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Phylogenetic and Taxonomic Studies on *Cortinarius caerulescens* (Schaeff.) Fr. a New Record for Turkish Mycota

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Abstract: *Cortinarius caerulescens* (Schaeff.) Fr. was given as a new record for the Turkish macromycota from Şemdinli district of Hakkari province, Turkey. Short description of the newly reported species was given together with its photographs related to macro and micromorphologies and described briefly. In addition to macro/micro characters, DNA sequences of nrDNA ITS (Internal Transcribed spacer) and LSU (D1/D2, Large subunit) regions were used to support the recognition of the studied specimen as a new record.

Key words: *Cortinarius*, fungal phylogeny, ITS, LSU, new record

Türkiye Mikotası İçin Yeni Bir Kayıt Olan *Cortinarius caerulescens* (Schaeff.) Fr. Üzerinde Filogenetik ve Taksonomik Çalışmalar

Öz: Bu çalışmada Hakkari ilinin Şemdinli ilçesinden *Cortinarius caerulescens* (Schaeff.) Fr. türü Türkiye makromikotası için yeni kayıt olarak verilmiştir. Türün kısa tarifi, makro ve mikromorfolojiyle ilgili fotoğraflarıyla birlikte verilip kısaca açıklanmıştır. Makro/mikro karakterlere ek olarak, incelenen örneğin yeni bir kayıt olarak tanınmasını desteklemek için nrDNA ITS (Transkripsiyonu yapılabilen aralayıcı bölgeler) ve LSU (D1 / D2, Büyük alt birim) bölgelerinin DNA dizileri kullanılmıştır.

Anahtar kelimeler: *Cortinarius*, fungal filogeni, ITS, LSU, yeni kayıt

Introduction

Cortinarius (Pers.) Gray is a species-rich and morphologically challenging fungal genus of family Cortinariaceae within the order Agaricales. The genus is an ecologically important macrofungus due to ectomycorrhizal associations with a large range of forest trees (Stefani et al., 2014; Garnica et al., 2016). A considerable number of the species has distribution in the temperate areas of the Southern Hemisphere (Brandrud et al., 1990-2018). About 5000 published *Cortinarius* names are observed in Index Fungorum (CABI Bioscience Databases, http://www.indexfungorum.org) and 116 of them have only been identified up to now in Turkey, (Sesli and Denchev, 2014; Akata et al., 2015; Güngör et al., 2015; Sesli and Moreau, 2015; Sesli et al., 2015; Sesli et al., 2016; Sesli, 2018; Sesli and Liimatainen, 2018).

The main problem within the genus is the appearance of different classification system particularly at the infrageneric level. For instance, Moser and Horak (1975) recognized the subgenera *Myxacium*, *Telamonia*, *Leprocybe*, *Phlegmacium*, *Dermocybe*, *Icterinula*,

Sericeocybe, *Cystogenes*, and *Paramyxacium* whereas Moser (1983) added *Cortinarius* as subgenera and regarded *Dermocybe* as a separate genus nearly one decade later. Bidaud et al. (1994) divided the genus into the six different subgenera while several authors proposed four subgenera based on macroscopic features (Knudsen and Vesterholt, 2012; Niskanen and Kytövuori, 2012; Brandrud et al., 1990-2018). Therefore, in the present study we wanted to use not only morphological characters but also DNA sequences of two different regions to describe species and indicate taxonomic position within the genus reliably.

One of the most recognizable features of the genus is the presence of cortina that is found between the pileus and the stipe, and cinnamon brown to rusty brown spore print (Arora, 1986; Kirk et al., 2008; Uzun et al., 2013). The basidiomes of *Cortinarius* species demonstrate a remarkable variety of forms and colours (Garnica et al., 2005; Stensrud et al., 2014). Macroscopic features referring to the consistency of both pileus and stipe surface are considered as crucial characters to decide the boundaries of major divisions in *Cortinarius* (Garnica et al., 2005). The base of the stem is another valuable character for identification of *Cortinarius*. It may be more or less equal, clavate or swollen dramatically at the base that cause a rim on the basal bulb. As microscopic features, spore shape, size and the degree of ornamentation (smooth to strongly verrucose) appear useful to circumscribe clades and identification. Cystidia are almost never heard for *Cortinarius* species except *C. violaceus* which has distinct cystidia (Kuo, 2011). Interpretations of morphological characters often varied among mycologists and resulted in disagreements. Therefore, the application of the morphological species concept has led to very different results in the same groups (Brandrud et al., 1990-2018). For instance, there are too many synonymous for *Cortinarius caerulescens* (Schaeff.) Fr. such as *C. coerulescens* (Schaeff.) Fr., *Agaricus caerulescens* Schaeff., *C. cyanus var. caerulescens* (Schaeff.) Gray (Index fungorum, CABI Bioscience Databases, http://www.indexfungorum.org). *Cortinarius caerulescens* locating in the subg. *Phlegmacium* grows in woodland, mainly under *Fagus* trees in late summer and autumn. This species is not edible and characterized by a striking blue-violet cap that turns brown in the center as it matures, amygdaloid-verrucose and rusty brown spores (Breitenbach and Kränzlin, 2000; Knudsen and Vesterholt, 2008).

