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Phylogenetic relationships and taxonomical positions of two new records *Melanoleuca* species from Hakkari province, Turkey

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Abstract

The aim of the current study is to contribute to the knowledge of the genus *Melanoleuca* in Turkey. Delimitation of species taxonomic position within the genus is invaluable to protect the genus. Therefore, both morphological and molecular characters were used for identification of *Melanoleuca* specimens. Samples collected from Hakkâri province of Turkey were studied morphologically based on pileus, lamellae, stipe, spore, basidia and cystidia structures. Then, DNA sequence data of the Internal Transcribed Spacer (ITS), the Large Subunit (LSU) of the nuclear ribosomal RNA (rRNA) gene complex and Translation Elongation Factor 1-α (TEF) regions were analysed to figure out phylogenetic positions within the genus. At the end of the study, two new records species of *Melanoleuca* (*M. communis* and *M. dryophila*) were reported from Hakkâri province. When ITS and LSU regions were analyzed, *M. communis* placed within subgenus *Melanoleuca* / section *Melanoleuca* and *M. dryophila* placed within subgenus *Urticocytis* / section *Grammopodiae*. Considering the lack of information of this genus in Turkey and the fact that no detailed study has been developed before, this work is valuable to the systematics of *Melanoleuca* and to the taxonomic knowledge of agarics in Turkey.

Key words: ITS, LSU, Morphology, *Melanoleuca*, Phylogeny

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Türkiye, Hakkari ilinde bulunan üç yeni kayıt *Melanoleuca* türünün filogenetik ilişkileri ve taksonomik pozisyonları

Özet

Çalışmamızın amacı Türkiye'de bulunan *Melanoleuca* cinsine ait literatür bilgisine katkıda bulunmaktır. Cins içindeki türlerin taksonomik konumlarının belirlenmesi, cinsi korumak için çok önemlidir. Bu nedenle toplanan *Melanoleuca* örneklerinin tanımlanması için hem morfolojik hemde moleküler karakterler kullanılmıştır. Hakkâri ilinden toplanan örnekler, şapka, lamel, spor, bazidya ve sistidya gibi yapılar kullanılarak morfolojik tanımlamaları yapılmıştır. Daha sonra, Transkribe Edilen Aralayıcı Bölgeler (ITS), Büyük Altünite (LSU) ve Translasyon Uzama Faktörü 1-α (TEF) bölgelerinin DNA dizi verileri türlerin filogenetik konumlarını cins içinde anlamak için analiz edilmiştir. Çalışmanın sonunda Hakkâri ilinden iki yeni *Melanoleuca* türü (*M. communis* ve *M.dryophila*) rapor edilmiştir. ITS ve LSU bölgeleri analiz edildiğinde, *M. communis*, alt cins *Melanoleuca* / seksiyon *Melanoleuca*'da ve *M. dryophila* alt cins *Urticocytis* / seksiyon *Grammopodiae*'da yer almıştır. Türkiye'de bu cins hakkında yetersiz bilgi olması ve daha önce hiçbir ayrıntılı araştırma yapılmadığı göz önüne alındığında, bu çalışma *Melanoleuca*'nın sistematiği ve Türkiye'de agariklerin taksonomik bilgisi açısından değerlidir.

Anahtar kelimeler: ITS, LSU, Morfoloji, *Melanoleuca*, Filogeni

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1. Introduction

The genus *Melanoleuca* Pat. (*Tricholomataceae*) comprises approximately 331 species worldwide (Mycobank database). It is a character-poor genus with many macroscopically similar species. The genus is characterized by white, brown, ocher or gray stipe with cylindrical or subcylindrical shape, white to pale yellowish lamellae and ellipsoid spores ornamented with amyloid warts. Four spored basidium have shapes varying from cylindrical to clavate. Pleurocystidia and cheilocystidia may exist, and they are urticoid, thin-walled or fusiform to lageniform, thick-walled (Vesterholt, 2008; Yu et al., 2014).

