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Two New Records from Türkiye with Morphological and Molecular Evaluations: *Gymnopus aquosus* and *Inocybe subporospora*

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Abstract: *Gymnopus aquosus* (Bull.) Antonin & Noordel and *Inocybe subporospora* Kuyper were recorded for the first time in Türkiye, among the fungal specimens collected during field studies in Kütahya. The specimens were identified based on morphological, microscopic features and analysis of ITS gene sequences. The habitats and characteristic features of the species are briefly explained and relevant photographs are presented. GenBank accession numbers were assigned to the samples in this study and phylogenetic trees were constructed to compare them with similar taxa. *Gymnopus aquosus* is mainly characterized by and distinguishes it from related species; have an almost to the center translucently striate, pileus center pale yellow to ochre, pallescent to almost white towards to margin and a distinctly bulbous base with pinkish-chromic rhizomorphs. *Inocybe subporospora*; have spores with an indistinct germ-pore, red-brown stipe and dark grayish brown pileus with rimulose margin is a very characteristic species that can be distinguished from its close species with its features.

Key words: Biodiversity, New records, Taxonomy, ITS gene, Phylogenetic

Morfolojik ve Moleküler Değerlendirmelerle Türkiye'den İki Yeni Kayıt: *Gymnopus aquosus* ve *Inocybe subporospora*

Öz: Kütahya ilinde yapılan arazi çalışmaları sırasında toplanan mantar örneklerinden *Gymnopus aquosus* (Bull.) Antonin & Noordel ve *Inocybe subporospora* Kuyper Türkiye'de ilk kez kaydedilmiştir. Örnekler, morfolojik, mikroskobik özelliklere ve ITS gen dizilerinin analizine göre teşhis edilmiştir. Türün habitatları ve karakteristik özellikleri kısaca açıklanmış ve ilgili fotoğraflar sunulmuştur. *Gymnopus aquosus*'u benzer türlerden ayıran özellikler; ortası soluk sarıdan koyu sarıya değişen renkte fakat kenarlara doğru beyaz renkte olan, neredeyse merkeze kadar yarı saydam çizgili bir şapkaya ve pembemsi-kromik ipliksi doku uzantılı belirgin soğansı bir sap tabanına sahip olmasıdır. *Inocybe subporospora*; belirgin olmayan çimlenme açıklığına sahip sporlar, kırmızı-kahverengi sap ve kenarları küçük çatlaklara sahip koyu grimsi kahverengi şapka özellikleriyle yakın türlerinden ayırt edilebilen oldukça karakteristik bir türdür.

Anahtar kelimeler: Biyoçeşitlilik, Yeni kayıt, Taksonomi, ITS gen, Filogenetik

Introduction

It has been determined that more than 2700 macrofungus species exist in Turkey, whose climatic conditions are suitable for macrofungal diversity (Sesli et al., 2020). There are few macrofungal taxonomy studies regarding the research area, Kütahya (Doğan & Karadelev, 2006; Doğan et al., 2011; Allı et al., 2017; Altuntaş et al., 2021). With this study, two new taxa were identified from Kütahya as new records for the Turkish macromycota.

The members of *Gymnopus* are mainly characterized by basidiomata, usually collybioid, marasmioid, and gymnopoid, which is a white spore print with smooth basidiospores that are commonly ellipsoid to oblong. The genus *Gymnopus* (Pers.) Roussel is known to contain approximately 300 species (Hu et. al. 2022; Index fungorum, 2024). The member of the genus, *G. aquosus*, is common throughout Europe (Antonin et al., 2013). In the current checklist of Türkiye fungi, it was reported that there were 55 taxa in the genus *Gymnopus* (Sesli et al., 2020). A study conducted subsequent to the publication of the checklist resulted in the addition of a new species to the genus Gymnopus in Türkiye, thereby increasing the number of taxa to 56. (Sesli, 2022).

Members of the genus *Inocybe* are generally characterized by the nonglutinous pileus, brownish mature lamellae, distinctive odors, pigmented basidiospores with a smooth exosporium and lack of or a indistinct germ pore, presence of pleurocystidia, and occurrence on soil (Matheny et. al. 2020). This genus has 1050 species described in the world (Matheny and Kudzma, 2019; Index fungorum, 2024).