Recent molecular studies (Frøslev et al., 2007; Niskanen et al., 2013; Garnica et al., 2016) indicate that there are a number of cryptic species in *Cortinarius* genus and this situation causes difficulties in the species identification within the genus when only morphological and ecological data were used. Molecular data may provide invaluable information to identify macrofungus correctly so rDNA ITS (ITS1-5.8S-ITS2) and nLSU (28S nuclear ribosomal large subunit rRNA) gene regions were used in the current study in addition to macroscopic/microscopic characters. Garnica et al. (2005; 2011) and Frøslev et al. (2007) concluded that the ITS region seems to be an appropriate marker for species level identification in *Cortinarius*. The suitability of ITS region has been indicated in many studies and the region has been proposed as barcode region for *Cortinarius* (Peintner et al. 2003; Ortega et al. 2008; Garnica et al. 2009; Garnica et al. 2011; Stefani et al. 2014; Garnica et al., 2016). In addition to the ITS region, the LSU gene located immediately downstream of the ITS was also analyzed to get more reliable results and compare usefulness of the regions. Whole length of the region was not used due to expectation of less nucleotide variations among sequences. Two hypervariable domains (D1 and D2) flanked by relatively conserved regions in most fungi were amplified and sequenced as indicated by several studies (Moncalvo et al., 2000; Peintner et al., 2004).

The purpose of this study is to describe a new record species of *Cortinarius* for Turkey based on ITS region including the gene coding the 5.8S ribosomal subunit and the D1-D2 regions of LSU.

Material and Method

Taxon sampling and morphological studies

The macrofungus samples were collected from Şemdinli district, Hakkari province of Turkey. Collected samples were deposited in the Fungarium of Van Yüzüncü Yıl University (VANF). During field work, specimens were photographed in situ using with a Canon (EOS 60D) camera equipped with Tokina 100 mm macro lens. Macroscopic characters (pileus, stipe, lamellae and cortina) were recorded using fresh materials. Microscopic characters (basidia, basidiospores and marginal cells) were observed in distilled water and 3% KOH solution under a Leica EZ4 stereo microscope while sections were examined under a Leica DM500 research microscope. Microscopic characters were measured with the Leica Application Suite (version 3.2.0) programme and described based on different studies (Ortega and Mahiques, 2002; Breitenbach and Kränzlin, 2000; Soop, 2014).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from dried basidiomata using the CTAB method with minor modifications (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. DNA amplification 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 (D1-D2) regions, primer pairs N-nc18S10 5'AGGAGAAGTCGTAACAAG3'/C26A 5'GTTTCTTTTCCTCCGCT3' (Wen et al., 1996) and LR0R 5'ACCCGCTGAACTTAAGC3'/LR5 5'TCCTGAGGGAAACTTCG3' (Vilgalys and Hester, 1990) were used, respectively. PCR products were run in a 1.0 % agarose gel and visualized by staining with Gelred dye. Positive reactions were sequenced with forward and reverse PCR primers using ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA).

Sequence alignment and phylogenetic analysis

Forward and reverse sequences were assembled and edited using Alibee Multiple Alignment 3.0 software from the GeneBee website (www.genebee.msu.su/genebee.html). Ambiguous sites were checked manually and corrected by comparing the strands. One sequence of each region generated from the present study and additional sequences retrieved from NCBI were analyzed together to see phylogenetic relationships among *Cortinarius* species in the constructed tree. The sequences downloaded from NCBI were selected considering results of BLAST searches and several valuable studies (Garnica et al. 2005; Stensrud et al. 2014). *Hebeloma mesophaeum* and *H. subtortum* were chosen as outgroup taxa and these sequences were obtained from another our study that has not been published yet. All sequences were aligned with the aid of the program ClustalW (Thompson et al., 1994) and adjusted manually where it was necessary.