According to the current checklists (Sesli and Denchev 2014; Solak et al., 2015), this genus comprises 21 species in Turkey. In the present study, samples were collected from Hakkâri province of Turkey and species *Melanoleuca communis* M. Sánchez-García&Cifuentes and *M. dryophila* Murrill were reported for the first time using morphological characters. After identification step, sequences of DNA regions were analyzed to see their taxonomical positions in the genus. Three regions, ITS, LSU of the nuclear ribosomal RNA (rRNA) gene and TEF, were selected to investigate taxonomical and phylogenetical relationships. DNA sequence of rRNA gene has been protected during evolutionary time and useful of ITS and LSU regions has been proved for different types of *Melanoleuca* species (Hinrikson et al., 2005). Especially, ITS region is accepted to be a valuable DNA marker for taxonomy of *Melanoleuca* (Schoch et al., 2012). TEF region encodes an essential part of a protein and it is widely used for phylogenetic application due to high information content at the species level and availability of universal primers (Geiser et al., 2004).

The aims of the present study were to i) describe all collected *Melanoleuca* species morphologically ii) adding new *Melanoleuca* records for Turkey iii) indicate phylogenetic relationship of the new records within the genus based on sequences of three different regions.

2. Materials and methods

2.1. Taxon sampling and morphological studies

The macrofungus samples were collected in 2014 and 2015 from different location of Hakkâri province (Table 1). Specimens were photographed with a Canon (EOS 60D) camera equipped with Tokina 100 mm macro lens. Microscopic studies were performed according to Clémençon (2009) in Molecular Biology and Genetics Department of Van Yüzüncü Yıl University. Dried specimens were sectioned with new bistouries under a Leica EZ4 stereo microscope; obtained sections were examined under a Leica DM500 research microscope after staining with Congo red, Melzer's reagent and KOH (5 %). Color images of microscopic characters were obtained with the Leica ICC50 HD camera, measurements were made with Leica Application Suite (version 3.2.0) programme. Identifications of the specimens and determination of subgenera / sections were done based on the following literature: Murrill (1913), Bresinsky and Stangl (1977), Bon (1991), Breitenbach and Kränzlin (1991), Dähncke (2004), Jordan (2004), Gerault (2005), Vizzini et al. (2011), Buczacki (2012), Garcia et al. (2013), Kuo and Methven (2014). Pileus, lamellae, stipe, spores, basidia and cystidia structures were used as morphological characters and detailed macro and microscopic descriptions of the species were given. All collected specimens were deposited in the Fungarium of Van Yüzüncü Yıl University (VANF).

Table 1. List of studied *Melanoleuca* species and their GenBank accession numbers.

Species	Number of samples	Location	Accession Number ITS	Accession Number LSU	Accession Number TEF
M. communis	5	Hakkâri, Turkey	KX507367	KX507364	KX507370
M. dryophila	5		KX507366	KX507363	KX507369

Spores from gill fragments of dried basidiomata (please, state origin) were measured in 2.5 % KOH (state type of mounting medium) using a light microscope (Nomarski interference contrast; oil immersion objective 100 x). Measurements are given as follows: (minimum) mean ± standard deviation (maximum), Q = length/width ratio (n = sample size; a statistically sufficient number of mature spores – usually more than 30 – should be measured. Example: Spores (10.5) 11.8 ± 0.7 (13.4) x (5.5) 6.2 ± 0.3 (6.7) µm, Q = (1.7) 1.9 ± 0.1 (2.1) (n = 31). Alternative notation: (10.5) 11.1-12.5 (13.4) x (5.5) 5.9-6.5 (6.7) µm (n = 31). For microscopic characters, numerical data should be rounded to one decimal place. For colour notations, the use of colour codes is strongly suggested (e.g. Methuen Handbook of Colours; Kornerup and Wanscher, 1978).