According to the checklist of the fungal taxa in Türkiye, there are 211 taxa in our country. (Sesli et al., 2020). In recent years, studies on the *Inocybe* genus have intensified in our country (Akata et. al., 2023), and even two new species have been introduced to the world (Kaygusuz et. al., 2022a; Kaygusuz et. al., 2022b). With these latest studies, the number of taxa belonging to the *Inocybe* genus has increased to 215.

Materials and Methods

In recent years, some challenges associated with the identification and taxonomy of fungi have been addressed through molecular analyses. Given that some members of the genus *Gymnopus* are morphotaxonomically very similar to each other, and phylogenetic studies are recommended for species identification (Çöl et.al. 2017). In most instances, results from molecular analyses furnish researchers with reliable and valuable information for identification. Consequently, in this study, both molecular and phenotypic methods were employed to identify the macrofungi specimens collected from Kütahya province.

Field and Laboratory Studies

Macrofungi specimens were collected from Kütahya province during routine field trips conducted from 2011 to 2013. Kütahya province, has a transitional climate that blends the Aegean, Mediterranean and Central Anatolian regions. This province has boast rich vegetation, including forests consisting of *Pinus* L., *Quercus* L., *Cedrus* Trew and *Castanea* Mill. The climatic and floral conditions of the region positively affect macrofungal diversity.

During the field study, the morphological and ecological characteristics of the samples were recorded and photographed. Coordinate and altitude information of the area where they were collected was noted. After their smell and taste were examined and noted then the samples were transferred to the laboratory at Muğla Sıtkı Koçman University.

The spore prints and microscopic properties of the samples brought to the laboratory were determined using a light microscope Leica DM750) and the samples were dried in the drying cabinet. The specimens were morphologically identified using macroscopic, microscopic, and ecological features with the help of Kuyper (1986), Antonin and Noordeloos (2010), Bellù (2012), Jacobsson & Larsson (2012), and Antonin et al. (2013).

The identified specimens are deposited at the Fungarium of Muğla Sıtkı Koçman University (MSKU).

Molecular Methods

Genomic DNA was isolated from 0.02-0.1 g mushroom specimens using the Qiagen Plant Mini Kit. PCR amplification was conducted using the primers ITS1F (Gardes & Bruns, 1993) and ITS4 (White et al., 1990). The PCR mix included 2 µl of DNA template (50-100ng), 5 µl of 10X buffer for Taq DNA polymerase, 4 µl of MgCl2 (25mM), 1 µl of dNTP mix (10mM), 1 µl of each primer (10 pmole each), 1 µl Taq polymerase (0.5 unit), and distilled water. The PCR program consisted of an initial denaturation at 95 ºC for 3 minutes, followed by 35 cycles of denaturation at 95 ºC for 1 minute, annealing at 52 ºC for 1 minute, and extension at 72 ºC for 2 minutes. An additional extension of 10 minutes at 72 ºC was performed before storage at 4 ºC. The success of PCR was confirmed by agarose gel electrophoresis on a 1% agarose gel stained with ethidium bromide and visualized using a gel imager (DNR Bio miniBis Pro, Israel).

The PCR products were purified using Fermentas PCR Purification Kit and sequenced by Macrogen (Holland) using the same primers. The sequences were edited using BioEdit program (Hall, 1999) to obtain the

full contig sequence. Phylogenetic analysis was performed using Mega 6 program (Tamura et al., 2013).

The evolutionary history was inferred using the Neighbor-Joining (Saitou & Nei, 1987), Maximum Likelihood (Tamura & Nei, 1993), and Minimum Evolution (Rzhetsky & Nei, 1992) methods. In Neighbor-Joining and Minimum Evolution methods (Nei & Kumar, 2000; Felsenstein, 1985), Evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are expressed in the units of the number of base substitutions per site. The Minimum Evolution tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei & Kumar, 2000) at a search level of 1. The Neighbor-Joining algorithm (Saitou & Nei, 1987) was used to generate the initial tree.

The Maximum Likelihood method was conducted based on the Tamura-Nei model (Tamura & Nei, 1993). 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 selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

The sequences have been deposited in GenBank.

Results

Identification and Taxonomy of the macrofungi species

Basidiomycota
Agaricomycetes
Agaricales
Omphalotaceae
Gymnopus aquosus (Bull.) Antonin & Noordel.

