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Two Newly Reported *Agaricales* Species from Türkiye with Morphological and Molecular Data

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Abstract

Aim of study: The main aim of this study is to contribute to the fungal diversity of Türkiye by presenting two newly documented species within the order of *Agaricales*.

Area of study: The samples of the newly reported species were collected under pine trees in Ankara Üniversitesi Beşevler 10. Yıl Campus, Ankara, and in fir forest at At Plateau, located 10 km from Bolu city center.

Material and method: The samples underwent detailed analysis, including morphological evaluation and DNA sequencing of the nrITS rDNA region and the *TEF1α* gene. This process involves examining both microscopic and macroscopic characteristics to perform phylogenetic analysis.

Main results: Fieldwork and laboratory analysis have led to the first report of the species *Agaricus nevoi* Wasser and *Hygrophorus abieticola* Krieglst. ex Gröger & Bresinsky, both belonging to the order Agaricales, from Türkiye.

Research highlights: The current study introduces a newly recorded *Agaricales* species in Türkiye, bringing the total number of known genera *Agaricus* and *Hygrophorus* species in Türkiye to 37 and 31, respectively.

Keywords: *Agaricales, Agaricus nevoi, Hygrophorus abieticola,* Mycobiota, Türkiye

Morfolojik ve Moleküler Verilerle Türkiye'den Yeni Bildirilen İki *Agaricales* Türü

Öz

Çalışmanın amacı: Bu çalışmanın temel amacı, *Agaricales* takımı içinde yeni belgelenen iki türü tanıtarak Türkiye fungal çeşitliliğine katkıda bulunmaktır.

Çalışma alanı: Yeni rapor edilen türe ait örnekler Ankara Üniversitesi Beşevler 10. Yıl Kampüsü (Ankara)'nde ağaçlarının altından ve Bolu şehir merkezine 10 km mesafede bulunan At Yaylası'ndaki göknar ormanından toplanmıştır.

Materyal ve yöntem: Örnekler, hem morfolojik değerlendirme hem de nrITS rDNA bölgesi ve *TEF1α* geninin DNA dizilimi dahil olmak üzere ayrıntılı analize tabi tutulmuştur. Bu süreç, filogenetik analiz yapmak için hem mikroskobik hem de makroskobik özelliklerin incelenmesini içermektedir.

Temel sonuçlar: Arazi çalışmaları ve laboratuvar araştırmaları ile *Agaricales* takımına ait *Agaricus nevoi* Wasser ve *Hygrophorus abieticola* Krieglst. ex Gröger & Bresinsky türleri Türkiye'den ilk kez rapor edilmiştir.

Araştırma vurguları: Mevcut çalışma, Türkiye'de yeni kaydedilen bir *Agaricales* türünü tanıtarak, *Agaricus* ve *Hygrophorus* cinslerinin Türkiye'deki bilinen toplam tür sayısını sırasıyla 37 ve 31'e çıkarmaktadır.

Anahtar Kelimeler: *Agaricales, Agaricus nevoi, Hygrophorus abieticola,* Mikobiyota, Türkiye

Introduction

The *Agaricales* Underw. occupies a central position in the fungal kingdom due to its extensive study and visibility. This prominence is not just because of their sheer numbers, with over 16000 species identified, but also due to their significant diversity, spreading across 374 genera and 40 families within the *Agaricales* order alone (He et al.,

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2019; Wijayawardene et al., 2020; Luna-Fontalvo et al., 2021). The *Basidiomycota* division, under which these fungi are classified, highlights the complexity and vastness of fungal life forms. The distinction between agarics and the broader *Agaricales* order is essential. While all agarics are part of the *Agaricales* order, not all members of this order are agarics (Akata et al., 2023). This nuance points to the variety within the *Agaricales*, known for encompassing various fungal types, from those with familiar pileus-and-stem morphologies to others with more unique and less commonly recognized forms (Kalichman et al., 2020).

Agaricus L., belonging to the family *Agaricaceae* Chevall. is a significant genus known for its saprophytic lifestyle and worldwide species distribution (Tarafder et al., 2022). Members of the genus are distinguished by their distinctive pileus, which ranges from furfuraceous to scaly in texture, and their dark-colored, free lamellae. The stipe is typically ringed, and the spores are purple-brown to cocoa-brown, notable for lacking a germ pore (Acar & Dizkırıcı, 2023). In terms of morphology, *Agaricus* species display a range of growth forms, including agaricoid and pluteiod, and their development can follow various patterns, from bivalvose-longicarpous-isocarpous to hymenocarpous (Saini et al., 2018). Its species are remarkably adaptable, thriving in diverse environments. They are commonly found in a communal setting in various habitats, including woodlands, forests, gardens, roadsides, fields, grasslands, and areas rich in organic material like rubbish dumps, manure heaps, and alluvial soils. Their habitat is not confined by elevation; they are found from sea level up to the highest vegetative zones in the mountains and even some desert regions (Lebel & Syme, 2012; Lebel, 2013; Karunarathna et al., 2016). The extensive genus encompasses approximately 580 species with a presence in virtually every part of the world (Medel-Ortiz et al., 2022). Currently, 36 species of the genus *Agaricus* have been reported in Türkiye (Akata, 2017; Sesli et al., 2020; Acar & Dizkırıcı, 2023; Halıcı & Güllü, 2023).

Hygrophorus Fr. is a widely distributed genus in the family *Hygrophoraceae* Lotsy, primarily thriving in the Northern Hemisphere's boreal and mild climatic zones (Larsson et al., 2014; Wang et al., 2020; 2023). Renowned for its ectomycorrhizal association, this genus presents a diverse array of basidiomata, ranging from petite to substantial sizes and from slender to robust textures (Bellanger et al., 2021). The pileus exhibits a spectrum from dryness to a pronounced glutinous quality. Furthermore, the lamellae are characterized by their waxy texture, substantial thickness, sparse distribution, and subdecurrent. The stipe is noted for its variable surface, which can be either dry or glutinous. It is typically smooth, with either a glabrous or a slightly fibrous texture, often adorned with delicate, pruinose, or grainy granulose. The spores are hyaline, smooth, and possess thin walls. The basidia are slender, and notably, cystidia are absent. A unique feature of this genus is its divergent lamellar trama, which sets it apart from other genera within the family *Hygrophoraceae* (Lodge et al., 2014; Wang et al., 2020; 2021). While the genus is globally widespread, with more than 130 known species (Wang et al., 2023), to date, only 30 species of *Hygrophorus* have been documented in Türkiye (Akata et al., 2018; Sesli et al., 2018; 2020; Sahin et al., 2021).

This research seeks to enhance knowledge of the *Agaricales* diversity in Türkiye by documenting two species newly recorded in the region, employing morphological and molecular methods.

Material and Methods

The study adopted an extensive approach, combining traditional techniques with advanced molecular methods to identify and classify samples collected from Ankara and Bolu provinces in Türkiye. It involved an in-depth examination of the specimens' macroscopic and microscopic features. This was further enhanced by comparing sequences and conducting phylogenetic analysis on nrITS rDNA and *TEF1α* gene sequences.

Morphological Characterization

Fungal specimens from Ankara and Bolu were collected and examined on-site for their macroscopic characteristics and environmental conditions. A Euromex Oxion

Trinocular light microscope in the laboratory facilitated a deeper inspection of the specimens, analyzing their microscopic details. To ensure the data's reliability, each microscopic feature was measured approximately 30 times, creating a robust dataset. After these measurements, the data underwent rigorous statistical analysis to confirm accuracy and uncover significant trends or variations. Furthermore, various specialized reagents were employed during the examination, each chosen to improve visibility, clarity, and detail in the observed features, enabling a more thorough analysis.

To prepare the fungal fragments for detailed examination, they were carefully affixed to small stubs using double-sided adhesive tape, ensuring stable positioning for the imaging process. After securing the samples, a thin layer of gold was applied to coat the fragments, a step essential for enhancing image clarity and reducing surface charge accumulation during electron microscopy. Visualization was conducted with an EVO 40XVP Scanning Electron Microscope (SEM) manufactured by LEO Ltd. in Cambridge, UK. SEM was set to operate at an accelerating voltage of 20 kV, a level optimized to produce high-quality, sharply detailed images of the fungal surface structure, allowing for in-depth analysis of its microscopic features.

The morphological identification was conducted using the techniques outlined in the research by Wasser (1996; 2000) and Wang et al. (2023). These studies were crucial in providing comprehensive and methodical procedures necessary for accurately determining the samples. Once correctly identified, the samples underwent meticulous preservation and were subsequently stored in the Fungarium at Ankara University within the Biology Department of the Science Faculty.

