

Diversity of *Melanoleuca* Pat. (Basidiomycota, Agaricales) in Çanakkale, Türkiye: Morphological and molecular perspectives

İsmail Acar^{1*}, Halide Karabıyık²

¹Van Yüzyüncü Yıl University, Başkale Vocational School, Department of Organic Agriculture, Van, Türkiye

²Trakya University, Arda Vocational School, Department of Food Processing, Edirne, Türkiye

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Abstract

The genus *Melanoleuca* Pat. (Basidiomycota or Agaricales) is taxonomically complex due to its remarkable morphological similarities with other genera. Consequently, any reliable species identification method requires a holistic approach that includes molecular phylogenetic data. In this study, five taxa, namely *M. excissa* (Fr.) Singer, *M. cf. graminicola*, *M. granadensis* Armada, *M. paedida* (Fr.) Kühner & Maire, and *M. stridula* (Fr.) Singer were identified from the Çanakkale province, Türkiye. These were described by integrating macro and micromorphological characters with phylogenetic analyses, which were based on Internal Transcribed Spacer rDNA sequences. *M. excissa*, *M. graminicola*, *M. paedida*, and *M. stridula* have already been documented in Türkiye. This study updated the detailed macro- and microscopic descriptions. *M. granadensis*, first described in Spain in 2020 and reported from only the Czech Republic and Slovakia in Europe, was confirmed as a novel record of the Turkish mycobiota. It represents the first known non-European specimen. The detailed habitat characteristics, geographic coordinates, and collection dates of all specimens were documented. The microscopic characters were supported with drawings; the description of the new record was supplemented with scanning electron microscope images. These findings contribute novel data for understanding the *Melanoleuca* spp. diversity in Türkiye, underscoring the significance of molecular data in distinguishing morphologically similar species. They also suggest that certain records should be re-evaluated within the context of current phylogenetics.

Özet

Melanoleuca Pat., Basidiomycota veya Agaricales, cinsi önemli morfolojik benzerliklerin varlığı nedeniyle taksonomik olarak karmaşık bir gruptur. Bu sebeple, güvenilir tür tanımlaması, moleküler filogenetik verilerle desteklenen bütünsel bir yaklaşım gerektirir. Bu çalışmada, Türkiye'nin Çanakkale ilinden tanımlanan beş takson, yani *M. excissa* (Fr.) Singer, *M. cf. graminicola*, *M. granadensis* Armada, *M. paedida* (Fr.) Kühner & Maire ve *M. stridula* (Fr.) Singer incelenmiştir. Bu türler, makro- ve mikromorfolojik karakterlerin İçsel Transkribe Edilmiş Aralık rDNA dizilerine dayalı filogenetik analizlerle entegre edilmesiyle tanımlanmıştır. *M. excissa*, *M. graminicola*, *M. paedida* ve *M. stridula* daha önce Türkiye'de belgelenmiştir; bu çalışmada, ayrıntılı makro- ve mikroskobik tanımlamalar güncellenmiş ve yeniden sunulmuştur. *M. granadensis*, ilk olarak 2020 yılında İspanya'da tanımlanmış ve Avrupa'da sadece Çek Cumhuriyeti ve Slovakya'da rapor edilmiştir. Bu tür, Türkiye mikrobiyotası için güvenilir bir şekilde doğrulanmış yeni bir kayıt olup, Avrupa dışından bilinen ilk örnek olarak kabul edilmektedir. Tüm örneklerin habitat gözlemleri, coğrafi koordinatları ve toplama tarihleri ayrıntılı olarak belgelenmiştir; mikroskobik karakterler çizimlerle desteklenmiştir ve yeni kaydın tanımı taramalı elektron mikroskobu görüntüleri ile desteklenmiştir. Bu bulgular, Türkiye'deki *Melanoleuca* türlerinin çeşitliliğinin anlaşılmasına yeni veriler katmakta, morfolojik olarak benzer türleri ayırt etmede moleküler verilerin kritik önemini vurgulamakta ve literatürdeki bazı kayıtların mevcut filogenetik bağlamda yeniden değerlendirilmesi gerektiğini göstermektedir.

Keywords: DNA, Internal Transcribed Spacer, new record, molecular taxonomy, *Melanoleucaceae*

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*Corresponding Author: İsmail Acar, E-mail: ismailacar@yyu.edu.tr

ORCID iDs of the authors: İA. 0000-0002-6049-4896, HK. 0000-0002-1778-2200



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Introduction

The genus *Melanoleuca* Pat. was first described in 1897 by Patouillard. It comprises > 440 species worldwide (Antonín et al., 2023). For a long time, the classification of the genus was ambiguous and was included in Tricholomataceae. Vizzini et al. (2024) placed five families into Pluteineae (a suborder of Agaricales): Amanitaceae (including *Leucocortinarius*), Limnoperdaceae, Melanoleucaceae, Pluteaceae, and Volvariellaceae. No clear, morphological synapomorphy unites the members of this suborder. The basidiomata are variously shaped: agaricoid, with open or closed hymenophore, or gasteroid/sequestrate. They are mostly fleshy; the way the lamellae attach to the stipe may vary (Vizzini et al., 2024).

For the first time, molecular analyses supported a monophyletic origin of *Giacomia* and *Melanoleuca* within Pluteineae (Vizzini et al., 2024). Thus, they were positioned within a novel family, Melanoleucaceae. Its members have pileostipitate basidiomata, mostly collybioid to tricholomatoid in structure, with subregular hymenophoral trama and monomitic hyphae.

Melanoleuca (Basidiomycota, Agaricales) comprises species with a diversity of stalked agarics, which are ecologically characterized as having a saprotrophic lifestyle. The genus can be distinguished by numerous morphological features, including basidiomata of collybioid–tricholomatoid habit, dull-colored tones, white–pale yellowish lamellae, and pale–whitish stipe bases. At the macroscopic level, these features comprise warty basidiospores with a robust amyloid reaction, prominent and variously shaped cheilocystidia, and the absence or rare presence of clamp connections, depending on the species (Vesterholt, 2008; Acar et al., 2017). However, the morphological classification of *Melanoleuca* spp. is cumbersome, especially due to overlapping macroscopic and microscopic characters and the blurring of interspecific boundaries. Indeed, many species are morphologically very similar, and the interspecific variations are often based on only a few microscopic features (Vizzini et al., 2011). This situation highlights the inadequacies of traditional identification methods, which lead to taxonomic confusion (Kalmer et al., 2022).

