MANTAR DERGİSİ/The Journal of Fungus **(2026)17(1)67-76**

This article is cited as: *Acar İ. and Karabıyık H. (2026). Hemimycena lactea on Pine Cones: First Molecular Confirmation in Türkiye, Mantar Dergisi, 17(1) 67-76.*

Geliş(Recevied) :14.03.2026
Kabul(Accepted) :17.04.2026

Research Article
Doi: 10.30708/mantar.1909607

Hemimycena lactea on Pine Cones: First Molecular Confirmation in Türkiye

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Abstract: In this study, a specimen of *Hemimycena lactea* (Acar 2041) was examined using morphological and molecular data. The specimen was collected on a pine cone from Çanakkale (Yapıldık Village, Derinkoy area) on January 28, 2025. The species is consistent with its description in the literature, with its completely white and delicate basidiomas, translucent-striate pileus margin, sparse lamellae, and thin stipe. Microscopically, the spores were found to be ellipsoidal–subfusiform in shape, and the spore wall was found to be inamyloid (Q ≈ 2). Microscopically, basidia with 2–4 sterigmata, clamp connections, and diverticulate-like pileipellis elements were observed; the spores were ellipsoidal–subfusiform and inamyloid (Q ≈ 2). In the context of Bayesian phylogenetic analysis, based on the ITS rDNA sequence (29 sequences; 27 in-groups, 2 out-groups), the sequence obtained in this study (GenBank: PZ091910) was placed within a highly supported (PP = 1.00) monophyletic clade with reference *H. lactea* sequences. The molecular results obtained from this study serve to confirm the species identification, and the observation of its growth on conifer cones during the winter period provides novel data on the ecological tolerance of the species.

Keywords: *Hemimycena lactea*, ITS rDNA, *Mycenaceae*, Türkiye, Pine cone

Çam Kozalaklarında *Hemimycena lactea*: Türkiye'de İlk Moleküler Doğrulama

Öz: Bu çalışmada, Çanakkale (Yapıldık Köyü, Derinkoy mevkii) lokalitesinden çam kozalağı üzerinde 28.01.2025 tarihinde toplanan *Hemimycena lactea* (Acar 2041) örneği morfolojik ve moleküler verilerle incelenmiştir. Tür; tamamen beyaz ve narin basidiomaları, translusent-striat pileus kenarı, seyrek lamelleri ve ince stipesi ile literatürdeki tanımıyla uyumludur. Mikroskobik olarak elipsoidal–subfusiform, inamiloid sporlar (Q ≈ 2), 2–4 sterigmalı basidiler, kıskaç bağlantıları ve divertiküllü pileipellis yapısı belirlenmiştir. ITS rDNA dizisine dayalı Bayesyen filogenetik analizde (29 sekans; 27 iç grup, 2 dış grup) bu çalışmada elde edilen dizi (GenBank: PZ091910), referans *H. lactea* dizileriyle yüksek destekli (PP = 1.00) monofiletik bir klad içerisinde yer almıştır. Sonuçlar, tür teşhisini moleküler olarak doğrulamakta ve örneğin kış döneminde konifer kozalağı üzerinde gelişmesi, türün ekolojik toleransına ilişkin yeni veriler sunmaktadır.

Anahtar Kelimeler: *Hemimycena lactea*, ITS rDNA, *Mycenaceae*, Türkiye, Çam kozalağı

Introduction

Mycenaceae, within the order *Agaricales*, is a family characterized by small and delicate basidiocarps, a predominantly saprotrophic lifestyle, and a morphologically "*mycenoid*" habitus. Current phylogenetic studies have revealed that *Mycenaceae* forms an independent lineage, distinct from the traditionally broad *Tricholomataceae,* and is specifically located within the marasmioid clade (Matheny et al., 2006; Dentinger et al., 2016; Vizzini et al., 2022). Nevertheless, the delineation of family boundaries and the classification of certain genera continue to be subjects of considerable debate.

