Geliş(Recevied) :11.11.2022 ***Research Article***
Kabul(Accepted) :21.12.2022 **Doi: 10.30708.mantar.1202827**

Comparison of Two DNA Barcoding Regions for Molecular Identification of Some *Helvella* Species from Türkiye

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Abstract: In the present study, phylogenetic relationships of some *Helvella* species from Türkiye (*Helvella acetabulum, H. arctoalpina, H. calycina, H. corium, H. crispa, H. elastica, H. lacunosa, H. leucomelaena, H. leucopus, H. solitaria)* were assessed using morphological and molecular characters. DNA sequences of internal transcribed spacer (ITS) and translation elongation factor 1-alpha (*TEF1-α*) were analyzed based on Bayesian Inference methods. Although, the *TEF1-α* region has less number of indels and consistent nucleotide variations, ITS region can be preferred for figuring out the phylogeny of *Helvella* species. However, none of the regions may be sufficient alone for the identification of some cryptic and morphospecies within the genus.

Key words: *Helvella*, Barcode, ITS, TEF1-α

Türkiye'den Bazı *Helvella* Türlerinin Moleküler Tanımlanması İçin İki DNA Barkodlama Bölgesinin Karşılaştırılması

Öz: Çalışmada iki nükleer DNA lokusu kullanılarak Türkiye'den bazı *Helvella* türlerinin (*Helvella acetabulum, H. arctoalpina, H. calycina, H. corium, H. crispa, H. elastica, H. lacunosa, H. leucomelaena, H. leucopus, H. solitaria)* filogenetik ilişkileri değerlendirilmiştir. Transkribe edilen aralayıcı bölgeler (ITS) ve translasyon uzama faktörü 1-alfa (*TEF1*-α) DNA dizileri Maksimum olasılık ve Bayes çıkarım yöntemleri baz alınarak analiz edilmiştir. *TEF1-α* bölgesi daha az indel ve tutarlı nukleotid varyasyonları içermesine ragmen, ITS bölgesi *Helvella* türlerinin filogenetik ayrımında daha iyi sonuç verebilir. Fakat her iki bölge de bu cins içerisinde yer alan bazı kriptik türlerin ya da morfotürlerin ayrımında tek başına yeterli olmayabilir.

Anahtar kelimeler: *Helvella,* Barkod, ITS, TEF1-α

Introduction

Helvella L. is a genus of apothecial *Ascomycetes*, widespread in terrestrial biomes of the Northern and Southern Hemispheres (Kirk et al., 2008). The *Helvella* species are characterized by a subsessile or stipitate fruiting body, cupulate to saddle-shaped and convex to campanulate apothecia, including species with folded and lobed caps seated on a simple ribbed or furrowed stipe (Skrede et al., 2017). The genus has approximately 520 (mycobank.org, 2022) species in the world and 24 species of which have been observed in Turkey (Sesli et al., 2020; Kesici and Uzun, 2021).

The term DNA barcoding was coined in 2003 as a molecular technique that uses a short, variable, and standardized DNA region for species identification and phylogeny (Dulla et al., 2016). Length of the region, PCR and sequencing success, presence of universal primer pairs, existence of a barcode gap (the difference between inter- and intraspecific genetic distances within a group of organisms), and nucleotide variations among sequences are important characters to select a proper barcode region for studied taxa (Stielow et al., 2015; Raja et al., 2017). RNA coding, non-coding and protein coding loci are used as molecular markers to infer phylogenies in the

fungal kingdom. Landvik and coworkers (1999) used nrDNA sequences to characterize a small subset of *Helvella* species from Norway and emphasized the general limitations of single locus analyses and the unsuitability of internal transcribed spacer (ITS) to infer phylogeny and discriminate species. Nguyen et al. (2013) used a combination of the ITS and *LSU* datasets for the discrimination of species in the *H. lacunosa* species complex. Nyguen et al. (2015) prefer the partial *LSU* sequences to study infrageneric groups of *Helvella* from Europe and North America. Zhao et al. (2015) used ITS and a concatenated multilocus dataset of ITS, *LSU*, *TEF-1α*, *RPB2* and *MCM7* for delimit of the phylogeny of the *Helvella* species. Zhao et al. (2016) preferred ITS, *LSU* and *TEF-1α* to describe new species of *Helvella* from China. Skrede et al. (2017) added the *HSP* region as an informative additional gene of utility in the species identification of *Helvella*. Skrede and colleagues (2020) sequenced DNA of *HSP*, *RPB2* and *LSU* regions to characterize *Helvella* species from Spain.

