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Molecular and Morphological Identification of *Cantharellus pallens* Pilát 1959 (Cantharellales, Basidiomycota), a New Record for Turkish Mycota

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ABSTRACT
Cantharellus pallens Pilát 1959 in *Cantharellus* genus is recorded for the first time from Tokat city province, Turkey. Morphological studies, phylogenetic data derived from both the DNA sequences of nuclear ribosomal internal transcribed spacer (ITS) and large ribosomal subunit (LSU) genes revealed that this species is a new record for the Turkish mycota.

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Türkiye Mikotası için Yeni Bir Kayıt Olan *Cantharellus pallens* Pilát 1959 (Cantharellales, Basidiomycota)'nın Moleküler ve Morfolojik Teşhisi

ÖZET
Cantharellus cinsine ait bir mantar türü olan *Cantharellus pallens* Pilát 1959, Türkiye'nin Tokat ilinde ilk kez kaydedilmiştir. Morfolojik çalışmalar, nükleer ribozomal internal transcribed spacer (ITS) ve large ribosomal subunit (LSU) genlerine ait DNA dizilerinden türetilen filogenetik veriler, bu türün Türk mikotası için yeni bir kayıt olduğunu ortaya koymaktadır.

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INTRODUCTION
The genus *Cantharellus* Adans. ex Fr. (Fries, 1821) contains species of commercial, medicinal and economical importance, and they are best known as edible ectomycorrhizal mushrooms. The species of this genus are morphologically distinguished by a well-developed hymenium or a complex lamellae-liked forked wrinkles, a solid and fleshy stipe, non-funnel-shaped pileus, smooth ellipsoid and non-amyloid spores, and spore deposits in cream to pinkish yellow or pale ochre color (Eyssartier and Buyck, 2000; Kibby, 2012). Many species mostly have five sterigmata per basidium and show differences in septal pore morphology (Hibbett et al., 2014). The type species of *Cantharellus* is *Cantharellus cibarius* which was first described by Fries (1821).

Molecular methods have been widely used to describe new species and reduce the possibility of misinterpretations in fungal taxonomy. The nuclear ribosomal internal transcribed spacer (ITS) region is the most commonly used fungal barcode (Schoch et al., 2012) for the exploration of fungal diversity in environmental samples and solving species delimitation problems among morphologically similar species. However, due to high evolutionary rate of nuclear ribosomal RNA genes in *Cantharellus* genus (Moncalvo et al., 2006), use of different gene regions, such as combination of ITS and ribosomal nuclear large subunit (LSU) regions are important for a better infrageneric classification in this genus. *Cantharellus* species delimitation problems can only be resolved with the availability of additional molecular data from different geographic locations.

In the last decade, taxonomic research on *Cantharellus* has increased and with the help of molecular studies, approximately 180 species of *Cantharellus* have been described worldwide in subtropical, tropical and temperate climate regions (An et al., 2017; Buyck and Hofstetter, 2011; Buyck et al., 2011, 2013, 2014, 2016a, 2016b, 2016c; De Kesel, 2011; Deepika et al., 2014; Shao et al., 2011, 2014, 2016a, 2016b; Buyck and Randrianjohany, 2013; Foltz et al., 2013; Kumari et al., 2013; Liu et al., 2015; Suhara and Kurogi, 2015; De Kesel et al., 2016; Leacock et al., 2016; Olariaga et al., 2017; Ogawa et al., 2018; Parad et al., 2018; Lao et al., 2019; Jian et al., 2020). In Turkey, nine *Cantharellus* species (*C. amethysteus* (Quél.) Sacc., *C. cibarius* Fr., *C. cinereus* (Pers.) Fr., *C. ferruginascens* P.D. Orton, *C. friesii* Quél., *C. ianthinoxanthus* (R. Maire) Kühner, *C. lutescens* (Fr.) Fr., *C. subalbidus* A.H. Sm. & Morse, *C. melanoxeros* Desm.) have been described from various regions but they all lack molecular evidences (Allı et al., 2017; Solak et al., 2007; Sesli and Denchev, 2008; Keleş et al., 2014; Akata and Kumbaşlı, 2014; Türkekul and Işık, 2016; Sesli et al., 2016; Bulam et al., 2018). No samples of *C. pallens* were recorded so far in Turkish mycobiota. More studies based on both morphological and molecular analysis are urgently needed for a better classification and correct description of *Cantharellus* species in Turkey and other locations.