Pileus 25-60 mm, convex to plane, when moist pale yellowish to ochre, pallescent on drying to almost white, strongly hygrophanous, translucently striate, smooth, glabrous, marginal zone often undulating with age. Gills adnate-emarginate, medium spaced, white to pale cream, edges pruinose. Stem 20-80 × 2-4 mm, cylindrical, smooth, glabrous, concolorous with or paler than cap. Odor fungoid, taste mild. Spores 4.8-7 × 2.9-3.6 µm, ellipsoid to oblong or dacryoid. Basidia, 18 – 27 × 5 – 8 µm, 4 spored.

Gymnopus aquosus is a widespread species found throughout Europe This species starts to appear in late April (Antonin et al., 2013).

Specimens Examined: Türkiye, Kütahya – Afyonkarahisar highway 30th kilometer, Altıntaş, Pusan Memory Forest, 39°09'49.1"N and 30°08'38.6"E, 1071m, *Quercus* sp. collected on 04.06.2011, Allı 3651; Gediz, *Pinus nigra* forest, collected on 25.05.2012, Allı 4198.

GenBank number for ITS sequence: KR264910.1

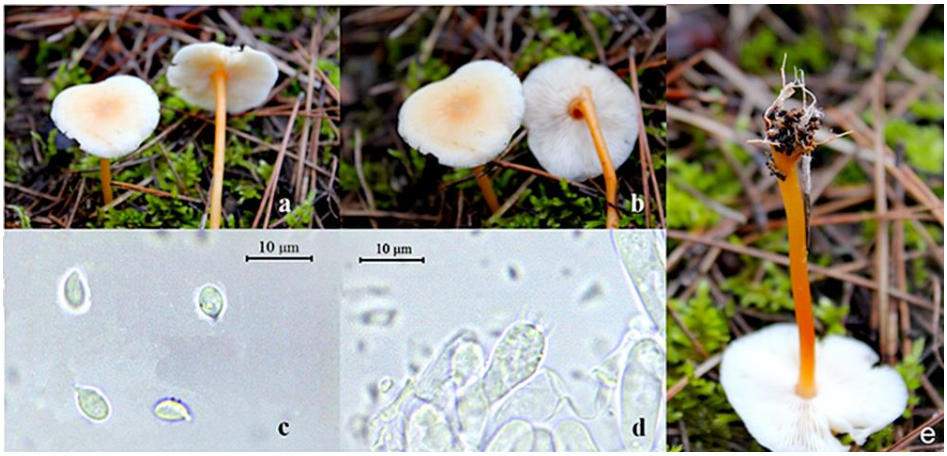

Figure 1. *Gymnopus aquosus*a, b, e. basidiocarp-s, c. basidiospores, d. basidium

Basidiomycota
Agaricomycetes
Agaricales
Inocybaceae
Inocybe subporospora Kuyper

Pileus 8-40 mm, convex to plane, with or without umbo, fibrillose tomentose, later breaking up to become finely scaly, not rimulose at the margin, dark red brown or brown. Gills narrowly adnate, pale brown to brown, edge fimbriate, whitish to concolorous. Stem 20 – 45 × 4 -7 mm, red brown, but at first covered with white fibrils, pruinose in the upper part, cortina present in very young specimens. Odor and taste spermatic. Spore 8.5-10.4 × 4.8-6 µm, ovoid to subamygdaloid with obtuse apex, with indistinct germ pore. Pleurocystidia 50-65 × 12-22 µm, broadly fusiform to subutriform, walls up to 2 µm thick with crystalliferousapex. Basidia, 26.54 - 33.4 × 8 - 11.7 µm, 4 spored.

It grows with conifers or *Salix* L. in sandy habitats (Knudsen and Vesterholt, 2008).

Specimens Examined: Türkiye, Kütahya, Simav, Çaysimav, Donuzkıran area, 39°07'23.5"N and 28°51'29.3"E, 801m,*P. nigra* forest, 28.04.2012, Allı 4103. GenBank number for ITS sequence: KR264912.1

