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Phylogeny of Some *Melanoleuca* Species (Fungi: Basidiomycota) in Turkey and Identification of *Melanoleuca angelesiana* A.H. Sm. As a First Record

Aysenur KALMER[1], İsmail ACAR[2], Ayten DIZKIRICI TEKPINAR[1*]

[1]Department of Molecular Biology and Genetics, Van Yüzüncü Yıl University, 65080, Van, Turkey
[2]Department of Organic Agriculture, Başkale Vocational High School, Van Yüzüncü Yıl University, 65080, Van, Turkey
*Corresponding author: aytendizkirici@gmail.com

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Abstract

Aim of Study: The purposes of the present study are to describe and identify all collected *Melanoleuca* samples using morphological characters, determine phylogenetic relationships among species using DNA sequences of ITS and LSU regions and check the number of subgenera of *Melanoleuca* genus.

Area of Study: Samples were collected from different parts of Turkey and the study was conducted at the Department of Molecular Biology and Genetics in Van Yüzüncü Yıl University.

Material and Methods: Twenty-two samples, representatives of fourteen species, were used. Structures of pileus, stipe, lamellae and basidia, cystidia, spores were used as macroscopic and microscopic features, respectively. The nuclear ribosomal internal transcribed spacer region (nrITS) and largest subunit (LSU) were amplified for molecular analyses.

Main results: Taxonomic positions of some *Melanoleuca* species from Turkey were evaluated based on both morphological characters and sequences of two different nuclear DNA regions. nrITS region was more informative (112 variable sites in ITS1, 1 in 5.8S and 95 in ITS2 subregions) compared to LSU (46 variable sites). Analyses of ITS region revealed that the genus *Melanoleuca* was monophyletic and divided into two subgenera as *Melanoleuca* and *Urticocystis*.

Research highlights: Within studied species, *Melanoleuca angelesiana* was firstly reported for mycobiota of Turkey

Keywords: ITS, LSU, *Melanoleuca,* Fungal taxonomy, New record

Türkiye'deki Bazı *Melanoleuca* türlerinin (Fungi: Basidiomycota) Filogenisi ve Yeni bir Kayıt Olarak *Melanoleuca angelesiana* A.H. Sm. Türünün Tanımlanması

Öz

Çalışmanın Amacı: Bu çalışmanın amacı toplanan *Melanoleuca* örneklerinin morfolojik karakterlerle tanımlanması, ITS ve LSU DNA sekanslarını kullanarak türlerin filogenetik ilişkilerinin belirlenmesi ve *Melanoleuca* cinsinin alt cins sayısının teyit edilmesidir.

Çalışma Alanı: Örnekler Türkiye'nin farklı bölgelerinden toplanmış ve çalışma Van Yüzüncü Yıl Üniversitesi Moleküler Biyoloji ve Genetik Bölümü'nde gerçekleştirilmiştir.

Material ve Yöntemler: On dört türü temsil eden yirmi iki örnek kullanılmışır. Şapka, sap, lamel yapıları ve bazidya, sistidya, spor yapıları sırasıyla makroskopik ve mikroskopik özellikler olarak kullanılmıştır. Nükleer ribozomal iç aralayıcı bölge (nrITS) ve ribosomal en büyük alt birim (LSU) moleküler analizler için amplifiye edilmiştir.

Ana sonuçlar: Bu çalışmada, Türkiye'den bazı *Melanoleuca* türlerinin taksonomik konumları hem morfolojik karakterleri hemde iki farklı nükleer DNA bölgesinin sekansları ile değerlendirilmiştir. nrITS bölgesi (ITS1'de 112, 5,8S'de 1, ve ITS2'de 95), LSU bölgesine (46 değişken alan) kıyasla daha bilgilendirici olmuştur. ITS bölgesinin analizleri *Melanoluca* cinsinin monofletik olduğunu ve *Melanoleuca* ve *Urticocystis* olarak iki alt cinse ayrıldığını göstermiştir.

Araştırma konuları: İncelenen türler içerisinde *Melanoleuca angelesiana* Türkiye mikobiyotası için ilk defa rapor edilmiştir.

Anahtar kelimeler: ITS, LSU, *Melanoleuca,* Fungal taksonomi, Yeni kayıt

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Introduction

Melanoleuca Pat. (Tricholomataceae R. Heim ex Pouzar) is a mostly edible saprotrophic fungal genus growing on soil in grasslands, evergreen forests and sand dunes (Boekhout, 1988; Phillips, 2006). Edible fungi have economic and ecologic importance (Yıldız, Gürgen & Can, 2017). The database of Mycobank lists 333 *Melanoleuca* species worldwide (http://www.mycobank.org, accessed; 25.02.2018) and only 23 of them have been identified up to now in Turkey (Sesli and Denchev, 2014; Solak, Işıloğlu, Erbil & Allı, 2015; Acar, Dizkirici Tekpınar, Kalmer & Uzun, 2017).

