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Molecular and Morphological Identification of *Melanoleuca cinereifolia* from Türkiye

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Abstract: *Melanoleuca cinereifolia* is identified based on macro/micro-morphological characters and DNA sequences of the nuclear ribosomal Internal Transcribed Spacer (nrITS) region and is reported here for the first time from Diyarbakır province of Türkiye. Macro- and micro-morphological description of the species is provided along with the photographs of fresh basidiomes in natural habitat and the line drawings. The species is mainly characterized by basidiome with a short stipe, grayish-brown pileus, grey lamellae and lageniform cystidia. In the phylogram, *M. cinereifolia* is clustered with its allies in the subgenus *Melanoleuca*. Morphological and molecular similarities and differences among the species and the close relatives were discussed in detail.

Key words: DNA, ITS, Molecular taxonomy, *Agaricales*

Türkiye'den *Melanoleuca cinereifolia* Türünün Moleküler ve Morfolojik Teşhisi

Öz: Bu çalışmada *Melanoleuca cinereifolia*, Türkiye'nin Diyarbakır ilinden ilk kez rapor edilmiş, makro/mikro-morfolojik karakterleri ile nükleer ribozomal Internal Transcribed Spacer (nrITS) bölgesinin DNA dizileri baz alınarak tanımlanmıştır. Türün makro ve mikro morfolojik tanımı, doğal ortamdaki taze bazidyomların fotoğrafları ve mikroskobik yapılarının çizimleri ile birlikte verilmiştir. Bu tür esas olarak kısa saplı, grimsi kahverengi şapka, gri lamelli ve şişe biçiminde sistityumlu bazidiyom ile karakterize edilir. Filogramda, *M. cinereifolia*, *Melanoleuca* alt cinsindeki akrabaları ile birlikte kümelenmiştir. Türler ve yakın akrabaları arasındaki morfolojik ve moleküler benzerlikler ve farklılıklar ayrıntılı olarak tartışılmıştır.

Anahtar kelimeler: DNA, ITS, Moleküler taksonomi, *Agaricales*

Introduction

Melanoleuca Pat. (*Agaricales*) is fungus distributed widely throughout the world with several edible species (Antonín et al., 2017). The genus is mainly characterized by a collybioid to tricholomatoid habit, dull color basidiomes, white to pale yellowish lamellae, strongly amyloid and warted spores, distinct cheilocystidia, and a lack of clamp connections (Vesterholt, 2008). Species of *Melanoleuca* are difficult to distinguish morphologically since many species appear very similar and differentiated by only using a few ambiguous features (Vizzini et al., 2011). Molecular techniques are proved to be successful approaches along with traditional methods for correct identification and reclassification. The sequence of the nrITS region is a superior molecular DNA barcode for molecular identification of Basidiomycetes (Schoch et al., 2012) so this region is used in the current study. In Turkiye, 24 *Melanoleuca* species have been reported until 2020 (Sesli et al., 2020) and 3 of them were listed in our

studies (Acar et al., 2017; Kalmer et al., 2018). In the current study, *Melanoleuca cinereifolia* Bon (Bon) was identified based on microscopic/macroscopic and molecular analyses. Some *Melanoleuca* species are reported to be edible while no information is found for the rest (Wu et al., 2019). *Melanoleuca cinereifolia* was first described in 1970 by a French mycologist, Marcel Bon (Saba and Khalid 2014). There is not any report that points out whether this species is edible or not. Furthermore, *Melanoleuca* species may be useful for pharmacological studies because of their antioxidant capacity (Bahadori et al., 2019). The purpose of the present study is to identify the collected materials by using morphological and molecular data and to publish a new species, *Melanoleuca cinereifolia*.

