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Morphological and Molecular Phylogeny of *Cortinarius rufo-olivaceus* (Pers.) Fr. (subgenus *Phlegmacium* sect. *Calochroi*) Collected from Tokat Region

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ABSTRACT
Cortinarius (Pers.) Gray samples were collected in the oak forests from Tokat province. Macro and micromorphological features as well as molecular phylogenetic analyses according to the DNA sequences corresponding to internal transcribed spacer region (ITS) and the large subunit (LSU) of nuclear ribosomal RNA gene regions indicated that the studied specimen is a *Cortinarius rufo-olivaceus* (Pers.) Fr. This study determined the first microscopic and macroscopic morphological description and molecular phylogenetic analysis of the *Cortinarius rufo-olivaceus* (Pers.) Fr. in Turkey.

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Tokat Yöresinden Toplanan *Cortinarius rufo-olivaceus* (Pers.) Fr. (subgenus *Phlegmacium* sect. *Calochroi*) Türünün Morfolojik ve Moleküler Filogenisi

ÖZET
Cortinarius (Pers.) Gray örnekleri Tokat ilinden meşe ormanlarında toplanmıştır. Makro ve mikromorfolojik özellikler ile nükleer ribozomal iç aralayıcı bölge (ITS) ve ribozomal en büyük alt birim (LSU) gen bölgelerine karşılık gelen DNA dizileri ile moleküler filogenetik analizler, çalışılan numunenin *Cortinarius rufo-olivaceus* (Pers.) Fr. türü olduğunu göstermiştir. Bu çalışma, Türkiye'de *C. rufo-olivaceus*'un mikroskobik ve makroskobik morfolojik tanımı ve moleküler filogenetik analizini belirleyen ilk çalışmadır.

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INTRODUCTION
The genus *Cortinarius* belongs to the order Agaricales and is one of the most diverse group of fungi. Members of this genus establish ectomycorrhizal associations with members of the genus *Quercus* and *Pinus*. They can be distinguished by the cortina between the cap and the stem especially when young, rusty brown lamellae and spore print and bulbous stipe. The subgenus *Phlegmacium* has characteristic features such as viscid cap especially when young, dry stem, arachnoid cortina and rust-yellow to rust brown spore print. In addition to morphological studies, molecular systematic analysis of the genus *Cortinarius* has provided clarification of the phylogenetic relationships among its species, and it has been possible to measure the taxonomic value of the morphological characters used in this genus (Orton, 1955; Breitenbach and Kränzlin, 2000; Ortega et al., 2008; Stensrud et al., 2014; Itoo et al., 2015). Worldwide, the genus *Cortinarius* is represented by more than 5000 records (Kirk, 2011). Approximate100 records of this genus have been reported from our country and more records are being added gradually with the support of molecular data (Sesli and Denchev, 2014; Akata et al., 2015; Sesli et al., 2015; Sesli et al., 2016; Sesli and Liimatainen, 2018; Sesli, 2018; Kalmer et al., 2019)

Cortinarius rufo-olivaceus has been previously reported in Turkey from Osmaniye province (Isıloglu and Öder, 1995). However, no morphological or

molecular data have been provided for this species. A detailed morphological interpretation is needed for the correct identification of this species. Moreover, molecular data will provide invaluable information to support morphological data and support the identification. Thus, DNA sequences corresponding to internal transcribed spacer region (ITS) and the large subunit (LSU) of nuclear ribosomal RNA genes were analyzed to understand the phylogenetic relationship of this species in *Cortinarius* genus. Although, the existence of *C. rufo-olivaceus* has been reported previously, this study contributes the first documentation of full description of the species and the new locality supported by both morphological and phylogenetic data.

MATERIALS and METHODS

Morphological studies

Mushroom samples were detected in the oak forest during a field trip in Akbelen village of Tokat in autumn 2019. Color photographs of basidiocarps were taken and brought to the laboratory in wrapped paper for microscopic studies. Spore print was obtained from a mature basidiocarp and the samples were dried using a heater. The samples were placed into polyethylene bags and kept in the fungarium of Tokat Gaziosmanpasa University, Department of Biology, for later studies. Microscopic studies were performed on dry samples using some chemicals (such as distillate water, KOH, Congo red, etc.). Comparing the microscopic, macroscopic and ecological features the sample were identified according to the literature such as Bon, 1987; Breitenbach and Kränzlin, 2000; Phillips, 1981; Moser, 1983.