Over the last few decades, the application of molecular data has emerged as an essential approach to solving such taxonomic ambiguities. In particular, the nuclear ribosomal internal transcribed spacer (nrITS) region has been proposed as the most reliable DNA barcode region for the molecular identification of Basidiomycota members due to its high variability and easy amplification with universal primers (Schoch et al., 2012). The use of this region has thus become common. However, studies on the diversity of *Melanoleuca* spp. in Türkiye are limited, and most records are based only on morphological data.

This study aims to reveal the morphological characteristics of certain *Melanoleuca* spp. from Çanakkale, Türkiye, in detail and confirm their identity with molecular data from the nrITS region. Thus, it aims to clarify the taxonomic position of related species and contribute to the knowledge of the macrofungal diversity of Türkiye.

Materials and Methods

Sampling

The macrofungus samples analyzed in this study were collected between 2023 and 2025 from the Çanakkale province, Türkiye (Figure 1). *Melanoleuca* samples collected from their natural habitat were identified and classified using an integrated approach that combined traditional methods and advanced molecular techniques. Macro and micromorphological characteristics of the specimens were examined in detail. Additionally, the nrITS sequences were compared and phylogenetically analyzed.

Morphological Studies

The study materials comprised macrofungus samples collected from various regions of the Çanakkale province, Türkiye. The macroscopic characteristics were recorded during field studies, and the specimens were photographed in their natural habitat with an EOS 60D digital camera (Canon). Ecological data, like the location and date of collection, and substrate type, were meticulously recorded in the field notebook. After field studies, the samples were transported to the laboratory, dried under controlled conditions at room temperature (~25°C), stored in polyethylene bags, and labeled. Color descriptions were based on the classification system developed by Kornerup and Wanscher (1978). The microscopic characters were analyzed with a DM500 light microscope (Leica), and the basidia, basidiospores, cystidia, and pileipellis were structurally evaluated in detail. To ensure that each character was within a reliable range, it was measured ≥ 30 times utilizing the Leica Application Suite (v3.4.0) software. Drawings of the microscopic structures were created

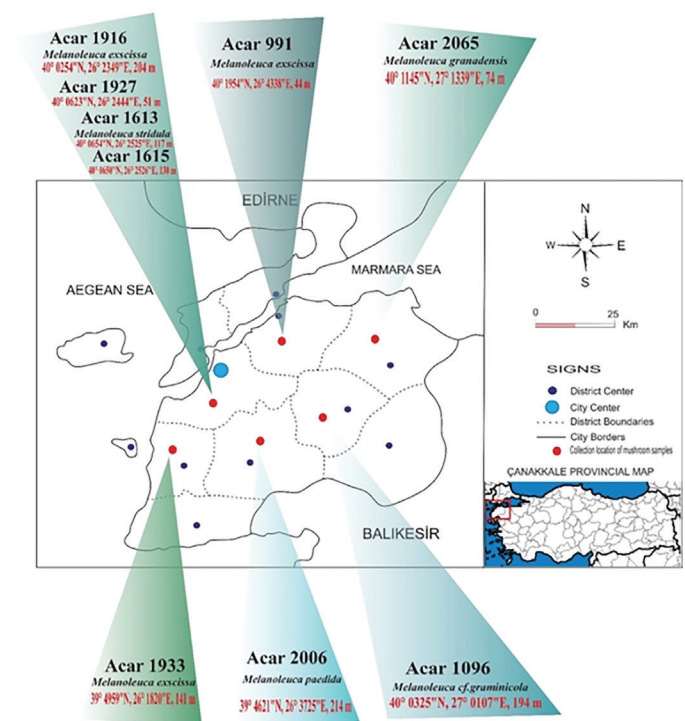


Figure 1. A map of the research area (Çanakkale, Türkiye).

using CorelDRAW (64-bit) (Corel Corporation, ON, Canada). Morphological identification was based on the literature, including Breitenbach and Kränzlin (1991), Kalmer et al. (2018), Xu et al. (2019), Acar et al. (2020), Armada (2020), and Antonín et al. (2022, 2023). After identification, the specimens were transformed into fungarium materials and stored under appropriate conditions in the Fungarium of the Department of Biology, Faculty of Science, Van Yüzüncü Yıl University, Van, Türkiye.

Sequencing of the ITS rDNA

Genomic DNA was isolated from the macrofungal samples by employing the CTAB method and following the protocols described by Rogers and Bendich (1994), Dizkırıcı et al. (2019), and Akata et al. (2024a, 2024b). The DNA purity and concentration were determined with a NanoDrop 2000c ultraviolet-visible spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). The DNA was stored at -20°C . Polymerase chain reaction (PCR) amplification of the ITS rDNA region was performed in a 25 μL reaction mix, containing 10 μM primers (forward and reverse), 5 U/ μL Taq polymerase, 10 ng/ μL genomic DNA, 10X PCR buffer, 25 mM MgCl_2 , 10 mM dNTP, and sterile water to make up the volume. The universal ITS1 and ITS4 primers were used; their

respective sequences were 5'-TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTTATTGATATATGC-3'. The PCR products were separated by agar gel electrophoresis at 100 V for 90 min, and an amplicon of ~ 700 bp was obtained; this single band confirmed amplification. The PCR products were purified using the HighPrep™ PCR Clean-up System kit (AC60005, MagBio Genomics, MD, USA), following the manufacturer's protocol. They were then sequenced at Macrogen Europe BV, Amsterdam, Netherlands. An ABI 3730XL Sanger sequencer and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) were used (Chen et al., 2014; Akata et al., 2024c, 2024d).

Molecular Phylogeny

The Sanger sequencing data obtained were assembled using the DNAMAN software v.10 (Lynnon Biosoft, CA, USA). The consensus sequences were analyzed with BLASTn (<https://blast.ncbi.nlm.nih.gov/>) to determine the species identity ratios. Based on the results, *Pluteus cervinus* (Schaeff.) P. Kumm. and *P. brunneidiscus* Murrill were selected as the ingroup and outgroup members, respectively, for further phylogenetic analyses. The related sequences were obtained from the NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>) and are listed in Table 1.

Table 1. Sequences used for the phylogenetic analysis. Examples of direct submissions are indicated with an “-.”