Internal transcribed spacer is accepted as a barcode marker for most of fungi (Schoch et al., 2012) but the success of species identification in *Ascomycota* is lower compared to *Basidiomycota* (Dizkirici and Kalmer, 2019). Using the ITS region alone might not be reliable for certain taxa so the possibility of a two-marker barcoding system for fungi is often discussed among mycologists, particularly researchers working on ascomycetous (Dizkirici and Kalmer, 2019). In the current study, ITS and *TEF-1α* regions were compared to find out their suitability for the construction of the phylogeny of *Helvella*. These regions were intentionally selected since the ITS is an official DNA barcoding marker for species-level identification of fungi and *TEF-1α* is a protein-coding gene used as supplement data for certain fungal groups.

Material and Method

Morphological studies

The macrofungus samples were collected from different part of Türkiye (Table 1) and deposited in the Fungarium of Van Yüzüncü Yıl University (VANF). Ten *Helvella* species were described based on morphological studies. Macroscopic and microscopic characters were observed in distilled water, IKI (iodine potassium iodine), KOH (potassium hydroxide) and Melzer reagent solutions. Sections were examined under a Leica DM500 research microscope and measured with the Leica Application Suite (version 3.2.0) programme. The terminology used for describing the morphological characters referred to Maia et al., (1996), Tedersoo et al., (2006), Healy et al., (2013), Hwang et al., (2015).

Molecular studies

DNA was extracted from dried herbarium collections according to 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 1% agarose gel electrophoresis. PCR was performed on Thermalcycler (ThermoScientific) using N-nc18S10 5'AGGAGAAGTCGTAACAAG3' / C26A 5'GTTTCTTTTC CTCCGCT3' (Wen and Zimmer, 1996) primer sets to amplify the ITS1-5.8S-ITS2 region (ITS), and EF1-983F 5’ACHGTRCCRATACCACCRATCTT3’ / EF1-1567R 5’GCYCCYGGHCAYCGTGAY3’ (Rehner and Buckley, 2005) for *TEF1-α* region. PCR reaction 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. The PCR condition for both regions was optimized as 95 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 50 sec, and elongation at 72 °C for 1 min, and final extension at 72 °C for 5 min to complete the primer-template extensions. PCR products were run in 1.0 % agarose gel and visualized by staining with Gelred dye. Positive reactions were sequenced with forward and reverse PCR primers using ABI 3730XL automated sequencer (BM Labosis, Ankara, Türkiye).

Pylogenetic analysis

DNA sequences were assembled and edited with Mafft 7.311 (Katoh and Standley, 2013) from the Mesquite 3.7 software (Maddison and Maddison, 2009). For initial comparison, Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) analysis was performed using the GenBank database. The sequences obtained from studied samples were compared to related sequences in GenBank via BLAST algorithm. To clarify the phylogenetic positions of the studied specimens, representative sequences of ITS and *TEF1-α* loci were retrieved from the GenBank database. For each region 16 sequences representing of *Helvella* were downloaded. For single dataset, 28 sequences were analyzed representing 10 *Helvella* taxa (Table 1). *Wynnella silvicola* (AF064596) and *W. supalpina* (MK113895) were chosen as outgroup samples for ITS and *TEF1-α* regions, respectively.

