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Mycorrhizal Associations between *Helvella bachu* and Its Host Plants

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Abstract: *Helvella bachu*, a prized edible and medicinal fungus, is primarily found in the forests of *Populus euphratica*, an ancient and endangered species crucial to desert riparian ecosystems. Despite extensive efforts, the isolation of pure cultures and cultivation of fruiting bodies of *H. bachu* have remained elusive. While some species within the *Helvella* genus have been confirmed as ectomycorrhizal fungi, others have been considered either saprotrophic or mycorrhizal. By integrating field observations of *H. bachu* habitat, macro- and micro-anatomical examination of plant root tips, and molecular data from fruiting bodies, mycorrhizae, and host plants, it has been confirmed that *H. bachu* forms ectomycorrhizal associations with *Populus* trees. The mycorrhiza of *H. bachu* displays a light earth color with a curved smooth cylindrical shape. It features a thick mantle and the presence of a Hartig net, accompanied by a small amount of epitaxy mycelia. Morphological observation of the root tips requires meticulous handling, and the paraffin section technique has yielded noteworthy results. Host plants encompass four *Populus* species, including *P. euphratica*, *P. pruinosa*, *P. nigra*, and *P. alba* var. *pyramidalis* (synonym *Populus bolleana*). A conservation area was established within the young *P. euphratica* forest at Tarim University, resulting in a 14.75% increase in the quantity of fruiting bodies during the second year. Establishing a conservation area and in situ propagation of *H. bachu* holds economic and ecological implications. This study will contribute to the conservation of resources related to *H. bachu* and *P. euphratica*.

Keywords: *Helvella bachu*; mycorrhiza; *Populus*; macro- and micro-anatomical characteristics

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1. Introduction

Bachu mushrooms are commonly enjoyed as delicious edible fungi in the Xinjiang Autonomous Region, China [1], as well as in Pakistan [2] and Iraq [3]. These mushrooms have received considerable attention in recent years due to their high economic, nutritional, and medicinal values [4]. They have been reported to efficiently enhance the phagocytic ability of leukocytes, lymphocyte conversion ratio, and antibody titer [5]. The polysaccharides from Bachu mushrooms exhibit strong antioxidant activities and demonstrate a relatively high inhibition rate on HepG2 (hepatocellular carcinoma cell) cells [6]. Additionally, the polysaccharides can attenuate high-fat-diet-induced high blood cholesterol and regulate the expression of lipid metabolism genes in vivo. This makes them a promising novel complementary alternative strategy for managing hyperlipidemia [7].

However, Bachu mushrooms have proven challenging to cultivate successfully, and obtaining a pure culture has also been elusive after years of effort. The annual yield of dried fruiting bodies of the Bachu mushrooms in China is up to 3000–5000 kg, averaging USD 150 per kilogram. In addition, Bachu mushrooms are distributed in forests of *Populus euphratica*, which is a rare, ancient, and endangered species and an essential component of desert riparian ecosystems [8]. Hence, the artificial domestication of Bachu

mushrooms holds both economic and ecological significance. Understanding the fungal nutritional mode is essential for the successful artificial domestication and cultivation of this valuable species.

Bachu mushrooms were confirmed to comprise two distinct new species, *Helvella bachu* and *H. subspadicea* [1]. *H. bachu* is a common and widespread species in the southern Xinjiang Autonomous Region, with "bachu" proposed as the trade name for Bachu mushroom. Pezizales species constitute a considerable proportion of the mycobionts in ectomycorrhiza (ECM) fungal communities in mature boreal deciduous and coniferous forests across several soil types. Two species of *Helvella*, *Helvella* aff. *Cupuliformis* and *H. crispa*, were detected on the root tips of oak and *Pinus armandii* [9,10]. Previous studies showed that *H. aestivalis* and two unidentified *Helvella* spp. formed ECM with *Dryas* sp. and *Salix* sp. [11]. The study by Hwang et al. [10] revealed that mycorrhizal lineages were distributed across the *Helvella* phylogeny. They confirmed that certain species of *Helvella*—such as *H. dovrensis*, *H. lacunosa*, *H. zhongtiaoensis*, *H. reflexa*, *H.* cf. *crispa*, *H. macropus* and *Helvella* cf. *Lacunosa* [12]—were ectomycorrhizal fungi. Meanwhile, *H. elastica* and *H. atra* were highly likely to be saprophytic fungi. *H. bachu* was reported to be scattered or gregarious on the sandy ground under *P. euphratica* or *P. bolleana* [1]. Whether it can form ECM with *Populus* is important for achieving the pure culture and artificial cultivation of this fungus and for the protection of *P. euphratica* forests.