Material and Metod

Morphological studies

Samples were determined during routine visits to the eastern region of Turkiye. Basidiomata were collected from Hani district of Diyarbakır in 2012 and photographed with a Canon (EOS 60D) camera equipped with Tokina 100 mm macro lens in their natural habitats. For micro-morphological studies, tissues from pileus, stipe, and gills were mounted in Melzer's reagent, and distilled water. Microscopic structures were stained with Melzer's reagent and KOH (5 %) and analyzed by using a Leica DM500 research microscope. Color images were obtained with the Leica ICC50 HD camera, and measurements were carried out with Leica Application Suite (version 3.4.0) programme. Methods used for morphological descriptions were performed based on the following literature; Boekhout, (1999); Breitenbach and Kränzlin (1991); Vizzini et al., (2011); Buczacki, (2012); Garcia et al., (2013); Kuo and Methven (2014). The voucher specimens were deposited in the Fungarium of Van Yüzüncü Yıl University (VANF).

Molecular studies

Total DNA was extracted from dried basidiomata using the CTAB method with minor modifications (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, MgCl2 (25 mM), dNTP mixture (10 mM), selected primer pair (10 µM), Taq polymerase (5u/µl) and sterile water. To amplify ITS (ITS1-5.8S-ITS2) region, primer pairs N-nc18S10 5'AGGAGAAGTCGTAACAAG3'/C26A 5'GTTTCTTTTCCTCCGCT3' (Wen and Zimmer, 1996) were used. PCR products were run in a 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 (Applied Biosystems, Foster City, CA, USA). Sequences were subjected to BLAST at GenBank and the *Melanoleuca* sequences with the highest homologies were retrieved for phylogenetic analysis. DNA sequences of nrITS region taken from the present study were aligned with 54 nrITS sequences (31 taxa) retrieved from GenBank using ClustalW (Thompson et al., 1994). Genetic parameters were calculated using Molecular Evolutionary Genetics Analysis software (MEGA 6; Tamura et al., 2013). To determine the phylogenetic positions of the sampled specimens, dataset was performed with IQ-TREE v.1.6.12 software (Nguyen et al. 2015) using TIM2+F+G4 model of evolution for ITS dataset. Branch support was obtained through 1000 replicates of ultrafast bootstrap (Hoang et al. 2018) and Shimodaira–Hasegawa (SH)-like approximate likelihood ratio tests (Guindon et al. 2010). Support values are given as SH-like approximate likelihood ratio test support (SH-aLRT) [%] / Ultrafast Approach Bootstrap (UFBoot) [%].

Results

Taxonomy

Melanoleuca cinereifolia Bon (Bon). Docums Mycol. 9(33): 71 (1978) (Fig. 1-2)

Synonymy: Synonymy: *Melanoleuca cinereifolia* var. *maritima* Huijsman ex Bon,–*Melanoleuca maritima* Huijsman, *Melanoleuca strictipes* var. *cinereifolia* Bon

Description: Pileus; 30–40 mm, plano-convex, thin, smooth, grayish-brown with slightly darker center. Lamellae; adnate, close, white to gray. Stipe; 20–30 × 4–7 mm, central, equal, striate, solid, sometimes bulbous at the base, fibrillose, brown to dark pale brown. Basidiospores; 7.6–9.0 × 4.0–5.8 µm, ellipsoid, apiculus absent, warty, amyloid, hyaline in KOH. Basidia; 8.0–10(12) × 25–30 µm, clavate, four-spored, hyaline in KOH. Cheilocystidia; 48–60(70) × 9–13(15) µm, lageniform, urticiform, sometimes septate, hyaline, with crystals at the apex. Pleurocystidia; similar to cheilocystidia. Pileipellis; a cutis, cylindrical, 8–15 µm wide, thin-walled, hyaline in KOH. Caulocystidia; cylindrical, 5–12(14) µm, hyaline to pale yellow in KOH. Clamp connections absent.

Specimens examined: TÜRKİYE, Diyarbakır, Hani, in grassland and meadows area, 38° 24' 08"N, 40° 23' 40"E, 846 m, 13.11.2012. Acar. 36 (VANF). Genbank accession numbers: OK356906-OK356907.