Molecular studies

DNA extraction, PCR amplification and Sequencing

The genomic DNA (gDNA) was extracted form dry samples using GeneMATRIX Plant & Fungi DNA purification kit (EURx, Poland) following manufacturer's protocol. For DNA amplifications, primer pairs ITS4-ITS5 (White et al., 1990) and LROR-LR5 (Vilgalys and Hester, 1990) was used to amplify ITS1-5.8S-ITS2 and 28S LSU rRNA gene regions, respectively. Each polymerase chain reaction (PCR) was performed in 30 µl volume mixture containing 3 µl 10X buffer, 3 µl dNTP mix, 3 µl degenerate primer pair (final concentration of 1 µM each), 0.3 µl Dream Taq DNA polymerase (Thermo), 10 µl gDNA and 7.7 µl sterile ddH_2O. A negative PCR control reaction was prepared in the absence of gDNA, which included sterile ddH_2O instead. ITS region was amplified using a programme containing 5 min initial denaturation at 95ºC followed by 40 cycles of denaturation at 95ºC for 30 sec, annealing at 53ºC for 30 sec and extension at 72ºC for 1 min and a final extension for 10 min. LSU region was amplified using a programme 3 min initial denaturation at 95ºC followed by 40 cycles of denaturation at 95ºC for 30 sec, annealing at 48ºC for 30 sec and extension at 72ºC for 1 min and a final extension for 10 min. PCR products were checked in a 1 % agarose gel electrophoresis and positive PCR products were gel purified by using Wizard SV Gel and PCR Clean-up System (Promega). Purified PCR products were sequenced in both directions using forward and reverse primers (BM Labosis Inc., Ankara).

Sequence and Phylogenetic analysis

Chromatograms for forward and reverse primer sequencing were checked for any nucleotide errors. All assembled rDNA sequences were examined using Basic Local Alignment Search Tool (BLAST) programme using the National Center for Biotechnology Information (NCBI) nucleotide database. For phylogenetic analysis, representative ITS and LSU sequences of *Cortinarius* species were retrieved from GenBank. The multiple sequence alignments and phylogenetic trees for each genomic region were done using Molecular Evolutionary Genetics Analysis software (MEGA 7.0; Kumar et al., 2016). Phylogenetic trees were constructed using the maximum likelihood (ML) and maximum parsimony (MP) methods. ML method was based on Tamura-Nei model (Tamura and Nei, 1993) with bootstrap support of 1000 replicates and default settings. Initial tree(s) for the heuristic search were automatically obtained by using Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and topology with superior log likelihood value was selected. MP trees were constructed using the Tree-Bisection-Reconnection (TBR) search method with 100 random addition replications. The bootstrap support values > 50% were marked on the branches of the tree.

RESULTS and DISCUSSION

Taxonomy

Description of macrofungi, photographs showing the morphological features of basidiocarps and microphotographs of basidia and basidiospores, locality and collection number are given below. The systematics of the macrofungi are in accordance with Index Fungorum (http://www.indexfungorum.org: accessed 14 December 2019).

Fungi

Basidiomycota

Cortinariaceae

Cortinarius rufo-olivaceus (Pers.) Fr., Epicr. Syst. Mycol. 268 (1838).

Pileus 5–10(13) cm across, hemispherical at first, then convex to plane, sometimes depressed, slightly

indented, pink- to purple-red or reddish-copper with paler margin. Flesh thick, whitish, light violet under cap cuticle. Stipe 4–9(10) × 1.2–2.0 cm, cylindrical, solid, pale violaceous, emarginated by a wine-red veil, bulbous, base with a purple-brown to wine-brown. Lamellae broadly attached, greenish to lemon-yellow when young then olivaceous-rusty, finally dark rusty, dense, fragile, narrow. Basidiospores (9-)11.5–13.5(-14) × (5.5-)6–7.5(-8) µm, lemon-shaped or almond-shaped. Basidia clavate, 40–45 × 10–12 µm, tetrasterigmatic and a basal clamp. Marginal cells cylindrical to clavate, 13–20 × 3–8 µm; no pleurocystidia seen (Figure 1). Spore print rust. KOH reaction green, then slowly red on cap cuticle, dark green at first, later dark red-brown on the flesh. Habitat under broadleaved trees, especially *Fagus* and *Quercus*. Growing season spring and autumn (Phillips, 1981; Moser, 1983; Bon, 1987; Breitenbach and Kränzlin, 2000; Garrido-Benavent et al., 2015; Mazza 2019).