Species	Specimen Voucher	Country	IST (NCBI number)	Source
<i>Melanoleuca</i> aff. <i>graminicola</i>	AMB:19613	Italy	OR863461	Vizzini et al. (2024)
<i>M. acystidiata</i>	RR22.020	Switzerland	PV074842	-
<i>M. acystidiata</i>	BRNM 772203	Italy	MW491319	Antonín et al. (2021)
<i>M. albomarginata</i>	BRNM 817818	Italy	NR_198097	Antonín et al. (2022)
<i>M. albomarginata</i>	BRNM 817818	Italy	MT270938	Antonín et al. (2022)
<i>M. albomarginata</i>	BRNM 817817	Czech Republic	MT270939	Antonín et al. (2022)
<i>M. angelesiana</i>	HAY-F-005291	USA	PV791591	-
<i>M. angelesiana</i>	OMDL iNat# 118476394	USA	OR802136	Canan et al. (2024)
<i>M. angelesiana</i>	FDS-CA-04464	USA	PV273201	-
<i>M. angelesiana</i>	FDS-CA-05212	USA	PV019524	-
<i>M. angelesiana</i>	Acar 971	Türkiye	MG989682	Kalmer et al. (2018)
<i>M. brachyspora</i>	SLO 1565	Slovakia	OP394189	Antonín et al. (2023)
<i>M. brachyspora</i>	BRNM 829071	Slovakia	OP394188	Antonín et al. (2023)
<i>M. brachyspora</i>	BRNM 772200	Czech Republic	OP394187	Antonín et al. (2023)
<i>M. cinereifolia</i>	QHU20156	China	MZ453083	-
<i>M. cinereifolia</i>	QHU20156	China	OM970925	-
<i>M. communis</i>	FCME3445	Mexico	JX429148	-
<i>M. communis</i>	HMJAU59694	China	OR468700	-
<i>M. exscissa</i>	Acar 991	Türkiye	PV459218	This study
<i>M. exscissa</i>	Acar 1916	Türkiye	PV459219	This study
<i>M. exscissa</i>	Acar 1927	Türkiye	PV459220	This study
<i>M. exscissa</i>	Acar 1933	Türkiye	PV459217	This study
<i>M. exscissa</i>	JAC11654	New Zeland	MN738640	-

Table 1. Continued.

Species	Specimen Voucher	Country	IST (NCBI number)	Source
<i>M. exscissa</i>	GB65476	Sweden	JX429121	-
<i>M. exscissa</i>	GB 65455	Sweden	NR_154163	Sanchez-Garcia et al. 2013
<i>M. exscissa</i>	GB65455	Sweden	JX429192	Sanchez-Garcia et al. 2013
<i>M. exscissa</i>	ML14	Türkiye	PV065669	-
<i>M. exscissa</i>	FBT1465	Australia	OL771712	-
<i>M. fontenlae</i>	H89	Tunisia	PP533056	-
<i>M. fontenlae</i>	BRNM 772194	Italy	MW491326	Antonín et al. (2021)
<i>M. fontenlae</i>	BRNM 772194	Italy	NR_172809	Antonín et al. (2021)
<i>M. graminicola</i>	Acar 1096	Türkiye	PV459221	This study
<i>M. graminicola</i>	ANC M0201	Italy	JN616438	Vizzini et al. (2011)
<i>M. graminicola</i>	BRNM 829064	Czech Republic	OP394192	Antonín et al. (2023)
<i>M. graminicola</i>	BRNM 829062	Slovakia	OP394193	Antonín et al. (2023)
<i>M. graminicola</i>	SLO 1523	Slovakia	OP394194	Antonín et al. (2023)
<i>M. graminicola</i>	SLO 1532	Slovakia	OP394195	Antonín et al. (2023)
<i>M. graminicola</i>	SLO 1623	Slovakia	OP394196	Antonín et al. (2023)
<i>M. graminicola</i>	SLO 1527	Slovakia	OP394197	Antonín et al. (2023)
<i>M. granadensis</i>	Acar 2065	Türkiye	PV459222	This study
<i>M. granadensis</i>	AC20220508	France	PV089839	-
<i>M. granadensis</i>	FR2018040	Spain: Granada	MW289911	Armada (2020)
<i>M. granadensis</i>	PRM 955782	Czech Republic	PQ098927	-
<i>M. luteolosperma</i>	SB051123A	France	PP111921	-
<i>M. luteolosperma</i>	M 0139486	Germany	MW491330	Antonín et al. (2021)
<i>M. luteolosperma</i>	SLO 1632	Slovakia	MW491331	Antonín et al. (2021)
<i>M. luteolosperma</i>	BRNM 817820	Czech Republic	MW491329	Antonín et al. (2021)
<i>M. melaleuca</i>	NSK 1014679	Russia	OQ216539	-
<i>M. melaleuca</i>	ANC M0161	Italy	MT270919	Antonín et al. (2022)
<i>M. melaleuca</i>	BRNM 807677	Slovakia	MT270918	Antonín et al. (2022)
<i>M. microcephala</i>	HMJAS00133	China	MK659987	-
<i>M. microcephala</i>	HMJAS00132	China	MK659986	-
<i>M. microcephala</i>	ANC M0196	Italy	JN616449	Vizzini et al. (2011)
<i>M. paedida</i>	Acar 2006	Türkiye	PV459223	This study
<i>M. paedida</i>	ANC M0190	Italy	JN616453	Vizzini et al. (2011)
<i>M. paedida</i>	ANC M0189	Italy	JN616452	Vizzini et al. (2011)
<i>M. paedida</i>	SB0521	Canada	OM338006	-
<i>M. paedida</i>	R. Para 010907-02	Italy	MW491337	Antonín et al. (2021)
<i>M. romanensis</i>	BRNM 772193	Italy	NR_198286	Antonín et al. (2023)
<i>M. romanensis</i>	BRNM 829060	Italy	OP394204	Antonín et al. (2023)
<i>Melanoleuca</i> sp.	HFRG_LG2304152	United Kingdom	OR907161	-
<i>M. stridula</i>	Acar 1615	Türkiye	PV459224	This study
<i>M. stridula</i>	Acar 1613	Türkiye	PV459225	This study
<i>M. stridula</i>	F-3760	Sweden	PQ639185	-
<i>M. stridula</i>	BRNM 825716	Slovakia	MW491340	Antonín et al. (2021)

Table 1. Continued.