In this study, we identified *Cantharellus pallens* Pilát (1959) from Tokat city province, Turkey, by both morphological examination and molecular phylogenetic studies. This species is identified with its pale orangish white to cream pileus, white to pale ochraceous cap surface, well-developed white to pale ochre-yellow hymenium, white to cream stipe, small and ellipsoid spores. This study presents a new *Cantharellus* species for Turkish mycological collections.

MATERIALS and METHODS

Study area and sampling

Mushroom specimens were collected from Akbelen plateau (Tokat). It has an elevation of 1600 to 1800 m with mild climate. This area is in the vicinity of Yaylacık Mountain. Due to its geographic location, it hosts many vegetations and a rich spot for fungal diversity. The sampling was made on June 29th, 2018. The fresh basidiomata were photographed in the field using a digital camera. Field notes were made and samples were dried for longer storage in boxes. They were deposited in the Fungarium of the Department of Biology, Tokat Gaziosmanpasa University, Tokat, Turkey (GOPUF) with a voucher number TTS46.

Morphological analyses

Macromorphological characters of fresh fruitbodies, colors of different parts of the basidiomata; shape, size, and color of the pileus and stipe; vegetation and odors were noted during field study. A mature sample was selected to obtain spore print of the sample. Microscopic studies were carried out on dry samples using a microscope. Some chemicals (such as 5% KOH, Congo red) were used to rehydrate and dye dry samples during the studies. The findings obtained in these studies were compared with the existing literature (Watling and Turnbull, 1998; Breitenbach and Kränzlin, 2000; Eyssartier and Buyck, 2000, 2001).

Molecular and phylogenetic analyses

Genomic DNA was extracted from about 20 mg of dried specimen using the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research Irvine, CA, USA) as described by the manufacturer's protocol. The primer pair ITS4/ITS5 (White et al., 1990) was used to amplify approximately 873 bp genomic sequence of the ITS1-5.8S-ITS2 region of the rDNA internal transcribed spacer region (ITS) and LROR-LR5 primer pair (Vilgalys and Hester, 1990) was used to amplify about 878 bp genomic sequence of the 28S nuclear ribosomal large subunit rRNA (LSU) gene region. The PCR conditions for ITS and LSU regions was performed as decribed in Şengul Demirak and Işik (2020). PCR amplifications were verified by using 1% agarose gel electrophoresis. PCR products were sequenced from both ends using forward and reverse primers (BM Labosis Inc., Ankara).

Sequences generated from both ends were edited and assembled to produce a final gene sequence for ITS and LSU regions of the studied sample and deposited in GenBank. Homology based searches using Basic Local Alignment Search Tool (BLAST) program was used to find the best matches for each genomic sequence. Representative ITS and LSU sequences of *Cantharellus* species were retrieved from GenBank for phylogenetic analysis. Sequences were aligned using ClustalW (Larkin et al., 2007) and adjusted manually. Phylogenetic trees for both ITS and LSU sequences were drawn using the Maximum Likelihood (ML) method based on Tamura-Nei model (Tamura and Nei, 1993) using MEGA 7.0 (Kumar et al., 2016) with bootstrap support of 1000 replicates and default settings. The bootstrap support values $\geq$ 50% were marked on the branches of the tree. Initial tree for the heuristic search was 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 selecting the topology with superior log likelihood value. The analysis involved 28 and 33 nucleotide sequences for ITS and LSU regions, respectively, including the studied specimen. All positions containing gaps and missing data were eliminated.