The genus is characterized by: collybioid to tricholomatoid basidiomata; emarginate to adnate to subdecurrent lamellae; a white to pale yellowish spore print; hyaline spores with ellipsoid, mostly amyloid ornamentation; absent or present cheilocystidia of two types; pleurocystidia similar to cheilocystidia; pileipellis a cutis to a trichoderm and an absence of clamp connections (Singer, 1986; Bon, 1991; Boekhout, 1999). Especially, structure of the cheilocystidia is a valuable character to identify taxonomic position of the studied species within the genus. Furthermore, the size, amount and ornamentation of spores are important features for delimitation of species (Kibby, 2016). The genus *Melanoleuca* can be distinguished from other genera with specific shapes of cystidia (thin walled and fusiform to lageniform). Even though these mentioned characters are useful to identify a sample at the genus level, lots of problems are encountered during identification of a sample at the species level because of morphological variabilities and macroscopic/microscopic similarities (Vizzini, Para, Fontenla, Ghignone & Ercole, 2011; Duriska, Antonin, Para, Tomsovsky & Jancovicova, 2017).

Some researchers divided *Melanoleuca* genus into three subgenera as *Acystis, Urticocystis* and *Melanoleuca*, using traditional classification which is based on presence and shape of cystidia (Bon 1991). *Acystis* is characterized by missing cystidia, *Urticocystis* with urticiform cystidia and *Melanoleuca* with macrocystidia (Bon, 1991; Bas, Kuyper, Noordeloos & Vellinga, 1999). However, the infrageneric classifications of the genus have always been unclear (Yu et al., 2014) since some morphological and ecological features can be changed due to geographic distribution. Interpretations of morphological characters often varied among mycologists and resulted in disagreements (Hyde et al., 2013). The molecular data may provide invaluable information to identify macrofungus correctly (Undan et al., 2016). Therefore, researchers have begun to use molecular and morphological data together. Vizzini et al. (2011) used sequence of *nrDNA* ITS region to check the traditional *Melanoleuca* classification. Their results indicated that *Melanoleuca* is a monophyletic genus including only two subgenera, *Urticocystis* and *Melanoleuca*.

In the present study, specimens were collected from different locations of Turkey and identified by using morphological characters. DNA sequences of ITS (Internal Transcribed Spacer) and LSU (Large Subunit of rRNA) regions were analyzed to understand phylogenetic relationships among *Melanoleuca* species. These two regions were selected for the study because rDNA gene has been conserved during evolutionary process and usefulness of ITS and LSU regions has been proved by several studies for the *Melanoleuca* genus (Hinrikson, Hurst, Lott, Warnock & Morrison, 2005; Brown, Rigdon-Huss, & Jumpponen, 2014). Especially, ITS region is accepted as a valuable DNA marker for fungal taxonomy (Schoch et al. 2012).

The purposes of the present study are to i) describe and identify all collected samples using morphological characters ii) determine phylogenetic relationships among species using DNA sequences of ITS and LSU regions iii) check the number of subgenera.

Materials and methods

Taxon sampling and morphological studies

The macrofungus samples were collected from different locations of Turkey (Table 1). Twenty-two samples, representatives of 14 species, were collected and used for morphological and molecular studies. All 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 were recorded using fresh materials. Pilea, cheilocystidia and basidia were observed in distilled water under a Leica EZ4 stereo microscope and sections were examined under a Leica DM500 research microscope. Microscopic characters were measured with the Leica Application Suite (version 3.2.0) program 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, Blanco & Matheney 2013; Kuo and Methven, 2014; Desjardin, Wood & Stevens, 2015).

Table 1. List of studied *Melanoleuca* species, their localities, collected date, subgenera and NCBI accession numbers. Subgenera were decided using study of Vizzini et al. (2011).