Phylogenetic inference was conducted using Bayesian inference (BI) method. The BI analyses were performed with MrBayes v.3.2.6 (Ronquist et al., 2003)

with four incrementally heated simultaneous Monte Carlo Markov Chains (MCMC) run for 5 million generations under GTR+G evolutionary model when the average standard deviations of split frequencies were <0.01 (the first 25% of generations were treated as burn-in). Branch support was determined by Bayesian Posterior Probabilities (BPP). For the remaining trees, a majority rule consensus tree showing all compatible partitions was computed to obtain estimates for Bayesian Posterior Probabilities (BPP). The final phylogenetic trees were visualized using Figtree 1.4.3 (Rambaut, 2016).

Table 1. Metadata of *Helvella* species. The studied samples were written with bold character. *The sequences downloaded from GenBank were cited at the end of the paper. **The accession numbers of *TEF1-α* were not assigned yet by GenBank.

Species	Sample and Fungarium ID	Locality	Collection Year	GenBank Accession Number*	
				ITS	*TEF1- α*
H. acetabulum (L.) Quél. Kuzukulağımantarı	KH1401	Sweden, Södermanland	2014	MK088073	MK113891
	H127, O-178005	Norway, Møre og Romsdal	2004	MK088072	KY772872
	VANF130 **VANF881**	**Erzurum** **Hakkari**	**2018** **2015**	**OQ065744** **OQ065743**	****** ******
H. alpina Skrede, T.A. Carlsen & T. Schumach.	H1124, C-F-55730	Greenland	1987	MN658193	MN658193
H. arctoalpina Harmaja	H030, O-253236	Norway, Oppland	2009	-	KY772835
	VANF127	**Hakkari**	**2010**	**OQ065745**	******
H. calycina Skrede, T.A. Carlsen & T. Schumach.	H022, O-253255	Norway. Oppland. Dovre.Grimsdalen	2009	MN656158	KY772833
	VANF100	**Erzurum-Kars**	**2013**	**OQ065747**	******
H. costifera Nannf. Boz semermantarı	AMH001	USA	2017	MG663264	
	HMAS 187120	Beijing, China	2019		MK652158
H. corium (O. Weberb.) Massee Semermantarı	H1958, TROM-F-610014	Norway. Troms. Salangen	2017	MN656172	
	H2184, O-255756 0-253259	Canada: Quebec, Chibougamau	2017	MN992597	
	H2184, O-255756	Svalbard. Longyearbyen	2017		MN658196
	O-253279	Norway,Hordaland	2014		MK113880
	VANF234	**Hakkari**	**2013**	**OQ065750**	******
H.crispa (Scop.) Fr. Delikli semermantarı	KH.09.186 (S)	Sweden,Gotland	2009	KU739895	MK113882
	VANF98	**Erzurum-Kars**	**2013**	**OQ065742**	******
H. confusa Harmaja	KH.12.75 (S)	Sweden,Jampland	2012		MK11390
	2484232	France	2016	ON622916	
H. elastica Bull. Esnek semermantarı	H066	Sweden	2009	KR019787	KY772858
	VANF351	**Erzurum**	**2018**	**OQ065749**	******
H. leucomelaena (Pers.) Nannf. Çukur semermantarı	KH.06.01 (FH)	USA, MA	2006		KC109207
	VANF806 **VANF1026**	**Erzurum-Kars** **Bingöl**	**2013** **2018**	**OQ065741** **OQ065740**	****** ******
H. lacunosa Afzel. Bet semermantarı	44625	United Kingdom: England, Nottinghamshire	2013	MZ159481	
	HKAS 87878	China	2016	KT894824	

	H1041, O-255761	Norway. Nordland. Saltdal. Junkerdalen	2016		MN689302
	O-253320	Norway, Oppland	2009		MK113883
	VANF784	Erzurum-Kars	2014	OQ065748	**
H. leucopus Pers. Top semermantarı	1287490	Italy	2012	JX462565	-
	VANF953	**Ordu**	**2015**	**OQ065751**	**
H. solitaria P. Karst. Ayrık semermantarı	2663	Norway	1987	AF046221	
	H080, O-25337	Norway	1987		KY772864
	VANF21	**Erzurum-Kars**	**2011**	**OQ065746**	**
H. spadicea Schaeff. Alaca semermantarı	HKAS83153	China	2016	KU739798	-
H.oblongispora Harmaja	34293	France	2012	ON622915	
	HMAS86051	Xinjiang, China	2019		MK652163