2.2. Molecular studies

2.2.1. DNA isolation, PCR amplification and DNA sequencing

Total DNA was isolated from dried basidiocarps using 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 0.8% agarose gel electrophoresis. Isolated stock DNA was stored at -20°C prior to PCR amplification. DNA amplification of each 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.

Primer pairs N-nc18S10 5'AGGAGAAGTCGTAACAAG3'/C26A 5'GTTTCTTTTCCTCCGCT3' (Wen et al., 1996), LROR 5'ACCCGCTGAACTTAAGC3'/LR5 5'TCCTGAGGGAAACTTCG3' (Stielow et al., 2015) and EF1-983F 5'GCYCCYGGHCAYCGTGAYTTYAT3'/EF11567R 5'ACHGTRCCRATACCACCRATCTT3' (Stielow et al., 2015) were used to amplify and get sequences for ITS, LSU and TEF regions, respectively. PCR reaction of each region consist of almost same amount of Buffer (2.5 µl), $MgCl_2$ (2.5µl) and Taq polymerase (0.20µl). Amount of each primer was 1.5 µl for ITS and 2.5 µl for LSU and 2.0 µl for TEF regions. Amount of diluted DNA were 2 µl for ITS region, 0.5µl for LSU region and 1.5 µl for TEF. The PCR program; for ITS region; 2 min at 95°C for one cycle, 1 min at 94 °C, 1 min at 54° C, 2 min at 72 °C for 30 cycles, 5 min at 72 °C for one cycle. For LSU and TEF regions; 2 min at 95°C for one cycle, 45 sec at 94 °C, 50 sec at 52° C, 2 min at 72 °C for 30 cycles, 5 min at 72 °C for one cycle. PCR products were run in a 1.0 % agarose gel and visualized by staining with Gelred dye (Figure 1). Purified PCR products were sequenced in both directions using an ABI 310 Genetic Analyzer (PE Applied Biosystems) and an Automatic Sequencer (RefGen Biotechnology, Ankara, Turkey).

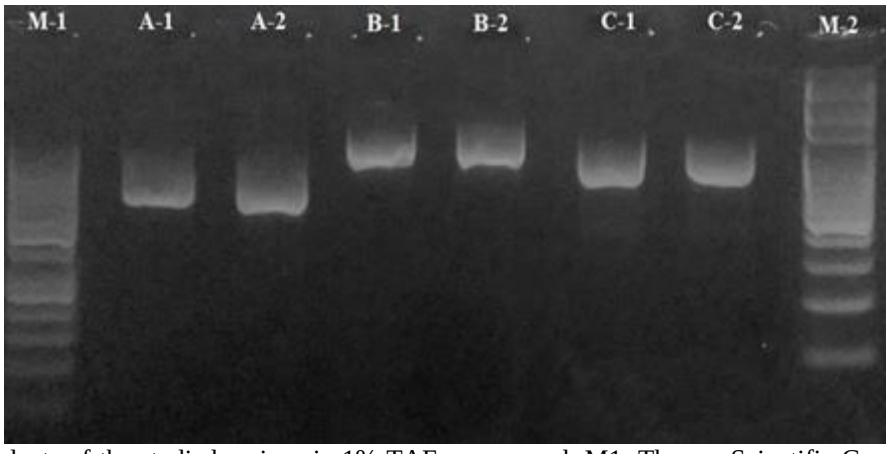

Figure 1. PCR products of the studied regions in 1% TAE agarose gel. M1: Thermo Scientific GeneRuler 50 bp, M2: Solis BioDyne 100 bp. A: TEF region, B: LSU region, C: ITS region. A1, B1, C1: *M.communis*, A2, B2, C2: *M. dryophila.*

