This article is cited as follows: Acar İ., Karabıyık H. and Özcan Ateş G. (2024). Morphological and Molecular Identification of *Sepultariella semi-immersa* : New Locality Record for Türkiye, Mantar Dergisi, 15(2), 94-102.

Geliş(Recevied) :30.05.2024 ***Research Article***
Kabul(Accepted) :27.08.2024 **Doi: 10.30708/mantar.1492590**

Morphological and Molecular Identification of *Sepultariella semi-immersa* : New Locality Record for Türkiye

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Abstract: *Sepultariella semi-immersa* (*Pyronemataceae*, *Ascomycota*) was identified in a new locality for macrofungal diversity in Türkiye from Çanakkale Onsekiz Mart University Terzioğlu Campus. *S. semi-immersa,* which constitutes a species comlex, is included in the *Sepultariella* clade. This study gives a detailed description of the species, geographical coordinates, photographs taken in its natural habitat, and its phylogenetic position in the genus Sepultariella based on molecular data. The Internal transcribed spacer (ITS) region sequence determined the evolutionary and phylogenetic relationship within the genus but *S. semi-immersa*, which might constitute a species complex.

Keywords: Ascomycota, *Sepultariella*, New Locality, Türkiye

Sepultariella semiimmersa 'nın Morfolojik ve Moleküler Tanımlaması: Türkiye için Yeni Lokalite Kaydı

Öz: *Sepultariella semi-immersa* (*Pyronemataceae*, *Ascomycota*), Türkiye'nin Çanakkale Onsekiz Mart Üniversitesi Terzioğlu Kampüsünden Türkiye makrofungal çeşitliliği için yeni lokalite olarak tanımlanmıştır. Bir tür kompeksi oluşturan *S. semi-immersa*, *Sepultariella* clade'ı içerisinde yer almaktadır. Çalışmada türe ait detaylı deskripsiyon, coğrafi koordinat, doğal habitatında çekilmiş fotoğralar ve moleküler verilere dayalı olarak yapılan cins içindeki filogenetik pozisyonu verilmiştir. Cins içindeki evrimsel ve filogenetik ilişkinin tespit edilmesinde Dahili kopyalanmış aralayıcı (ITS) bölge dizisi belirleyici olmuştur.

Anahtar kelimeler: Ascomycota, *Sepultariella*, Yeni Lokalite, Türkiye

Introduction

The two most important classes of *Pezizomycotina*, the largest subdivision of Ascomycota, are Pezizomycetes (cup fungi or operculate Discomycetes) and Orbiliomycetes (round to wavy, cupulate to flat or convex apothecia) (Spatafora et al., 2006; Schoch et al., 2009). Of these two important classes, a few members of the *Pezizomycetes* are plant and bryophyte parasites, others are saprobes and a small number are mycorrhizal. It is estimated that there are more than 2000 species worldwide (Pfister and Healy, 2021). This class is characterised by a valve-like structure (operculum) at the apex of the asci that opens during spore discharge (Hansen et al., 2013).

Inside *Pezizomycetes*, the genus *Sepultariella* Van Vooren, U. Lindem. & Healy is represented by two species: *Sepultariella patavina* (Cooke & Sacc.) Van Vooren, U. Lindem. & Healy and *S. semi-immersa* (P. Karst.) Van Vooren, U. Lindem. & Healy. *Sepultariella semi-immersa* was first described by Karsten (1869) as *Peziza semi-immersa* P. Karst. *Peziza patavina* Cooke & Sacc., first named by Saccardo (1877), was renamed as *Pustularia patavina* (Cooke & Sacc.) Boud. in 1907. Later, both species were combined in *Leucoscypha* Boud. by Svrček (1974). However, Perry et al. (2007) reported that some tricharinoid species in the genera *Pustularia* Boud. and *Leucoscypha* do not belong to either of these genera. The analyses of Van Vooren et al. (2017) also supported Perry et al.'s results. Both species do not belong to the same clade as *L. leucotricha* (Alb. & Schwein.) Boud., the type specimen of the genus *Leucoscypha*, although it was accepted in this genus by Saccardo (1889), Le Gal (1957), Rifai (1968), Eckblad (1968). The name *Sepultariella* was first used by Kutorga (2000), but the publication of the name is invalid. These two species were placed in the genus *Sepultariella* by Van Vooren et al. (2017) and *S. patavina* was selected as the type of the genus. The reason why *L. semi-immersa*, which constitutes our study sample, was not selected as the type specimen; it was suggested that this species may be a species complex (Van Vooren et al., 2017).