Species	Locality	Collected date	Subgenera	Accession no.	
				ITS	LSU
1. *M. brevipes* (Bull.) Pat.	Bingöl	2009	*Urticocystis*	MG989678	MG989689
	Tatvan-Bitlis	2010-2011			
2. *M. exscissa* (Fr.) Singer	Hani-Diyarbakır	2009-2010	*Urticocystis*	MG989683	MG989694
	Erzincan	2006			
	Bingöl	2009			
	Artvin	2009			
3. *M. graminicola* (Velen.) Kühner & Maire	Baykan-Siirt	2012	*Urticocystis*	MG989684	MG989695
4. *M. grammopodia* (Bull.) Murrill	Hani-Diyarbakır	2010	*Urticocystis*	MG989681	MG989692
5. *M. paedida* (Fr.) Kühner & Maire	Bingöl	2009			
	Tatvan-Bitlis	2010	*Urticocystis*	MG989679	MG989690
6. *M. substrictipes* Kühner	Selim-Kars	2013	*Urticocystis*	MG989680	MG989691
	Tatvan-Bitlis	2011			
7. *M. dryophila* Murrill	Şemdinli-Hakkari	2014	*Urticocystis*	KX507366	KX507363
8. *M. microcephala* (P. Karst.) Singer	Hakkari	2014	*Urticocystis*	MG989677	MG989688
9. *M. angelesiana* A.H. Sm.	Soğanlı-Kars	2011	*Urticocystis*	MG989682	MG989693
10. *M. arcuata* (Bull.) Singer	Siirt	2016	*Urticocystis*	MG989686	MG989697
11. *M. melaleuca* (Pers.) Murrill	Erzincan	2006			
	Bingöl	2008	*Melanoleuca*	MG989685	MG989696
12. *M. polioleuca* (Fr.) Kühner & Maire	Hani-Diyarbakır	2009	*Melanoleuca*	MG989687	MG989698
	Hakkari	2014			
13. *M. heterocystidiosa* (Beller & Bon) Bon	Yüksekova - Hakkari	2014	*Melanoleuca*	KX507368	KX507365
14. *M. communis* M. Sánchez-García&Cifuentes	Hakkari	2015	*Melanoleuca*	KX507367	KX507364

Molecular studies

DNA isolation, PCR amplification and DNA sequencing

Genomic DNA was isolated from fungal material using the CTAB (Cetyltrimethylammonium bromide, PubChem ID 5974) method (Doyle and Doyle, 1987). The purity and quantity of extracted DNA were determined with NanoDrop2000c UV–Vis Spectrophotometer (Thermo Scientific) and 0.8% agarose gel electrophoresis. Isolated stock DNA was stored at -20°C prior to PCR amplification. DNA amplification of each studied region 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.

To amplify ITS (ITS1-5.8S-ITS2) and LSU regions, primer pairs N-nc18S10 5'AGGAGAAGTCGTAACAAG3'/C26A 5'GTTTCTTTTCCTCCGCT3' (Wen et al. 1996) and LROR 5'ACCCGCTGAACT TAAGC3'/LR5 5'TCCTGAGGGAAACTT CG3' (Vilgalys & Hester 1990) were used,

respectively. ITS region was amplified under a program consisting of a hot start at 95 ºC for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 54° C for 1 min, extension at 72 °C for 2 min, and a final 72 ºC step for 10 min. Same temperature and time conditions were used for amplification of LSU region except annealing (52 °C). PCR products were checked in 1% TAE agarose gels staining with Gelred dye, and positive reactions were sequenced with forward and reverse PCR primers. Chromatograms were checked for putative reading errors and corrected. The sequences of species were deposited in NCBI database and their accession numbers were given in Table 1.

Sequence alignment and phylogenetic analysis

BLAST (Altschul, Gish, Miller, Myers & Lipman,1997) was used to select the most closely related ITS and LSU rDNA sequences from NCBI database (Appendix 1) and all sequences were aligned with the aid of the program ClustalW (Thompson, Higgins & Gibson, 1994). Alignments were checked and manually adjusted where it was necessary. Totally 40 sequences (26 of them retrieved from NCBI) of the genus were included in the phylogenetic analysis of ITS regions. LSU region was less studied compared to ITS so 10 LSU sequences could be downloaded from NCBI and analyzed with our samples. *Pluteus cervinus* (Schaeff.) P. Kumm. was used as outgroup for testing the monophyly of *Melanoleuca* (ITS: JX857448, LSU: HM562221, Garcia et al., 2013). Borders of regions were decided considering sequences downloaded [ITS (JX429148, Garcia et al., 2013) and LSU (JX429179, Garcia et al., 2013)].

Phylogenetic trees were constructed using both Maximum Likelihood (ML) and Maximum Parsimony method with aid of Molecular Evolutionary Genetics Analysis software (MEGA 6; Tamura, Stecher, Peterson, Filipski & Kumar, 2013). Tamura-Nei model (Tamura and Nei, 1993) and bootstrap analysis with 1000 replications (Felsenstein, 1985) were selected to construct ML tree. The tree with the highest log likelihood was shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join 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

Taxonomy

Macroscopic and microscopic characters of studied *Melanoleuca* species were given in Table 2. Within the studied specimens, *Melanoleuca angelesiana* A.H. Sm. was reported firstly for Turkey and its characterization was done by using morphological characters as follow.