2.2.2. Sequence alignment and phylogenetic analysis

Sequences of *Melanoleuca communis* and *M. dryophila* were generated in this study and other sequences were retrieved from GenBank database. GenBank sequences were intentionally selected from different subgenera such as *Melanoleuca*, *Urticocystis* and *Acystis* (Appendix 1) to see phylogenetic positions of our species in the phylogenetic trees. The sequence data of each region were deposited in GenBank database and their accession numbers are given in Table 1. Borders of regions were decided using sequences downloaded from GenBank database [ITS (JX429148, Garcia et al., 2013), LSU (JX429179, Garcia et al., 2013) and TEF (KT279053, Antonin et al., 2015)]. Completed sequences were aligned with the aid of the program ClustalW (Thompson et al., 1994). Alignments were checked and manually adjusted where it was necessary. *Pluteus cervinus* was utilized as out-group (ITS: JX857448, LSU: HM562221, TEF: KJ009889).

Prior to construction of phylogenetic trees, total nucleotide length (bp) and variable sites were calculated using Molecular Evolutionary Genetics Analysis software (MEGA 6; Tamura et al., 2013). Number of deletion/insertion (indel) was not taken into consideration because they were not included in the analysis. Phylogenetic tree of each studied region was constructed using two different methods; Maximum Likelihood (ML) and Maximum Parsimony (MP). Tamura-Nei model (Tamura and Nei, 1993) and bootstrap analysis with 500 replications (Felsenstein 1985) were selected to construct ML tree. In the method initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. 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.

3. Results

3.1. Taxonomy

Melanoleuca communis M. Sánchez-García&Cifuentes, in meadow, Şemdinli, near Şabatan gateway, 37º21′687″N, 44º32′461″E, 1723m, 01.05.2015, VANF Acar. 925 (Figure 2)

Pileus 30-150 mm diam., plane-convex to plane, sometimes umbonate, margin decurved, brown, greyish brown, slightly paler or yellowish-brown towards the margin, surface moist, glabrous. Lamellae sinuate, uncinateoradnate, close to crowded, white to yellowish with entire edges. Stipe 40-170× 4-16 mm, cylindrical to slightly attenuated towards the base, solid, white to pale yellow, with yellowish longitudinal striates, fleshy-fibrous. Basidiospores 6-9(10) × 4-6 µm, ellipsoid to oblong, ornamented with amyloid, isolated warts, hyaline. Basidia 20-40 × 5-10µm, clavate, tetrasporic. Cheilocystidia 50-80(90)×8-18(20) µm, fusiform, sometimes lageniform usually with crystals at the apices. Pleurocystidia similar to cheilocystidia.

Figure 2. *Melanoleuca communis* a) Basidiocarp, b) basidiospores (distilled water), c) basidiospores (Melzer's reagent), d) basidia (KOH), e) cystitis (KOH), f) caulocystidia (KOH).

Melanoleuca dryophila Murrill, under oak (*Quercus* sp.) and poplar (*Populus* sp.) trees, Şemdinli, Derya village, 37°21'271"N, 44° 31'282"E, 1525 m, 24.10.2014, VANF Acar. 478 (Figure 3)

Pileus 30-150 mm diam., convex, gibbous, becoming almost expanded, scattered; surface glabrous, viscid when flesh, subshining, nearly smooth, whitish, stained with rusty-brown, margin paler, somewhat lobed or irregular; context white, with farinaceous. Lamellae deeply sinuate to adnexed, close, narrow, plane, white, scarcely changing on drying. Stipe 60-130 mm tall, 10-45 mm thick, stuffed at maturity, equal to enlarged at the base, surface moist, white, finely striate at the apex, fibrillose brown over a pallid background below, bruising and discoloring brown in age; flesh white unchanging; veil absent. Spores 5-7(8) × 3,5-4,5 µm, globose, smooth, hyaline.

Figure 3. *Melanoleuca dryophila* a) Basidiocarp, b) basidiospores (distilled water), c) basidiospores (Melzer's reagent), d) basidium (KOH), e) hyphae (KOH), f) cystitis (Congo red).