Historically, *Hemimycena* Singer was considered to belong to the *Tricholomataceae* family for a considerable period and was at times considered conspecific with *Mycena* (Pers.) Roussel (Singer, 1986; Miersch & Antonín, 2013). However, the systematic position of the genus has been reinterpreted with different views emerging over time. Redhead (1987) transferred *Hemimycena* to *Xerulaceae* Jülich based on the presence of sarcodomic tissue. Subsequently, Moncalvo et al. (2002) incorporated *Hemimycena* into the clade "/*hemimycena*" through their molecular phylogenetic analysis, aligning it with *Calyptella*. Conversely, Matheny et al. (2006) proposed that the genus might constitute a distinct lineage, along with *Pleurotopsis* (Henn.) Earle, within the marasmioid clade. In accordance with contemporary taxonomical frameworks, the genus *Hemimycena* is widely recognised as a member of the *Mycenaceae* (Kirk et al., 2008).

Morphologically, the genus *Hemimycena* is characterised by slender and elegant basidiocarps that are predominantly white or pale in colour and are perishable. The spores are inamyloid, thin-walled and smooth, and the pileipellis structure consists of frequently diverticulous hyphae. The presence of prominent cheilocystidia and pincer attachments is also a distinguishing feature of most species (Antonín & Noordeloos, 2004). These characteristics represent the primary morphological basis for distinguishing the genus from both classical *Mycena* and similar-looking taxa within Tricholomataceae sensu lato (Niveiro et al., 2014).

Malysheva and Morozova (2009) expanded the diversity of the genus based on materials from European Russia and emphasised that *Hemimycena* species mostly thrive on leaf litter, conifer needle litter, and fine woody debris. This ecological framework indicates that the genus represents a saprotrophic group primarily specialising in the decomposition of fine organic residues. However, it is noteworthy that the mycohabitat preferences of individual species are rarely discussed in detail in the literature, and distinctions at the micro-substrate level remain poorly documented.

The type species of the genus, *Hemimycena lactea* (Pers.) Singer occupies a central position in the taxonomic classification system. As Antonín & Noordeloos (2004) describe in detail, this species is a taxon with the following characteristics: completely white, thin-fleshed, small caps; dense and narrow lamellae; a thin, cylindrical and generally pruinous stipe; inamyloid spores, smooth and thin-walled; and typical cheilocystidia morphology, with pleurocystidia usually absent. The species is regarded as a cosmopolitan macrofungus, exhibiting a wide distribution that extends from Europe to Asia and the Americas.

Phylogenetic studies suggest that at least the core group of *Hemimycena* (particularly the species around *H. lactea*) may be monophyletic, but the entire genus has not been adequately sampled molecularly (Moncalvo et al., 2002; Antonín & Noordeloos, 2004; Vizzini et al., 2022). This renders the combined assessment of morphological identification and molecular barcoding of particular significance.

The present study aims to evaluate a specimen of *H. lactea* obtained from Türkiye in light of morphological and molecular data. This species has previously been reported from various localities in Türkiye, typically occurring on needle litter in pine forests (Kaya, 2015; Çevik et al., 2021; Berber et al., 2022). Specifically, the objective is to document the species' development on cone substrates within the context of its microhabitat preferences and to compare the obtained ITS data with reference sequences in the literature. In this context, an examination was conducted of the correlation between morphological identification and molecular barcode data, with the objective of contributing to a more detailed understanding of the species' ecological boundaries.

Material and Method

The macrofungus specimen examined in this study was collected on 28 January 2025, during systematic fieldwork in the Derinkoy locality of Yapıldık Village, Çanakkale Central District, Çanakkale Province, Türkiye (see Figure 1). The specimen exhibited the presence of fruiting on pine cones in an area predominantly characterised by *Pinus brutia* trees. The species was identified through a combination of detailed morphological evaluations of macroscopic and microscopic characteristics and molecular analyses based on the ITS region (ITS1/ITS4); thus, a reliable and comprehensive species-level identification was achieved.