Specimen examined: Turkey, Kars, Soğanlı, under conifers, 40°24'053" K, 42°29'117"D, 2525 m, 03.10.2011, Acar, I., 971, VANF. NCBI numbers: MG989682 (ITS region), MG989693 (LSU region).

Description of *Melanoleuca angelesiana* A.H. Sm.

Pileus: 60-100 mm across; convex to young, then flatted, often with a superficial or evident central lump, smooth, damp when fresh, dark brown, discoloration to tan. **Lamellae**: notched, attached to the stipe, close to cramped, at first greyish-tan, grayish to whitish, sometimes growing lightly pinkish color. **Stipe**: 30-60 × 5-15 mm, more or less equal, sometimes lightly bulb at the base, fairly smooth, surface fibrillose-striate when maturity, whitish at the apex but brown like the pileus below, basal mycelium white and partial veil absent. **Basidia**: 23.5-37.2 × 6.8-10.7 µm. **Spores**: 7-10 × 5-6.5 µm, more or less elliptical, amyloid, finely warts. Pleurocystidia and cheilocystidia absent (Figure 1).

Figure 1. *Melanoleuca angelesiana*, a) Basidiocarps b) Spores (distilled water) c) Basidia d) Basidioles e) Hyphae

Table 2. Considerable morphologic characters of studied *Melanoleuca* species

Pileus	Lamellae	Stipe	Basidia	Cystidia	Spores	References
		M. brevipes (Bull.) Pat.				
50-90 mm, convex when young, then depressed center somewhat umbonate, radially fibrillose, beige to gray-brown, grading to black-brown toward the center,	Light cream, later with gray to lilactints, slightly notched to subdecurent	30-50 × 8-15 mm, usually with a clavate base and widened apex, surface with ocherish on a brown background, covered with white to ocherish powder	30-40 × 8-9 µm, clavate	Cheilocystidia of the stinging hair type urticifom, Caulocystidia at the stipe apex	Elliptic, irregularly verrucose, hyaline with drops, 6.9-9.7 × 4.6-7.0 µm	Boekhout, 1988; Breitenbach & Kränzlin,1991
		M. exscissa (Fr.) Singer				
40-70 mm, convex when young, later plane and sometimes with a depressed center, obtusely umbonate in the center	White to cream, edges slightly undulating to crenate	40-60 × 5-8 mm, somewhat enlarged toward the apex, surface fibrillose, apex white-pruinose, dark-brown toward the base	22-25 × 6.5-8 µm, cylindric to cylindric clavate	Cheilo- and pleurocystidia fusiform to lageniform	Elliptic, verrucose, hyaline, 7.1-8.8 × 4.2-5.5 µm	Breitenbach & Kränzlin,1991

Table 2. (continued)

M. graminicola (Velen.) Kühner & Maire						
20-30 mm, convex-campanulate when young, with a distinct papilla, later plan to slightly depressed, dark-brown to dark-gray brown, center darker	White, later cream-whitish, sometimes also with a warm pink tint	40-50 × 3-4 mm, slightly clavate toward the base, surface cream to pink-whitish, with white fibrils	23-35 × 7-8.5 µm clavate,	Cystidia absent	Elliptic, verrucose, hyaline, with drops, 5.6-7.9 × 4.6-6.0 µm	Breitenbach & Kränzlin,1991
M. grammopodia (Bull.) Murrill						
70-150 mm, bell-shaped then expanded, often becoming centrally depressed; light to dark gray-brown	Crowded sinuate, rather thick, triangular-ventricose, pale cream-beige	50-120 × 10-15 mm, swollen at the base; grey-brown, longitudinally fibrillose	30-40 × 8- 13 µm, clavate	Cheilocystidia with swollen and long, narrow neck	Elliptical, minutely warted, amyloid, 8.5-9.5 × 5-6 µm	Boekhout, 1988; Phillips, 2006
M. melaleuca (Pers.) Murrill						
30-80 mm, dark-brown, hygrophanous, drying buff; at first flattened convex, becoming umbonate-depressed	White, emarginated, broad, crowded	55- 90 × 5- 8 mm, cylindrical, slightly broader towards base, sometimes becoming pale beige to brown-grey longitudinally fibrillose with pruinose apex	25- 40 × 7- 10 µm, clavate	Cystidia absent	Hyaline, warty, ellipsoid, amyloid, droplets, 7-8.5 × 5-5.5 µm	Boekhout, 1988; Jordan, 2004
M. paedida (Fr.) Kühner & Maire						
35-50 mm, hemispherical when young, later convex to plane, gray-brown with a silver tinge when dry, gray-beige when wet	Gray-whitish to gray, with yellowish-brown edges,	30-50 × 7-10 mm, cylindric, some with a bulbous base or enlarged apex, surface longitudinally fibrillose, cream to gray-brown	27-30 × 6-8 µm, cylindric to cylindric-clavate	Cheilocystidia 40-50 × 5-8 µm, stinging-hair shaped, sometimes sparse to absent	8.1-9.6 × 5.5-6.3 µm elliptic, finely verrucose, hyaline	Breitenbach & Kränzlin,1991
M. polioleuca (Fr.) Kühner & Maire						
25-70 mm, at first convex, finally with somewhat depressed centre, mostly with low broad umbo, when moist yellowish brown to dark grey-brown	Crowded, adnate to sinuose, mostly with subdecurrent toothlet, ventricose to triangular, whitish	35-85 × 3-8 mm, cylindrical clavate base, stuffed, at first whitish, becoming pale to sordid grey-brown	15- 40 × 6-10 µm, clavate	Cheilocystidia 45-75 × 8-15 µm, fusiform to lageniform, at apex encrusted with crystals	6.3-9 × 4-5 µm ellipsoid to elongate, rather ornamented	Boekhout,1988