3.2. Molecular phylogeny

ITS data matrix comprises a total of 32 sequences (including 30 from GenBank). DNA sequences of *M. communis* was intentionally downloaded from GenBank to see evolutionary relationships with our species. The amplified DNA fragment of the region was approximately 650 bp long encompassing complete ITS1, 5.8S and ITS2 sub-regions. The data set of ITS region included a total of 701 positions, of which 508 were conserved, 190 were variable as determined in MEGA 6.

As expected, nucleotide variation was not observed in 5.8S sub-region. There was almost same number of variations in the sequences of ITS1 and ITS2.

The second region, LSU, includes 12 sequences (10 from GenBank) and yielded total lengths of 875 nucleotides with 12 nucleotide variations. And finally, the length of TEF region was about 500 bp with 105 polymorphic sites. Unfortunately, there were not enough sequences for LSU and TEF region in GenBank. Only four sequences could be downloaded for TEF region. In the study, we mainly used the results taken by using ITS region and wanted to support them by using results of LSU and TEF regions. MP and ML tree topologies were congruent so only ML results are presented in Figures 4-6. Two major clades, A and B, were distinguished within *Melanoleuca* when ITS (Figure 4) and LSU (Figure 5) regions were used for analyses.

In the ITS tree, Clade A and B were well supported with 99 and 85% bootstrap while these values were lower (56 and 64%) in the LSU tree. Clade A consists of 2 main clades (A1-A2) and clade B of 2 clades (B1–B2). Clades A1 was formed mainly by species located in subgenus *Melanoleuca*/section *Melanoleuca*. Clade A2 was formed mainly by species, *M. candida, M. subalpina, M. strictipes*, found in subgenus *Melanoleuca*/section *Alboflavidae.* Species *M. dryophila* clustered outside of *M. communis* when ITS and LSU regions were analysed. This is expected because *M. communis* is morphologically different from *M. dryophila.* Clade B comprised species found in subgenus *Acystis* / section *Acystis* (B1) and subgenus *Urticocytis* / section *Grammopodiae* (B2). *Melanoleuca dryophila* grouped with *M. rasilis* and supported by 99% bootstrap value. LSU sequence of *M. rasilis* was not found in GenBank database. So, *M. dryophila* showed close relationship with *M. grammopodia* (Figure 5). Therefore, *M. dryophila* is expected to be in subgenus *Urticocytis* /section *Grammopodiae*.