Genomic DNA Isolation From Fungal Specimens

In the genomic DNA isolation procedure, 50 mg of dried sporophore samples were first ground into a fine powder using a mill grinder. The powdered samples were then transferred into 1.5 mL microcentrifuge tubes and mixed with 700 µL of CTAB lysis buffer (pH 8.0). This buffer solution contained 3% w/v cetyltrimethylammonium bromide (CTAB), 1.4 M sodium chloride (NaCl), 20 mM ethylenediaminetetraacetic acid (EDTA), 100 mM Tris, 3% w/v polyvinylpyrrolidone (PVP), and 0.2% v/v β-mercaptoethanol. After adding the buffer, the mixture was vortexed vigorously for 1 minute to ensure complete homogenization. The tubes were then incubated at 65°C for 30 minutes to facilitate cell lysis. Following lysis, the samples were centrifuged at 13000 rpm for 10 minutes, after which 500 µL of the clear supernatant was carefully transferred to new tubes (Akata et al., 2024a).

To purify the DNA, an equal volume of chloroform-isoamyl alcohol (in a 24:1 ratio) was added to the supernatant. The mixture was briefly vortexed and centrifuged at 13000 rpm for another 5 minutes. The resulting upper aqueous layer was pipetted into fresh tubes, combined with an equal volume of cold isopropanol, and stored at -20°C for 30 minutes to precipitate the DNA. After this incubation period, the samples were centrifuged again at 13000 rpm for 10 minutes to obtain genomic DNA pellets. The supernatants were discarded, and the pellets were washed twice with 70% ethanol to remove impurities. Any residual ethanol evaporated by incubating the tubes at 60°C until the DNA pellets were almost dry. The semi-dried DNA pellets were dissolved in an appropriate volume of nuclease-free distilled water (Akata et al., 2024b).

The DNA concentration and purity were determined using the Nanodrop Lite (Thermo Scientific), while DNA integrity was assessed by agarose gel electrophoresis. A TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) with 0.8% agarose was prepared, and electrophoresis was conducted at a constant voltage of 5 volts per centimeter. The DNA bands were visualized using a safe blue light transilluminator with a safe green dye. A 1 kb Plus DNA Ladder was used as a molecular weight marker to verify the size and quality of the genomic DNA in the gel (Akata et al., 2024c).

PCR Amplification of the Internal Transcribed Spacer (ITS) rDNA and Translation Elongation Factor 1α (TEF1α)

Gene Regions and Determination of Nucleotide Sequences for Molecular Phylogeny of Macrofungal Samples

The genomic DNA of macrofungal sporophores was extracted using the CTAB (cetyltrimethylammonium bromide) method and subsequently served as a template for amplifying the internal transcribed spacer (ITS) region and the *TEF1α* gene region, both crucial for conducting molecular phylogenetic analyses in fungi. A hot start DNA polymerase enzyme was employed to ensure high specificity during PCR, effectively reducing the formation of non-specific products such as primer dimers, which can compromise amplification accuracy. The PCR reactions were prepared in 200 µL polypropylene tubes, with a reaction volume set to 50 µL. Within each reaction mixture, the components included 5 µL of a 10X DNA polymerase buffer (supplying 25 mM $MgCl_2$), 1 µL of a 10 mM deoxynucleotide triphosphate (dNTP) mixture (each nucleotide present at 10 mM), between 300 and 400 ng of genomic DNA template, 1 µL of each oligonucleotide primer at 10 µM, 5 units of DNA polymerase enzyme, and nuclease-free distilled water added to adjust the final volume to 50 µL. The PCR process required optimization of thermocycling parameters, which was achieved by considering the melting temperatures (Tm) specific to each primer pair, the length of the targeted gene regions, and the copy numbers of these regions within the genome. Additionally, the "Touchdown" PCR technique was employed to enhance amplification fidelity, gradually lowering the annealing temperature to diminish non-specific binding and reduce the occurrence of undesired primer dimers. Detailed information regarding the sequences and melting temperatures of the primers used in the PCR process is provided in Table 1, ensuring reproducibility and precision in the molecular phylogenetic studies (Akata et al., 2024c).

The standard PCR protocol was conducted in several stages, beginning with an initial denaturation phase set to 95°C for 2 minutes to ensure the DNA template strands were fully separated. Following this initial step, the reaction underwent 35 cycles of thermal cycling, each comprising three main stages: denaturation at 95°C for 30 seconds, annealing at a temperature range of 65°C to 50°C for 15 seconds (utilizing the touchdown approach to gradually lower the annealing temperature and increase primer specificity), and elongation at 72°C for 15 to 30 seconds. The elongation time at 72°C was adjusted according to the length of the amplicon and the specific requirements of the DNA polymerase enzyme being used, ensuring efficient replication of the target sequence. After completing the 35 cycles, a final extension phase of 7 minutes at 72°C was performed to ensure the complete synthesis of any remaining partial DNA strands. The PCR amplification was executed on a MiniAmp Plus Thermal Cycler manufactured by Applied Biosystems, allowing for precise temperature control throughout the reaction. To verify the quality and success of the amplification, the resulting PCR products were analyzed through electrophoresis on a 1% agarose gel. This process revealed distinct bands representing the target amplicons, while the absence of additional bands confirmed minimal non-specific amplification. The agarose gel electrophoresis was conducted following established protocols, with a GeneRuler 100 bp Plus DNA Ladder from Thermo Scientific included on the gel as a reference marker to enable accurate sizing of the amplified DNA products (Akata et al., 2024a).

Amplicons that satisfied the predetermined quality standards proceeded to a purification and cleaning stage, carried out using the GeneJET Gel Extraction and DNA Cleanup Micro Kit from Thermo Scientific, adhering strictly to the manufacturer's protocol. After this purification step, the concentration and purity of the amplicons were quantitatively evaluated through spectrophotometric analysis using a Nanodrop Lite device, ensuring that the samples met the necessary standards for sequencing. The Sanger dideoxy chain termination method was employed for the sequencing process, a reliable approach for accurate DNA sequencing. The same oligonucleotide primers initially used during PCR amplification were also used in the sequencing reactions to ensure consistency in targeting the desired DNA regions. DNA sequencing and subsequent analysis were

outsourced to a specialized external facility to handle sequencing tasks and data analysis, ensuring precise and high-quality sequence data for downstream applications (Akata et al., 2024a; 2024b; 2024c).

Table 1. Sequences and Tm temperatures of Oligonucleotide Primers Used in the PCR

Locus	Primer	Oligonucleotide Sequence (5'-3')	Tm Temperature (°C)
ITS	ITS1	TCC GTA GGT GAA CCT GCG G	63
	ITS4	TCC TCC GCT TAT TGA TAT GC	54
TEF1α	EF1-983F	GCY CCY GGH CAY CGT GAY TTY AT	57-72
	EF1-1567R	ACH GTR CCR ATA CCA CCR ATC TT	55-65

Molecular Phylogenetic Analyses of the Fungal Specimens

The specimens underwent molecular phylogenetic analyses using MEGA-X software, accessible at https://www.megasoftware.net/, focusing on their nucleotide sequences. Initially, the amplicon sequences were scrutinized using NCBI's Nucleotide BLAST (Basic Local Alignment Search Tool) to pinpoint analogous sequences. The sequences from the GenBank DNA database that exhibited the most remarkable resemblance to the analyzed amplicon sequences were designated as the ingroup for the phylogenetic analyses. Furthermore, sequences from distantly related macrofungi that did not display similarity to the analyzed amplicon sequences were specifically chosen as the outgroup for the analyses (Akata et al., 2024a; 2024b).

To build a phylogenetic tree, the sequences were initially aligned with both the ingroup and outgroup sequences using the MUSCLE algorithm. Subsequently, the most suitable nucleotide substitution model was determined, and the phylogenetic trees were constructed using the Neighbor-Joining algorithm. To evaluate the confidence of the tree branches, 1000 bootstrap replicates were employed (Akata et al., 2024c).

Results

The newly reported species have been carefully documented, including critical information such as the dates of collection, locations of collections, observations of their natural habitats, geographical coordinates, and unique collection identifiers. The description includes both macroscopic and microscopic morphological features of the species. Furthermore, images of the spores captured through a scanning electron microscope (SEM) are included to provide deeper insights into their intricate traits. These images enhance our understanding of the complex characteristics defining the species.

Systematic overview

Family: *Agaricaceae* Chevall.

Genus: *Agaricus* L.

Species: *Agaricus nevoi* Wasser (1995), (Figure 1-6).