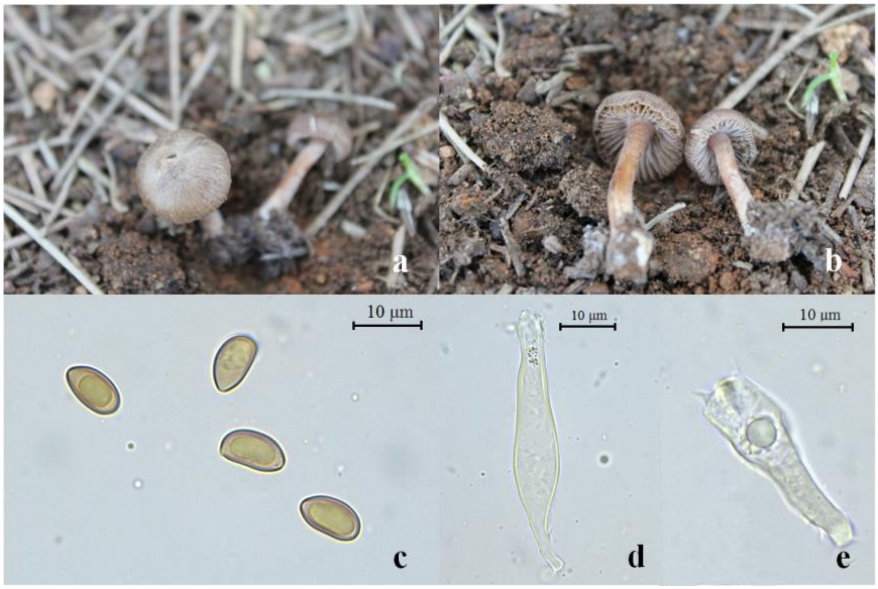

Figure 2. *Inocybe subporospora*; a – b. basidiocarps, c. basidiospores, d. pleurocystidium, e. basidium

Discussion

Gymnopus aquosus and *G. dryophilus* are very similar species, but they differ from each other in the color of the basidiocarp and stem features. *Gymnopus aquosus* has a rather pale basidiocarp and a distinctly bulbous stipe and base with pinkish rhizomorphs, distinguishes it from *G. dryophilus* (Knudsen and Vesterholt, 2008; Antonin and Noordeloos, 2010) (Figure 1). In addition to these morphological differences, it has been demonstrated that *G. aquosus* and *G. dryophilus* can be clearly distinguished by ITS gene sequences (Figure 3).

In the present study, we report *Gymnopus aquosus* as the 57th -taxon of Turkish *Gymnopus*. *Inocybe subporospora*, belonging to Subgen. *Inocybe* and Sect. *Tardae* Bon, is characterized by its spores. Each spore has a small germ pore, distinguishing it from other *Inocybe* species. However, observing this tiny germ pore under a light microscope can be challenging, so it should be examined carefully. *Inocybe luteipes* J. Favre also has germ pores, but *I. subporospora* can be distinguished from *I. luteipes* by its dark grayish-brown pileus with a rimulose margin (Jacobsson and Larsson, 2012) (Figure 2).

In a recent study, it was stated that the type collection of *Inocybe subporospora* was mixed and the ITS gene sequences of the lectotype of this collection are quite similar to *Inocybe tjallingiorum* Kuyper. In the same study, it was suggested that *I. subporospora* should be considered a synonym of *I. tjangilliorum*(Bandini et al., 2021). The ITS gene sequence of the sample presented in this study undoubtedly matches with *I. subporospora*. It has been shown that *I. subporospora* separately branched with high bootstrap value (Fig. 4). The ITS gene sequence of our sample was compared with the gene sequence of *I. tjallingiorum* that mentioned in the study and it was determined that they showed 63% similarity. Furthermore, *I. subporospora* (Sect. Tardae) and *I. tjallingiorum* (Sect. Splendentes) are two distinct species that are located in different sections. They exhibit notable morphological and microscopic differences. For instance, *I. tjallingiorum* is distinguished by a more conical cap and a grayish brown or pale ochraceous stipe. In contrast, *I. subporospora* is characterized by a convex cap and a dark red brown stem (Knudsen and Vesterholt, 2008; Cervini, 2021). Another clear distinction is that *I. tjallingiorum* have a dark umbo on the cap center (Matheny and Kudzma, 2019). For these reasons, our research sample is *I. subporospora*. Additionally, Index Fungorum, which we have already cited as a source for naming macrofungi, accepts these two as separate species. To eliminate such confusion, further molecular studies on the Inocybe genus and comparisons according to the characteristics of the species are recommended. This study is of significant importance in this context.