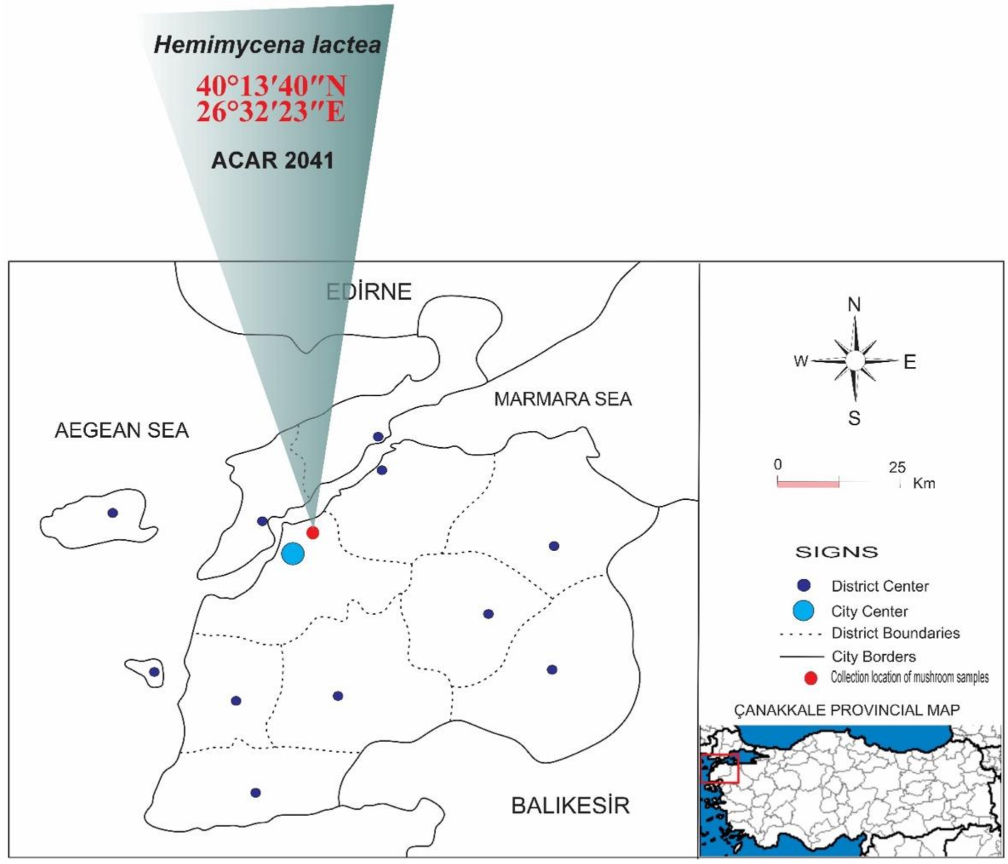

Figure 1. Map of research area

Morphological Studies

During field studies, locality information (including coordinates and altitude), collection date, habitat type, accompanying vegetation, substrate characteristics, and macroscopic findings were meticulously recorded for each specimen. Photographs of the basidiomas were captured in their natural environment using a Canon EOS 60D digital camera to accurately reflect their colour, developmental stage, and habitus characteristics. Following collection, the specimens were dried under controlled conditions at 40–45 °C to preserve their morphological characteristics. They were then labelled and stored appropriately until the analysis stage. Micromorphological examinations were performed using a Leica DM500 light microscope. Thin sections of the material, desiccated before examination, were prepared in a 5% KOH solution, and the basidium, basidiospore, cheilocystidia, caulocystidia, and pileipellis structures were examined in detail. To ensure the reliability of the data for each microscopic character, a minimum of 25 measurements were obtained, and these were analysed using Leica Application Suite (v3.4.0) software. The preparation of drawings of microscopic structures was undertaken using the CorelDRAW (64-bit, Corel Corporation, Canada) program, in accordance with the standards stipulated in the scientific publication. The identification of species was conducted through a comparative evaluation of macro- and micromorphological characteristics. In particular, the European species concept belonging to the genus *Hemimycena* was utilised as a foundation for this study. In this particular context, the primary sources with references Antonín & Noordeloos (2004), Malysheva & Morozova (2009) and Breitenbach & Kränzlin (1991) were utilised. The identified specimen were deposited as fungarium material in the Fungarium Collection of the Biology Department, Faculty of Science, Van Yüzüncü Yıl University (VANF).