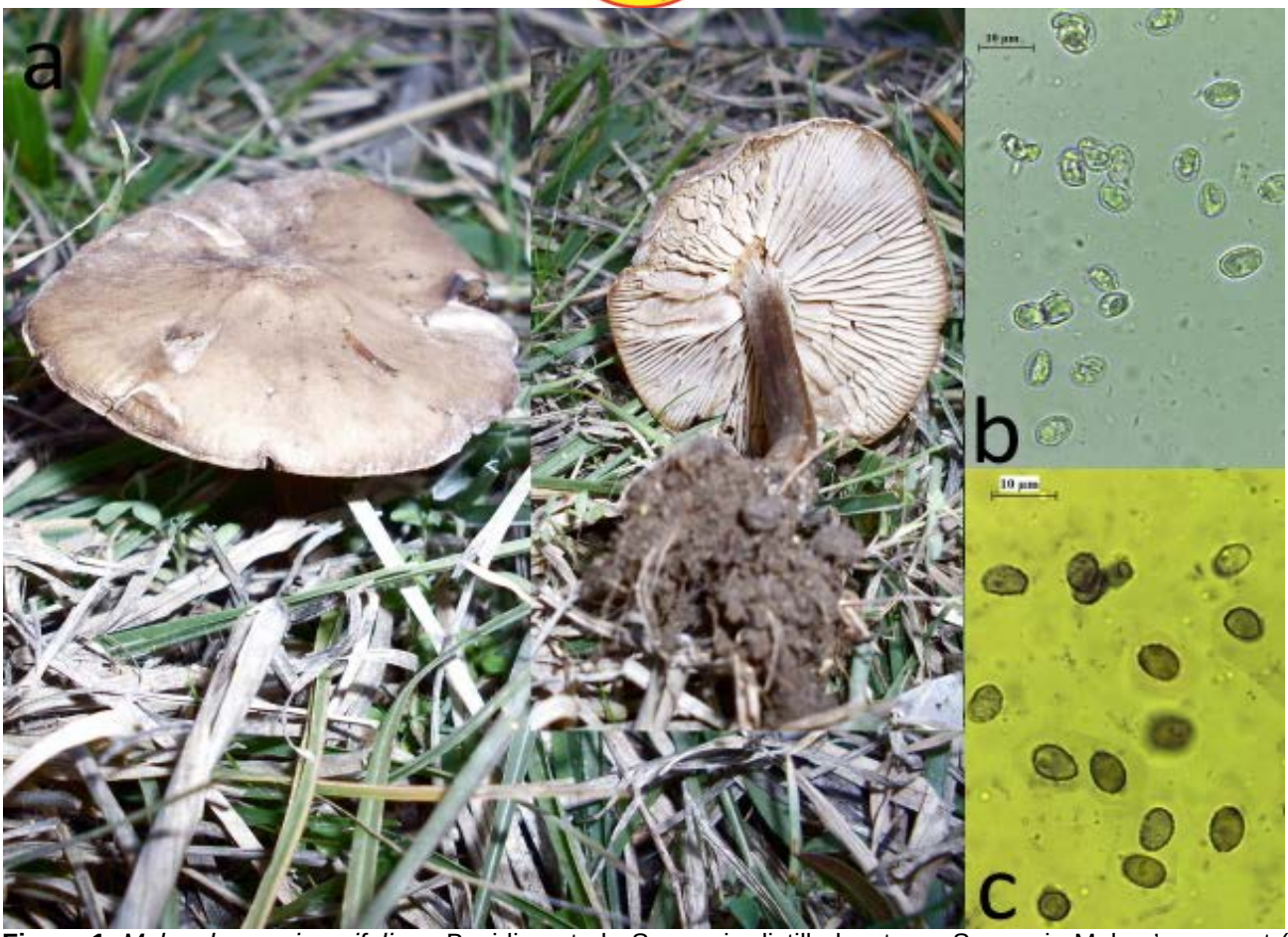

Figure 1. *Melanoleuca cinereifolia* a. Basidiomata b. Spores in distilled water c. Spores in Melzer's reagent (Scale bar=10 µm).