Figure 1. Basidiomata of *Agaricus nevoi* (Illustrated by Meltem Kurt)

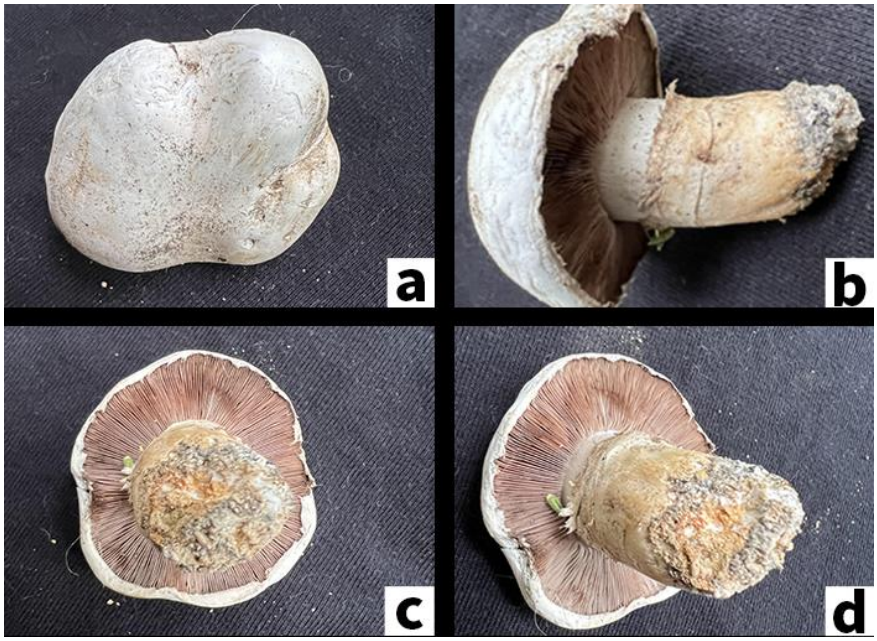

Figure 2. *Agaricus nevoi:* a-d. Basidiomata

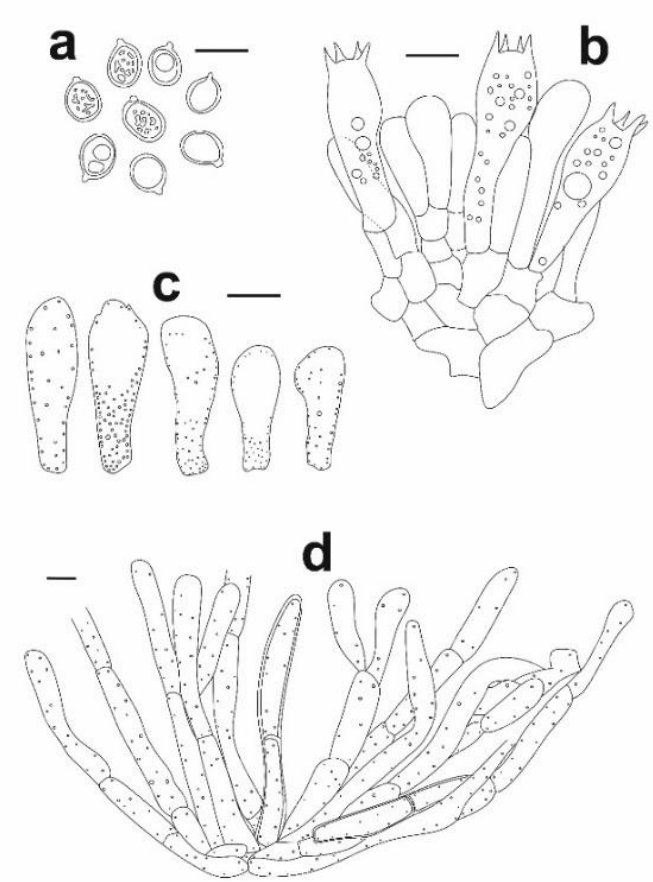

Figure 3. *Agaricus nevoi*: a. spores, b. basidia, c. cheilocystidia, d. pileipellis (scale bars: 10 µm)

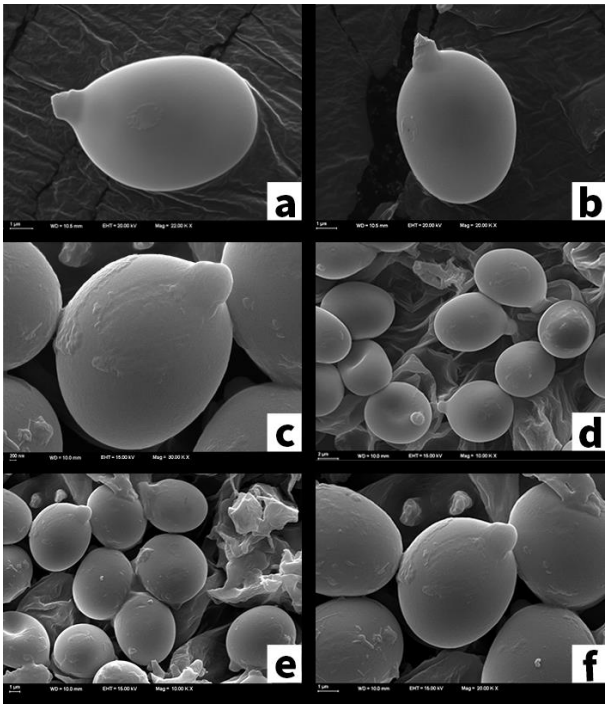

Figure 4. *Agaricus nevoi*: a-f spores (SEM), (scale bars: a,b: 1 µm, c: 200nm, d,e: 2 µm, f: 1 µm)

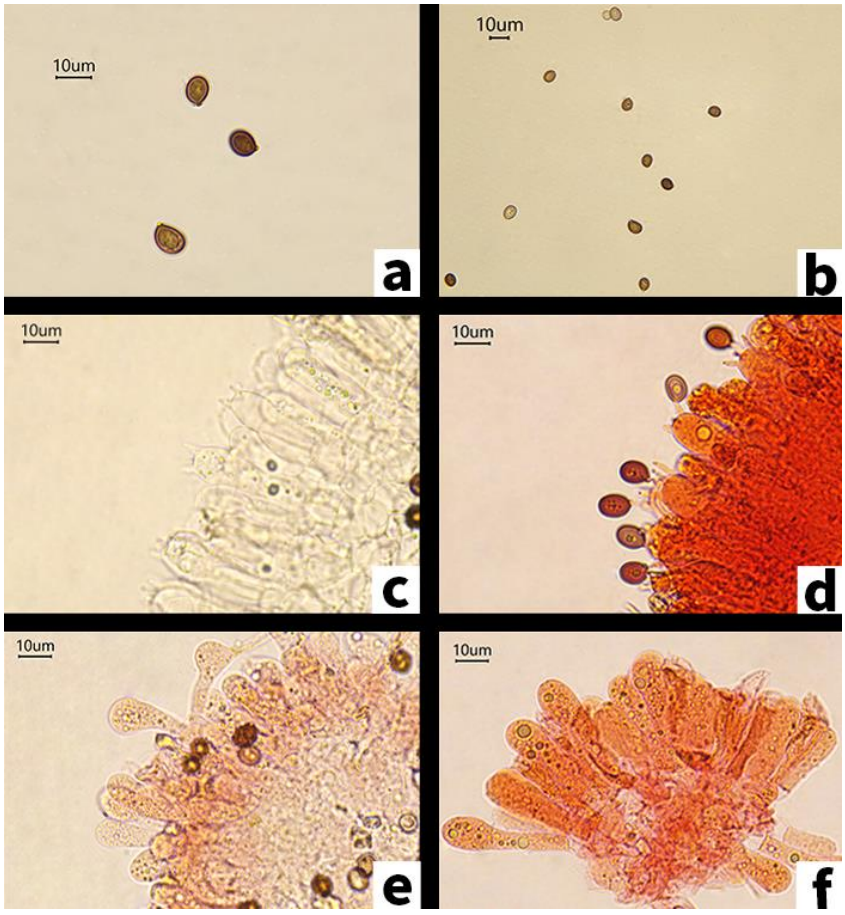

Figure 5. *Agaricus nevoi*: a,b. spores (LM, in 5% KOH), c. basidia (LM, in 5% KOH), d. basidia with spores (LM, in Congo red), e-f. cheilocystidia (LM, in Congo red)

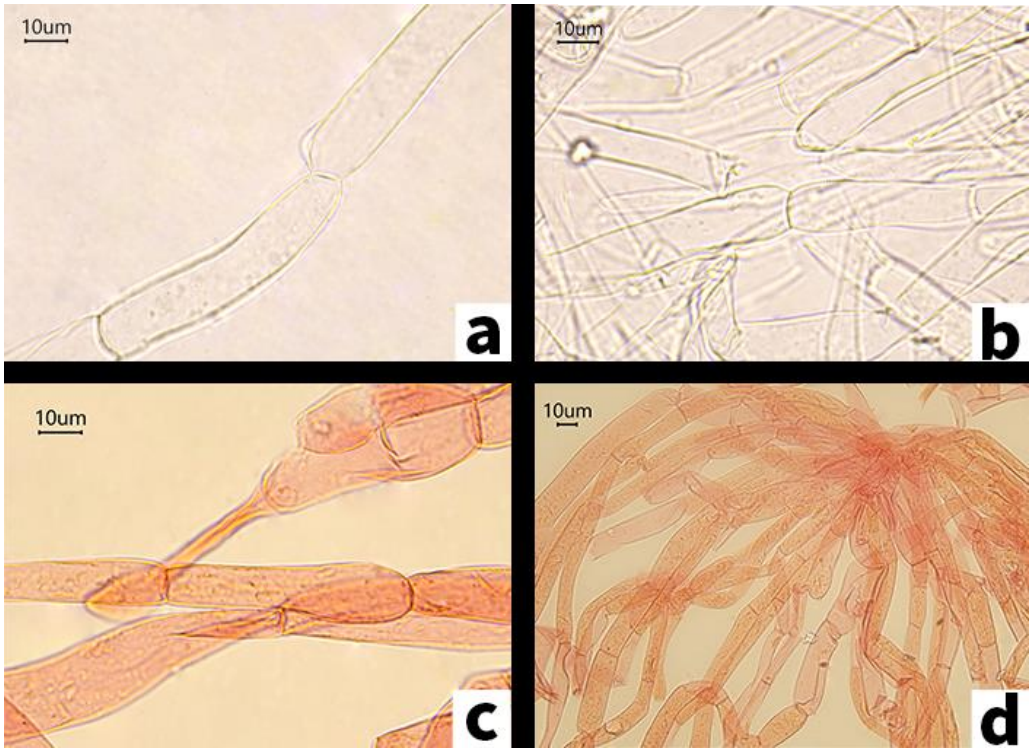

Figure 6. Pileipellis of *Agaricus nevoi*: a,b. (LM, in 5% KOH), c-d. (LM, in Congo red)