Prior to construction of phylogenetic tree, total nucleotide length (bp) and variable sites were calculated using Molecular Evolutionary Genetics Analysis software (MEGA 6.0; Tamura et al., 2013). Phylogenetic tree of each studied region (ITS and LSU) was constructed using three different methods; Maximum Likelihood (ML), Maximum Parsimony (MP) and Neighbor Joining. The sequence data was analyzed by using the Maximum Likelihood (ML) method based on the Tamura-Nei model (Tamura and Nei, 1993). To test branch support, bootstrap analysis was used with 500 replicates (Felsenstein, 1985). In the ML 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 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

Macroscopic and microscopic characters

Pileus: 50-100 mm, hemispherical when young, later convex to plane and somewhat indented in the center, surface silky-dull when dry, slimy and shiny when moist, blue-violet, later discoloring gray-ocher to pale ocher, covered with dingy white fugacious velar floccus when young, margin incurved and connecting to the stipe by a white-violet filamentous cortina when young. **Flesh:** light blue, thick in the center of the pileus, thin toward to margin. **Lamellae:** blue-violet when young, later gray-violet to ocher-brown. **Stipe:** 40-70 × 10-20 mm, cylindrical, base with a marginate bulb up to 45 mm, surface gray-violet and longitudinally fibrillose when young, later glabrescent. **Spores:** 8.8-11.5 × 5-6.5 µm, amygdaliform, weakly to moderately verrucose, yellow-brown. **Basidia:** 30-43 × 10-12 µm, clavate, with 4 sterigmata and a basal clamp. **Marginal cells:** 12-15 × 5-8 µm, basidiole-like. **Pleurocystidia:** not seen. **Hyphae:** 2-9 µm, yellow, some septa with clamp (Figure 1). **Ecology:** Solitary to gregarious in montane hardwood forest. Under *Fagus* sp., Hakkari, Şemdinli, Durak village, 37° 24'210"N - 44° 30'661"E, 1640 m, 24.10.2014, Acar 471.