Here, through investigating the field habitat of *H. bachu*, examining the macro- and micro-anatomical characteristics of the host root tip, and conducting molecular biological identification of the fruiting body and mycorrhiza, this study has initially demonstrated that *H. bachu* is an ectomycorrhiza. This finding helps to understand the nutritional mode of *H. bachu* and offers guidance for its artificial cultivation. Additionally, these results hold great significance for the restoration of degraded poplar forests and desert ecosystem restoration in Xinjiang.

2. Materials and Methods

2.1. Sampling, Processing, and Observation

Fungal samples were collected in Aral City, Bachu, and Maigaiti Counties of Kashi Prefecture, Xinjiang Uygur Autonomous Region of China, in April 2022 and 2023. Sample details, including fruiting bodies of *H. bachu* and soils and leaves of host plants, are listed in Table 1. The growth state of *H. bachu* in each location was photographed and observed. Additionally, the occurrence quantity of *H. bachu* and the distance between *H. bachu* and plants were measured. The soil pH, nitrogen (N), phosphorus (P), and potassium (K) contents were tested using a seven-in-one soil parameter speedometer (Prsens, Jinan, China).

Root samples in the soil with a diameter of 20 cm and depth of 20 cm were collected at the location where the fruiting bodies of *H. bachu* occurred. These samples were carefully transported back to the laboratory in a sampling box within 2 h and were subsequently preserved at 4 °C for no more than one week. The root tips were handled very carefully following the method of Wang et al. [13]. First, the loose soil outside was removed by gentle shaking. Then, the roots were delicately washed in tap water to eliminate most of the soil and organic debris, minimizing any damage to ECM roots. Tightly adhering materials were removed carefully using forceps. Subsequently, the cleaned roots were cut into 2 cm long segments and placed in a Petri dish filled with tap water. The enlarged thickened root tips were meticulously selected and examined under a stereo microscope (SMZ1500, Nikon, Minato-ku, Tokyo, Japan) to observe the color, shape, size, texture, branching, emanating elements, and other taxonomically relevant morphological features [14,15].

The microstructures of mycorrhiza were identified using the paraffin section technique. The cleaned root tips were immersed in FAA fixed liquid (formaldehyde/glacial acetic acid/alcohol/distilled water at a ratio of 6:1:20:40), subjected to vacuum pumping, and then placed into 4 °C refrigerators. After fixing for more than 24 h, the root was treated with 10% KOH in a water bath at 90 °C until it became translucent. Subsequently, it was de-

hydrated using various alcohol gradients, followed by transparency treatment with xylene, wax soaking, embedding in molds, solidification in a freezer, and subsequent slicing. After cutting into different wax strips, they were placed in a constant temperature water bath at 40 °C to adhere to slides coated with adhesive. The slides were then put in an incubator overnight to dry, followed by dewaxing with xylene. Staining was performed using trypan blue, and then a decolorizing solution was applied [16,17]. Following microscopic examination of the lateral distribution of the mantle and the Hartig net in the intercellular space using a Nikon Eclipse 80i microscope (Nikon, Minato-ku, Tokyo, Japan), a permanent sheet was created using neutral resin for sealing.

Table 1. Collection of fruiting bodies of *H. bachu* and host plants.