Specimen examined: Tokat, central district, Akbelen village, among leaf litters in *Quercus* sp. forest, 40°27'498"N- 36°39'226"E, 1022 m, 15.12.2019, HIS 19.

Cortinarius rufo-olivaceus var. *vinosus* (Cooke) species has been found in Barcelona (Spain) (Garrido-Benavent et al., 2015). The morphological features of this species resemble our sample of *Cortinarius rufo-olivaceus* (Pers.) Fr. such that they have similar spore size, red to purplish pileus. However, we found that *C. rufo-olivaceus* (Pers.) Fr. has greenish to lemon yellow lamellae and not purplish yellow.

Morphologically, *Cortinarius prasinus* (Schaeff.) Fr. and *C. cupreorufus* Brandrud also resemble *C. rufo-olivaceus*. However, *C. prasinus* has a smaller basidiocarp, greenish yellow lamellae, slightly smaller spores (9–11,5 × 6–7) when compared to that of *C. rufo-olivaceus*. Additionally, *C. prasinus* cap colour is hazel or ferruginous to reddish tawny at the centre and greenish yellow at the margin. KOH reaction shows dark red color on the cuticle (Garrido-Benavent et al., 2015). *Cortinarius cupreorufus* has yellowish bulbous stipe and copper-brown pileus. But, *C. cupreorufus* grows mainly in coniferous forests, usually near *Picea* (Breitenbach and Kränzlin, 2000). These differences clearly distinguish *Cortinarius rufo-olivaceus* (Pers.) Fr. from other species that are morphologically similar.

Molecular Phylogeny

Cortinarius rufo-olivaceus (Pers.) Fr. was first identified in 1995 by Işıloğlu and Öder (1995) in Osmaniye region of Turkey. However, this study did not examine the macro- micro morphological properties of this fungus species. Additionally, no molecular study has been conducted to evaluate the molecular phylogeny of this species.

In this study, an approximately 688 bp long region for ITS1-5.8S-ITS2 and 948 bp long region for the 28S LSU rRNA gene were amplified and both sequences were deposited at GenBank under the accession numbers MN814239 and MN889515, respectively. We obtained the ITS genomic region sequence data for 53 species that were representatives of the Rufoolivacei clade and constructed both MP and ML trees using *C. aureifolius* as the outgroup species. Since similar topologies were observed, only ML tree was given to indicate phylogenetic relationship of the studied species based on ITS region (Figure 2). The *Cortinarius* species were distributed in separate phylogenetic units. Previously identified sequences belonging to *Cortinarius rufo-olivaceus* (Pers.) Fr. species were well conserved with a bootstrap support of 99 % and our sequence was included among them. The tree also separated *C. prasinus* and *C. rufo-olivaceus* with a bootstrap support of 76 %. There are few representative LSU sequences in the Rufoolivacei clade and no significant matches were observed from our BLAST results. Phylogenetic relationship based on LSU region was not well resolved due to low bootstrap values. Thus, no phylogenetic separation at the subgenus level was observed for LSU region.

As mentioned above, morphological similarities exist within *Cortinarius* species that could support their close relationship. However, microscopic variations also in congruent with our molecular data and well separate members of the Rufoolivacei clade. Our ITS tree provides further detail on the evolutionary relationship of most studied species that belong to section Fulvi and phylogenetic data mostly agree with other studies (Froslev et al., 2007; Garnica et al., 2009).

CONCLUSIONS

The genus *Cortinarius* is the largest genus of Agaricales and systematic studies could be overwhelming. Integrating macro- and micromorphological characters as well as molecular phylogenetic analysis are important to well characterize a species within this genus. Molecular analyses are very helpful in determining the relationship in closely related species. As more nuclear rDNA sequences are available, it is more possible to solve species delimitation and identification in the *Cortinarius* genus. In this study, we not only used ITS region but also LSU sequence to confer molecular identification of a *Cortinarius* species in Turkey. Accordingly, the identified species was found to be a *Cortinarius rufo-olivaceus* (Pers.) Fr. and supported by morphological results. Our finding contributes significantly to the *Cortinarius* mycobiota of Turkey.