Results

The analysis involved a total of 29 sequences including outgrup for each region (Table 1). The length of sequence was about 600 bp for ITS and 500 bp for *TEF-1α* regions, and both regions had relatively clear barcoding gaps. The resolution of the ITS tree was better than that of *TEF1-α* because of greater number of nucleotide variations (Figure 1). The analysis based on variable characters of the two regions yielded phylogenetic trees with well supported clusters (Figures 2 and 3). However, the resolution of the *TEF-1α* tree was less compared to ITS resolution so phylogenetic separation of some taxa such as *H. corium* (N234) and *H. alpina* (MN658193); *H. calycina* (N100) and *H. costifera* (MK652158); *H. arctoalpina* (N127) and *H. acetabulum* (N130) could not be observed clearly.

Species were grouped in keeping with their sections (*Solitarie, Leucomelaenae, Acetabulum, Helvella, Macropodes, Lacunosae, Elasticae*) so seven clusters were observed in both trees. The studied species grouped with their representatives retrieved from the database. This result suggests that the morphological and molecular data are sufficient for identification of the studied samples.

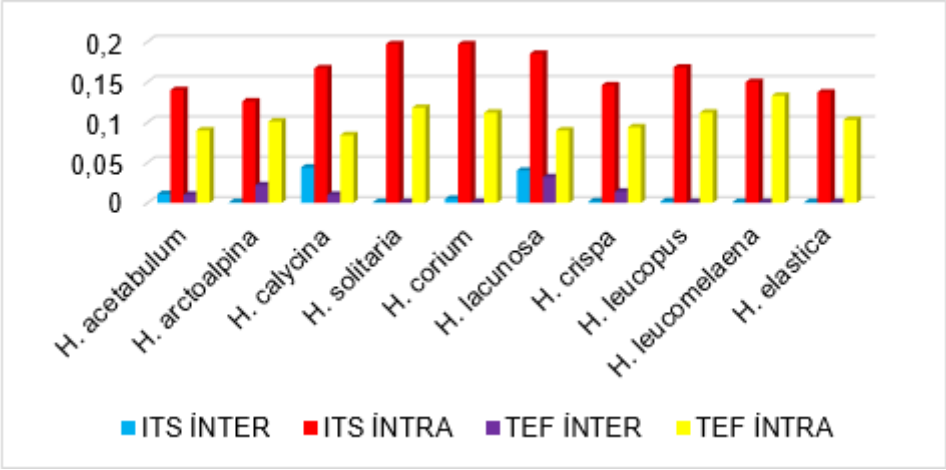

Figure 1. Comparisons of intra- and inter-specific variation pairwise distances among ITS and *TEF-1α* genes of *Helvella* generated by MEGA and Excel.