Macroscopic and Microscopic Features

Pileus 50–60 mm diam., initially hemispherical, then widening to a broad convex, occasionally featuring a slight depression at the center hemispherical and then widening to a broad convex, featuring a slight depression at the center. **Surface** whitish to pale grey, with a silky and shiny texture, and adorned with broad scales ranging from greyish to light brown, thin in a radial direction and becoming fibrillose towards the margin. **Margin** incurved, frequently displaying remains of the universal veil. **Lamellae** free and crowded, initially pinkish and becoming dark brown at maturity. **Stipe** 50–60 × 20–25 mm, solid, cylindrical, sometimes tapering towards the base, white to creamy white, without annulus **Volva** wide, predominantly whitish to light pinkish or light pinkish-brown, decorated with broad, delicate scales. **Context** primarily white, occasionally slight pink hue above the lamellae. **Odor** not distinctive, and **Taste** mild. **Spore print** chocolate brown. **Spores** (5.7-) 6.4-8.5 (-9) × (4.8-) 5.5-6.7 (-7) µm, Q = 1.14-1.28 (-1.47), Qav = 1.22, ellipsoid thick-walled, smooth, and light brown. **Basidia** 28–33 × 9–10.5 µm, clavate with long sterigmata, measuring 4-5 µm long, and four spored. **Cheilocystidia** 26–35 × 7–9.5 µm, and clavate. **Pleurocystidia** not observed. **Hymenophoral trama** regular and composed of cylindrical hyphae. **Pileipellis** composed of hyaline, cylindrical, and septate hyphae, measuring 6-18 µm broad. **Clamp connections** absent. **Schaeffer reaction** negative.

Material examined: TÜRKİYE— Ankara, Ankara Üniversitesi Beşevler 10. Yıl Campus, under pine, 39° 56' N-32° 50' E, 860 m, 05.05.2023, ANK AKATA 8711.

Family: *Hygrophoraceae* Lotsy
Genus: *Hygrophorus* Fr.
Species: *Hygrophorus abieticola* Krieglst. ex Gröger & Bresinsky (2008), (Figure 7-12).

Figure 7. Basidiomata of *Hygrophorus abieticola* (Illustrated by Meltem Kurt)

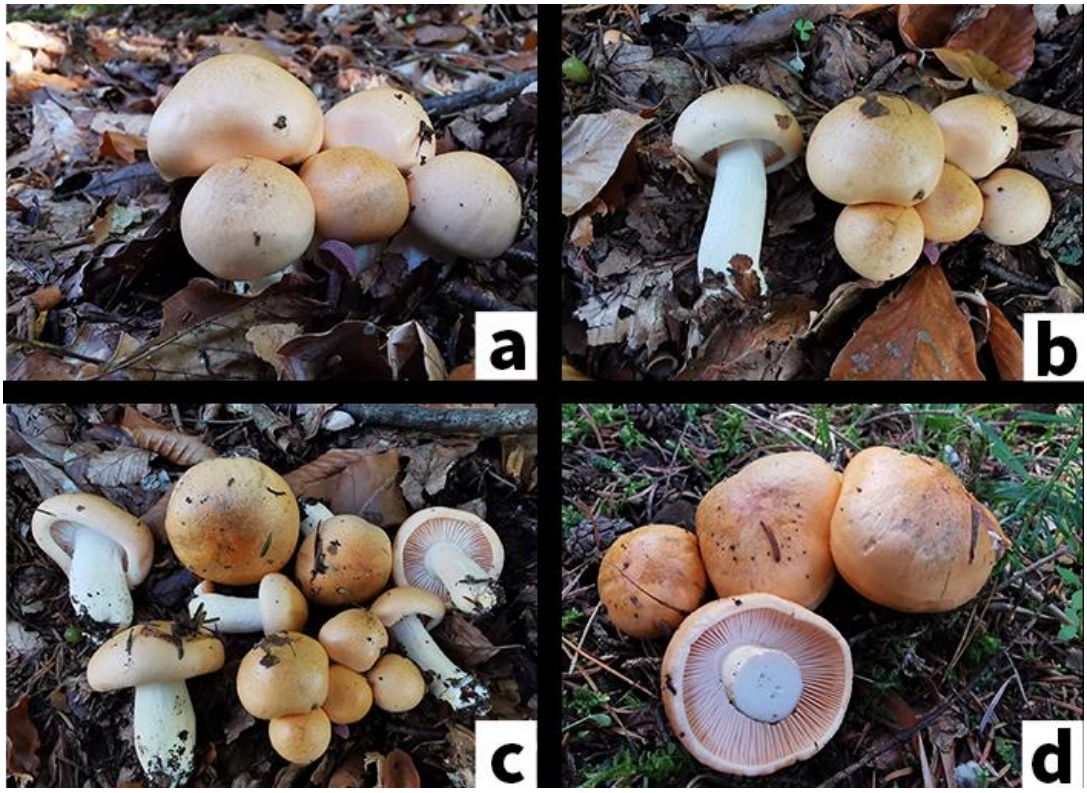

Figure 8. *Hygrophorus abieticola*: a-d. Basidiomata

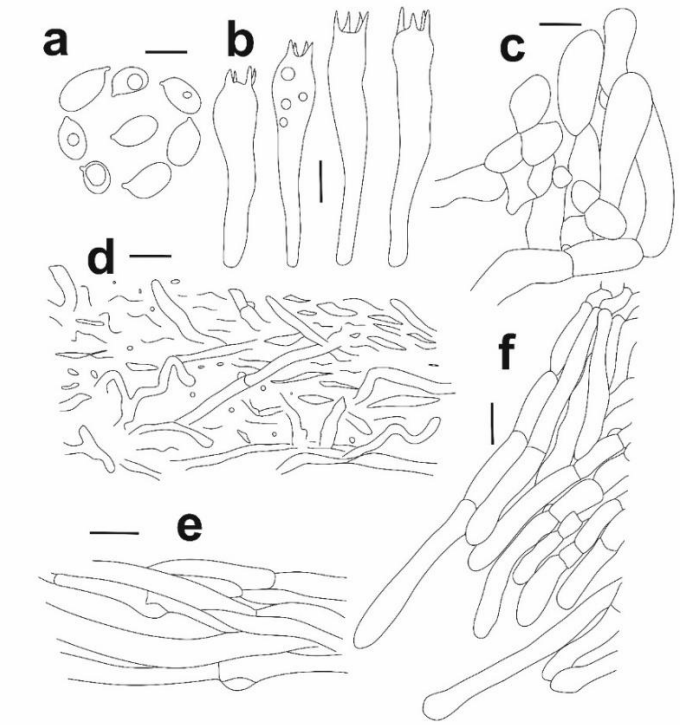

Figure 9. *Hygrophorus abieticola*: a. spores, b. basidia, c. hyphae of pileipellis, d. pileipellis, e,f. stipitipellis (scale bars: 10 µm)

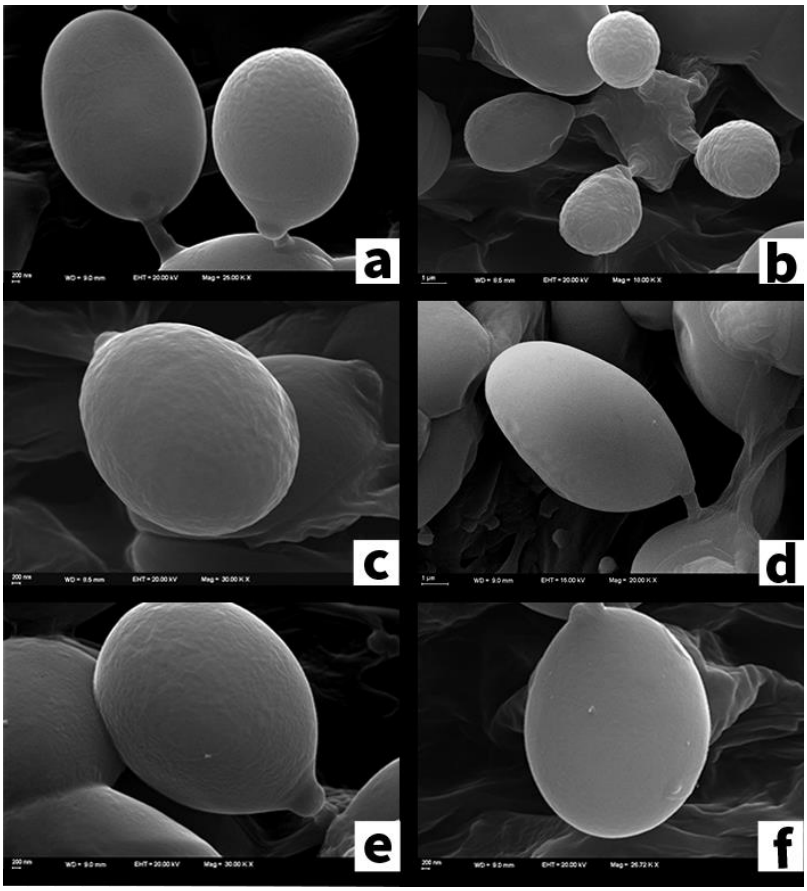

Figure 10. *Hygrophorus abieticola:* a,b. spores on a basidium, c-f spores (SEM)