Table 2 (continued)

			M. substrictipes Kühner			
25-70 mm, convex when young, later plane to irregularly undulating, with an umbonate center, satiny, white when young, later cream-whitish	White, later cream colored with a pink tint	40-70 × 3-8 mm, cyclindric, base bulbous and up to 10 mm thick, sometimes twisted, surface smooth, longitudinally fibrillos, white when young later yellow-brownish	33-38 × 7-9 µm, clavate,	Cheilocystidia fusiform to subulate, almost like stinging hair	8.1-10 × 5-6.3 µm, elliptic, finely verrucose, hyaline, with drops	Breitenbach & Kränzlin, 1991
			M. dryophila Murrill			
30-150 mm, convex, gibbous, viscid when flesh, subshining, whitish, stained with rusty-brown, margin paler, context white, with farinaceous	Deeply sinuate to adnexed, close, narrow, plane, white	60-130 × 10-45 mm, stuffed at maturity, equal to enlarged at the base, fibrillose brown over a pallid background below		Not seen	5-7 × 3.5-4.5 µm, globose, smooth, hyaline	Acar et al., 2017
			M. microcephala (P. Karst.) Singer			
10-30 mm, low convex to plane with a small umbo, smooth, yellow brown to dark brown	white or tinged pale buff			Cheilocystidia 40-60 × 6-10 µm, urticoid septate, often with crystals at apex	6-8 × 4-5 µm, ellipsoid, verrucose	Vesterholt and Knudsen, 2008
			M. heterocystidiosa (Beller & Bon) Bon			
50-70 mm., convex to smooth, slightly pruinose, gray-dark brown with olive in the center, then greyish ochre	Somewhat fragile, closed, white	50-80 × 4-7 mm, slightly pruinose, grey-sepia, pale greyish, downwards striate, at the base dark		Cheilocystidia 35-90 × 8-20 µm, abundant and variable in size and shape, mostly vertical-shaped	7-9 × 4-5 µm, elliptical, warty	Acar et al., 2017
			M. communis M. Sánchez-García&Cifuentes			
30-150 mm, plane-convex to plane, sometimes umbonate, margin decurved, brown, greyish brown, slightly yellowish-brown towards the margin	Sinuate, uncinateoradnate, close to crowded, white to yellowish with entire edges	40-170 × 4-16 mm, cylindrical to slightly attenuated towards the base, white to pale yellow, with yellowish longitudinal striates	20-40 × 5-10µm, clavate	Cheilocystidia 50-80 × 8-18 µm, fusiform, usually with crystals	6-9 × 4-6 µm, ellipsoid, ornamented with amyloid	Acar et al., 2017

Table 2 (continued)

M. arcuata (Bull.) Singer					
50-100 mm, convex, then plane, pale brownish, brownish clay or coffee and milk	Emarginate, deccurrent with a long tooth, yellowish	40-80 × 7-14 mm, fibrillose, base thickened	Cystidia 40-60 × 10-12 µm, lanseolate	9-10 × 5-6 µm, punctate	Carleton, 1922

Molecular phylogeny

ITS data matrix comprised a total of 40 sequences including 26 from NCBI. The amplified DNA fragment of the region was approximately 650 bp long encompassing complete ITS1, 5.8S and ITS2 subregions. The data set of the region included a total of 731 positions, of which 508 were conserved, and 208 were variable (112 variable sites in ITS1, 1 in 5.8S and 95 in ITS2 subregions). Number of variations was higher in ITS1 (112) compared to ITS2 (95).