ID	Location	Habitat	Host Specimens	Soil Texture	Longitude and Latitude
A	Conservation Land, Tarim Uni., Aral	Lawn and *Populus*	HB-ALE-TUR-H9	Sandy loam	N: 40.323320 E: 81.18219
B	North Gate, Tarim Uni., Aral	Poplar forest	HB-ALE-TUN-H10	Sandy loam	N: 40.5488299 E: 81.2929233
C	6th Company, 11th Regiment, Aral	Mixed windbreak	HB-ALE-1106-H1 HB-ALE-1106-H5	Sandy soil	N: 40.35179 E: 81.412649
D	4th Battalion, 11th Regiment, off State Route 217, Aral	*Populus*	HB-ALE-1104-H2	Sandy soil	N: 40.35179 E: 81.412649
E	Entrance to 217 Route, 11th Regiment, Aral	*Populus*	HB-ALE-11217-H6	Sandy soil	N: 40.35179 E: 81.412649
F	Tarim Bridge head, Next to the 12th Regiment, Aral	*Populus*	HB-ALE-1200-H11	Sandy soil	N: 40.323320 E: 81.18219
G	Beside the road of 5th Company, 13th Regiment, Aral	*Populus*	HB-ALE-1305-H3	Sandy soil	N: 40.401973 E: 81.295430
H	Kokochal Village, 14th Regiment, Aral	*Populus*	HB-ALE-1400-H4	Sandy loam	N: 40.6437333 E: 81.8031466
I	Alongside the road of 14th Regiment, Aral	Mixed windbreak	HB-ALE-1405-H7 HB-ALE-1405-H12	Sandy soil	N: 40.384379 E: 81.482014
J	217 Route Junction between 14th regiment and 10th regiment 7, Aral	Mixed windbreak	HB-ALE-1410-H8 HB-ALE-1410-H13	Sandy soil	N: 40.384379 E: 81.482014
K	Xiamale Forest Farm, Bachu county, Kashi	*Populus*	-	Sandy soil	N: 39.5768316 E: 79.4363783
L	Humudan village, Bazhajiemi Town, Maigaiti County, Kashi	*Populus*	-	Sandy soil	N: 38.8943850 E: 77.6115016

2.2. DNA Analyses of *H. bachu* Ectomycorrhizae and Host Plants

The mycorrhizal samples were carefully selected and then frozen in a 2 mL centrifugal tube using liquid nitrogen. Subsequently, they were placed in a −80 °C refrigerator in preparation for DNA extraction. The improved CTAB method was utilized to extract mycorrhizal DNA, followed by the use of ITS1-F and ITS4 primers (ITS1-F: CTTGGTCATTTAGAGGAAGTAA; ITS4: TCCTCCGCTTATTGATATGC) [18] for PCR amplification. The system comprised 25 μL of green mix (Cat No. P222-w1, Vazyme Biotech Co., Ltd., Nanjing, China), 2 μL of ITS1-F and 2 μL of ITS4, 2 μL of template DNA, and 19 μL of distilled water. The PCR amplification reaction procedure involved pre-denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 15 s, extension at 72 °C for 15 s, and final extension at 72 °C for 10 min. The PCR products were analyzed using 1% agarose gel electrophoresis at a setting voltage of 120 V and current of 100 mA for 40~50 min. The 780 bp target band was then purified and recovered using a Vazyme kit, and the PCR products were sequenced by Sangon Biotech Co., Ltd. (Shanghai, China).

Leaves of the host plant were collected from 10 locations where *H. bachu* with different hosts were found (Table 1). The ITS2 sequence of Salicaceae nuclear gene was amplified using S2F and S3R primers (S2F: 5 'ATGCGATACTTGGTGTGAAT 3': S3R 5

'GACGCTTCTCCAGACTACAAT 3') [19]. The PCR reaction conditions for 25 µL comprised 12.5 µL of mix, 1 µL of S2F, 1 µL of S3R, 1 µL of template DNA, and 9.5 µL of distilled water. The PCR amplification procedure involved initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 45 s, and final extension at 72 °C for 10 min. The PCR products were then sent to Sangon Biotech Co., Ltd. for sequencing.