These two species are known in our country: *Pustularia patavina* was cited by Kaya and Uzun (2015) and *Sepultariella semi-immersa* by Uzun et al. (2018). Both were described based on morphological data. In this study, in addition to morphological data, DNA sequences of the nuclear ribosomal internal transcribed spacers (nrITS) region including ITS1/ITS2 subregions were used as molecular characters to determine the relationship and position of *Sepultariella semi-immersa* within the genus.

Recently, many molecular studies have been carried out in our country. Some of them are; Aktaş and Karaselek, 2019; Acar, 2021; Akata et al, 2021; Acar et al., 2022; Dizkirici et al., 2022; Dizkirici and Acar, 2022; Akata et al., 2023; Acar et al., 2024; Akata et al., 2024. In many platforms, mycologists agree that molecularly supported descriptions would be healthier for the scientific world. This study aims to contribute to the knowledge of the distribution of *Sepultariella semi-immersa* in Türkiye and to redefine the species using both morphological and molecular data.

Material and Methods

Taxon sampling and morphological studies

On 11.03.2024, mushroom samples were collected at the Terzioglu Campus of Çanakkale Onsekiz Mart University (Figure 1). Macrofungi samples were photographed in their natural habitat and their morphological characteristics were noted in the field notebook. Specimens were preserved as fungarium material (deposited in the Mycology Laboratory of Çanakkale Onsekiz Mart University, Vocational School of Health Services) and were used for the examination of microscopic data. The preparations were analysed under a Leica DM2500 (Germany) research microscope. Ascus, ascospores, hairs, ectal excipulum and paraphyses were measured at least 20 replicates using Leica Application Suite (version 4.8). Macro- micromorphological analyses were performed following the methods described by Dougoud (2002), Medardi (2006), Ribes and Pancorbo (2010) and Van Vooren et al. (2017). Microscopic data were plotted using CorelDRAW (64-bit) (Canada) to ensure accuracy and clarity in the depiction of the observed characteristics of *Sepultariella*.

Molecular studies

EurX GeneMATRIX Plant & Fungi DNA isolation kit (Poland) was used for DNA isolation. After DNA isolation, spectrophotometric measurement was performed on Thermo Scientific Nanodrop 2000 (USA) to check the amount and purity of the DNA obtained. In the PCR study, ITS1 - ITS4 primers were used as universal primers to amplify the gene regions targeted for species identification. The primer sequences used were ITS1 5'TCCGTAGGTGAACCTGCGG 3' and ITS4 5' TCCTCCGCTTATTGATATGC 3' primers. DNA amplification was performed in a 35 µl mixture containing genomic DNA (10 ng/µl), 10× PCR Buffer, MgCl2 (25 mM), dNTP mix (20 mM), selected primer pair F. Primer (10 µM), R. Primer (10 µM), Taq polymerase (5u/µl) and sterile water. The amplification results obtained by PCR (kyratec thermocycler) were electrophoresed in a 1.5% agarose gel prepared with 1x TAE buffer at 100 volts current for 90 min and visualised under UV light using ethidium bromide stain. A one-step PCR was performed to amplify a region of approximately 700 bases. The PCR reaction was performed with Solis Biodyne (Estonia) FIREPol® DNA Polymerase Taq polymerase enzyme. For our sample, a single band was obtained on agarose gel after PCR, indicating that the PCR process was successful. At the PCR product purification stage, MAGBIO 'HighPrep™ PCR Clean-up System' (AC-60005) purification kit was used for the single band samples obtained and purification was carried out following the procedures of the kit. For Sanger Sequencing, an ABI 3730XL Sanger sequencer (Applied Biosystems, Foster City, CA) and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) were used at Macrogen Netherlands laboratory. The reads obtained with the ITS1 and ITS4 primers were

contig-formed to generate a consensus sequence. The CAP contig assembly algorithm in BioEdit software was used to perform this process. Ambiguous sites were manually checked and corrected. Sequence data for the ITS region were deposited in GenBank and accession numbers were added to the manuscript.

Sequence alignment and phylogenetic analysis

The sequence of *Sepultariella semi-immersa* from the present study and additional sequences from the NCBI database (Appendix 1) were combined and analysed together to determine the phylogenetic relationship and the position of the studied species within the genus *Sepultariella*. *Tricharina gilva* (Boud. ex Cooke) Eckblad was selected as the outgroup. All sequences were aligned with the ClustalW program (Thompson et al., 1994).