The second region, LSU, comprised 24 sequences (10 from NCBI) and yielded total lengths of 885 nucleotides with 21 nucleotide variations. In the study, we mainly used the results taken from ITS region that was more informative than LSU. The topologies of the MP and ML phylogenetic trees had no considerable differences, so only one tree (ML) was given to indicate phylogenetic relationships. Phylogenetic tree constructed based on ITS region (Figure 2) composed from two major clades which were named according to the study of Vizzini et al. (2011) and called as clade A (subgenera *Melanoleuca*) and clade B (subgenera *Urticocystis*). Clade A and B were well supported with 99 and 98% bootstrap, respectively. Clade A consisted of four of studied species; *M. communis*, *M. heterocystidiosa*, *M. arcuata* and *M. polioleuca*. *Melanoleuca arcuata* from these species was placed unexpected clade. Clade B consisted of studied *M. brevipes*, *M. dryophila*, *M. substrictipes*, *M. paedida*, *M. grammopodia*, *M. exscissa*, *M. angelesiana*, *M. graminicola*, *M. microcephala* and *M. melaleuca*. In this clade, *Melanoleuca melaleuca* was placed unexpected clade. There was no discrimination at the section level in the tree.

LSU tree was not given due to less informativeness to understand phylogeny and low bootstrap values. The species could not be separated at the subgenus level (*Melanoleuca* and *Urticocystis)* and they scattered in the tree so taxonomic relations could not be resolved. Only *M. paedida*, *M. substrictipes*, *M. grammopodia* and *M. exscissa* evolutionarily separated from other species and caused a small cluster which was also observed in ITS tree.