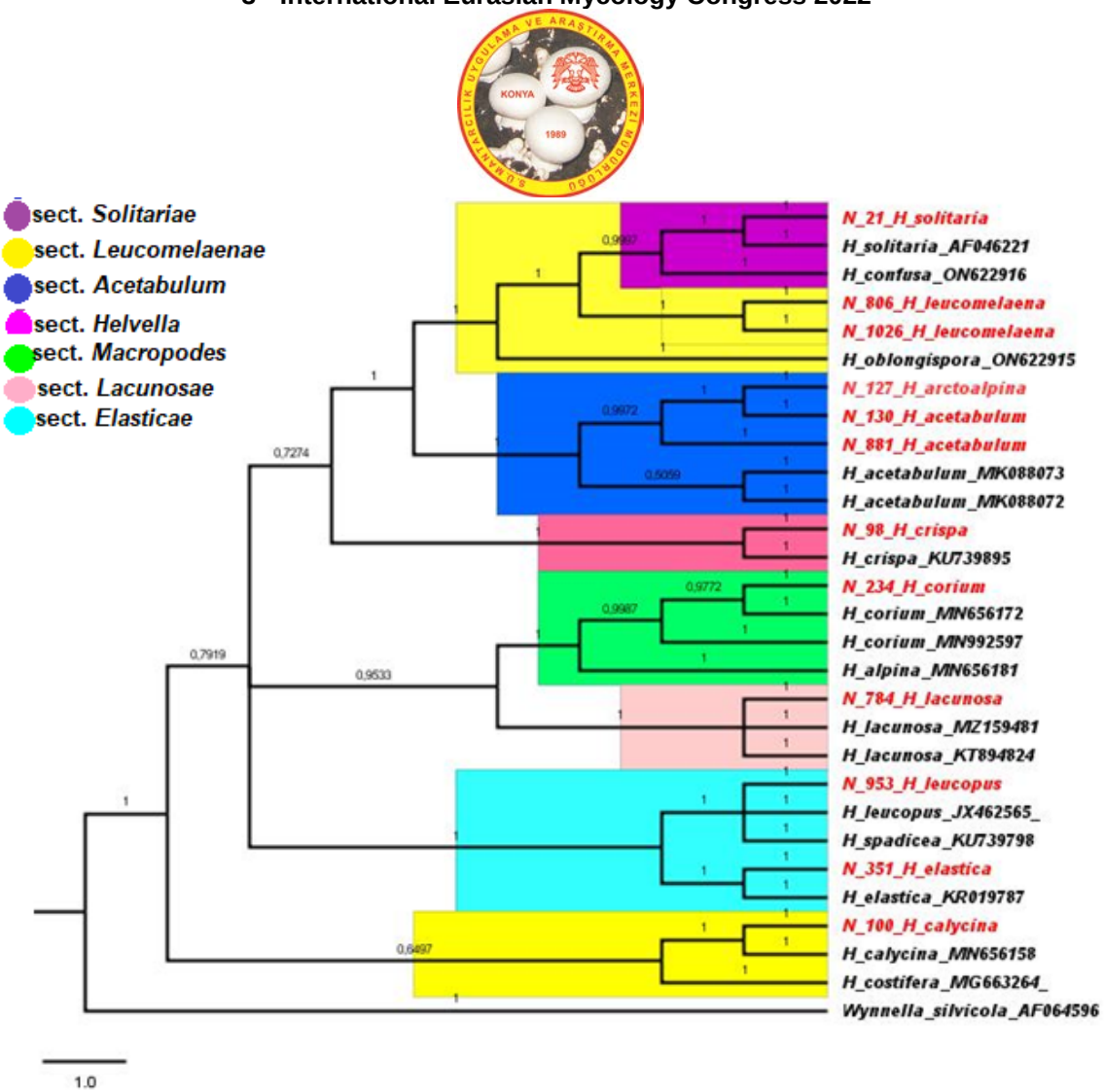

Figure 2. Phylogenetic tree of ITS region conducted by Bayesian analysis (Taxa marked in red indicate studied examples).