RESULTS and DISCUSSION

Taxonomy

Hydnaceae Chevall. 1826

Cantharellus pallens **Pilát,** Omagiu lui Traian Savulescu: 600 (1959)

Mycobank: MB 327488

Descriptions: Pileus 25–95 mm across, pale orangish white to orange cream, with the cap surface varying from white to pale ochraceous, sometimes yellowish at the margin (Figure 1a). Hymenophore well-developed, forked veins to anastomosing. Stipe robust, whitish cream, 15–60 × 11–25 mm. Flesh white to whitish cream, pale violet in the stipe. Taste mild. Basidia 75-85 × 7-9 µm, slenderly clavate, 4 spored (Figure 1c). Basidiospores 7.5–9.5 × 3.5–5.5 µm, ellipsoid, hyaline, thin walled and smooth, with granular contents (Figure 1b).

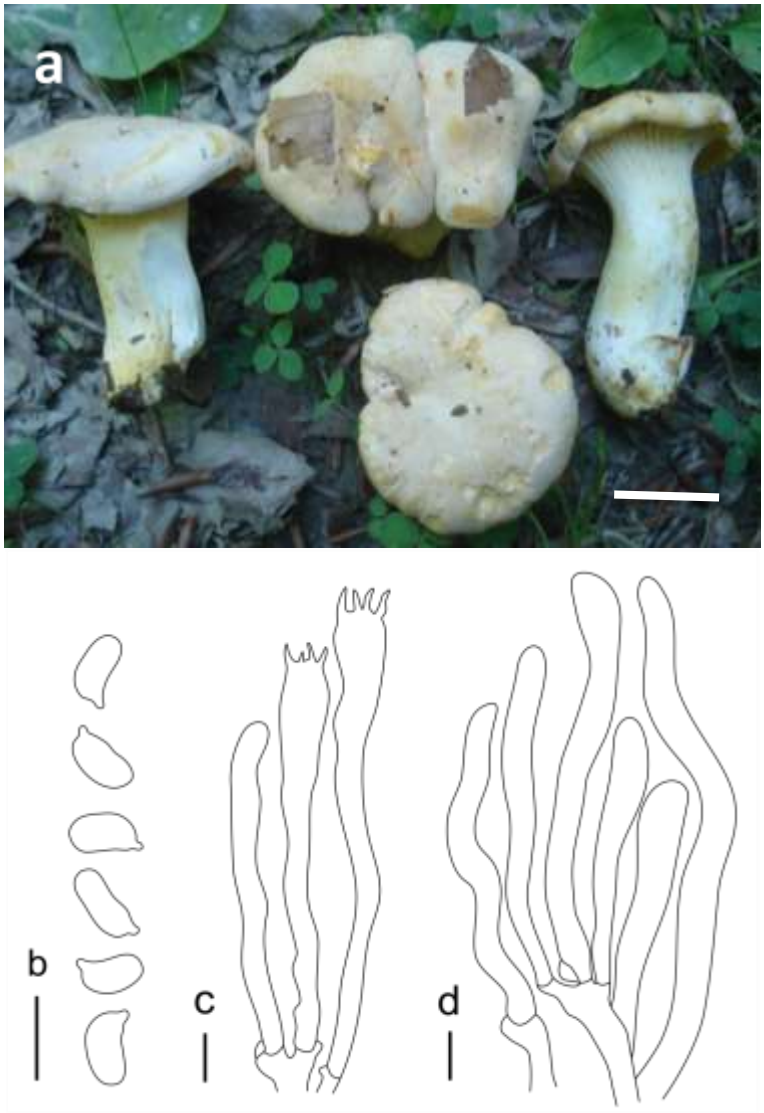

Figure 1. *Cantharellus pallens*. a. basidioma, b. basidiospores, c. basidia and basidioles. d. hyphal extremities of the pileipellis. Scale bars: a= 20 mm; b, c, d = 10 µm.

Şekil 1. Cantharellus pallens. a. bazidiyokarplar, b. bazidiyosporlar, c. bazidiyol ve bazidiyumlar, d. pileipellisde hif yapıları. Ölçek çubuğu: a= 20 mm; b, c, d= 10 µm.

Edibility: Edible, occasionally collected and sold on local markets in the world (Pilz et al., 2003).

Ecology: Rare, summer to fall, on rich, calcareous soil, mycorrhizal with beech (*Fagus* L.) and oak (*Quercus* L.) trees (Hansen and Knudsen, 1997).