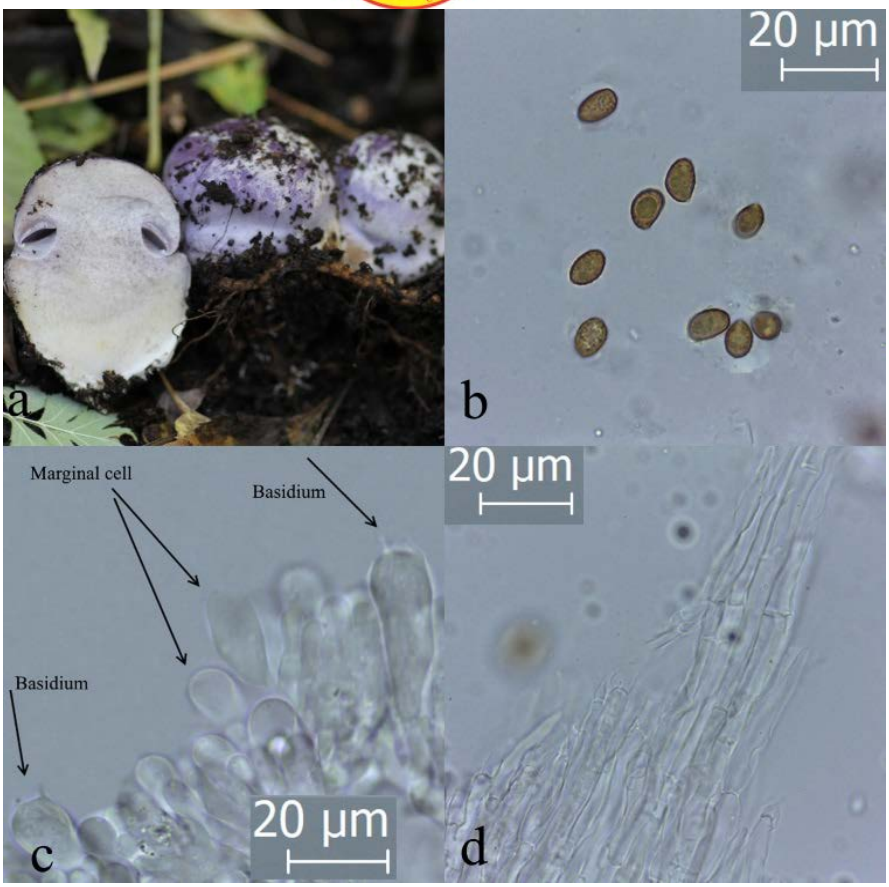

Figure 1. Macroscopic and Microscopic characters of *Cortinarius caerulescens* a. Basidiocarps b. Basidiospores (dH_2O) c. Basidium and marginal cells (%3 KOH) d. Hyphae (dH_2O)

Molecular phylogeny

Accession numbers for sequences of ITS and LSU gene regions were assigned as MH718791 and MH718792, respectively. The amplified DNA fragment of the ITS region was approximately 650 bp length encompassing complete ITS1, 5.8S and ITS2 subregions. ITS data matrix comprised a total of 34 sequences including 33 from NCBI. The aligned data included a total of 675 positions, of which 481 were conserved, and 177 were variable (91 variable sites in ITS1, 2 in 5.8S and 84 in ITS2 subregion) nucleotides. The second region, LSU, comprised 23 sequences (22 from NCBI) and yielded the total lengths of 871 nucleotides with 54 nucleotide variations. The results received from ITS region were more informative compared to outcomes of LSU because of higher nucleotide variation number. LSU region is generally less variable than the ITS region and this situation may limit taxonomic resolution at the species levels and diversity analysis.

The Maximum Likelihood analysis resulted in similar phylogenetic topologies with Maximum Parsimony and Neighbor Joining analyses so only ML tree was given to indicate phylogenetic relationships and taxonomic position of studied species and discussed. The trees constructed based on ITS and LSU regions showed no phylogenetic separation at the subgenus or section levels (Figure 2 and Figure 3).

The studied sample, *Cortinarius caerulescens,* grouped with only one of its representatives (AF389134) and several retrieved samples of *C. terpsichores* with high bootstrap value (100%) in the ITS tree (Figure 2). Intentionally, lots of *C. caerulescens* sequences were downloaded from NCBI to increase genetic diversity and figure out taxonomic position within the genus. Unexpectedly, all representatives but only one (AF389134) located in different clusters of the tree. The LSU tree showed close relationship between studied specimen and *C. terpsichores* as well (Figure 3). *Cortinarius caerulescens* and *C. terpsichores* can be distinguished from each other based on macroscopic/microscopic and ecologic characters even though phylogenetically located closely. For instance, *C. caerulescens* is generally found under *Fagus* whereas *C.*

terpsichores associated with primarily *Quercus* trees. Macroscopically, *C. caerulescens* has a dark blue violet cap whereas *C. terpsichores* has a light blue cap. Microscopically, they have different ornamented spores; *C. caerulescens* has amygdaloid and verrucose spores while the other has ellipsoid ones.