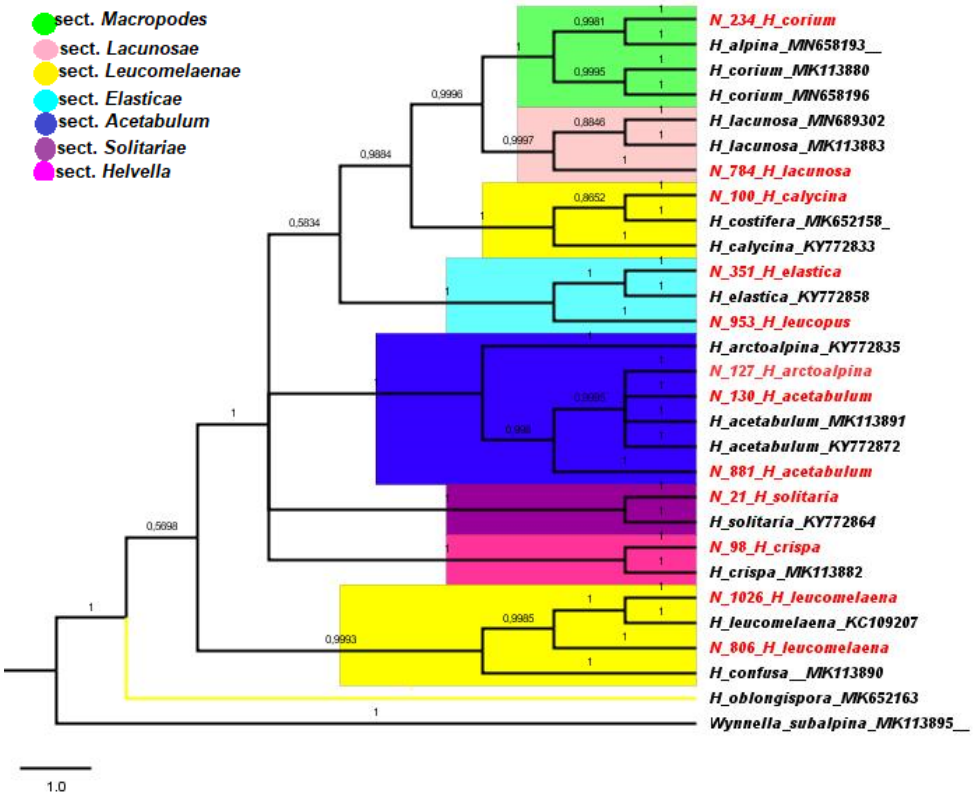

Figure 3. Phylogenetic tree of *TEF1-α* region conducted by Bayesian analysis (Taxa marked in red indicate studied examples).

Discussions

RNA coding, non-coding and protein coding loci are used as molecular markers to infer phylogenies in the fungal kingdom. Several studies have pointed out that single-copy protein-coding markers are more useful and may perform better than multi-copy ribosomal genes (Raja et al., 2011; Stielow et al., 2015; Hansen and Olariaga, 2015). Since *Ascomycetes* is the last evolving class of the fungal kingdom, working with conserved regions in addition to ITS may be valuable to get more informative results. Therefore, secondary DNA barcodes have been suggested to overcome the limitations of the ITS region (Stielow et al. 2015). The single copy protein coding gene such as *TEF1-α* has been suggested as a secondary barcode for fungi because of its ability to resolve closely related species (Hansen and Olariaga 2015; Stielow et al. 2015).

The procedures such as DNA isolation and PCR were easier for *TEF1-α region* compared to ITS. For instance DNA was isolated from old-dated samples with high quality even though Skrede and coworkers (2017) indicated that fresh specimens are needed to isolate DNA. Moreover, *TEF1-α* region was amplified easily and did not give any non-specific band pattern; only one band was observed after PCR but more than one was seen for ITS region. Even though the *TEF1-α* region has less number of indels and consistent nucleotide variations, the ITS region has better resolution to determine the phylogeny of *Helvella* species. Discrimination of some species was not sufficient at the tree constructed based on *TEF1-α* region. One of them is *Helvella corium* which belongs to the morphospecies complex and this species is determined pseudocryptic species. For discrimination of these cryptic species additional regions are needed such as *LSU* (Large Subunit) (Løken et al., 2020). *Helvella costifera* and *H. calycina* are sister species and protein coding gene such as *HSP* (Heat Shock Protein) and *RPB2* (RNA Polymerase 2) regions are needed for getting be better resolution in the tree (Skrede et al., 2020).

As a result, it is safe to say that ITS region may be more useful to study phylogeny of the genus. This region can be used alone or combined with protein coding gene such as *TEF1-α* to better understand relations between species.

Acknowledgements

This study was financially supported by Van Yüzüncü Yıl University (Scientific Research Project Foundation, FHD-2022-10113), Van, Türkiye.

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