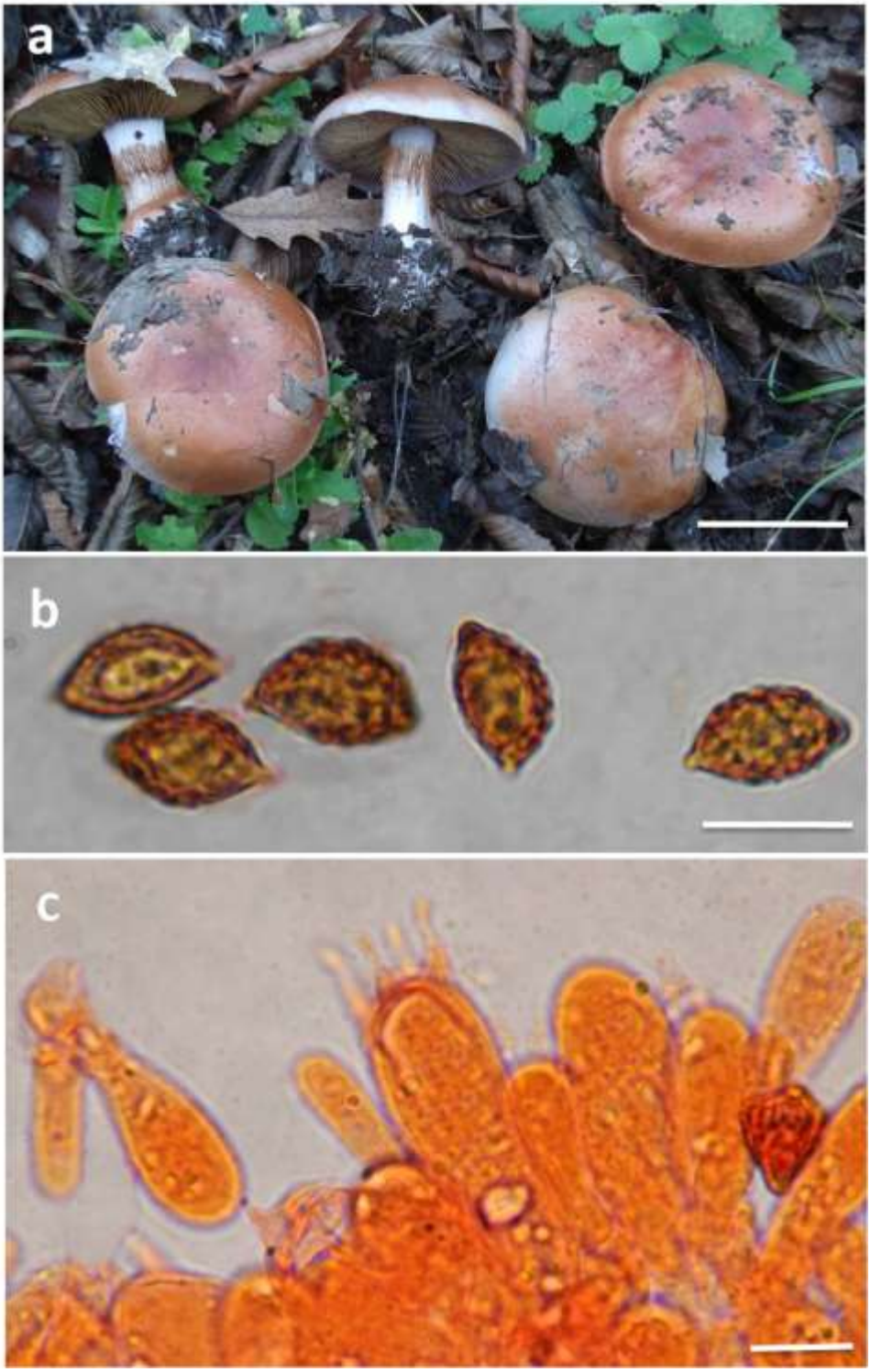

Figure 1. *Cortinarius rufo-olivaceus*: a-basidioma; b- basidiospores (in congo red); c- basidium and marginal cells (in congo red) (scale bars: a= 70 mm; b, c = 10 µm).

Şekil 1. Cortinarius rufo-olivaceus: a-bazidiokarplar; b- bazidiosporlar (kongo kırmızısı ortamında); c- bazidium ve marjinal hücreler (kongo kırmızısı ortamında) (ölçek çubuğu: a= 70 mm; b, c = 10 µm).