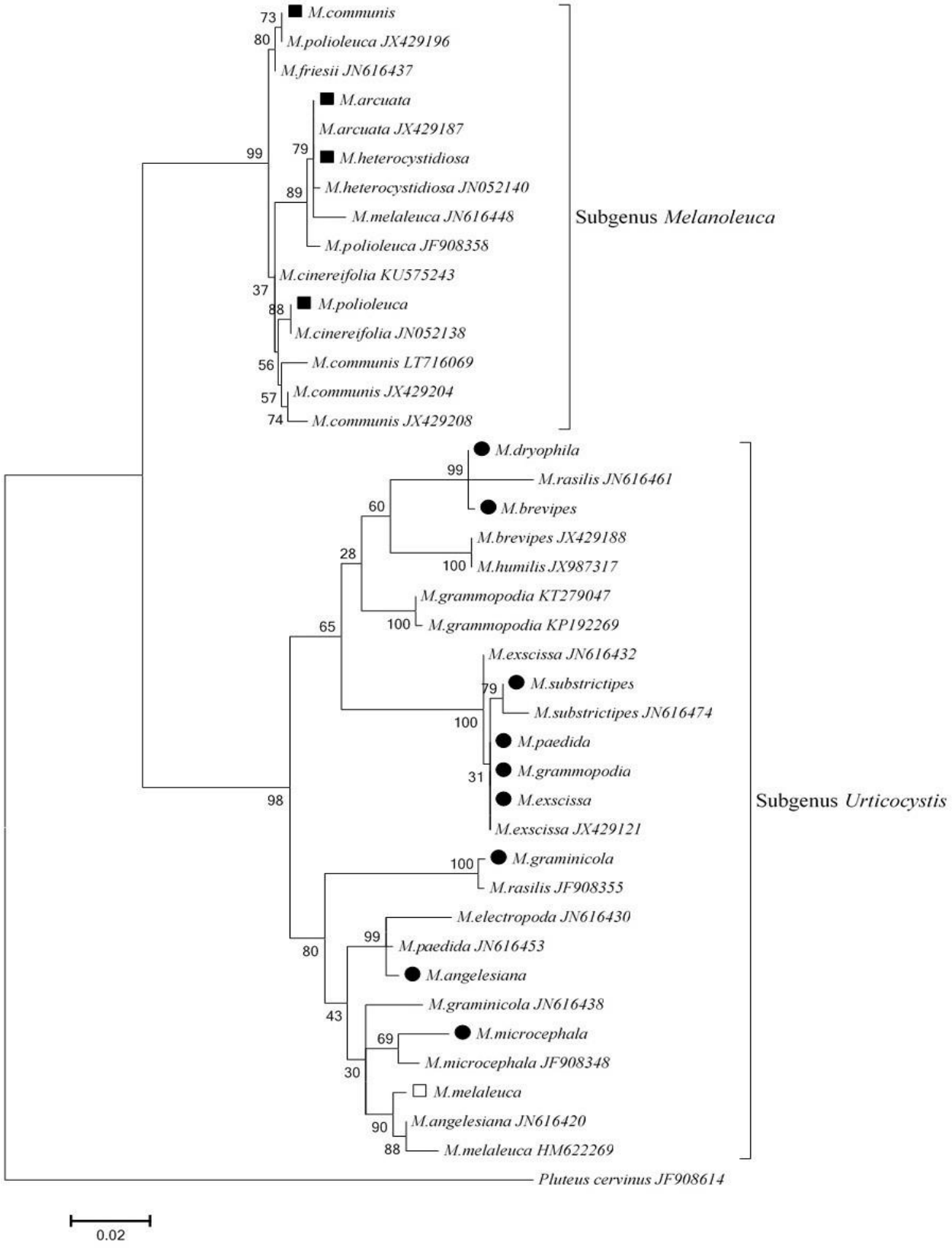

Figure 2. Maximum Likelihood phylogeny of nrITS sequences of *Melanoleuca* taxa. *Pluteus cervinus* was used as outgroup taxon. Studied species were marked with black square in Clade A and black circles in Clade B. Species whose position was incorrect from the studied species were marked with white square. Clade A; subgenus *Melanoleuca*. Clade B; subgenus *Urticocytis*.

Discussions

Identification of *Melanoleuca* species is controversial due to similarities of morphological characters. Vizzini *et al*. (2011) indicates that environmental factors affect these characters so molecular analyses are required for delimitation of the species. For molecular analysis, one of the most important steps is to select useful region that changes according to studied genus. Therefore, two regions were used in the present study to compare and select the most useful one. Results indicated that ITS region was more useful compared to LSU to figure out phylogenetic relationships among *Melanoleuca* species. Genetic variation observed in the LSU was lower than that of ITS and insufficient to figure out phylogeny. In our previous study, *Melanoleuca* species were separated at both subgenus and section level (Acar *et al*., 2017) but separation was occurred at only subgenus level in the current study. The fact that number of species is increased, analysis of taxonomy is complicated.

Two major clades (Clade A and B) with high bootstrap values were observed in the constructed ITS tree. Clade A mainly included species *Melanoleuca* subgenus and Clade B generally composed from species *Urticocystis* and *Acystis* subgenera. Bon (1991) claimed that three subgenera are found in the genus and one of them is called as *Acystis.* In the present study, this subgenus was represented by only two species, *M. graminicola* and *M. angelesiana,* and they located distantly to each other. Therefore, our outcome supported the results of Vizzini and his colleagues (2011) where *Acystis* was rejected to be a subgenus of *Melanoleuca* and number of the subgenus was decreased from three to two as *Melanoleuca* and *Urticocystis*.

Clade A (subgenus *Melanoleuca*) consisted of species downloaded from NCBI and four of studied species; *M. communis*, *M. heterocystidiosa*, *M. arcuata* and *M. polioleuca*. This subgenus characterized by a dark-brown blackish, slate-grey pileus, whitish lamellae and mainly lageniform cystidia or rarely without cystidia (Bon, 1991; Boekhout, 1988; 1999; Vizzini *et al*., 2011). Sample determined as *M. arcuata* placed in this clade with *M. arcuata* (JX429187) and other species of subgenus *Melanoleuca*. *Melanoleuca arcuata* differs mainly by a very dark pileus and yellow-ochre lamellae only at maturity (Boekhout, 1988; Vizzini *et al*., 2011) so discrimination of *M. arcuata* is expected to be accurate. *M. heterocystidia* clustered with representative NCBI sample (JN052140) with 79% bootstrap value. The studied *M. polioleuca* and its representative retrieved from NCBI located distantly. Similar relations were seen for other species such as *M. communis, M. paedida, M. grammopodia* and *M. graminicola*. This type relationship can be originated from environmental differences. *M. communis* were given as new records in our previous study and detailed information (spores, basidia, cystidia *etc.*) can be seen (Acar *et al*., 2017).

Clade B (subgenus *Urticocystis*) consisted of studied *M. brevipes*, *M. dryophila*, *M. substrictipes*, *M. paedida*, *M. grammopodia*, *M. exscissa*, *M. angelesiana*, *M. graminicola*, *M. microcephala*, *M. melaleuca* and their representatives. This subgenus characterized by mainly with urticocystidia but also with macrocystidia and brightly colored pilei or lacking cystidia and absent or very rare pleurocystidia (Bon, 1991; Vizzini *et al*., 2011). *M. angelesiana* and *M. graminicola* species placed in this clade with *M. paedida* (JN616453), *M. electropoda* (JN616430) and *M. rasilis* (JF908355). These studied species previously were accepted to be members of subgenus *Acystis* (Bon, 1991) however according to Vizzini *et al*. (2011) *Acystis* is a polyphletic taxa and nests with *Urticocystis*. *Melanoleuca angelesiana* was firstly reported in Turkey with current study. It is distinguished from other species by greenish-brown stipe, amyloid warts, grey lamellae and central umbo pileus (Table 3) (Smith, 1944; Desjardin et al., 2015; Vizzini et al., 2011).