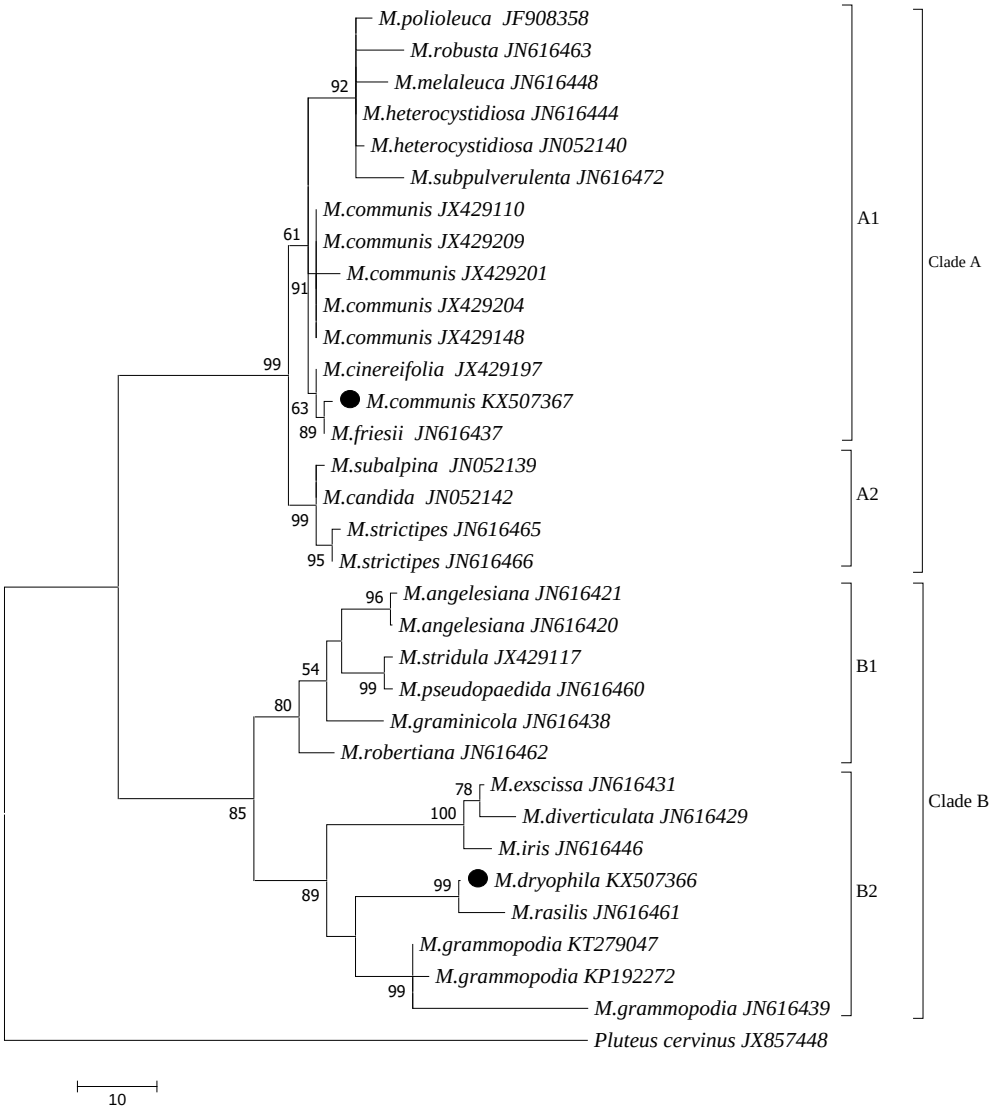

Figure 4. Maximum Parsimony phylogram obtained from the ITS nrDNA sequences of *Melanoleuca* taxa. *Pluteus cervinus* was used as outgroup taxon. Support values were given at branches. *Melanoleuca* species that are new records for Turkey were marked with black circles. Clade A; subgenus *Melanoleuca,* A1; section *Melanoleuca,* A2; section *Alboflavidae.* Clade B; subgenus *Acystis* / section *Acystis* (B1) and subgenus *Urticocytis* / section *Grammopodiae* (B2).

4. Conclusions and discussion

Results of our phylogenetic analyses indicated that the most useful region to figure out phylogenetic relationships among *Melanoleuca* species was ITS. Species were separated at the section and subgenus level in the phylogenetic tree. Phylogeny of ITS was supported that by LSU tree. DNA sequence of TEF region showed high variation and few sequence was there in GenBank database. Therefore, the tree was not informative to understand phylogeny of the species based on this region.

Melanoleuca communis showed close phylogenetic relationships with *M. cinereifolia* (Bon) Bon, *M. friesii* (Bres.) Bon and *M. melaleuca* (Pers.) Murrill (Figure 4, 5) in Clade A. Although mature *M. cinereifolia* samples are morphologically similar to fresh *M. communis* samples, they can easily be differentiated by using microscopic features of *M. cinereifolia;* spores -8 × 4-5 µm, basidia; 23-33 × 8-10 µm, cystidia; (41) 49-64 × (8)10-13 µm (Garcia et al., 2013). Even though *M. melaleuca* is morphologically different from *M. communis,* they are similar in terms of their spore dimensions (7-10 × 4-5,5 µm) and fusiform cystidia structures (Gerault, 2005). *Melanoleuca friesii* is slightly similar to *M. communis* but it can easily be differentiated microscopically (cheilocystidia (35) 45-65 × 10-16 µm and pleipellis an ixotrichoderm, up to 40 µm thick, made up of slender) from *M. communis* (Gerault, 2005; Bas et al., 1999). *Melanoleuca communis* is included in subgenus *Melanoleuca* / section *Melanoleuca* when morhological characters were studied and its position is also proved genetically with the current study.