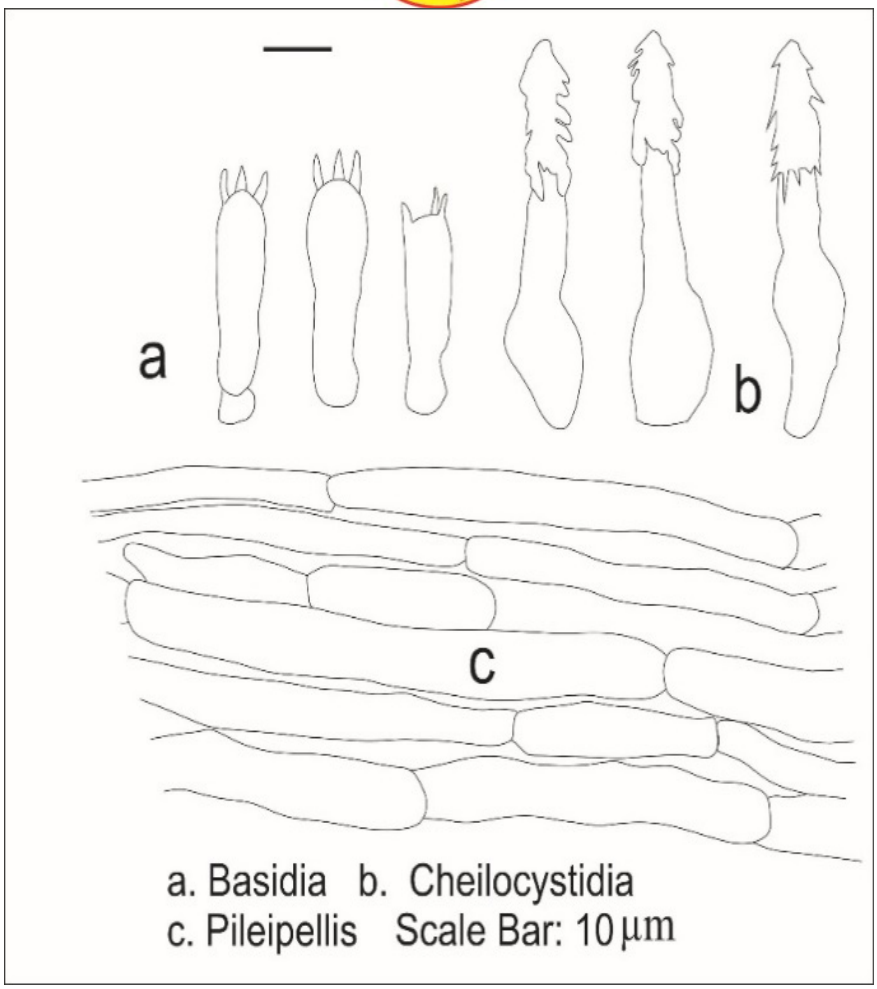

Figure 2. *Melanoleuca cinereifolia* a. Basidia b. Cheilocystidia c. Pileipellis (Scale bar=10 µm).

Molecular identification

Two nrITS sequences were used for phylogenetic analysis. The sequences were submitted to GenBank and the accession numbers were given in the text. The ITS data matrix included 54 sequences in total. The amplified DNA fragment of the region was approximately 650 bp length encompassing complete ITS1, 5.8S and ITS2 sub-regions. The final data set included a total of 636 positions, of which 452 were conserved, 181 were variable.