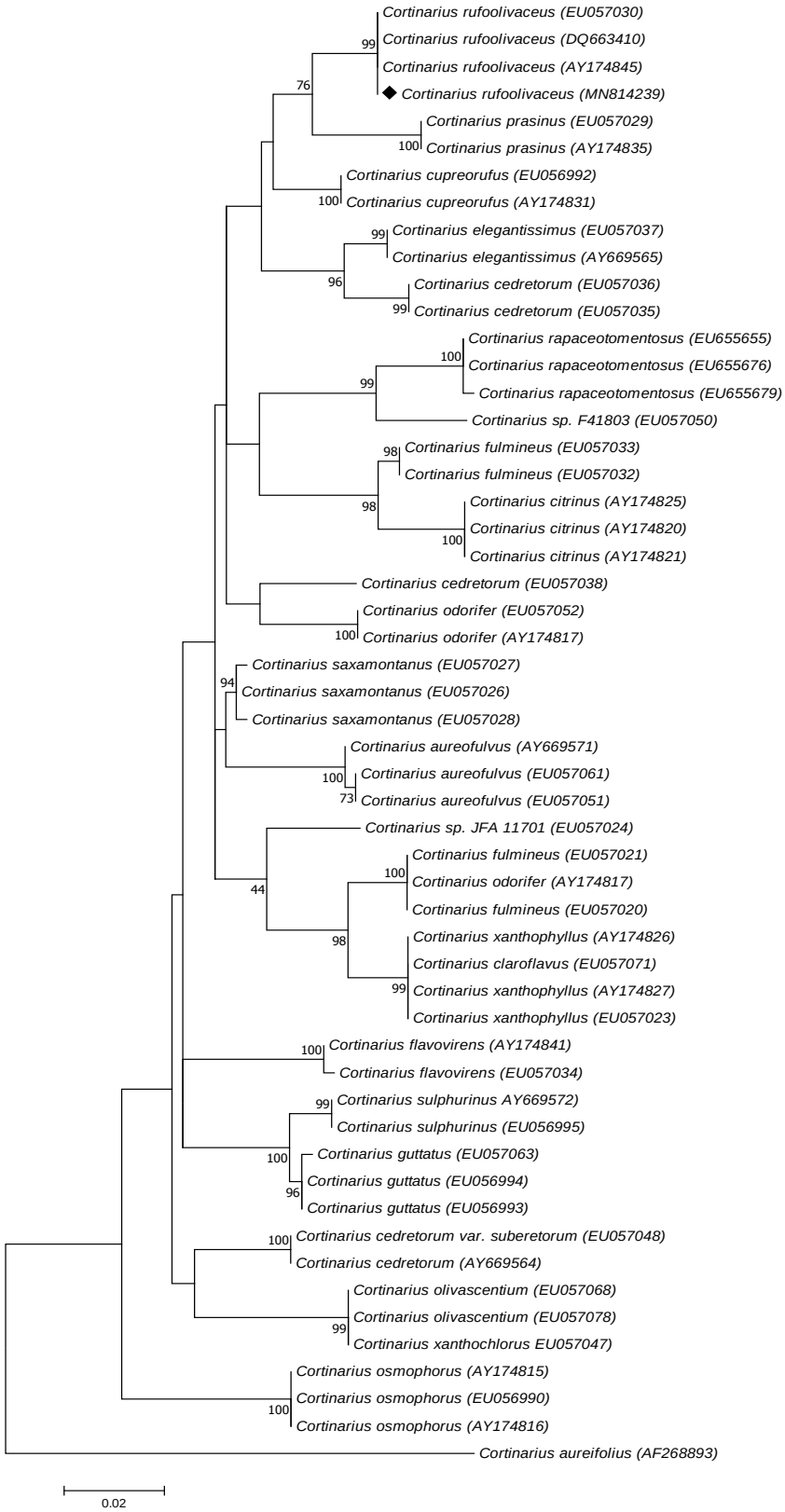

Figure 2. Phylogenetic relationship of *Cortinarius* species inferred from ITS genomic region using ML method. Diamond label indicate the Turkish *Cortinarius rufo-olivaceus* (Pers.) Fr identified in this study. *Cortinarius aureifolius* was used as the outgroup species. Bootstrap support values ≥ 50% from ML analysis were shown on the branches. Bar indicates 0.02 expected change per site per branch.

Şekil 2. ML yöntemi kullanarak Cortinarius türleri arasında ITS genomik bölgeden çıkarılan filogenetik ilişki. Elmas ile gösterilen simge, bu çalışmada belirlenen Türk Cortinarius rufo-olivaceus (Pers.) Fr'yi göstermektedir. ML analizlerinde, ≥ %50 bootstrap destek değerleri olanlar dalların üstünde gösterilmiştir. 0.02 çizgisi, her daldaki her noktaya ait tahmini değişimi ifade etmektedir.

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Statement of Conflict of Interest

Authors have declared no conflict of interest.

Author's Contributions

The contribution of the authors is equal.

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