Determination of ITS rDNA Sequences

Genomic DNA was extracted from dried basidiomata using a cetyltrimethylammonium bromide (CTAB)-based protocol following Rogers & Bendich (1994), with minor modifications according to Acar & Karabıyık (2025) and Akata et al. (2024a, b; 2025). DNA quantity and purity were assessed using a Thermo Scientific NanoDrop 2000c UV–Vis spectrophotometer (USA). DNA samples with suitable purity ratios were stored at −20 °C until further analyses. For molecular identification, the internal transcribed spacer region of ribosomal DNA (ITS rDNA), the formal fungal barcode marker, was amplified using the universal primer pair ITS1 and ITS4 described by White et al. (1990). Primer sequences were ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATATGC-3′). Polymerase chain reaction (PCR) amplifications were carried out in a final volume of 25 µL containing 10× PCR buffer, $MgCl_2$ (25 mM), dNTP mixture (10 mM each), 10 µM of each primer, approximately 10 ng template DNA, 5 U/µL Taq DNA polymerase (FIREPol®, Solis BioDyne, Estonia), and sterile distilled water. Negative controls lacking template DNA were included in each PCR run to monitor possible contamination. PCR amplifications were performed under the following cycling conditions: initial denaturation at 95 °C for 3 min; followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min; with a final extension step at 72 °C for 5 min. Amplification success was verified by electrophoresis on 1% agarose gels run at 100 V for approximately 90 min. Successful reactions yielded a single amplicon of approximately 650–700 bp. PCR products were purified using the MAGBIO HighPrep PCR Cleanup System (AC60005). Purified amplicons were sequenced bidirectionally by Macrogen (Netherlands) using an ABI 3730XL automated sequencer with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) (Chen et al., 2014). Forward and reverse chromatograms were visually inspected and edited in FinchTV, then assembled into consensus contigs using DNAMAN v10. Preliminary taxonomic affinities were assessed using BLASTn searches against the GenBank database. Newly generated sequences were aligned together with closely related reference sequences retrieved from GenBank using the MUSCLE algorithm implemented in MEGA X, and were subsequently used for phylogenetic analyses.

Molecular Phylogeny

In this study, the ITS rDNA sequence of the *H. lactea* (Acar 2041) specimen was generated by combining Sanger read data obtained with the ITS1/ITS4 primer pair in the DNAMAN v10 (Lynnon Corporation) environment. The editing and consensus generation were performed by IA. The resulting consensus sequence was then compared with the NCBI GenBank database using BLASTn analysis to support species identification, and the reference sequences showing the highest similarity were determined. Accordingly, representative ITS sequences were retrieved from GenBank for utilisation in phylogenetic analyses, and a dataset was created (see Appendix 1). The dataset used for the phylogenetic analysis consists of a total of 29 ITS sequences. One of these sequences is the *H. lactea* (Acar 2041) sequence generated in this study, and the remaining 28 sequences were obtained from GenBank. The ingroup was formed from *Hemimycena* species; two sequences (*Mycenella trachyspora* and *M. minima*) downloaded from GenBank were selected as outgroups for tree rooting. The range of raw sequence lengths in the FASTA dataset is from 553 base pairs (*H. lactea* Acar 2041; shortest) to 1597 base pairs (*H. lactea* PQ652379; longest). This length discrepancy can be attributed to the presence of longer ITS regions, accompanied by flanking regions, in certain GenBank records. Consequently, all sequences were aligned and made comparable before phylogenetic analysis. Bayesian phylogenetic analyses were performed using MrBayes v3.2.7a [19]. Markov Chain Monte Carlo (MCMC) analysis was executed over 3,000,000 generations (Ronquist et al., 2012), with two independent runs and four chains (1 cold, 3 hot). Sampling was performed at 100 generation intervals, and the initial 25% of samples were excluded as burn-in. The GTR+I+Γ nucleotide substitution model was applied (Tavaré, 1986; Yang, 1994), and the support values of the branches were reported as posterior probability (PP). The tree was rooted by identifying *Mycenella* sequences as outgroups.

Results

The macrofungus specimen collected in this study was examined using morphological and phylogenetic methods and identified as *H. lactea* at the species level. Macroscopic and microscopic features were evaluated in detail, supported by high-resolution images and drawings. The ITS rDNA region was sequenced at the molecular level, and the results of the analysis performed using the Bayesian method confirmed the species identification. The data obtained contribute to the macrofungus diversity of Türkiye and the regional molecular characterization of the taxonomy of the genus *Hemimycena*.