Figure 1. Photo of the research area

A phylogenetic tree was constructed using the Maximum Likelihood method to determine species distinctions. The appropriate nucleotide evolution model for phylogenetic analyses was determined using MEGA v7 (Kumar et al., 2016). The model with the lowest BIC (Bayesian Information Criterion) score was used as the model that best describes the substitution model (Tamura et al. 2013). Bootstrap analysis with 1000 replications was used to test branch support (Felsenstein, 1985). In the Maximum Likelihood (ML) method, the first tree for heuristic search was automatically obtained by applying the Neighbour-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then the topology with the highest log likelihood value was selected. All positions with gaps and missing data were eliminated.

Result

Ascomycota Caval. -Sm.
Pezizomycotina O.E. Erikss. & Winka
Pezizomycetes O.E. Erikss. & Winka
Pezizales J. Schröt.
Pyronemataceae Corda
Sepultariella Van Vooren, U. Lindem. & Healy
Sepultariella semi-immersa (P. Karst.) Van Vooren, U. Lindem. & Healy **(Figure 2, 3.)**

Apothecia 2–4 mm diam., sessile, discoid to cup-shaped, hymenium orange to ochre-orange, margin crenulate, with upper small whitish hairs, ones are longer and multicellular, the outer surface is of the same colour and slightly tomentose. **Asci** 180–250 × 13–20 µm, cylindrical, eight-spored, ascus base with croziers, inamyloid. **Ascospores** uniseriate, 21–25 × 9.5–11.5 µm, smooth, with two guttules, sometimes only one, accompanied by smaller droplets, narrowly ellipsoidal to

slightly fusiform, with acute ends. **Paraphyses** 2.5–3.5 µm, towards the apex 3.5–6.5 µm wide, cylindrical, forked, septate, and slightly thickened at the apex. **Excipulum** two-layered: inner layer in various shapes, outer layer –of *textura globulosa/angularis*, with cells 10–20 × 13–35 µm.

Specimen examined: Çanakkale Onsekiz Mart University, Terzioğlu Campus, behind Medical Faculty, 40° 06′ 34"N, 26° 24′ 48"E, 34 m, on soil, near *Pinus brutia* Ten. and *Quercus coccifera* L. trees, 11.03.2024, Acar 1841.

Figure 2. *Sepultariella semi-immersa.* a-d. Ascomata, e. Ascospores (in water), f. Asci (in water), g. Asci (in Melzer's reagent), i. Paraphyses (in water) **Scale bar** = 10 µm

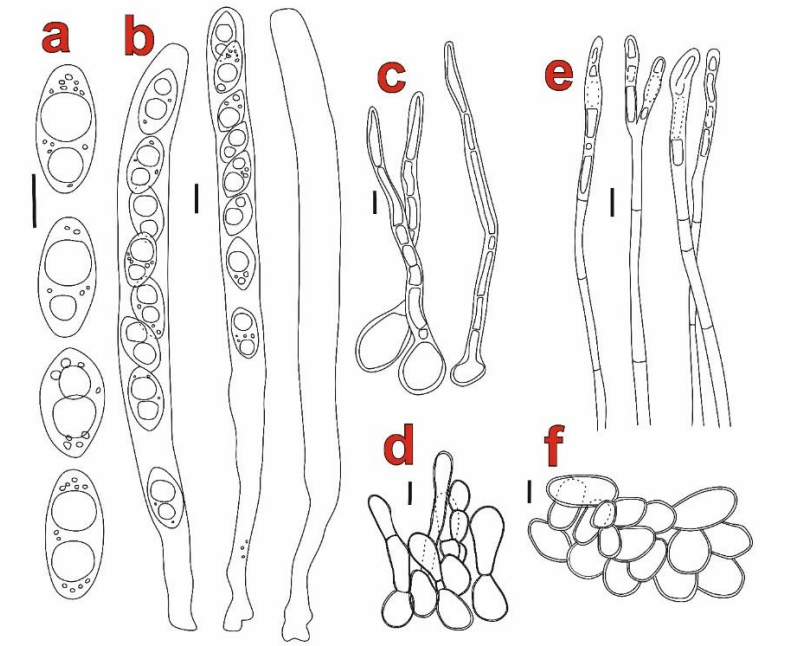

Figure 3. *Sepultariella semiimmersa* a. ascospores, b. asci, c. long hairs, d. short hairs, e. paraphyses, f. cells of ectal excipulum **Scale bar** = 10 µm

Molecular analysis

The amplified DNA fragment of the ITS region was approximately 700 bp long, covering the entire ITS1/ ITS2 subregions. Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997) analysis was performed to determine sequence homologies using the National Centre for Biotechnology Information (NCBI) database. The ITS data matrix consisted of a total of 22 sequences, including the studied sample and three outgroup samples.