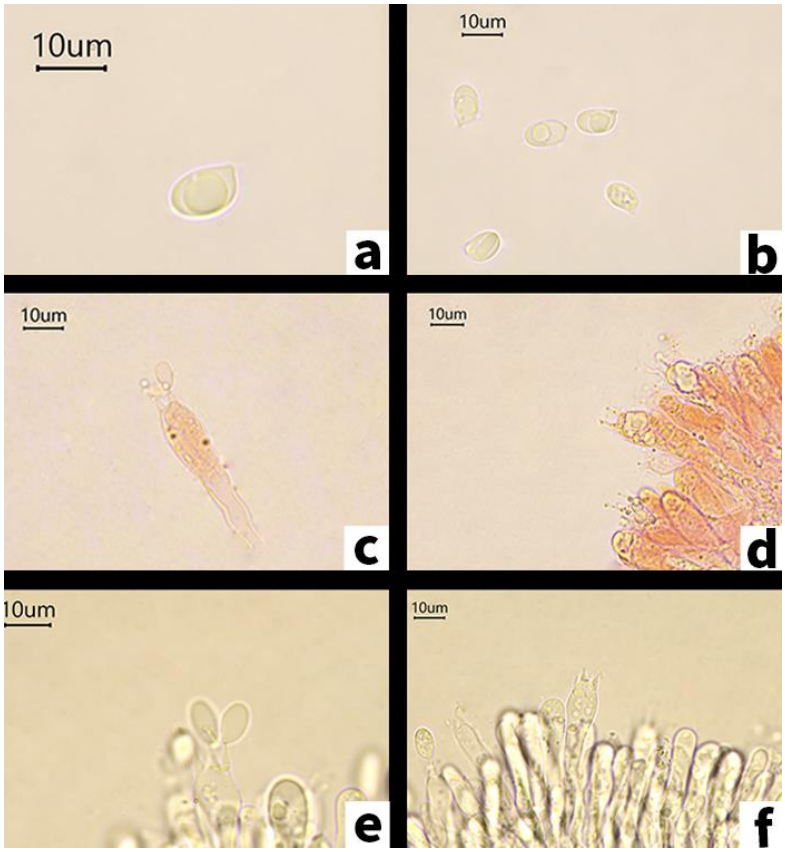

Figure 11. *Hygrophorus abieticola*: a. a single spore (LM, in 5% KOH), b. spores (LM, in 5% KOH), c. a single basidium with spores (LM, in Congo red), d. basidia (LM, in Congo red), e. single basidium with spores (LM, in 5% KOH), f. basidia (LM, in 5% KOH)

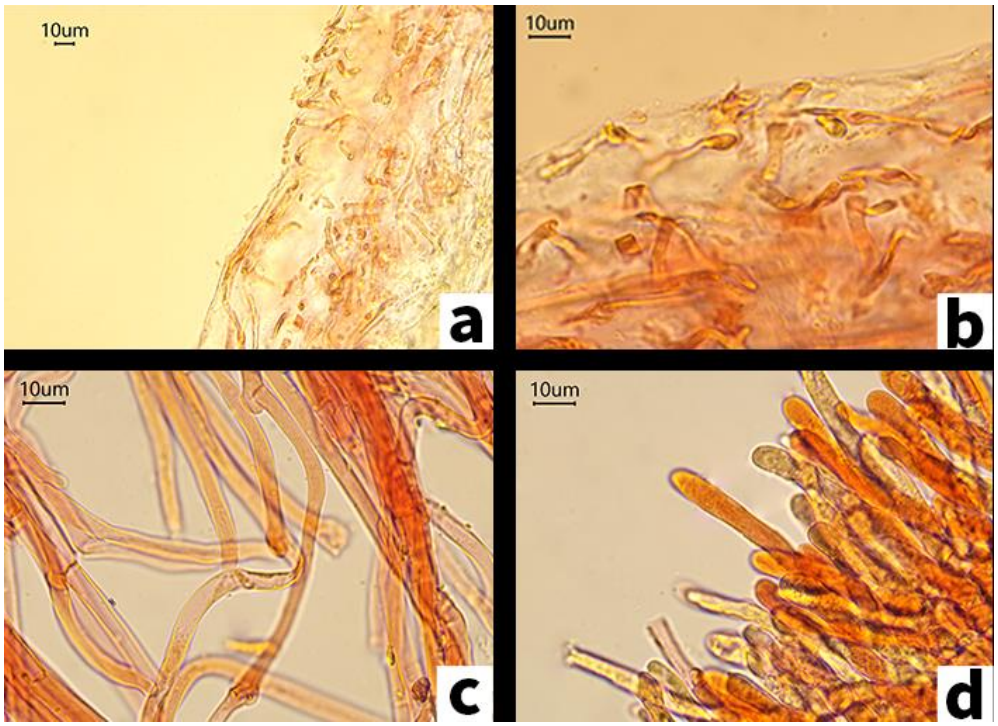

Figure 12. *Hygrophorus abieticola*: a,b. pileipellis, c,d. stipitipellis

Macroscopic and Microscopic Features

Pileus 40–70 mm diam., initially hemispherical to convex, later becoming broadly expanded to convex or flat-convex, sometimes featuring a prominent umbo at maturity. **Surface** subviscid, ochraceous orange to salmon, occasionally displaying a brownish hue and rarely somewhat squarrose towards the center at maturity. **Margin** initially incurved, later becoming straight or slightly decurved. **Lamellae** initially subdecurrent, becoming adnate at maturity, waxy, delicate, thick, orange cream to light orange, often displaying a pinkish to salmon hue and the margin somewhat pointed. **Lamellulae** subdistant to dense. **Stipe** 70–130 × 10–20 mm, solid, cylindrical, relatively uniform in thickness, often widening slightly toward the base, frequently narrower at the apex and usually curved, dry or subviscid, whitish or cream to pale orange, typically turning white at the apex and the base, sometimes displaying vivid yellowish spots on the lower part, fibrillose to slightly striate, somewhat squarrose in mature specimens. **Context** ranging from whitish to faint pink, yellowish, or orange near the base. **Odor** not distinctive, and **Taste** mild. **Spore print** white. **Spores** (8.5-) 8.8-10,5 (-11.5) × (5.7-) 5.9-6.8 (-7) µm, Q = (1.4-) 1.43-1.164 (-1.66), Qav = 1.52, ellipsoid to elongate, thin-walled, smooth, hyaline, and inamyloid. **Basidia** 48–60 × 8–10 µm, narrowly clavate, with long sterigmata, measuring 5-8 µm long, and four spored. **Cystidia** absent. **Hymenophoral trama** divergent, composed of cylindrical and hyaline hyphae. **Pileipellis** an ixotrichoderm, coated with a strongly gelatinized substance and composed of slender, cylindrical hyphae measuring 3.5–8 µm broad. **Stipitipellis** consisting of thin, gelatinized hyphae measuring 4-8 µm broad. **Clamp connections** observed in all tissues.

Material examined: TÜRKİYE— Bolu, At Plateau, in fir forest, 1210 m, 40° 46" N - 31° 30 'E, 25.10.2023, ANK AKATA 8980.

Evolutionary History ANK AKATA 8711 and ANK AKATA 8980

The ANK AKATA 8711 and ANK AKATA 8980 specimens were subjected to an investigation regarding their evolutionary lineage. This investigation involved analyzing their nrITS rDNA and *TEF1α* gene sequences. These genetic sequences were obtained using traditional molecular methods and have been recorded in the NCBI GenBank with specific accession numbers. The accession numbers for the ITS rDNA region are PP967962.1 and PP967963.1, while for the *TEF1α* gene, they are PP983238.1 and PP983237.1. In order to explore the evolutionary connections of ANK AKATA 8711 and ANK AKATA 8980, we selected various nrITS rDNA and *TEF1α* gene sequences from members of the *Agaricus* and *Hygrophorus* genera. These sequences were used as comparators within the group. Additionally, we included the nrITS rDNA region and *TEF1α* gene sequences of *Terfezia claveryi* and *Amanita vidua* as representatives of the outgroup members in the phylogenetic trees.