Taxonomy

Hemimycena lactea (Pers.) Singer (Figure 2 – 3)

GenBank Number: PZ091910

Pileus 2–9 mm in diam., campanulate when young, later convex to plano-convex; in most specimens with a slight umbo. Surface smooth, matte, entirely white; hygrophanous when moist, with translucent-striate margin. Context thin and fragile. **Lamellae** adnate to slightly subdecurrent, rather distant, white and concolorous with the pileus; edges even, sometimes appearing slightly sterile. **Stipe** 10–20(–30) × 0.4–1 mm, cylindrical, slender and delicate; straight or slightly curved, entirely white; surface smooth above, with conspicuous white rhizomorph tufts at the base. **Odour and taste** indistinct or very faint. **Basidiospores** (6.7–)7.4–10(–13.5) × (2.4–)3.3–4.8(–5.6) µm; mean approximately 8.6 × 4 µm; Q ≈ 1.7–2.7 (mean ~2.2); ellipsoid to subfusiform, smooth, thin-walled, hyaline, inamyloid, with a distinct apiculus; often guttulate. **Basidia** 18.3–28(–35) × 4.4–6.9 µm, clavate, 2- or 4-

sterigmate, with basal clamp connections. **Cheilocystidia** 20–45 × 3–8 µm, fusiform, lageniform or cylindrical, thin-walled. **Pleurocystidia** absent. **Pileipellis** a cutis of radially arranged, thin-walled, hyaline hyphae; hyphae 3–8(–10) µm wide; surface elements with conspicuous digitate (finger-like) diverticula. **Pileocystidia** 15–40 × 3–6 µm, cylindrical, narrowly fusiform or lageniform, thin-walled, hyaline; scattered to locally abundant on the pileus surface. **Caulocystidia** 15–50 × 3–8 µm, fusiform, cylindrical or narrowly lageniform, thin-walled, hyaline; abundant near the stipe apex, sparse to absent toward the base. **Clamp connections** present.

Ecological Distribution

The saprotrophic species *H. lactea* has been observed to thrive on leaf and needle litter, small woody residues, and humus layers. European monographic studies indicate that the species appears in small groups, especially on conifer litter and finely lignified materials (Antonín & Noordeloos, 2004). The presence of the fungus has been documented on *Picea abies* (L.) H.Karst. needles in European Russian records (Malysheva & Morozova, 2009), as well as on dune and conifer litter (Breitenbach & Kränzlin, 1991). From a phenological perspective, the species in question is characterised by its tendency to bear fruit during the period spanning from May to November, with the exception of certain regions where early summer observations have been documented. In temperate and coastal regions, winter fruiting has also been observed under conditions conducive to moisture. Its geographical distribution is Holarctic, with a prevalence in Europe and North America. In Türkiye, it has been recorded on needle litter and is a typical micro-saprotrophic species that contributes to the decomposition of lignocellulosic residues in forest undergrowth ecosystems (Breitenbach & Kränzlin, 1991; Antonín & Noordeloos, 2004; Malysheva & Morozova, 2009; Kaya et al., 2019; Sesli et al., 2020).

Specimen examined

Türkiye, Çanakkale, Yapıldık village, Derinkoy area, on pine cone, 33 m a.s.l., 40°13′40″N 26°32′23″E, 28.01.2025, leg. İ. Acar, voucher number: Acar 2041 (VANF).

Figure 2. *Hemimycena lactea* A-D. Basidiomata in natural habitat Scale bar: 1 cm (for A) (Photo by İsmail Acar)