The accession number of the studied ITS region was assigned as 'GenBank ID: PP783916'. Our sample clustered in the *Sepultariella* clade and was grouped with *Sepultariella* samples downloaded from NCBI having a bootstrap value of 87% (Figure 4).

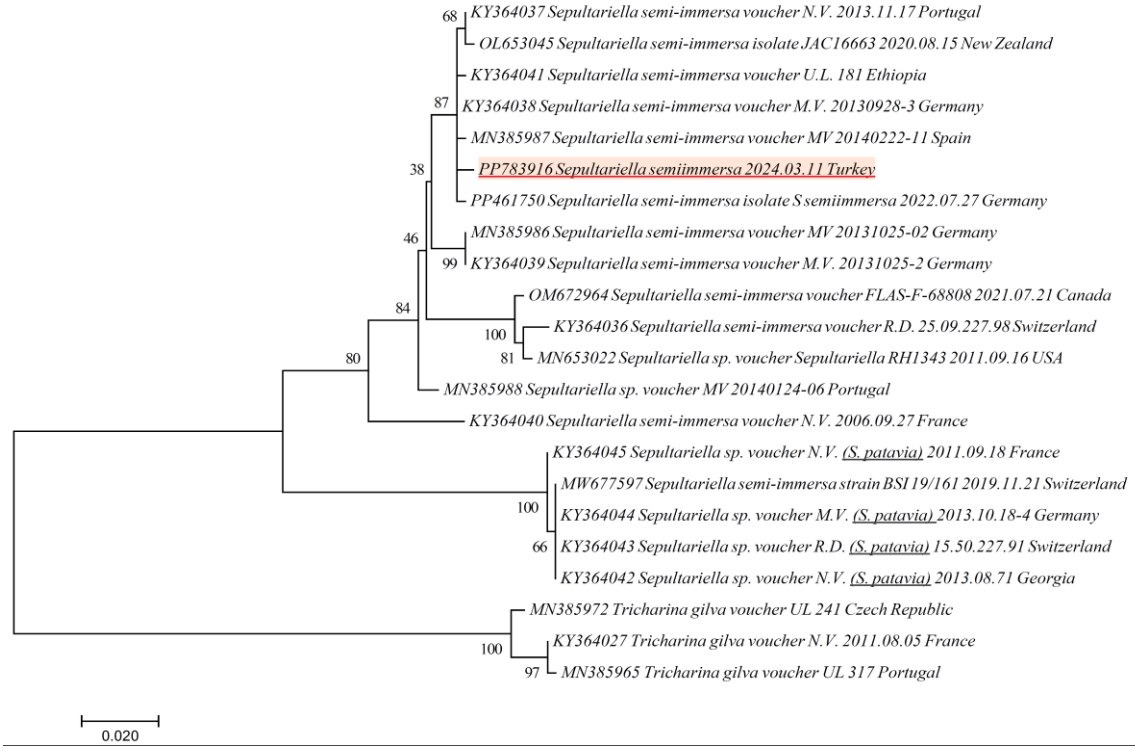

Figure 4. Phylogenetic tree of *Sepultariella* species based on ML analysis of the ITS region. The underlined light orange colour indicates the studied specimen. Sequences of *Tricharina gilva* were used as the outgroup.

Discussions

The species to which our studied specimens are morphologically and molecularly closest is *Sepultariella patavina*. Both species are carefully examined morphologically (Dougoud, 2002; Medardi, 2006; Ribes and Pancorbo, 2010). There are differences in macro- and micro-characters, albeit with minor nuances. Although they are quite close in evolutionary perspectives, their separation is clear in molecular analyses.

Dougoud (2002) and Ribes and Pancorbo (2010) comprehensively documented the morphological characteristics of *S. semi-immersa*. Medardi (2006) did not illustrated this species but mentioned some of its characteristics due to its similarity with *S. patavina*. Table 1 provides a comprehensive comparison between the specimens of *S. semi-immersa* analysed in this study and the specimens included in the works of the aforementioned authors (Dougoud, 2002; Medardi, 2006; Ribes and Pancorbo, 2010; Uzun et al., 2018). We meticulously summarise the dimensions of the various structures observed in our specimens, macroscopic features and microscopic characters, as well as habitat. This comparison highlights similarities and differences in measurements and characteristics between the present specimens and other collections reported in the literature.