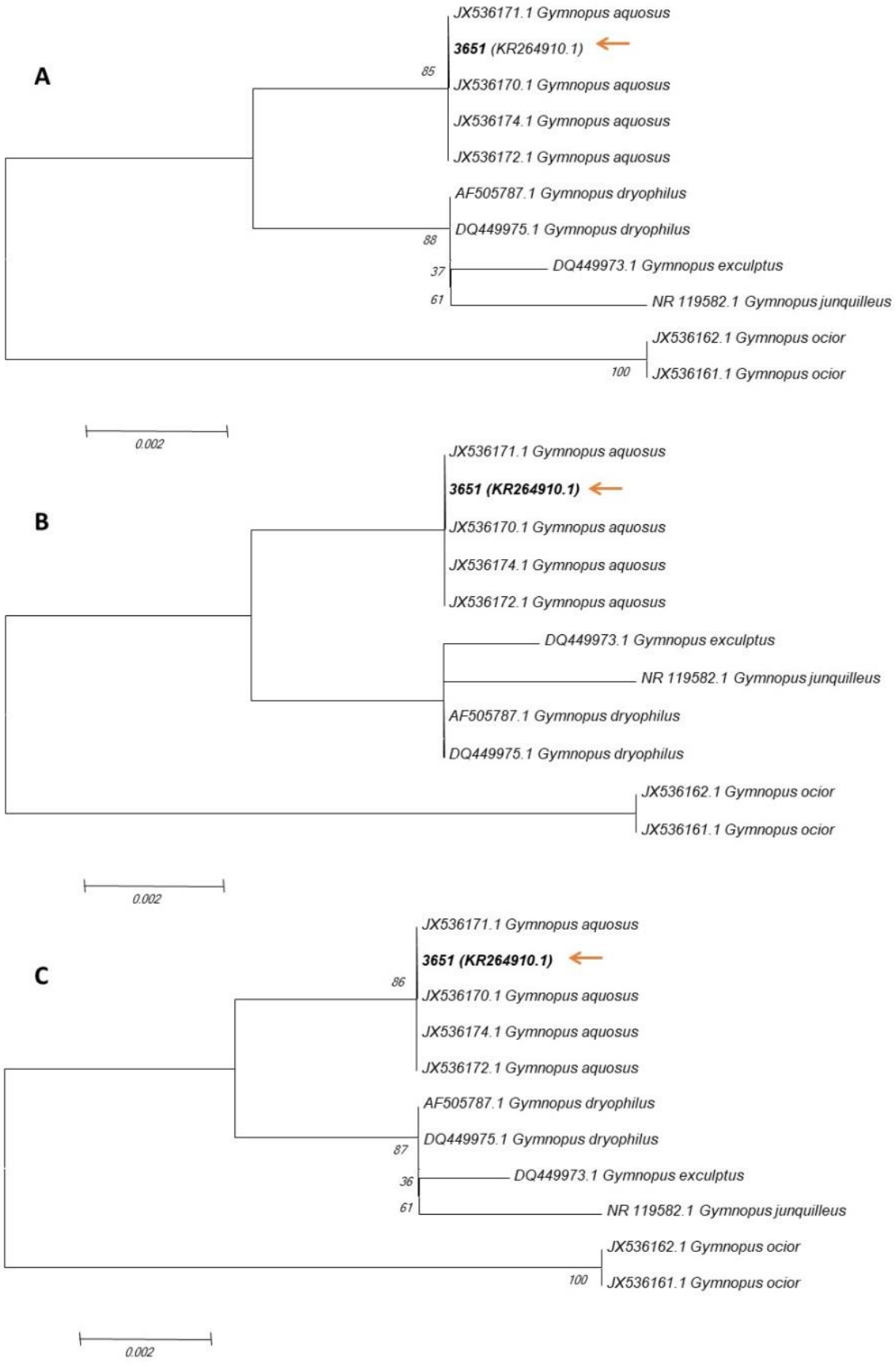

Figure 3. Phylogenetic position of *Gymnopus aquosus*, a. Neighbour Joining tree. b. Maximum Likelihood tree. c. Minimum Evolution tree. All analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 724 positions in the final dataset.