The molecular phylogenetic analysis, which incorporated the ITS rDNA and *TEF1α* gene sequences, revealed that ANK AKATA 8711 grouped with different members of *Agaricus nevoi* (Figures 13 and 14). The remaining samples within the ingroup, utilized for constructing both phylogenetic trees, consisted of diverse isolates of closely related *Agaricus* species. Among them are isolates of *A. gennadii*, the morphologically closest species of *A. nevoi*, separately branched from the clade comprised ANK AKATA 8711 and different isolates of *A. nevoi*. In the gene trees of ITS rDNA and *TEF1α*, *Terfezia claveryi* and *Amanita vidua* formed a separate branch distinct from the primary clades, indicating their expected role as the outgroup. On the other hand, ANK AKATA 8980 was clustered with different members of *Hygrophorus abieticola* in the molecular phylogenetic analysis, which included the sequences of both the ITS rDNA and *TEF1α* genes (Figure 15 and 16). The other samples within the same group, which were utilized to construct the phylogenetic trees, consisted of various isolates of closely related *Hygrophorus* species. *H. pudorinus*, the species most similar in appearance to *H. abieticola*, diverged independently from the clade consisting of ANK AKATA 8980 and various isolates of *H. abieticola*. In ITS rDNA and *TEF1α* gene trees, *Terfezia claveryi* and *Amanita vidua* constituted a distinct branch separate from the main clades, suggesting

their anticipated position as the outgroup. BLAST examinations of the nuclear ITS rDNA and *TEF1α* gene sequences of ANK AKATA 8711 and ANK AKATA 8980 revealed similarity rates exceeding 99% with distinct isolates of *A. nevoi* and *H. abieticola*, respectively. The phylogenetic analyses further supported the close association of these two specimens with *A. nevoi* and *H. abieticola*, with a high branch bootstrap rate indicating the reliability of the grouping.

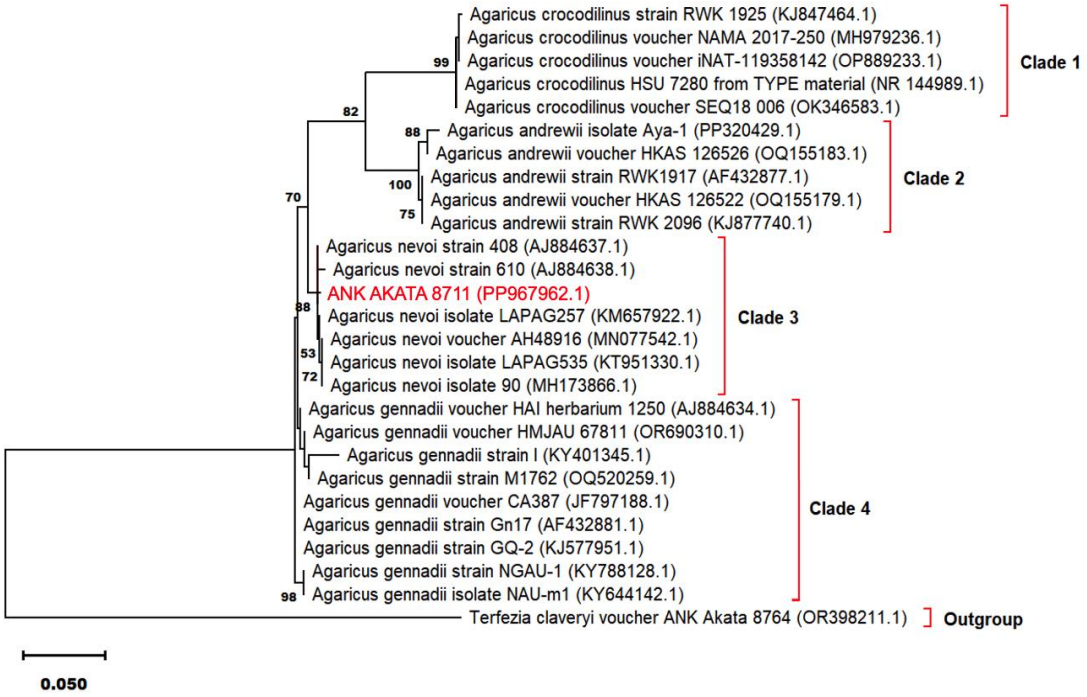

Figure 13. A phylogenetic tree was constructed to show the evolutionary relationships among 27 fungal specimens using the nrITS rDNA region and the maximum likelihood (ML) method with T92 + G nucleotide substitution model. Confidence levels were indicated by assigning bootstrap values to each branch. All sequences used in building the tree were obtained from the NCBI GenBank, with the exception of ANK AKATA 8711. Additionally, *Terfezia claveryi* was included in the phylogenetic tree as the outgroup representative. GenBank accession numbers were provided for each sequence, and a scale bar in the lower left corner represented a genetic distance of 0.05

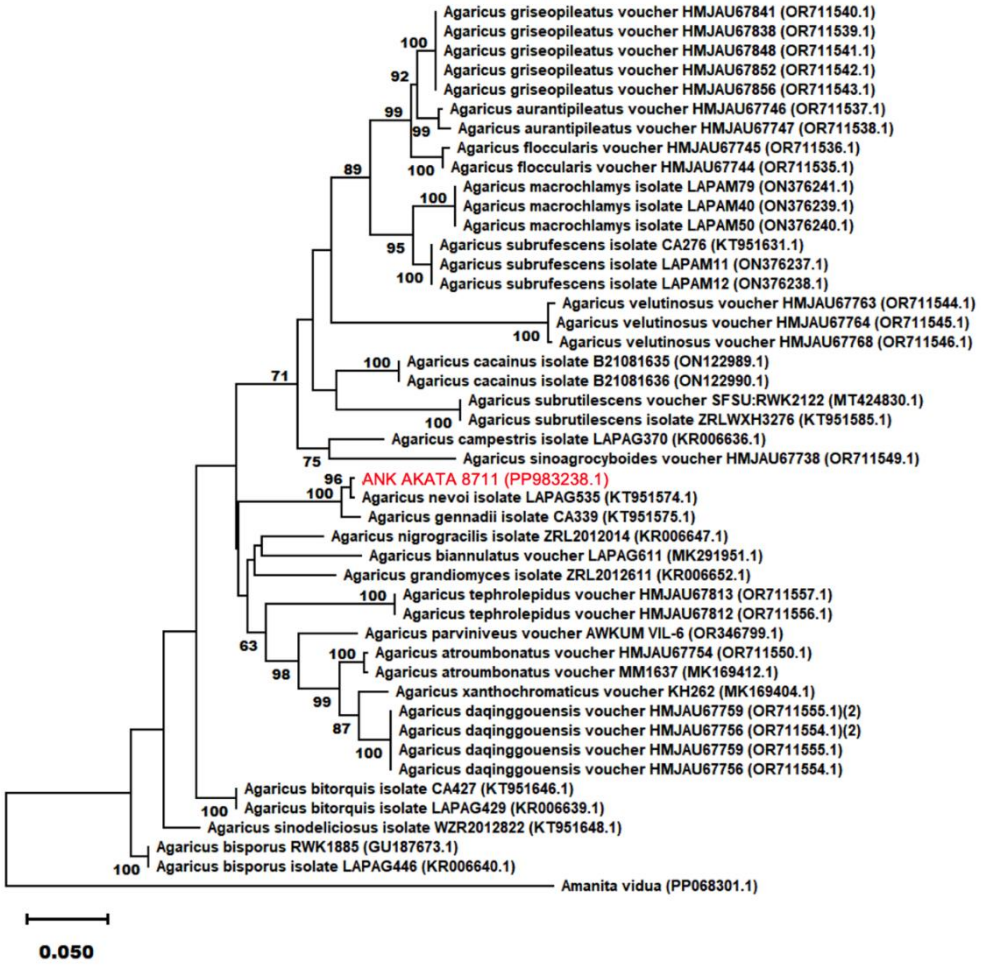

Figure 14. The evolutionary relationships among 46 fungal specimens are illustrated in a phylogenetic tree using the partial sequence of the *TEF1α* gene and the maximum likelihood (ML) method with K2 + G nucleotide substutition model. Confidence levels are indicated by bootstrap values assigned to each branch. All sequences used in constructing the tree were obtained from the NCBI GenBank, with the exception of ANK AKATA 8711. Additionally, *Amanita vidua* was included in the phylogenetic tree as the outgroup representative. GenBank accession numbers are provided for each sequence, and the scale bar in the lower left corner represents a genetic distance of 0.05