Species	Specimen Voucher	Country	IST (NCBI number)	Source
<i>M. stridula</i>	SLO 1543	Slovakia	MW491339	Antonín et al. (2021)
<i>M. stridula</i>	F-3756	Sweden	PQ639184	-
<i>M. striimarginata</i>	ANC M0202	Italy	JN616468	Vizzini et al. (2011)
<i>M. subcinereiformis</i>	CA FUNDIS	USA	PP975555	-
<i>M. subcinereiformis</i>	ONT WCMB23	USA	OR167957	-
<i>M. subpulverulenta</i>	ANC M0178	Italy	JN616473	Vizzini et al. (2011)
<i>M. subpulverulenta</i>	ANC M0004	Italy	JN616472	Vizzini et al. (2011)
<i>M. subpulverulenta</i>	MCVE:9821	Italy	JN392453	-
<i>M. cf. subpulverulenta</i>	SOMF30862	Bulgaria	OQ398593	Assyov and Slavova (2023)
<i>Pluteus brunneidiscus</i>	HMJAU 602011 i	China	OM991893	-
<i>P. brunneidiscus</i>	HMJAU 60206	China	OM943513	-
<i>P. cervinus</i>	REG 13641	Germany	HM562152	Justo et al. (2011)

The assembled sequences were aligned with those of the ingroup and outgroup members for phylogenetic analyses. Bayesian inference was performed employing the Markov Chain Monte Carlo (MCMC) + MC³ algorithm in the MrBayes v3.2.7a software (Ronquist et al., 2012). It allowed an exploration of the posterior probability distributions and uncertainty in the tree topologies. Such information is particularly relevant given the limited sequence divergence within the dataset. In this model, Dirichlet priors were used to calculate the rates of change: uniform (0.00 and 1.00) for the fixed regions and exponential (1.00) for the γ -shaped parameter. Independent MCMC runs were conducted twice, each with four chains. The analyses were run for 3,000,000 generations and sampled every 1,000 generations. The phylogenetic analyses performed in this study provided valuable insights into the evolutionary history of the *Melanoleuca* spp. in Türkiye. ITS-based phylogeny distinguished *M. granadensis* from closely related species such as *M. albomarginata*, supporting their recognition as distinct evolutionary lineages by Antonín et al. (2022). Furthermore, grouping the Turkish *M. granadensis* specimens with their European counterparts suggests a recent common ancestry, highlighting the potential significance of historical biogeographic connections across the Mediterranean. These evolutionary insights were corroborated by the posterior probability distributions, enabling a robust sampling of the phylogenetic tree space and bolstering the reliability of the evolutionary relationships inferred. These results emphasize the importance of incorporating molecular data into taxonomic and evolutionary research, particularly in genera where morphological characteristics demonstrate a high overlap and plasticity.

The General Time Reversible model + I + Γ model with uniform ratios was applied for nucleotide substitution during phylogenetic analyses (Höhna et al., 2017; Barba-Montoya et al., 2020). The bootstrap method (Felsenstein, 1985) was used, and 1,000 bootstrap replications were performed, to assess the reliability of the findings.

Results

The phylogenetic and morphological identification of the collected macrofungal specimens as *M. excissa*, *M. cf. graminicola*, *M. granadensis*, *M. paedida*, and *M. stridula* based on the methodology described in the literature is presented. It includes descriptions of the specimens, macromorphological illustrations, micromorphological drawings, locations and dates of collection, scanning electron microscopy images of the new spore records, ITS rDNA sequences, and phylogenetic analyses.

Morphological Taxonomy

Melanoleuca excissa (Fr.) Singer (Figure 2)

Description. **Pileus:** 25–80 mm, convex when young, semi-convex, flattening as it matures, sometimes curving outwards in mature specimens, with a small, broad umbo, pale white to creamy white, grayish brown to gray, light brown to dark brown, usually darker in the center, finely tomentose, shiny when moist. **Context:** white and unchanging throughout, becoming slightly darker and more compact at the stipe base. **Lamellae:** white to cream, margin slightly undulating to crenate. **Stipe:** 20–70 × 3–8 mm, cylindrical, somewhat enlarged toward the pileus, surface fibrillose, dirty white to creamy white, with a white-pruinous apex, dark brown toward the base. **Basidia:** 25–38 × 6–10 (apex) × 5–8 (base) μ m, cylindric, cylindric-clavate, clavate to sub-clavate, hyaline, guttulate, without clamp connections and with four sterigmata. **Spores:** 7–11 × 4.5–7 μ m, ellipsoid to cylindric or subovoid, verrucose, hyaline, amyloid. **Cheilocystidia:** 30/35–55 × 5–10 μ m, fusiform to lageniform, mostly transversely septate, often with crystals at the apex; both macrocystidia and urticoid cystidia (excissa-type) present. **Pleurocystidia:** similar to cheilocystidia and very sparse. **Caulocystidia:** similar to cheilocystidia but narrower, extending up to 90 μ m. **Pileipellis:** 20–50(–100) μ m thick trichoderm, irregular hyphae up to 10 μ m long, sometimes slightly gelatinized, somewhat ascending hyphae with obtuse or clavate terminal cells, occasionally at the apex encrusted with crystals, septa without clamp connections.



Figure 2. *Melanoleuca excissa*. (a–b) basidiomata (a: in meadows; b: in forest clearings); (c) spores; (d) basidia; (e) pleurocystidia; and (f) cheilocystidia. Scale bar = 10 µm.

Specimens examined: Türkiye, Çanakkale, Lapseki, around the Yeniceköy village, 40°19'54"N and 26°43'38"E, 44 m, in meadows, 05.IV.2023, Acar 991; above Çınarlı village, around Sefer Bozkurt Fountain, 40°02'54"N and 26°23'49"E, 204 m, under *Pinus brutia* L., in meadows, 08.V.2025, Acar 1916; Çanakkale Onsekiz Mart University (ÇOMÜ), Hasan Mevsuf sports hall vicinity, 40°06'23"N and 26°24'44"E, 51 m, under *P. brutia*, in meadows, 09.I.2025, Acar 1927; Ezine, around the Çamköy village, 39°49'59"N and 26°18'20"E, 141 m, in meadows, 10.I.2025, Acar 1933.