Material examined: TURKEY—Tokat, Akbelen plateau, on soil, under *Quercus* L. sp., 40° 30' 41"N - 36° 37' 22"E, 1510 m, 29 June 2018, TTS46.

The holotype specimen of *C. pallens* Pilát (PRM 655551) is described from Czech Republic found on soil associated with conifers (Pilát, 1959). This species is quite robust and well characterized by its pale-colored morphological features, including a light colored, white to pale ochraceous cap, white flesh, pale orange to

white pileus, pale ochre white to pale ochre stipe, and a well-developed hymenophore with bright orange yellow to pale ochre yellow color, and thick margin (Pilát, 1959; Eyssartier and Buyck, 2000; Kibby, 2012; Olariaga et al., 2017). Among the European species of *Cantharellus*, *C. pallens* has the smallest spores, generally of 7–9 × 4–5 µm, with ellipsoid and smooth appearance (Bertolini, 2014).

Cantharellus pallens is mostly distributed in Europe, especially in the Mediterranean area, but also found in Great Smoky Mountains of North Carolina in the United States. (Buyck et al., 2016; Olariaga et al., 2017). *C. cibarus* is also greatly distributed in Europe, but not present in the Mediterranean climate (Olariaga et al., 2017). They have some differences in their ecological preferences such that *C. pallens* is associated with *Quercus*, other evergreen oaks, deciduous oaks and *Pinus*, while *C. cibarius* prefers cooler climates, and is found with broad-leaved trees such as *Castanea sativa* Mill., deciduous oaks or conifers, and shows preference for acidic soils (Olariaga et al., 2017). *C. pallens* and *C. cibarius* also exhibit some variable features. *C. cibarus* is characterized by an orange-yellow hat, pileus, stipe and uniform colored hymenophore, while *C. pallens* has a whitish to pale orange-yellow colored pileus, stipe, and has a brighter orange-yellow hymenophore near the margin (Eyssartier and Buyck, 2000; Olariaga et al., 2017). Moreover, *C. pallens* is often a very robust species.

Based on morphology, *C. ferruginascens* P.D. Orton (1969) is a species that has been synonymised with *C. pallens* (Pegler et al., 1997). Later, Watling and Turnbull (1998) indicated that they are different species exhibiting differences in their color and staining. Kibby (2012) also reported a larger spore size and predominant staining feature for *C. ferruginascens*. Moreover, it is indicated that *C. ferruginascens* differs from *C. pallens* due to different colors of young pileus and hymenophore (Olariaga et al., 2017). In northern Europe, the name *C. pallens* has been more commonly used, while *C. subpruinosus* Eyssart. and Buyck (2000) has been used in southern Europe (Olariaga et al., 2017). Based on morphological characters, *C. subpruinosus* was described to show strong staining and have young basidiomata with white pileus, but *C. pallens* is described as the non-staining species (Eyssartier and Buyck, 2000).

Phylogenetic analyses

We have successfully amplified about 880 bp ITS1-5.8S-ITS2 and LSU rRNA gene sequences which were deposited in GenBank with accession numbers MW386299 and MW386300, respectively. An initial BLAST searches using the ITS and LSU sequences as query retrieved sequences up to 99% identity belonging to *Cantharellus* subg. *Cantharellus* species described from various collections in different geographic locations. Best matches from BLAST results and *C. pallens* sequences described in Olariaga et al. (2017) were included in the phylogenetic analysis.

We present ML trees based on the sequences for ITS and LSU regions to indicate phylogenetic relationship of the studied species with other *Cantharellus* species (Figures 2 and 3). In the ITS and LSU phylogenies, two distinct clades were recognized that separated the clade including *C. pallens* from *C. cibarius* with a high bootstrap support (Figures 2 and 3). They are both supported as monophyletic groups. Phylogeny reveals that *C. pallens* is closely related and sister to *C. cibarius*, where the sequence divergence between the two species was estimated to be approximately 0.02%. Recent molecular studies also indicate that they are closely related but phylogenetically distinct species (Olariaga et al., 2017; Ogawa et al., 2018). The results showed a high degree of sequence similarity in both ITS and LSU genomic regions for *C. cibarius* and *C. pallens* specimens, which may indicate that these two species can be conspecific. The phylogenetic analysis suggested that the two species were distinguished as separate lineages with high statistical support. Although not observed in the ITS phylogeny, *C. cibarius* specimen (KX828796) appear to be a basal clade in the LSU phylogeny. It is highly possible that DNA sequence generated for the LSU region of this specimen is incorrect and needs validation by additional sequencing data.