This study of *S. semi-immersa* specimens highlights subtle but important macroscopic and microscopic variations that do not appear to affect species identification. These variations may be indicative of a broader spectrum of intraspecific variability or perhaps an evolutionary adaptation within the species. Consequently, these small differences that do not affect species identification may be an important opportunity to investigate the genetic and environmental factors underlying micro- and macromorphological diversity.

In fungal taxonomy, morphological diversity significantly exceeds genetic diversity (Akata et al., 2024). For a more reliable identification of fungal species, phylogenetic analyses should be useful. Among the markers used in phylogenetic analyses, ITS is widely used for molecular taxonomic studies in fungi and provides valuable information. In our study, the nuclear ITS rDNA gene sequence was used for the molecular identification of our sample Acar 1841 and a similarity of more than 99% was found with the reference sequences of *S. semi-immersa* available in GenBank (Figure 4).

As a result, the number of species belonging to the genus *Sepultariella* in Turkey was recorded as two (Sesli et al., 2020). However, these two species were previously identified based on morphological data. In this study, the *Sepultariella* specimens collected in Çanakkale in 2024 and identified as *Sepultariella semi-immersa* are based on both morphological and molecular studies.

Author contributions

The authors have equal contribution.

Conflicts of interest

The authors declare no competing interests.

Ethical Statement

It is declared that scientific and ethical principles have been followed while carrying out and writing this study and that all the sources used have been properly cited (İsmail Acar, Halide Karabıyık, Gülçin Özcan Ateş).

Acknowledgement

Many thanks to Nicolas Van Vooren for language checking and finalising the manuscript.

Appendix 1

ITS sequences downloaded from NCBI database

Tricharina gilva (KY364027), *Tricharina gilva* (MN385965), *Tricharina gilva* (MN385972), *Sepultariella semi-immersa* (KY364037), *Sepultariella semi-immersa* (KY364041), *Sepultariella semi-immersa* (MN385987), *Sepultariella semi-immersa* (KY364038), *Sepultariella semi-immersa* (KY364036), *Sepultariella* sp. (MN385988), *Sepultariella semi-immersa* (MN385986), *Sepultariella semi-immersa* (KY364039), *Sepultariella semi-immersa* (KY364040), *Sepultariella semi-immersa* (MW677597), *Sepultariella* sp. (MN653022), *Sepultariella semi-immersa* (PP461750), *Sepultariella semi-immersa* (OM672964), *Sepultariella semi-immersa* (OL653045), *Sepultariella* sp. (KY364045), Sepultariella sp. (KY364044), *Sepultariella* sp. (KY364043), *Sepultariella* sp. (KY364042)

Table 1. Comparison of some measurements of the morphological structures of *S. semi-immersa*

	Dougoud (2002)	Medardi (2006)	Ribes and Pancorbo (2010)	Uzun et al. (2018)	Current study
Apothecia	1–3.5 mm	up to 3.5 mm	1–2.5 mm	1.5–5 mm	2–4 mm
Asci	230–260 × 18–20.5 (23) µm	unspecified	cylindrical, octosporic, uniseriate	180–200 × 10–15 µm	180 – 250 × 13 – 20 µm
Ascospores	19.5–22 × 9–10.5 µm	20.5–23 × 9–10.5 µm	(19.1)–21.5–22.1–(24.5) × (9.4)–10.2–10.4-(11.3) µm	21–23 × 10.5–12 µm	21–25 × 9.5–11.5 µm
Paraphyses	(2.5)3.5–4 µm, in diameter at the bottom, widened to 4-5 µm at the apex, hyaline, septate, simple or forked towards the apex	unspecified	cylindrical, forked, segmented and slightly thickened at the apex.	cylindrical, septate, slightly thickened at the apex	2.5–3.5 µm, towards the apex 3.5–6.5 µm wide, cylindrical, forked, septate, smooth, and slightly thickened at the apex.
Excipulum	external surface cells 8 – 20(35) × 10– 20 (25) µm	unspecified	globose-angular cells.	unspecified	two-layered, inner layer in various shapes, outer layer globulose - angularis, cells 10 – 20 × 13 – 35 µm.
Margin	often irregularly crenulate	crenulate	crenulate	crenulate with small whitish teeth	crenulate
Habitat	On clayey and bare soil of a rut in a forest road, as well as on the ground of a fire pit, but not on the ashes, under *Quercus* sp.	grows in the same environments as *L. patavina*	on nearly bare soil with some bryophytes and in a burnt pine forest (*Pinus canariensis* C.Sm. ex DC)	on soil among woody debris on burnt ground	near *Pinus brutia* Ten. and *Quercus coccifera* L. trees, on soil

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