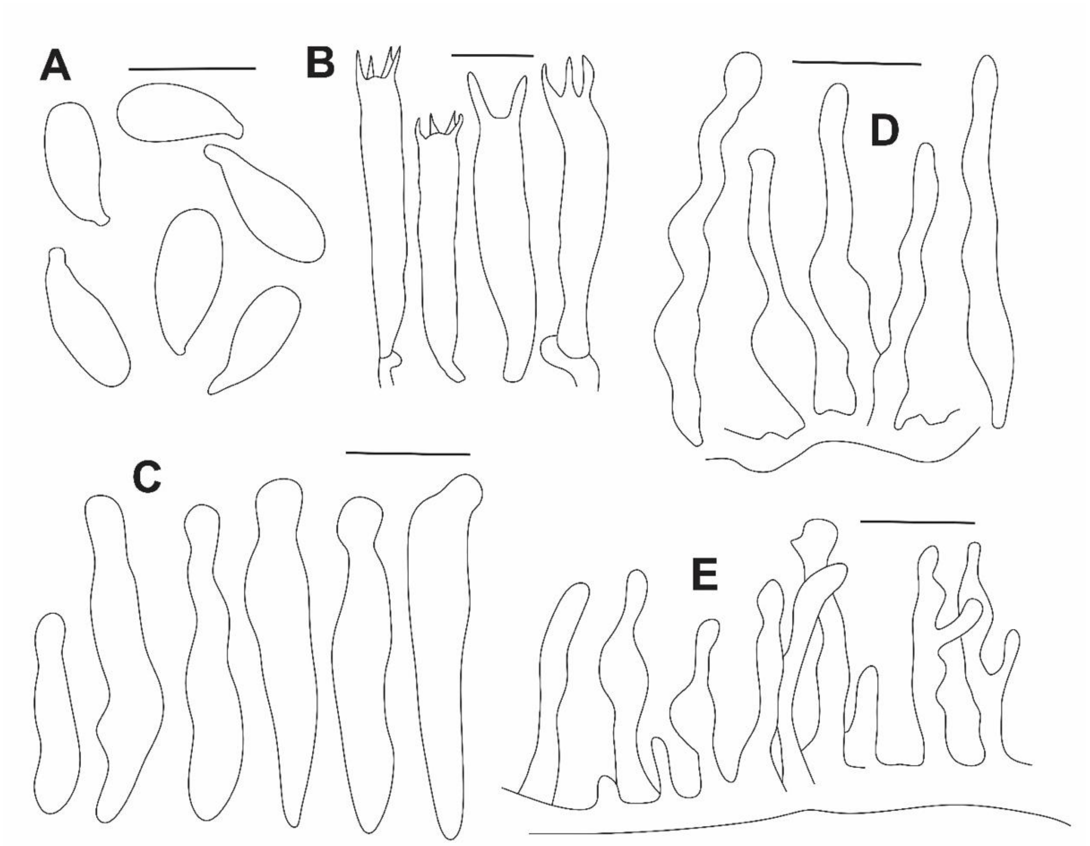

Figure 3. *Hemimycena lactea* A. Spores, B. Basidia, C. Cheilocystidia, D. Caulocystidia, E. Pileipellis

Phylogenetic Results

The Bayesian phylogenetic analysis revealed that the *H. lactea* specimen (PZ091910; Acar 2041) obtained in this study is located within a highly supported (posterior probability, PP = 1.00) monophyletic *H. lactea* clade according to ITS rDNA sequences (see Figure 4 for details). This clade is robustly supported by reference *H. lactea* sequences downloaded from GenBank (MH718253, OR863440, PQ652379, PX966754, PQ644387, MN992168 and PP464447), clearly demonstrating that the Acar 2041 specimen is genetically compatible with this species. The *H. lactea* clade is positioned in a sister relationship to the *H. cucullata* (Pers.) Singer clade in the phylogenetic tree, and these two species collectively constitute a higher lineage group that is well-supported. Conversely, the *H. pseudocrispula* (Kühner) Singer, *H. crispula* (Quél.) Singer, and *H. hirsuta* (Tode) Singer sequences were grouped within a separate but closely related clade, for which there was strong support (PP ≥ 0.96).

Furthermore, it was observed that the *H. delectabilis, H. persimilis* (Malençon ex Redhead) Antonín & Noordel., *H. mairei* (E.-J. Gilbert) Singer, and *H. albicolor* sequences formed more distant but statistically significant branches. Specifically, the *H. mairei* and *H. albicolor* sequences exhibited the formation of highly supported subclades within themselves (PP = 1.00). The phylogenetic tree was rooted using the *Mycenella trachyspora* **(**Rea) Bon, in Bon & Chevassut, and *M. minima* Singer, Beih. sequences as outgroups. The clear distinction between these two taxa and the *Hemimycena* clade further supports the efficacy of the employed rooting method in the analysis. In conclusion, the ITS-based phylogenetic results corroborate the morphologically defined *H. lactea* specimen. Furthermore, the species is situated within the same genetic lineage as the European and other reference sequences in GenBank.