Melanoleuca cf. *graminicola* (Figure 3)

Description. **Pileus:** 20–65 mm, convex to campanulate when young, then flat, usually in mature specimens, the edges are curved outward, the center becomes more flattened, usually with a shallow central umbo, smooth, dull, grayish-brown, light brown to dark brown, with a darker center. **Context:** white, brownish under the cuticle, stipe base somewhat white tomentose. **Lamellae:** white when young, then cream to whitish cream, sometimes developing slightly pinkish hues, crowded, notched, and slightly uncinat,

margin smooth. **Stipe:** 40–55 × 3–6 mm, more or less cylindrical, sometimes thicker near the pileus, slightly clavate toward the base, occasionally longitudinally white fibrillose, firm, powdery near the apex, whitish to light brown, grayish brown to dark brown, sometimes white tomentose at the base. **Basidia:** (18–)21–32(–37) × 7–10 (apex) × 5–7 (base) µm, cylindric to cylindric-clavate, hyaline, guttulate, without a clamp connection and with four sterigmata. **Spores:** 6–8(–9) × 4.7–6(–6.5) µm, fusoid-ellipsoid to subcylindrical or subovoid, hyaline, with verrucose ornamentation, guttulate. **Cheilocystidia:** 20–55 × 4.8–10 × 3–5.3 µm, urticoid, scattered, lageniform, often irregular, with a subulate, obtuse apex and thin walls. **Marginal cells:** 15–40 × 5–11 µm, sometimes irregular, clavate, subcylindrical, or subutriform, with thin walls. **Pleurocystidia:** absent. **Pileipellis:** initially appears in the form of an ixocutis, which develops into an ixotrichoderm structure in the center, composed of thin-walled, cylindrical, non-dextrinoid hyphae up to 10 µm in width; terminal cells are usually flattened to erect, cylindrical, subclavate, or rarely branched in shape, ca 65 × 8 µm, yellowish-gray colored in KOH.



Figure 3. *Melanoleuca graminicola*. (a–b) basidiomata; (c) spores; (d) basidia; and (e) cheilocystidia. Scale bar = 10 µm.

Specimens examined: Türkiye, Çanakkale, Çan, around Mallı village, 40°03'25"N and 27°01'07"E, 194 m, near *Pinus* spp. trees, in meadows, 25.I.2024, Acar 1096.

***Melanoleuca granadensis* Armada (Figures 4–6)**

Description. **Pileus:** 35–85 mm, campanulate when young, then plano-convex with a vaguely broad umbonate margin, surface smooth and dull, margin white, light brown to dark brown or yellowish brown when young, with equal colors or slightly darker in the center. **Context:** white to hyaline gray, fragile, thin or thick, becoming yellowish-gray toward the stipe base. **Lamellae:** white, fairly close, thin, fragile, greasy to the touch, intervened in the sinuses, notched to slightly decurrent on a narrow base; margin very irregular to eroded, paler. **Stipe:** 30–75 × 4–11 mm, 14 mm at the base, very fragile and brittle, hollow, longitudinally fibrous, whitish near the apex and base, cylindrical, slightly swollen at base, whitish at first, then dirty white to yellowish-gray, brown to grayish-brown. **Basidia:** 22–35 × 7–11 (apex) × 5–7 (base) µm, hyaline, guttulate, cylindrical to clavate, without a basal clamp and with four sterigmata. **Spores:** 7.5–10 × 4.8–6 µm, ellipsoid, slightly or moderately ornamented, with relatively small warts, occasionally with rough, truncate, or blunt warts, amyloid. **Cheilocystidia:** 50–80 × 10–16 µm, lageniform to fusiform, urticoid, some metuloid or plain, with a pointed or blunt apex, hyaline, and non-guttulate. **Pleurocystidia:** 43–75 × 13–17(–19) µm, lageniform to fusiform, sometimes cylindrical, urticoid, occasionally non-crystalliferous, some with tomentose apices, and a pointed or blunt apex, hyaline, and non-guttulate. **Caulocystidia:** 40–80 × 10–15 µm, mainly lageniform to fusiform, sometimes ventricose to cylindrical, occasionally 1 or 2-septate, urticoid, with some metuloid or plain, and a pointed or blunt apex, hyaline, non-guttulate. **Hairs:** 20–32 × 8–11, clavate, occasionally 1 or 2-septate, mostly among caulocystidia and sometimes in clusters near them. **Pileipellis:** develops on the surface of long and relatively smooth hyphae, sometimes quite elongated at the tip or short and blunt in morphology (× 4–16.5 µm). It is characterized by a yellowish coating with slight parietal pigmentation, or in some cases, it may show a thin encrustation, particularly evident when stained with

Congo red. The substrate is characterized by sausage or sausage-like structures, which may reach 22 µm in diameter and consist of densely intertwined hyphae of homogeneous size.

Specimens examined: Türkiye, Çanakkale, Biga, Around Sarıkaya village, 40°11'45"N and 27°13'39"E, 74 m, under *P. brutia* trees, 02.II.2025, Acar 2065.

***Melanoleuca paedida* (Fr.) Kühner & Maire (Figure 7)**

Description. **Pileus:** 30–60 mm, initially almost hemispherical but soon flattening with an irregularly wavy margin and a broad, low umbo; cuticle slightly hygrophanous, smooth, grayish-brown, lighter toward the margin and darker toward the center. **Lamellae:** numerous, dense, lobed, white, becoming creamy with age, with an entire to slightly fimbriate edge. **Stipe:** 35–70 × 6–10 mm, cylindric, sometimes with a bulbous base or enlarged apex, surface longitudinally fibrillose, densely pruinose, cream to gray-brown. **Context:** white on the pileus, slightly hygrophanous on the lamellae, cream to ochre, thick in the center and thinning toward the margin, becoming slightly darker toward the stipe base. **Spores:** (6–)7–9.3 × 4.5–7 µm, subglobose to ellipsoid, hyaline, with drops, rounded tubercles, and strongly amyloid. **Cheilocystidia:** 37–52 × 4.5–8.3 µm, more or less frequent, urticoid to hair-shaped, typical or fusiform. **Pleurocystidia:** not seen. **Pileipellis:** hyphae 2–5 µm, arranged into cutis, with more/less interwoven hyphae from which some clavate tips emerge, which may give the impression of a trichodermium. **Stipitipellis:** of the middle zone without differentiated elements.

Specimens examined: Türkiye, Çanakkale, Bayramiç, Around Ören village, 39°46'21"N and 26°37'25"E, 214 m, garden area, in meadows, 25.I.2025, Acar 2006.

***Melanoleuca stridula* (Fr.) Singer (Figure 8)**

Description. **Pileus:** 25–50(–70) mm diameter, campanulate to convex when young, later flattening and sometimes curling outwards or wavy at the margin in mature specimens, and also somewhat obtusely umbonate; surface smooth, dull, with shades of brown ranging from light to dark, usually darker in the center



Figure 4. *Melanoleuca granadensis*. (a–b) basidiomata.