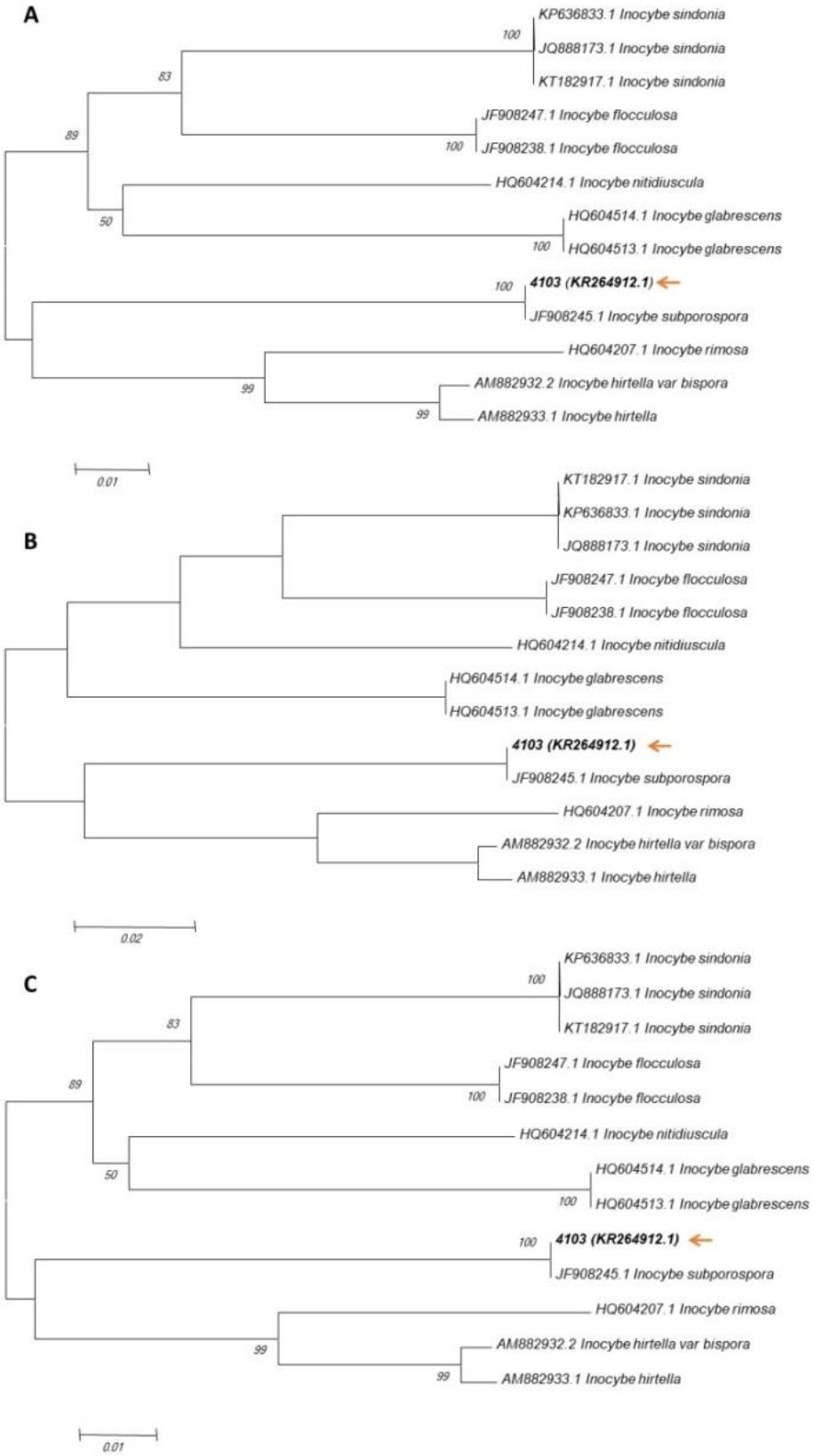

Figure 4. Phylogenetic position of Inocybe subporospora, a. Neighbour Joining tree. b. Maximum Likelihood tree. c. Minimum Evolution tree. All analysis involved 13 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 460 positions in the final dataset.

The results of this study, which included macroscopic, microscopic, and phylogenetic analyses, demonstrated the presence of *G. aquosus* and *I. subporospora* in our country and contributed to the diversity of macrofungi in Türkiye.

Additionally, in a study examining the relationship between *I. subporospora* and plants, it was found that the fungus selected *P. nigra* as its host, and an ectomycorrhizal association was established between them (Seress et al., 2016). The specimen presented in this study was obtained from a *P. nigra* forest, providing further evidence of this mycorrhizal association.

The number of taxa belonging to the *Inocybe* genus distributed in Türkiye has increased to 216 with the *I. subporospora* reported in this study.

Author Contributions

All authors have equal contribution.

Conflict of Interest

There is no conflict of interest with any institution or person within the scope of this study.

Ethical Statement:

It is declared that the scientific and ethical principles have been adhered to throughout the conduct of this study and that all sources utilized have been duly referenced (Hakan ALLI, İsmail ŞEN, Ezgin TIRPAN, Bekir ÇÖL).

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