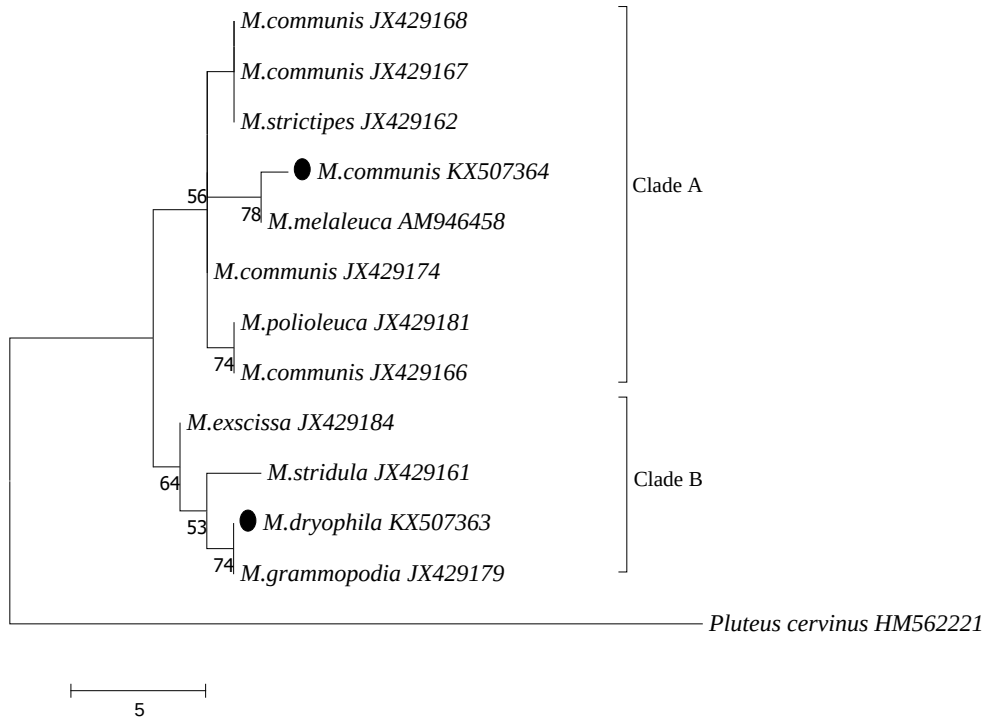

Figure 5. Maximum Parsimony phylogram obtained from the LSU nrDNA sequences of *Melanoleuca* taxa. *Pluteus cervinus* was used as outgroup taxon. Support values were given at branches. *Melanoleuca* species that are new records for Turkey were marked with black circles. Clade A; subgenus *Melanoleuca*, Clade B; subgenus *Urticocystis*.