Cantharellus ferruginascens samples also formed a separate clade distant from *C. pallens* clade but sister to the clade of *C. alborufescens* (Malençon) Papetti and Alberti (1998). Similar phylogenetic results have also been shown by other studies (Buyck et al., 2014; Olariaga et al., 2017; Ogawa et al., 2018; Jian et al., 2020). We confirmed that *C. ferruginascens*, *C. alborufescens* and *C. pallens* are separate taxa within the *Cantharellus*.

Distinction based on morphological features alone is not enough for *C. pallens* and *C. subpruinosus*. Buyck et al. (2014) reported that *C. subpruinosus* is monophyly with *C. cibarius* based on the sequence analysis of the largest subunit of the RNA polymerase II (RPB2) region. In Figure 3, LSU based phylogeny shows that *C. subpruinosus* collection from France (KF294660) is very closely related to *C. pallens* species described from different localities, and also supports the monophyly of *C. subpruinosus* and *C. cibarus*. Zamora et al. (2018) propose that two different species may share identical DNA sequences at a given locus, even for already tested barcoding markers. This could explain why *C. subpruinosus* is grouped with the sequences of *C. pallens* in the same clade. Since ITS sequence data is not available for *C. subpruinosus*, we only made our interpretations according to the LSU

phylogeny. Additional collections from various localities and additional gene regions for molecular analysis has to be studied to solve the species delimitation of the infrageneric group, including *C. subpruinosus* and *C. pallens*.