Table 3. Comparison of Turkish specimens of *Melanoleuca angelesiana* and data of another authors.

Pileus	Lamellae	Stipe	Basidia	Spores	References
60-100 mm convex, smooth, central umbo, dark brown	attached to the stipe, grayish	30-60 × 5-15 mm smooth, fibrillose, partial veil absent	23.5-37.2 × 6.8-10.7 µm	7-10 × 5-6.5 µm, elliptical, amyloid, finely warts	This study
50-70 mm, broad, central umbo, brown	adnate, grayish	50-60 × 10-12 mm, fibrillose	Four spored	7-9 × 4.5-6 µm, elliptical, amyloid warts	Smith, 1944
20-110 mm, convex, broad, sometimes umbo, dark brown	adnate, grayish	15-75 × 3-15 mm, fibrillose	20-50 × 6-13 µm	7.5-10 × 5-6 µm, elliptical, verrucose, amyloid warts	Bessette et al. 1995
30-90 mm convex, low umbo, dark brown,	adnate, grayish	30-50 × 5-15 mm fibrillose, partial veil absent	-	8-10.5 × 5-6.5 µm, elliptical, amyloid, finely warts	Desjardin et al., 2015

Although *M. graminicola* clustered with *M. rasilis* (JF908355) with 100% bootstrap value morphologically they are different. *Melanoleuca graminicola* has not cystidia while *M. rasilis* has an urticoform cystidia (Boekhout, 1999). *M. rasilis* (JN616461) and *M. brevipes* clustered together with 99% bootstrap value in the tree. *M. brevipes* is differentiated from *M. rasilis* with its fusiform to lageniform cystidia and small spores covered by coarse warts. *M. exscissa*, *M. grammopodia*, *M. paedida* and *M. substrictipes* clustered together in both ITS and LSU (not shown) trees and morphologically they share resemble characters. They have fibrillose-smooth stipe and urticoform cystidia. However, *M. grammopodia* is characterized and separated from others by a large pileus and a long stipe (Antonin, Duriska, Jancovicova & Tomsovsky, 2015). So, it is expected that the diagnosis is correct. *M. dryophila* found in *Urticocystis* subgenus was given as new record in our previous study and detailed information (spores, basidia, cystidia *etc.*) can be seen Acar et al. (2017).

Melanoleuca melaleuca is belonging to subgenus *Melanoleuca* but placed in subgenus *Urticocystis* (clade B) with *M. melaleuca* (HM622269) in the tree. Interestingly, one of *M. melaleuca* sample retrieved from NCBI (JN616448) placed in clade A while the other sequence (HM622269) placed in clade B with our sample *M. melaleuca*. This species is characterized by grey-brown or dark-brown pileus, amyloid warts spores and lacking cystidia (Kuo and Methven, 2014). However, morphologic characters may not enough due to homoplasy and may cause incorrect identification.

As a conclusion, morphological characters of *Melanoleuca* species may show high level of similarities due to convergent evolution. Therefore, only morphology may not be enough to study taxonomy of this genus. Molecular techniques are also needed to resolve some problems and selecting useful region is important. ITS region may be a proper marker to solve some taxonomic problems but it is only enough to solve problems at the subgenus level. So, additional region should be used to figure out phylogenetic relations at the species level. This study is valuable because *Melaleuca* specimens from Turkey have never been studied by using morphological and molecular characters. Moreover, *Melanoleuca angelesiana* was firstly reported for mycobiota of Turkey and detailed information about this species was also given in the paper.

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Appendix 1

Accession numbers of species downloaded from NCBI database.

ITS region:
Melanoleuca angelesiana (JN616420), *M. brevipes (*JX429188), *M. humilis* (JX987317), *M. cinereifolia* (JN052138, KU575243), *M. graminicola* (JN616438), *M. melaleuca* (HM622269, JN616448), *M. grammopodia* (KT279047, KP192269), *M. polioleuca* (JF908358, JX429196), *M. communis* (LT716069, JX429204, JX429208), *M. heterocystidiosa* (JN052140), *M. rasilis* (JN616461, JF908355), *M. friesii* (JN616437), *M. exscissa* (JX429121, JN616431), *M. substrictipes* (JN616474), *M. microcephala* (JF908348), *M.arcuata* (JX429187), *M. electropoda* (JN616430), *M. paedida* (JN616453), *Pluteus cervinus* (JF908614)

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