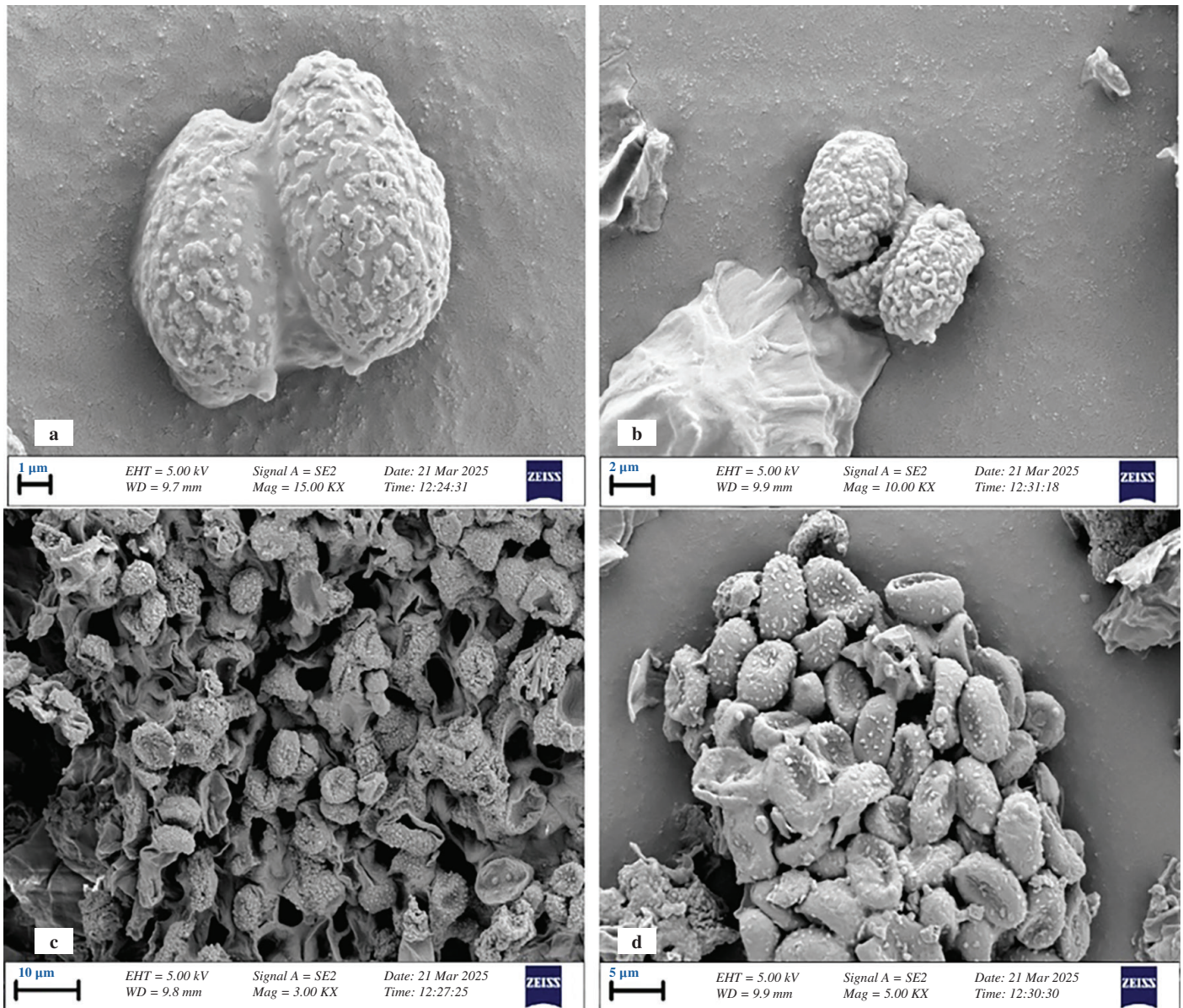


Figure 5. SEM images of basidiospores of *Melanoleuca granadensis*. (a) individual spores with verrucose ornamentation (scale bar = 1 µm); (b) paired spores with surface ornamentation (scale bar = 2 µm); (c) clustered spores (scale bar = 10 µm); and (d) aggregated spores showing surface sculpture (scale bar = 5 µm).

and occasionally with a white margin. **Context:** white to whitish cream, thin, becoming pale brownish in the stipe cortex and hollow interior. **Lamellae:** white, off-white to cream, broadly adnate to adnexed, occasionally notched. **Stipe:** 40–80 × 4–7 mm, cylindrical, bulbous, and white tomentose at the base, fragile with a longitudinally fibrillose white surface on an ocher-brown background. **Spores:** 7–8.2 × 4.5–5.5 µm, ellipsoid, hyaline, verrucose or slightly verrucose, with one or two drops. **Basidia:** 24–35 × 7–10 (apex) × 5–8 µm (base), cylindrical to clavate, with (1–2) 4 sterigmata, and without clamp connections. **Cystidia:** not seen. **Pileipellis:** composed of parallel to slightly irregularly arranged, brownish pigmented hyphae up to 10 µm in diameter, lacking clamp connections; the overall structure corresponds to a cutis.

Specimens examined: Türkiye, Çanakkale, ÇOMÜ, Opposite the Sports Faculty, 40°06'54"N and 26°25'25"E, 117 m, under *P. brutia*, 24.XII.2023, Acar 1613; behind Campus AVM, 40°06'50"N and 26°25'26"E, 130 m, under *P. brutia*, 24.XII.2023, Acar 1615.

Phylogenetic Results

The ITS rDNA sequences were amplified and used for the molecular identification of the nine *Melanoleuca* samples included in this study. After trimming the termini of the aligned forward and reverse sequences, the lengths of the newly generated sequences indicated efficiency (Acar 991 = 654 bp, Acar 1916 = 678 bp, Acar 1927 = 694 bp, Acar 1933 = 697 bp, Acar 1096 = 482 bp, Acar 2065 = 670 bp, Acar 2006 = 632 bp, Acar 1515 = 758 bp,