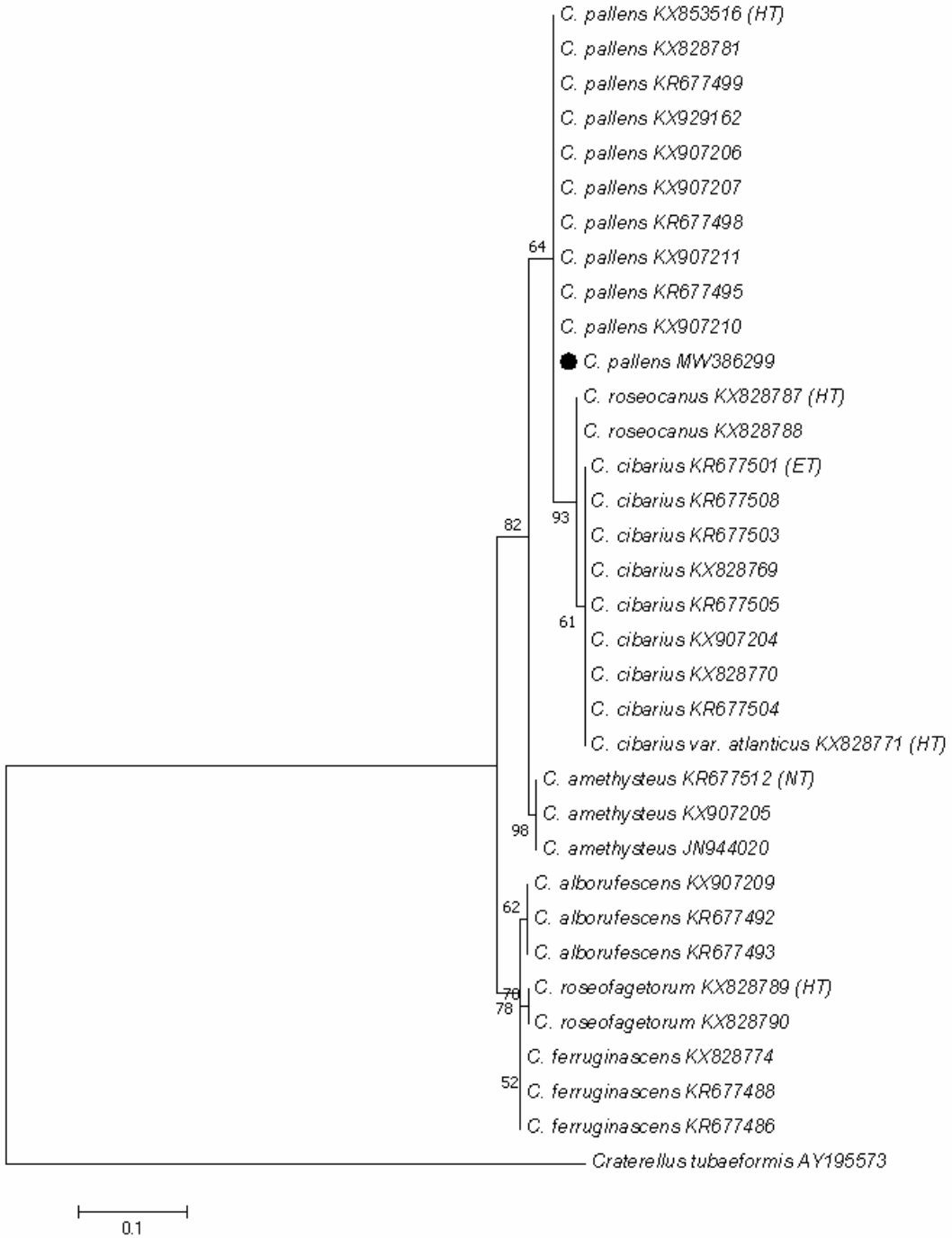

Figure 2. Molecular phylogenetic analysis using ML method based on ITS sequences. The studied specimen was indicated with black circle. Bootstrap support values ≥ 50% from ML analysis were shown on the branches. Sequences from type collections were marked. Bar indicates 0.1 expected change per site per branch. *Craterellus tubaeformis* (AY195573) was the outgroup species. C.: *Cantharellus*; ET: epitype; HT: holotype; NT: neotype

Şekil 2. ITS dizilerine dayalı ML yöntemi ile moleküler filogenetik analiz. Çalışılan numune siyah daire ile belirtilmiştir. ML analizlerinde, ≥ %50 bootstrap destek değerleri olanlar dalların üstünde gösterilmiştir. Tip koleksiyonlar şekilde belirtilmiştir. 0.1 bar çizgisi, her daldaki her noktaya ait tahmini değişimi ifade etmektedir. Craterellus tubaeformis (AY195573) dış grup türdür. C.: Cantharellus; ET: epitip; HT: holotip; NT: neotip