The studied *Melanoleuca cinereifolia* (OK356906-OK356907) sequences showed 100% homology with *M. cinereifolia* (JN052137-JN052138-JF908356). Some of the DNA sequences of our samples, especially the clean and reliable ones were blasted in NCBI and the samples showing the highest homologies were retrieved and added to our dataset.

Two major clades, supporting recognition of two subgenera (*Urticocystis* and *Melanoleuca*), were observed in the phylogram (Figure 3). Clade *Melanoleuca*, which was supported by 92% SH, 90% UF support, included the taxa with mainly lageniform cystidia or rarely without cystidia. Clade *Urticocystis* was supported by a robust support values (100% SH, 100% UF) and included the taxa mainly with mainly with urticocystidia but also with macrocystidia.

The studied specimens of *M. cinereifolia* is the part of the clade around *M. cinereifolia* and *M. ammophilum* (91% SH, 97% UF). The position of *M. cinereifolia* in this major clade was supported by the absence of the large brevipes-type cystidia.

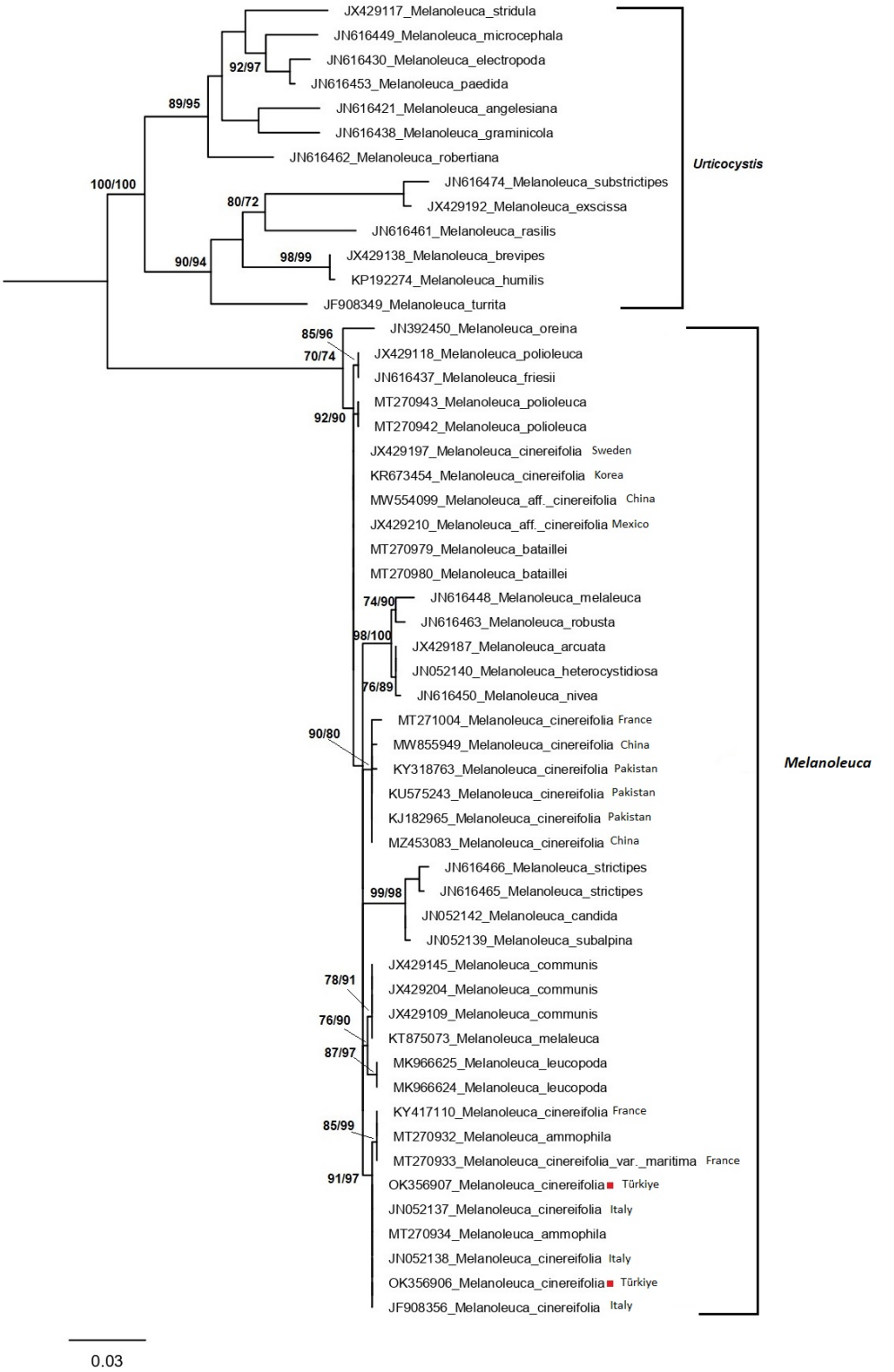

Figure 3. Phylogenetic relationship of *Melanoleuca cinereifolia* with other *Melanoleuca* spp. based on the ML method. Sequences generated from the local collection are marked with red bullets.

Discussions

Identification of *Melanoleuca* species is controversial since the morphology of the species are easily affected by environmental factors. Therefore, just traditional taxonomy may not be enough to determine the species correctly.

Melanoleuca cinereifolia is reported here for the first time from Diyarbakır province of Turkiye. Key characteristics of the species are as follow; convex, thin and greyish brown pileus with dark brown center; concolorous short solid stipe often with subbulbous base and the lower part of the stipe darker brownish, greyish close lamellae, quite lageniform cystidia and usually dunes habitats (Bon, 1991; Vesterholt, 2008; Saba and Khalid 2014; Duriska et al., 2017). Morphologically, *M. cinereifolia* can be confused with *M. polioleuca* (Fr.) Kühner & Maire however it differentiates from *M. polioleuca* in having a