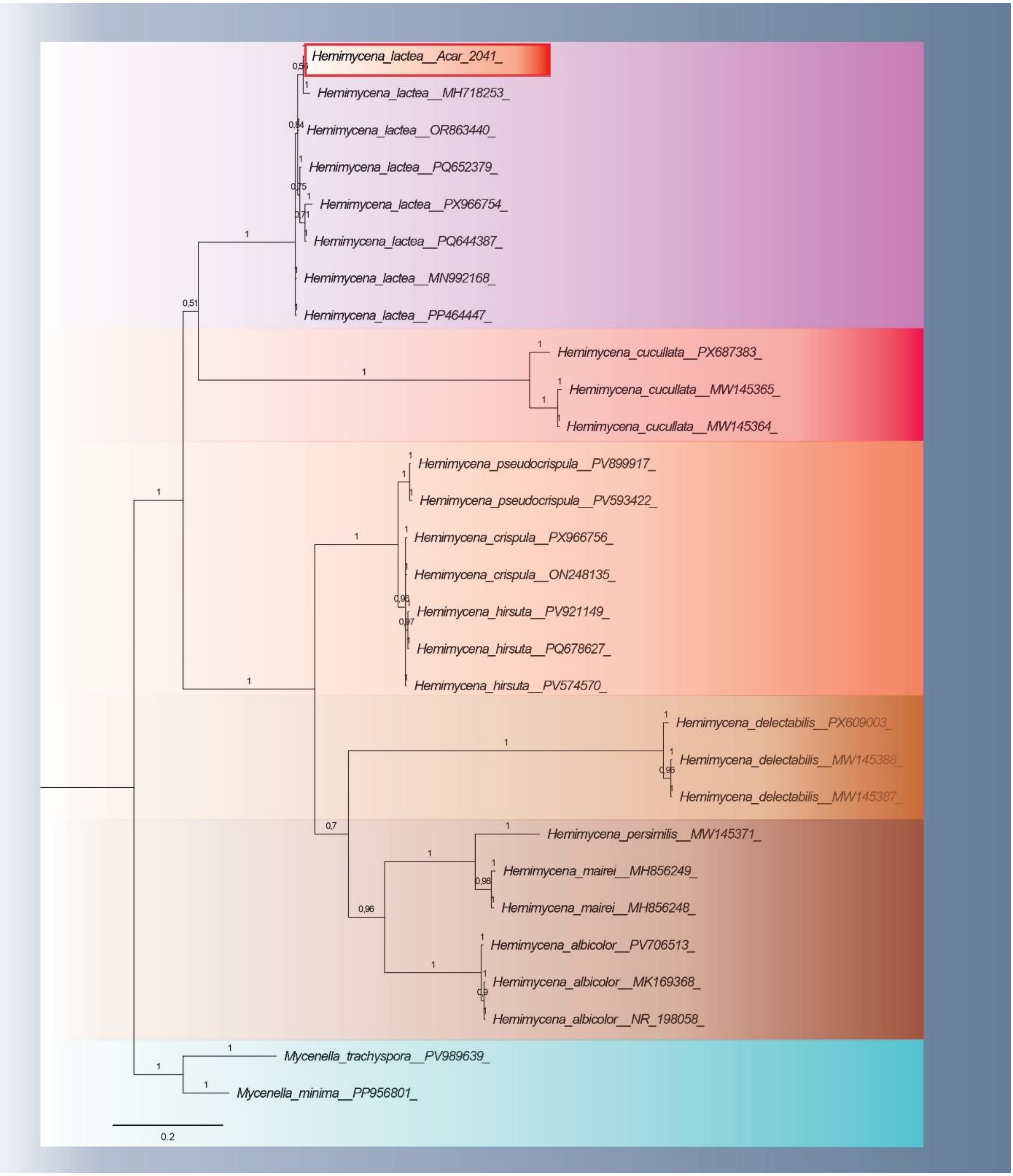

Figure 4. Bayesian 50% majority-rule consensus tree of *Hemimycena* based on ITS rDNA sequence data. The taxon generated in this study (*H.lactea* Acar 2041) is highlighted in red. Bayesian posterior probabilities (PP) are indicated above the branches. *Mycenella trachyspora* and *M. minima* were used as outgroup taxa. Scale bar indicates the number of nucleotide substitutions per site.