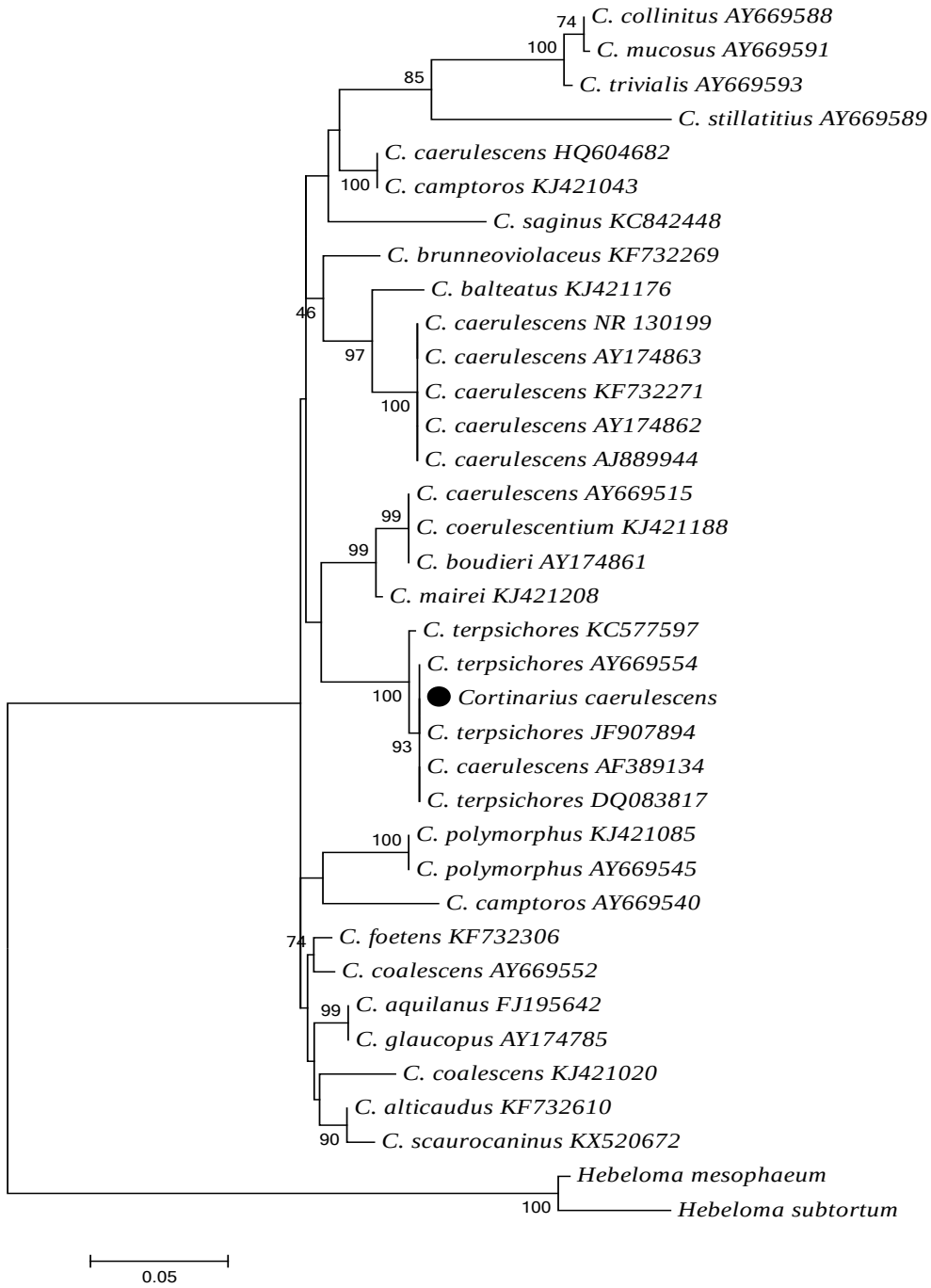

Figure 2. Phylogenetic tree of *Cortinarius* species based on ML analysis of the ITS region. Black circle indicates studied specimen. *Hebeloma subtortum* and *H. mesophaeum* were used as outgroups. Bootstrap analysis of ML was based on 500 replicates and values higher than 40% were indicated on branches.