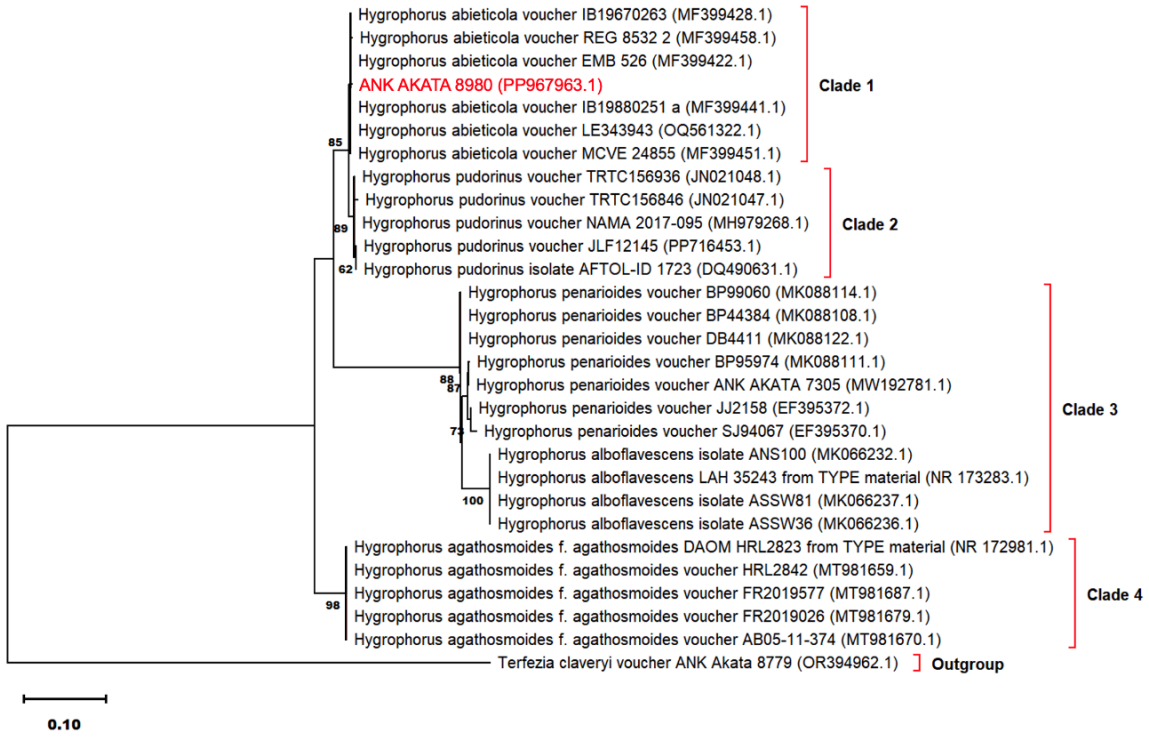

Figure 15. A phylogenetic tree was constructed to show the evolutionary relationships among 29 fungal specimens using the nrITS rDNA region and the maximum likelihood (ML) method with T92 + G nucleotide substitution model. Confidence levels were indicated by assigning bootstrap values to each branch. All sequences used in building the tree were obtained from the NCBI GenBank, except ANK AKATA 8980. Additionally, *Terfezia claveryi* was included in the phylogenetic tree as the outgroup representative. GenBank accession numbers were provided for each sequence, and the scale bar at the lower left corner represents a genetic distance of 0.1.

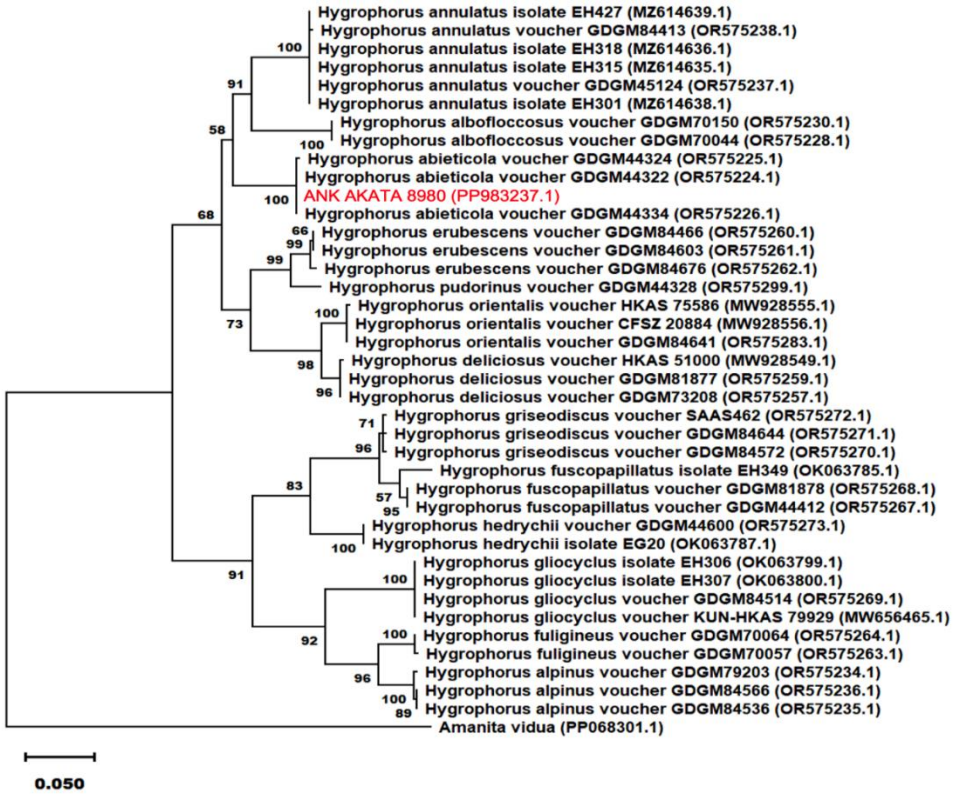

Figure 16. The evolutionary relationships among 40 fungal specimens are illustrated in a phylogenetic tree using the partial sequence of the *TEF1α* gene and the maximum likelihood (ML) method with K2 + G nucleotide substitution model. Confidence levels are indicated by assigning bootstrap values to each branch. All sequences used in constructing the tree were obtained from the NCBI GenBank, except ANK AKATA 8980. Additionally, *Amanita vidua* was included in the phylogenetic tree as the outgroup representative. GenBank accession numbers are provided for each sequence, and a scale bar in the lower left corner represents a genetic distance of 0.05.

Discussion

Agaricus nevoi, *A. gennadii*, and *A. pequinii* form a closely related cluster, as evidenced by morphological characteristics and internal transcribed spacer (ITS) sequence data. This grouping suggests a high degree of genetic and phenotypic similarity among these species, underscoring the importance of integrating molecular and traditional taxonomic approaches in delineating species boundaries within the genus *Agaricus* (Didukh et al., 2005).

The differentiation among these species is notably marked by the diverse appearances of their universal veil remnants, including variations in shape, size, and texture, hinting at a volva-like formation (Wasser, 1995; Wasser et al., 2002). Additionally, these species exhibit distinctive traits such as the color change of the flesh upon injury or with age and the particular forms of their spores and cheilocystidia. This blend of morphological and genetic data emphasizes the rich variety within these closely related groups, pointing to the significance of visible and microscopic characteristics for their identification and categorization (Cappelli, 1984; Wasser et al., 2002; Didukh et al., 2005).

Agaricus nevoi is recognized by its broad, smooth, and substantial basal volva featuring a dual margin that curves outward, along with flesh that remains white, potentially turning pink near the lamellae. Under the microscope, it shows short clavate cheilocystidia, with dimensions of 26-33 × 6.5-9 µm, and ellipsoid spores, measuring on average 7.3 × 5 µm (Wasser, 2002; Didukh et al., 2005). In contrast, *A. gennadii* presents a slender basal volva, resembling a whitish veil sock that

reaches up to mid-stipe, with a margin extending 3 mm wide. The flesh is white, showing minimal to no browning when sliced, with the stipe base turning orange or yellow. Its cheilocystidia are cylindrical to narrowly clavate, with sizes ranging from 20-36 × 8.5-10 µm, and its spores are ellipsoid to broadly ellipsoid, averaging 8.6 × 6.5 µm (Capelli, 1984; Nauta, 2001; Didukh et al., 2005). Meanwhile, *A. peguini* displays a whitish-greyish, short volva with a ragged margin and flesh that shifts to pink or lightly brown. Its cheilocystidia are clavate to fusiform, measuring 23-32 × 7-9 µm, and its spores are ovoid, with an average size of 6.6 × 4.7 µm (Wasser et al., 2002; Didukh et al., 2005).

Although *A. nevoi* and *A. gennadii* exhibit white flesh, the coloration reaction to cutting between *A. gennadii* and *A. peguini* varies in intensity and hue. Additionally, the dimensions and forms of cheilocystidia and spores distinguish each species, with *A. nevoi*'s microscopic characteristics differing from those of *A. gennadii*, which diverge from *A. peguini*. These morphological differences and specific adaptations underscore the distinct identities within the *Agaricus* genus categorization (Cappelli, 1984; Wasser, 1995; Nauta, 2001; Wasser et al., 2002; Didukh et al., 2005).