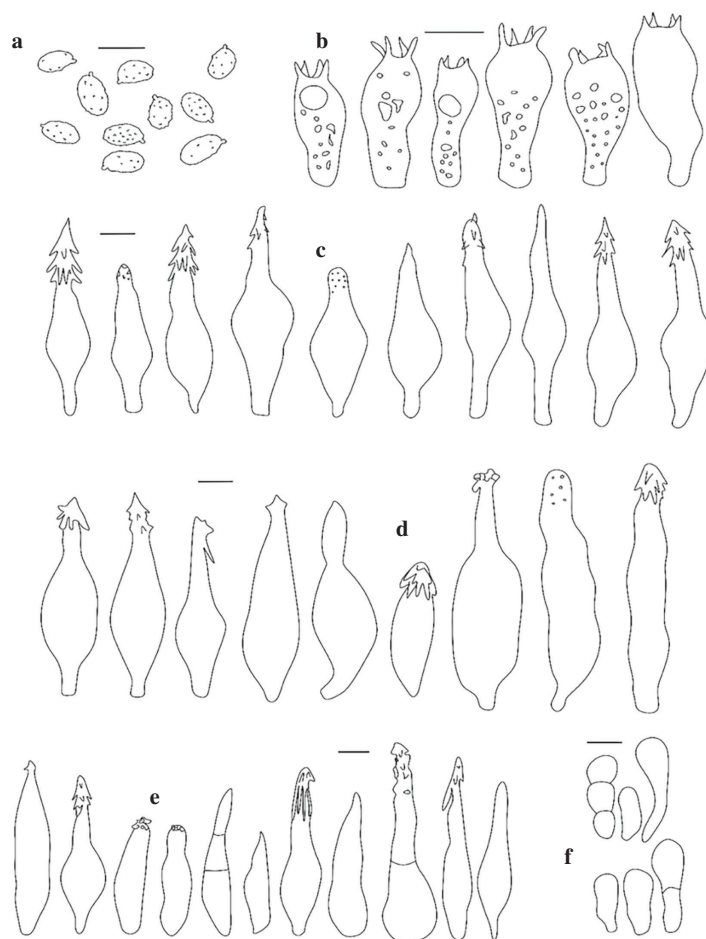


Figure 6. *Melanoleuca granadensis*. (a) spores; (b) basidia; (c) cheilocystidia; (d) pleurocystidia; (e) caulocystidia; and (f) hairs of stipe. Scale bar = 10 µm.



Figure 7. *Melanoleuca paedida*. (a–b) basidiomata; (c) spores; (d) basidia; and (e) cheilocystidia. Scale bar = 10 µm.

Acar 1613 = 543 bp, and Acar 1615 = 715). In addition to the sequences generated in this study (GenBank accession numbers: PV459217, PV459218, PV459219, PV459220, PV459221, PV459222, PV459223, PV459224, and PV459225), 68 sequences (including 3 outgroups) were obtained from GenBank; a total of 77 sequences were analyzed (Table 1). The sequences obtained from NCBI ranged from 380 (MW491330) to 1,624 (PQ639184) bp.

The rediscovered *Melanoleuca* specimens were in the appropriate clades of the phylogenetic tree (Figure 9). In addition, the ITS region sequence obtained from the possibly new record “Acar 2065” was compared with those in the NCBI GenBank database using the BLASTn algorithm. The highest similarity was observed with *M. granadensis* (PVO98839). In total, 713 bp of the 855 bp sequence were aligned, with 99.55% similarity (667/670 matched



Figure 8. *Melanoleuca stridula*. (a–b) basidiomata (under *Pinus brutia*); (c) spores; and (d) basidia. Scale bar = 10 µm.

bases) and no gaps, indicating a high level of homology. This result demonstrates that our specimen was most likely *M. granadensis*. The high similarity rate (99%) and gap-free matching obtained through BLASTn analysis were over the threshold values (usually > 97–98%) commonly applied for the molecular identification of species. Along with morphological findings, the molecular data support the identification of the sample as *M. granadensis*.

Discussion

The present study unequivocally demonstrates that the taxonomic boundaries within the genus *Melanoleuca* cannot be reliably delineated using only morphology-based approaches, and that integrating molecular data is decisive for species-level identification (Vizzini et al., 2011; Kalmer et al., 2022). The findings obtained were of scientific significance for two principal reasons. The reliable documentation of *M. granadensis* from Türkiye for the first time indicates an expansion of its known ecological and diagnostic scope. Second, the phylogenetic separation of the Acar1096–OR863461–JN616438 lineage from the *M. graminicola* s. str. core suggests its independence.

Notably, the identification of the *Melanoleuca* specimen Acar 2065, collected from Çanakkale, as *M. granadensis* is supported by the high congruence between detailed morphological assessments and ITS-based molecular data. This specimen was recorded at a low elevation (~74 m) under a *P. brutia* tree, significantly extending the known altitudinal range of the species compared to the holotype and other records from higher elevations (Armada, 2020; Assyov & Slavova, 2023). Furthermore, it provides a novel ecological context in terms of habitat associations. The high degree of DNA sequence similarity and the absence of gaps with the reference sequences serve to reinforce the molecular-level identification.

The phylogenetic tree reaffirms the reported affinity with *M. albomarginata*. Additionally, the expanded measurement ranges of the diagnostic microcharacters (e.g., pleurocystidia and stipe hairs) support this relationship, enabling the inclusion of *M. granadensis* as an emended description (Table 2). In parallel, the placement of the specimen OQ398593, reported as *M. cf. subpulverulenta* by Assyov and Slavova (2023), within the *M. granadensis* clade and its separation from the *M. subpulverulenta* specimens in our phylogenetic tree strongly indicate the likelihood of a misidentification (Figure 9 and Table 2).

Table 2. A comparison of *Melanoleuca granadensis* and its holotype (Armada, 2020).

<i>Melanoleuca granadensis</i>	Armada (2020)	Current study
Pileus	37–43 mm	35–85 mm
Stipe	42–51 × 7–9 mm	30–75 × 4–11 mm, at the base 14 mm
Basidia	23–34 × 9.5–13 µm	22–35 × 7–11 (apex) × 5–7 (base) µm
Spores	8–9 (9.5) × 4.75–5.5 (6) µm	7.5–10 × 4.8–6 µm
Cheilocystidia	50–82 × 11–17 µm	40–80 × 10–15 µm
Pleurocystidia	Similar or more lageniform with an obtuse apex	43–75 × 13–17(–19) µm
Caulocystidia	50–82 × 11– 17 µm	40–80 × 10–15 µm
Hairs on the stipe	Size unspecified	20–32 × 8–11, clavate, occasionally 1 or 2-septate
Habitat	Among several Cistaceae, under <i>Pinus pinaster</i> , <i>P. halepensis</i> , with rare <i>Quercus ilex</i> , very young, 1250–1300 m, November	Under <i>P. brutia</i> trees, 74 m, February

Second, although the Acar1096 sequence clustered within the same clade as *M. aff. graminicola* (OR863461) and *M. graminicola* (JN616438), it was markedly distant from the *M. graminicola* s. str. core (see Figure 9 for topology). Full-length ITS comparisons revealed only 74–75% identity between Acar1096 and the *M. graminicola* s. str. core specimens (OP394192–OP394197). These

values fall well below the commonly accepted 97–99% threshold for species-level delimitation in Basidiomycota. OR863461 was collected in Italy (Emilia-Romagna, Imola) under *Cedrus deodara* (voucher AMB:19613, coll. S. Morini, 01 Jan 2021) and assessed recently via multi-locus phylogenies; JN616438 represents a classical record from Piedmont, Italy (voucher ANC M0201).