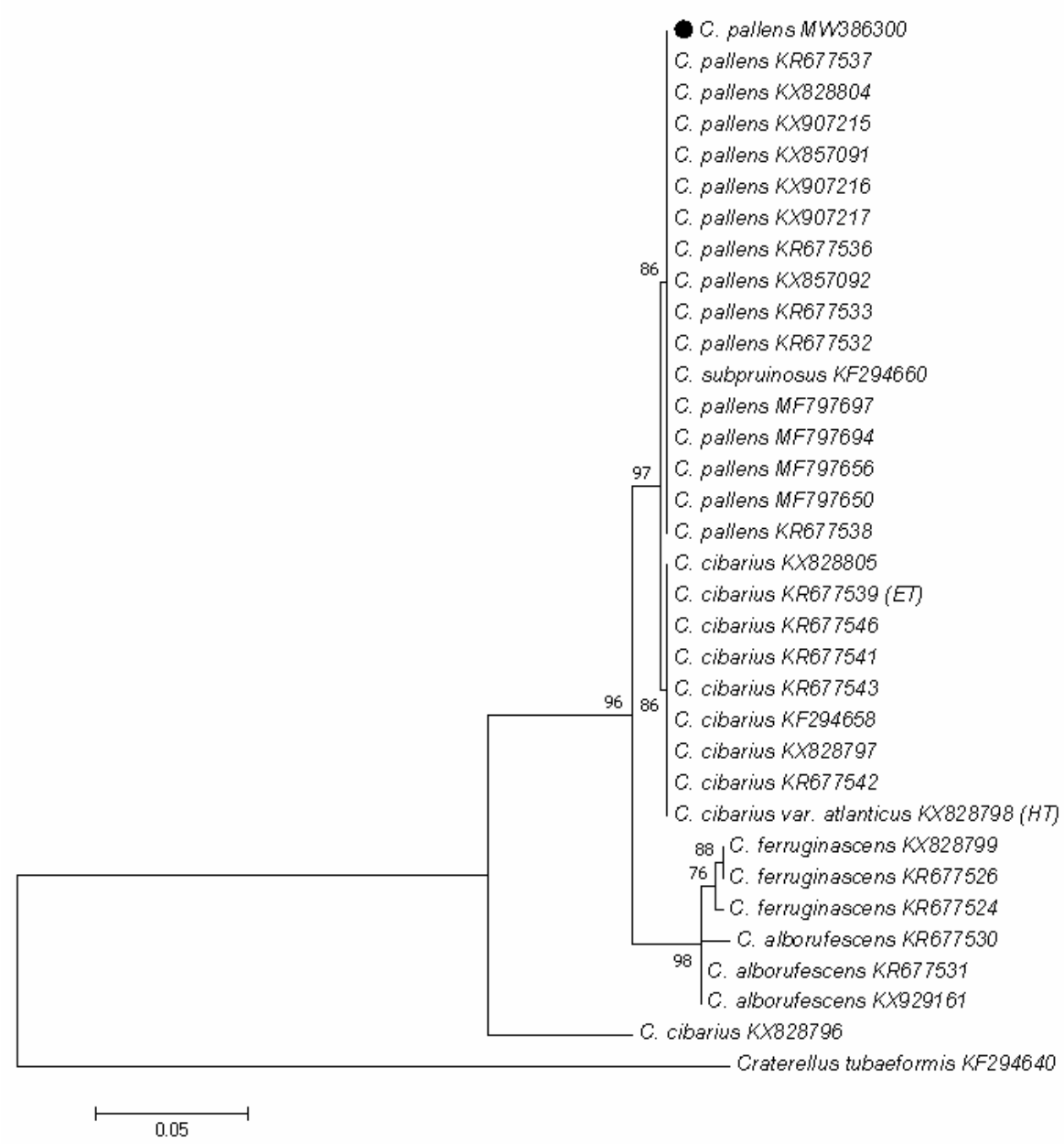

Figure 3. Molecular phylogenetic analysis using ML method based on LSU sequences. The studied specimen was indicated with black circle. Bootstrap support values ≥ 50% from ML analysis were shown on the branches. Sequences from type collections were marked. Bar indicates 0.05 expected change per site per branch. *Craterellus tubaeformis* (KF294640) was the outgroup species. C.: *Cantharellus*; ET: epitype; HT: holotype

Şekil 3. LSU dizilerine dayalı ML yöntemi ile moleküler filogenetik analiz. Çalışılan numune siyah daire ile belirtilmiştir. ML analizlerinde, ≥ %50 bootstrap destek değerleri olanlar dalların üstünde gösterilmiştir. Tip koleksiyonlar şekilde belirtilmiştir. 0.05 bar çizgisi, her daldaki her noktaya ait tahmini değişimi ifade etmektedir. Craterellus tubaeformis (AY195573) dış grup türdür. C.: Cantharellus; ET: epitip; HT: holotip

CONCLUSIONS

Nomenclature of a new species displaying similar morphological features with the existing species and in the absence of molecular evidences causes ambiguity among *Cantharellus*. Thus, taxonomic studies with the hep of molecular evidences have increased in the last decade to clarify species delimitation of this genus. This study reports *Cantharellus pallens* from Tokat province, Turkey, with morphological and molecular evidences, including ITS and LSU gene regions. A taxa adopted to a different location can only be resolved with additional descriptions based on morphological, ecological features, and more importantly, with the inclusion of reliable molecular studies.

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Author's Contributions

The contribution of the authors is equal.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

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