less color pileus with flexed to down at margin, a darker brown stipe, and a different shape of cheilo- and pleurocystidia. Phylogenetically, the Turkish specimens of *M. cinereifolia* closely grouped with *M. ammophila*. *Melanoleuca ammophila* has more robust and larger basidiomes (pileus 40–50 mm diam., stipe 30–70 mm long vs. 30–40, 20–30) (Antonín et al. 2021). Micromorphologically, the cheilocystidium of *M. ammophila* is thin-walled with slightly thick walled apex compared to *M. cinereifolia*. Another difference between these taxa is the pileipellis structure which in the new species presents a thicker ixocutis (15 µm vs. 11 µm).

In the phylogeny obtained, the sampled sequences of *M. cinereifolia* species appear in three clades. One of them, with collections from Sweden, Korea, China and Mexico. *Melanoleuca cinereifolia* closely clustered with sequences of Antonín et al. (2017) (KY417110), Antonín et al. (2021) (MT270933), Garbelotto et al. (unpublished-JN052137-JN052138), Osmundson et al. (2013) (JF908356) in the phylogram. The other two clades are composed of specimens from China and Pakistan included the type specimen (MT271004) form France.

Melanoleuca cinereifolia was originally described from Europe; usually develops in dunes (Vesterholt, 2008). Sánchez-García et al. (2013) reported *M. cinereifolia* from Mexico City and collected from mountain mesophilic forest. Also, *M. cinereifolia* collected from *Pinus wallichiana* forest in Pakistan (Saba and Khalid, 2014). However, Turkish specimens were collected from grassland and meadow. There are no previous reports of *M. cinereifolia* being associated with grassland. This report serves as the first documentation of this association. According to the reports of *M. cinereifolia*, it shows different ecological range.

In the present study, *Melanoleuca cinereifolia* specimens collected from Eastern Turkiye are identified by using morphological and molecular data. Morphological observation and phylogenetic analyses confirm that *Melanoleuca cinereifolia* is a new record for Turkish mycobiota.

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