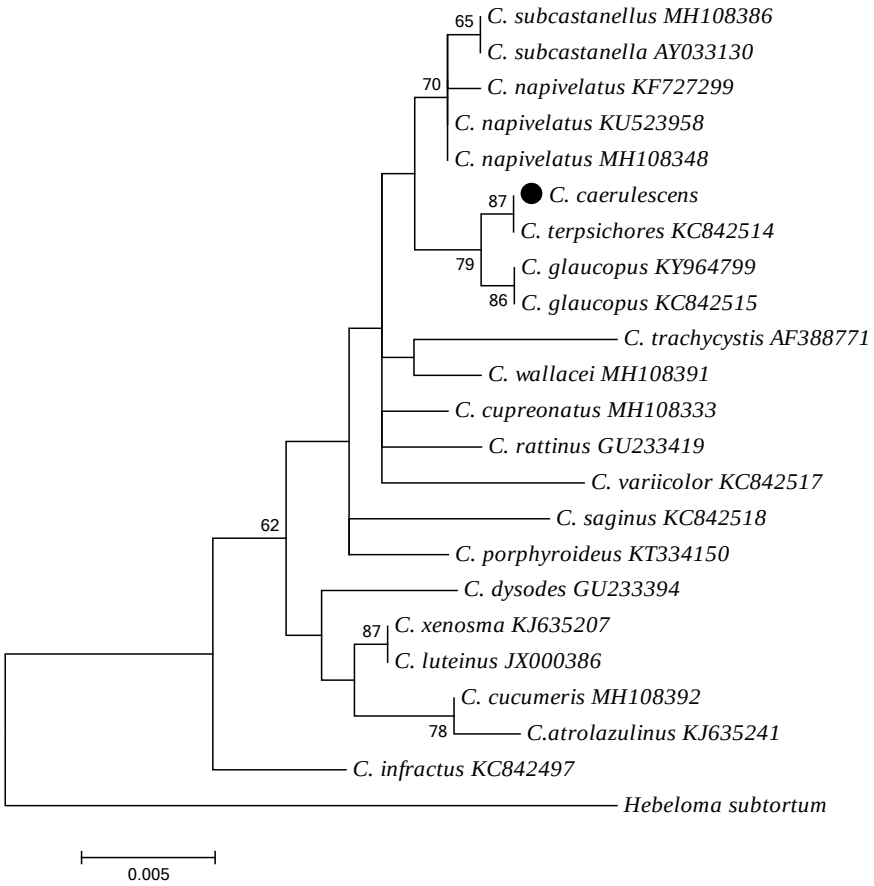

Figure 3. Phylogenetic tree of *Cortinarius* species based on ML analysis of the LSU region. Black circle indicates studied specimen. *Hebeloma subtortum* was used as outgroup. Bootstrap analysis of ML was based on 500 replicates and values higher than 40% were indicated on branches

Discussion

Species delimitation within the *Cortinarius* genus is debatable due to high level of homoplasy and phenotypic plasticity for morphological and ecological characters. This circumstance causes poor resolution power while dividing the genus into subgenera and lower taxonomical levels. The delimitation of species based on both ITS and LSU sequences may not be enough to determine boundaries of *Cortinarius* so not only DNA sequences but also morphological features must be used to resolve boundaries of *Cortinarus* species correctly. The tree constructed based on DNA sequence of ITS region was more informative than the tree constructed by DNA sequence of LSU region due to presence of more nucleotide variation in ITS region. Similar situation was also proved by several researchers (Garnica et al., 2005; Schoch et al., 2012)

Cortinarius caerulescens belongs to subgenus *Phlegmacium*, and this subgenus is proved to be polyphyletic by several studies (Peintner et al., 2004; Garnica et al., 2005; Liimatainen et al., 2014). Our results supported this phenomenon; some species belonging different subgenus located within samples of subgenus *Phlegmacium* in the both trees. Some *Cortinarius caerulescens* samples downloaded from NCBI located distantly to each other in the ITS tree and our sample grouped with only one of representatives (AF389134). According to Garnica et al. (2016), *Cortinarius* is a complex genus and has many cryptic species which need both molecular and morphological data for correct identification. *Cortinarius caerulescens* is one of the complex species that needs detailed morphological and molecular analyses for reliable identification. So, we used both morphological and molecular data for correct identification of the species.

Interestingly, *C. caerulescens* and *C. terpsichores* grouped together in both ITS and LSU trees with 100% and 87% bootstrap values, respectively. Formerly, *Cortinarius caerulescens* sensu Marchand, Moser, NCL (1960) was accepted as synonym with *C. terpsichores* but the sample used for comparison was not holotype (*Cortinarius caerulescens* (Schaeff.) Fr. 1838 is holotype) one so these two species are not accepted as synonymous and showed red line in Index Fungorum database. These two species have different morphological and ecological properties. *Cortinarius caerulescens* grows in broad leaf forest (primarily *Fagus*) whereas *C. terpsichores* grows primarily with *Quercus* (sometimes also with *Pinus*). Macroscopically, *Cortinarius caerulescens* has a dark blue violet cap whereas *C. terpsichores* has a light blue cap. Microscopically, they have different ornamented spores; *C. caerulescens* has amygdaloid and verrucose spores and the other has ellipsoid ones. Furthermore, *C. caerulescens* has a thick a whitish filamentous cortina forming whitish remnants at bulb margin of young species.

We tried to mention the importance of not only molecular data but also morphological one considering the enormous diversities and cryptic species within the genus *Cortinarius*. Especially, close phylogenetic relationship between *C. caerulescens* and *C. terpsichores* proved that only molecular or morphological data may not be enough to determine species and resolve phylogenetic relationship within the tree.

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