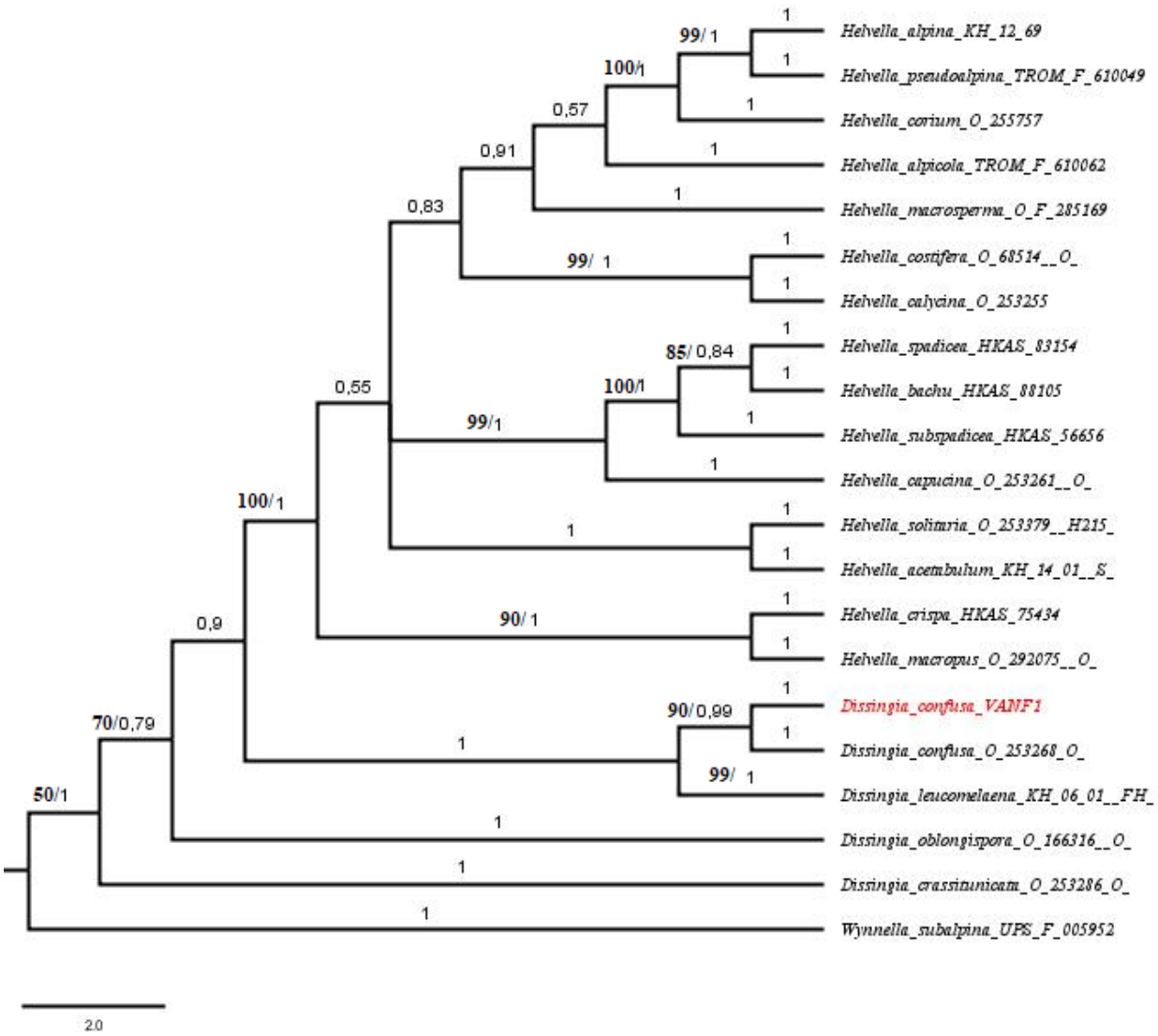


**Fig. 5.** Majority Rule Consensus tree of concatenated (LSU+TEF1-α) data conducted by Bayesian analysis. Branches are labelled with Maximum parsimony bootstrap value and Bayesian posterior probabilities, respectively. The specimen of the current study, *Dissingia confusa*, is shown in red.

The division of *Helvella* and *Dissingia* based on ascus development was also confirmed by the phylogenetic analyses of LSU region (Landeros *et al*. 2015, Hansen *et.al.* 2019). LSU region is usually preferred to delimit *Helvella* species (Nguyen *et al*. 2013, Landeros *et al*. 2015, Skrede *et al*. 2017), so the region was preferred as a barcode in the current study. Carbone *et al*. (2021) accepted *D. confusa* as a priority synonym of *H. pedunculata* by using the same region. Our results also clearly supported the phylogenetic separation of *Dissingia* from *Helvella* (Figs 3-5). Even if both names appear as valid in the Index Fungorum database, our collection of *D. confusa* grouped with its representatives and *H. pedunculata* downloaded from database (Fig. 3), suggesting that these two latter species may be synonymous (as already reported in Carbone *et al*. 2021). In this study, this synonymy has been shown by using macro/micro characters and DNA sequences of LSU in particular, after the study of Carbone et al. (2021). However, it should be noted that supplementary DNA barcodes could corroborate the synonymy with *H. pedunculata.*

As a result, the presence of *D. confusa* in Türkiye has been demonstrated, increasing the number of *Dissingia* species in Türkiye to 2.

**Ethics Committee Approval:** Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

**Data Sharing Statement:** All data are available within the study.

**Author Contributions:** Concept: Ş.S.T., A.D., M.E.A., C.S., Design: Ş.S.T., A.D., M.E.A., C.S., Execution: Ş.S.T., A.D., M.E.A., C.S., Material supplying: C.S., Data acquisition: Ş.S.T., A.D., M.E.A., C.S., Data analysis/interpretation: Ş.S.T., Writing: Ş.S.T., M.E.A., Critical review: A.D.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

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