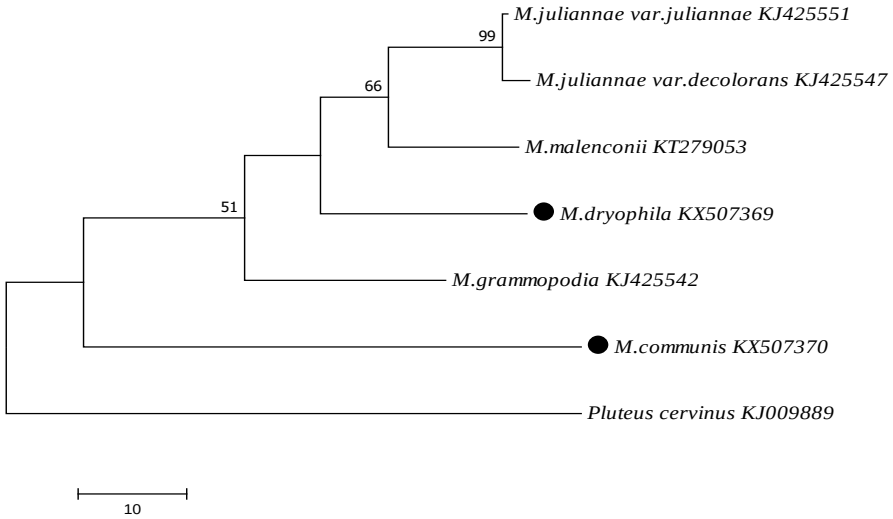

Figure 6. Maximum Parsimony phylogram obtained from the TEF sequences of *Melanoleuca* taxa. *Pluteus cervinus* was used as outgroup taxon. Support values were given at branches. *Melanoleuca* species that are new records for Turkey were marked with black circles.

Melanoleuca dryophila located in Clade B and showed close relationship with *M. exscissa* (Fr.) Singer, *M. grammopodia* (Bull.) Murrill, *M. rasilis* (Fr.) Singer and *M. stridula* (Fr.) Singer (Figure 4, 5 and 6). However, *M. dryophila* does not resemble to these species either microscopically or macroscopically**.** *Melanoleuca excissa* spores are elliptic, verrucose (Breitenbach and Kränzlin, 1991). *M. grammopodia* is verrucose and it has larger spores, its warts are round or irregular (Kumar et al., 2013; Antonin et al., 2015). *M. rasilis* spores are broadly ellipsoid to ellipsoid, rather densely ornamented with larger amyloid warts (Bas et al., 1999). As shown in literature, spores of all kinds have warty except *M. dryophila.* By using molecular data it can be true that this species should be included in subgenus *Urticocytis* / section *Grammopodiae.*

Melanoleuca communis and *M. dryophila* were decided to be new records for Turkey. Moreover, uncertain taxonomic position of *M. dryophila* was clarified and decided to be found in subgenus *Urticocystis* based on ITS and LSU trees. For further studies, more *Melanoleuca* species that is represented with low number in Turkey may be identified and phylogenetic positions of all records should be understood better using both molecular and morphological data..

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Appendix 1

Accession numbers of species downloaded from GenBank database.

ITS region:

M.angelesiana (JN616420, JN616421), *M.cinereifolia* (JX429197), *M.graminicola* (JN616438), *M.melaleuca* (JN616448), *M.gramopodia* (KT279047, KP192272, KP192272), *M.subalpina* (JN052139), *M.stridula* (JX429117), *M.polioleuca* (JF908358), *M.communis* (JX429148, JX429110, JX429201,JX429204,JX429209), *M.heterocystidiosa* (JN616444, JN052140), *M.iris* (JN616446), *M.rasilis* (JN616461), *M.subpulverulenta* (JN616472), *M.friesii* (JN616437), *M.strictipes* (JN616466), *M.robusta* (JN616463), *M.robertiana* (JN616462), *M.exscissa* (JN616431), *M.diverticulata* (JN616429), *M.candida* (JN052142), *M.pseudopaedida* (JN616460), *Pluteus cervinus* (JX857448)

LSU region:

M.communis (JX429174, JX429168, JX429167, JX429166), *M.grammopodia* (JX429179), *M.excissa* (JX429184), *M.polioleuca* (JX429181), *M. melaleuca* (AM946458), *M.stridula* (JX429161), *M.strictipes* (JX429162), *Pluteus cervinus* (HM562221)

TEF region:

M.juliannae var. juliannae (KJ425551), *M.juliannae var. decolorans* (KJ425547), *M.grammopodia* (KJ425542), *M.malenconii* (KT279053), *Pluteus cervinus* (KJ009889)

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