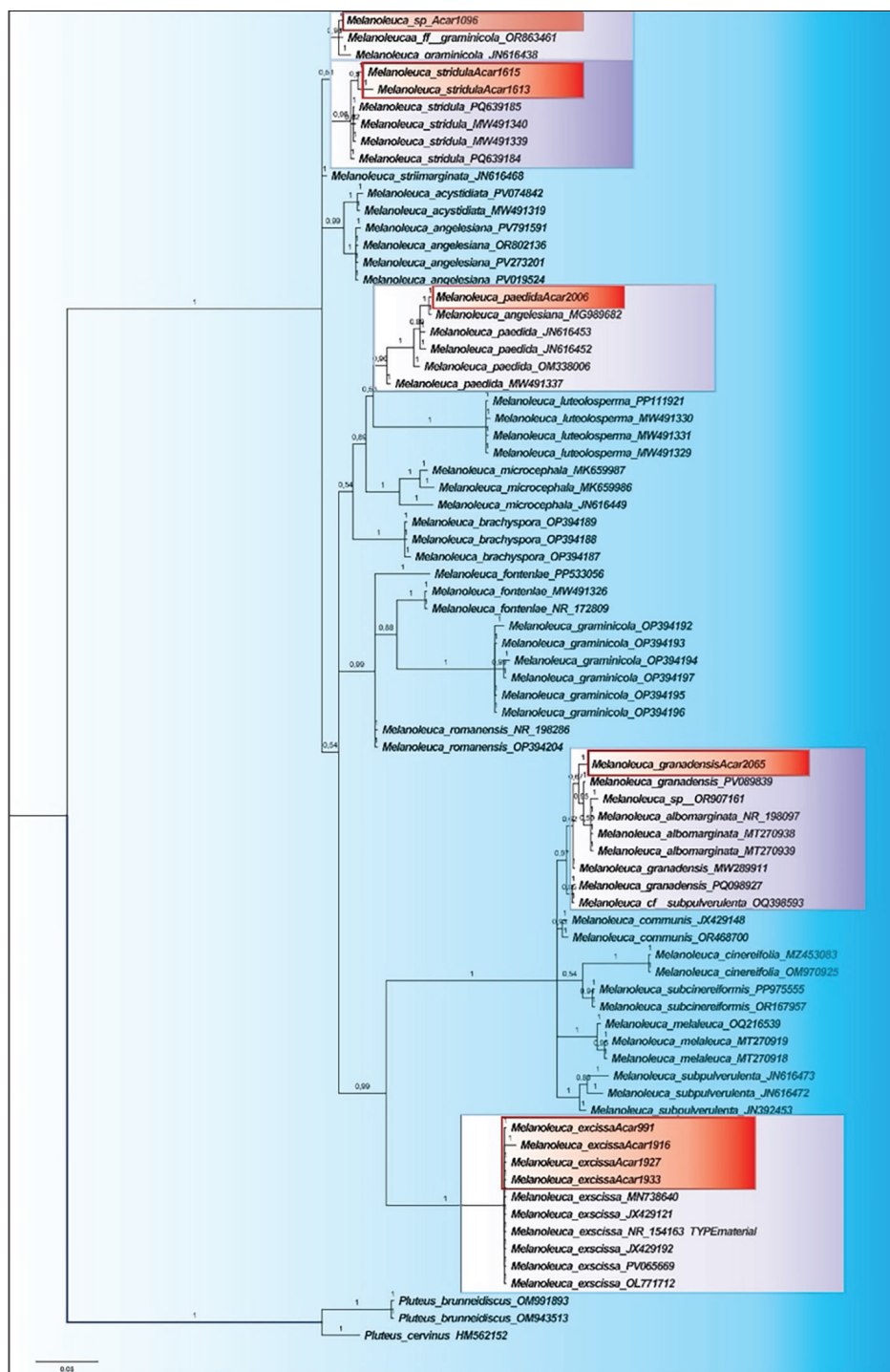


Figure 9. Bayesian 50% majority rule-based consensus tree constructed for *Melanoleuca* using ITS sequence data. The taxa recorded are indicated in the red boxes. Bayesian posterior probabilities are shown above the branches. *Pluteus cervinus* and *P. brunneidiscus* were the outgroups.

The findings suggest that this lineage may represent a Europe-derived variation line. However, the affinity observed does not imply a direct equivalence with *M. graminicola* s. str. Rather, it indicates that the Acar1096–OR863461–JN616438 trio forms a lineage closely related to, yet distinct from, the *graminicola* complex. In this context, the broad interpretations in certain studies, such as Antonín et al. (2023), and early GenBank annotations require a re-evaluation in light of updated sampling and analyses.

Technically, the high local similarity values (99–100%) observed in BLASTn searches may reflect matches within the conserved ITS subregions, potentially masking full-length variations. Therefore, species-level interpretations should be based on the alignment of complete sequences within a robust phylogenetic framework. Regarding the taxonomic designation of Acar1096, as the current dataset is ITS-based, its placement is best assessed in conjunction with other specimens within the same clade whenever available. Until then, it is recommended that Acar1096 be reported cautiously as *M. cf. graminicola*.

In conclusion, the present study not only confirms *M. granadensis* as a new record for Türkiye, but also suggests that the Acar1096–OR863461–JN616438 lineage should be regarded as a distinct and isolated clade, separate from *M. graminicola* s. str. based on robust morphological and molecular evidence. It also expands the known distribution, altitude, and habitat range of *M. granadensis*. These dual findings emphasize the pivotal role of integrating morphological and molecular data in establishing species limits within *Melanoleuca*. They underscore the need to re-evaluate specific records, notably early GenBank annotations, within a revised phylogenetic framework.

Ethics

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.

Footnotes

Authorship Contributions: Conceptualization: İ.A., and H.K.; Design/methodology: İ.A., and H.K.; Execution/investigation: İ.A., and H.K.; Resources/materials: İ.A., and H.K.; Data acquisition: İ.A., and H.K.; Data analysis/interpretation: İ.A., and H.K.; Writing – original draft: İ.A., and H.K.; Writing – review & editing/critical revision: İ.A., and H.K.

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

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