Wasser (1996) detailed the morphological traits of *A. nevoi* samples, including specific features of various macroscopic and detailed properties and dimensions of microscopic structures. Table 2 thoroughly compares the *A. nevoi* specimens analyzed in this study and those documented by Wasser. It summarizes the observed dimensions of various structures in our samples, emphasizing the similarities and differences in measurements and features compared to the type specimens.

A thorough comparison of the macroscopic and microscopic characteristics of *Agaricus nevoi* specimens from our study with those described by Wasser (1996) reveals several points of similarity and some notable differences. Macroscopically, the dimensions of our specimens closely align with those reported in Wasser's findings, providing a consistent framework for identifying these structures. This alignment supports the reliability of using these macroscopic traits for accurate identification. However, our detailed analysis has revealed significant discrepancies, particularly in the dimensions of some microscopic elements.

Table 2. Comparison of various morphological structure measurements of *A. nevoi*

Dimensions	Wasser (1996)	Present study
Pileus	50-70 mm broad	50-60 mm broad
Stipe	40–60 × 18–22 mm	50–60 × 20–25 mm
Spores	(6)7-8.5 x 4.5-5.5 µm	(5.7-) 6.4-8.5 (-9) × (4.8-) 5.5-6.7 (-7) µm
Spores (Q-values)	not provided	Q = 1.14-1.28 (-1.47), Qav = 1.22
Basidia	24-27 x 7.5-8.5 µm	28–33 × 9–10.5 µm
Sterigmata	3-4 µm long	4-5 µm long
Cheilocystidia	26-33 x 6.5-9 µm	26-35 × 7-9.5 µm
Pileipellis	4-7 µm broad	6-18 µm broad

In our study, we observed that the spore measurements of our specimens were notably larger than those previously reported. Specifically, the dimensions ranged from (5.7-) 6.4-8.5 (-9) × (4.8-) 5.5-6.7 (-7) µm, with a mean aspect ratio (Qav) of 1.22. This surpasses the previously reported range of 6-8.5 x 4.5-5.5 µm, for which Q values were not provided. Additionally, the basidium dimensions measured in our study, which were 28–33 × 9–10.5 µm, exceeded those documented by Wasser, which were 24-27 x 7.5-8.5 µm. These differences suggest significant variations in the fungal reproductive structures, critical for making precise taxonomic distinctions. Further examination revealed that the cheilocystidia in our specimens, described as clavate to short-clavate, were measured to be 26-35 x 7-9.5 µm, which is similar to the dimensions reported in the previous study (26-33 x 6.5-9 µm). This similarity indicates that cheilocystidia dimensions may be stable across different specimens. Moreover, we found that the hyphae of the pileipellis in our specimens were broader, measuring 6-18 µm

in width, compared to the 4-7 µm width reported by Wasser (1996). This broader range in our specimens suggests a stable characteristic that can be consistently observed across various samples.

Initially, the name *Hygrophorus pudorinus* (Fr.) Fr. was erroneously used for a *Hygrophorus* species associated with *Abies* trees. However, Krieglsteiner recognized the distinct species associated with *Abies* as *H. abieticola*, a classification later validated by Gröger and Bresinsky (Bresinsky, 2008). This validation confirmed *H. abieticola* as a distinct species and established it as a type of subgen. *Colorati*, sect. *Pudorini*, subsect. *Salmonicolores* (Lodge et al., 2014).

Hygrophorus abieticola, first recognized in Europe (Larsson & Jacobsson, 2014), exhibits distinctive features that aid in its identification. The pileus displays shades of orange, while the lamellae range from an orange to a pinkish hue. This species is often found in habitats covered with moss and establishes a symbiotic relationship with fir (Wang et al., 2023).

H. abieticola and *H. magnisporus* C.Q. Wang, Xiao Lan He & T.H. Li can be confused due to their similar morphological features. However, they can be distinguished by specific characteristics. The former species is known for its more robust basidiomata and distinctive orange color on the pileus and stipe. In contrast, the latter presents slender and white basidioma, with a pileus typically convex to plano-convex when mature and has distant lamellae. Furthermore, the spores (11.5-15 x 6.5-9 µm) of *H. magnisporus* are notably larger (Wang et al., 2023).

A comparison of the macroscopic and microscopic features of *Hygrophorus abieticola* specimens with those described by Wang et al. (2023) reveals notable similarities. The dimensions of the pileus, stipe, basidiospores, and hyphae in both the pileipellis and stipitipellis of our specimens closely match the measurements reported by Wang et al. (2023), offering a reliable basis for identifying these structures. The dimensions of the pileus, stipe, basidiospores, and hyphae in the pileipellis and stipitipellis in our specimens align closely with the measurements reported by Wang et al. (2023), providing a dependable basis for identifying these structures (Table 3). There are some minor differences, however. In our specimens, the basidiospores measured (8.5-) 8.8-10.5 (-11.5) × (5.7-) 5.9-6.8 (-7) µm, with a mean aspect ratio (Qav) of 1.52. This is slightly smaller than the reported range of 9–10.5 (11.5) × 5.5–6.5 (7) µm and a Qav of 1.64. Additionally, the basidia dimensions in our study were 48–60 × 8–10 µm, which are smaller than those documented by Wang et al. (2023) at 50–86 × 8–11 µm. Furthermore, we found that the hyphae of the pileipellis and stipitipellis in our specimens were slightly broader, measuring 3-10 µm and 3.5–8 µm respectively, compared to the 2.5–6.5 µm and 4-8 µm width reported by Wang et al. (2023).

Table 3. Comparison of various morphological structure measurements of *H. abieticola*

Dimensions	Wang et al. (2023)	Present study
Pileus	20–70 mm broad	40–70 mm broad
Stipe	60–150 × 7–17 mm	70–130 × 10–20 mm
Spores	9–10.5(11.5) × 5.5–6.5(7) µm	(8.5-) 8.8-10.5 (-11.5) × (5.7-) 5.9-6.8(-7) µm
Spores (Q-values)	(1.43)1.46–1.82(1.92), Qav = 1.64	Q = (1.4-) 1.43-1.164 (-1.66), Qav = 1.52
Basidia	50–86 × 8–11 µm	48–60 × 8–10 µm
Sterigmata	up to 7 µm long	5-8 µm long
Stipitipellis	4-8 µm	3-10 µm broad
Pileipellis	2.5–6.5 µm	3.5–8 µm

These findings highlight the critical need for re-evaluating and updating taxonomic descriptions as specimens of newly reported species are analyzed. The variations observed in spore size, basidium dimensions, pileipellis, and stipitipellis hyphae width underscore the necessity for continuous study and comparison to enhance our understanding of the taxonomy of these species. The differences may be crucial for defining species boundaries and improving the accuracy of distinguishing morphological

characteristics among different species samples.

The morphological diversity observed among fungal species is greatly exceeded by their genetic diversity, prompting the need to integrate genetic data with traditional morphological methods to achieve more precise species identification. Molecular systematics has relied on an array of genetic markers for this purpose, particularly regions within the rRNA genes, such as nrITS, nrSSU, and nrLSU, alongside protein-coding genes like TEF1α (Raja et al., 2017; Akata et al., 2024a; 2024b; 2024c). The ITS rDNA and TEF1α regions within this range of markers are precious for fungal molecular taxonomy, offering critical insights that enhance species classification and identification accuracy. Advances in high-throughput sequencing technologies and bioinformatics have transformed the field by enabling comprehensive whole-genome comparisons and phylogenomic analyses across fungal taxa, marking a significant shift from traditional molecular phylogenetic approaches that rely on only a limited number of marker genes (Marian et al., 2024). In our study, we utilized nuclear ITS rDNA and TEF1α gene sequences to perform the molecular identification of ANK AKATA 8711 and ANK AKATA 8980. The results revealed over 99% sequence similarity with previously documented specimens in GenBank, specifically PP983238.1 and PP983237.1. These findings confirmed the identity of ANK AKATA 8711 as *A. nevoi* and ANK AKATA 8980 as *H. abieticola* (Figure 13-16).

Conclusion

The recent research has verified for the first time the occurrence of *Agaricus nevoi* and *Hygrophorus abieticola* within Türkiye, thereby enhancing the known diversity of the Turkish mycobiota. The reports of these species were grounded on the morphological examination of the specimens labeled ANK AKATA 8711 and ANK AKATA 8980, which aligned with the diagnostic traits of *A. nevoi* and *H. abieticola*, respectively. Additionally, molecular phylogenetic assessments using ITS rDNA and *TEF1α* sequences corroborated the identifications made through traditional morphological methods. These records have increased the total species counts of the genera *Agaricus* and *Hygrophorus* in Türkiye to 37 and 31, respectively.

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Ethics Committee Approval

N/A

Peer-review

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Author Contributions

Conceptualization: I.A., E.Ş.; Investigation: I.A., E.K, G.E. E.Ş.; Material and Methodology: I.A., E.K, G.E. E.Ş.; Supervision: I.A.; Visualization: E.K, G.E. İ.A.; Writing-Original Draft: I.A., E.K, G.E. İ.A.; Writing-review & Editing: I.A., E.Ş. The authors have seen and agreed to publish the version of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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