Discussions

In this study, a specimen of *H. lactea* (Acar 2041; GenBank: PZ091910), collected on a pine cone on 28 January 2025 from the Çanakkale (Yapıldık Village, Derinkoy area), was confirmed with morphological and molecular data. In the context of ITS-based Bayesian analysis, the presence of our specimen within a highly supported (PP = 1.00) monophyletic clade, in conjunction with the reference *H. lactea* sequences in GenBank, provides substantial support for the species identification. Moreover, the presence of *Mycenella* species in the tree's root system serves to substantiate the integrity of the *Hemimycena* clade. The morphological findings are largely consistent with the classical literature description of the species. These include small, completely white, and delicate basidiomas; a translucent-striate pileus margin; sparse lamellae; a thin, long stipe and basal rhizomorph; and ellipsoidal–subfusiform, inamyloid spores (Q ≈ 2). The 2–4 sterigma basidia and pincer attachments, as well as the pileipellis with a diverticulum (digital exrescent) cutis structure, correspond to the *H. lactea* concept as outlined in the extant literature (Kühner, 1938; Desjardin et al., 2015; Aronsen & Læssøe, 2016; Wei et al., 2024). The variability of cheilocystidia between fusiform–cylindrical forms, and the general absence of pleurocystidia are also consistent with the findings of previous studies.

From an ecological standpoint, the collection is noteworthy. *H. lactea* has been documented in the extant literature predominantly in association with leaf litter, fine woody residues, and moist forest humus (Geesteranus, 1986; Desjardin et al., 2015; Aronsen & Læssøe, 2016). However, there are scattered records of the species growing on lignified and resinous substrates, such as coniferous cones. The finding that the specimen under study exhibited direct growth on pine cones suggests that the species' substrate tolerance may be more extensive than previously hypothesised, indicating its capacity to adapt to diverse lignocellulosic materials. This supports the species' saprotrophic flexibility and opportunistic strategy at the microniche level. In terms of phenology, *H. lactea* is typically documented in the European literature during the spring-autumn period (Geesteranus, 1986; Aronsen & Læssøe, 2016). The occurrence of fruiting in January at a low altitude (33 m) and during mild winter conditions in the coastal zone of the Marmara Region demonstrates the species' phenological plasticity. This is significant because it demonstrates that macrofungal phenology can deviate from the classic Central European model in the Mediterranean and transitional climate zones.

In conclusion, the Acar 2041 collection reveals the morphological variation and ecological tolerance of *H. lactea* in Türkiye and confirms its identification, supported by strong phylogenetic positioning based on ITS data. The present study contributes to the understanding of the diversity and adaptation capacity of the *Hemimycena* genus in Anatolia by drawing attention to relatively underemphasised ecological characteristics of the species, such as its winter phenology and development on coniferous cones.

Author contributions

All authors have contributed equally.

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 KARABIYIK)

Acknowledgement

This study was supported by the Scientific Research Projects Coordination Unit of Van Yüzüncü Yıl University (VAN YYÜ BAP) under project number *FHD-2025-12036*. The authors gratefully acknowledge the financial support provided by VAN YYÜ BAP.

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APPENDIX 1

Hemimycena albicolor (MK169368), *H. albicolor* (NR_198058), *H. albicolor* (PV706513), *H. pseudocrispula* (PV899917), *H. pseudocrispula* (PV593422), *H. persimilis* (MW145371), *H. mairei* (MH856249), *H. mairei* (MH856248), *H. crispula* (PX966756), *H. crispula* (ON248135), *H. lactea* (OR863440), *H. lactea* (MH718253), *H. lactea* (MN992168), *H. lactea* (PQ652379), *H. lactea* (PX966754), *H. lactea* (PQ644387), *H. lactea* (PP464447), *H. cucullata* (PX687383), *H. cucullata* (MW145365), *H. cucullata* (MW145364), *H. delectabilis* (PX609003), *H. delectabilis* (MW145388), *H. delectabilis* (MW145387), *H. hirsuta* (PV921149), *H. hirsuta* (PV574570), *H. hirsuta* (PQ678627), *Mycenella trachyspora* (PV989639), *Mycenella minima* (PP956801).