2.3. Phylogenetic Analyses

Relevant nucleotide sequences were downloaded from the GenBank database to determine the taxonomic status of mycorrhizae and host plants. Homologous ITS sequences obtained from GenBank were aligned using the MultAlin program version 5.4.1 [20] from the INRA server with a DNA-5-0 score matrix. Alignments were thoroughly reviewed and manually edited as needed. For phylogenetic analyses, sequence distances were calculated using Kimura's two-parameter model in the MEGA7 program [21], with pairwise deletion of gaps. Phylogenetic trees were constructed via maximum likelihood in the MEGA7 program and evaluated through bootstrapping with 1000 replicates. The resulting phylogenetic trees were visualized and edited using the Tree Explorer tool within MEGA7 version 7.0.26 [21].

3. Results

3.1. Occurrence of Helvella bachu Mushroom

We collected Bachu mushrooms from two regions of Xinjiang Uygur Autonomous Region—Alar City and Kashi Prefecture—from 2022 to 2023. A comprehensive investigation was conducted in twelve main production locations (Table 1). The fruiting bodies were identified as *H. bachu* (Figure 1A,B) using universal primers for fungi (ITS4 and ITS5), and the sequence of samples from Bachu county was deposited in GenBank under accession number PP082042. It was found that *H. bachu* occurs only in April and May each year around the Yeerqiang River, Tarim River, and Hotan River basin, growing under *Populus* trees or aspen trees (Figure 1C). The area is situated in a warm temperate zone with an extreme continental arid desert climate characterized by an annual average temperature ranging from 9.8 to 11.4 °C. The temperature during the occurrence of the mushroom varied from 15 to 31 °C. Additionally, the soil composition in the region was predominantly sandy. The Aral area experienced an average annual rainfall of 40.1~82.5 mm.

H. bachu was found scattered or clustered on the sandy ground near *Populus* (Figure 1D,E), with some even found beneath the *Populus* (Figure 1F). Additionally, we observed the mushroom in the vicinity of *Medicago sativa* L., but several meters away there were also *Populus* trees (Figure 1G). The soil pH was recorded between 6.5 and 7.2, with N, P, and K content measured at 18.89, 25.67, and 64.22 mg/kg, respectively, and a conductivity of about 267 µs/cm. Fresh *H. bachu* mushrooms are commonly traded in the market from April to May each year and have gained popularity (Figure 1H,I).

In order to observe the occurrence of *H. bachu* and its surrounding environments, a quadrat measuring about 300 square meters was selected in Tarim University, Alar City (Figure 2). There were 24 *P. euphratica* trees approximately five to ten years old within the selected quadrat. In 2022, the diameter of *Populus* ranged from 5.23 cm to 23 cm, with an average diameter of 11.13 cm. The average distance between *Populus* trees was measured at 107.48 cm, with the farthest distance recorded as 451 cm and the closest as 1 cm. During April and May in both 2022 and 2023, a detailed observation of the mushroom *H. bachu* in this quadrat was conducted. A total of 122 mushrooms was observed in 2022, while 140 were observed in 2023. The distance between the mushrooms varied from 0 cm to 395 cm, with an average distance of 47.98 cm. Additionally, the distance between the mushrooms and the trees ranged from 0 cm to 274.5 cm, with an average distance of 113.55 cm. Interestingly, although there were no *H. bachu* mushrooms around some *Populus* trees in 2022 (tree W and U in Figure 2), a significant harvest of mushrooms occurred in 2023. This highlights the fluctuating nature of fungal distribution in relation to host trees. This

experiment conducted at Tarim University provided valuable insights into the occurrence of *H. bachu* mushrooms and their relationship with the surrounding environment.



Figure 1. Habitats and market of *Helvella bachu.* Bar: 1 cm. (**A**,**B**) fruiting body of *H. bachu*; (**C**) *Populus euphratica* forest; (**D**–**F**) *H. bachu* and *P. euphratica* indicated by red and blue arrows, respectively; (**G**) *Medicago sativa* L. and *H. bachu*; (**H**,**I**) *H. bachu* traded in the market.

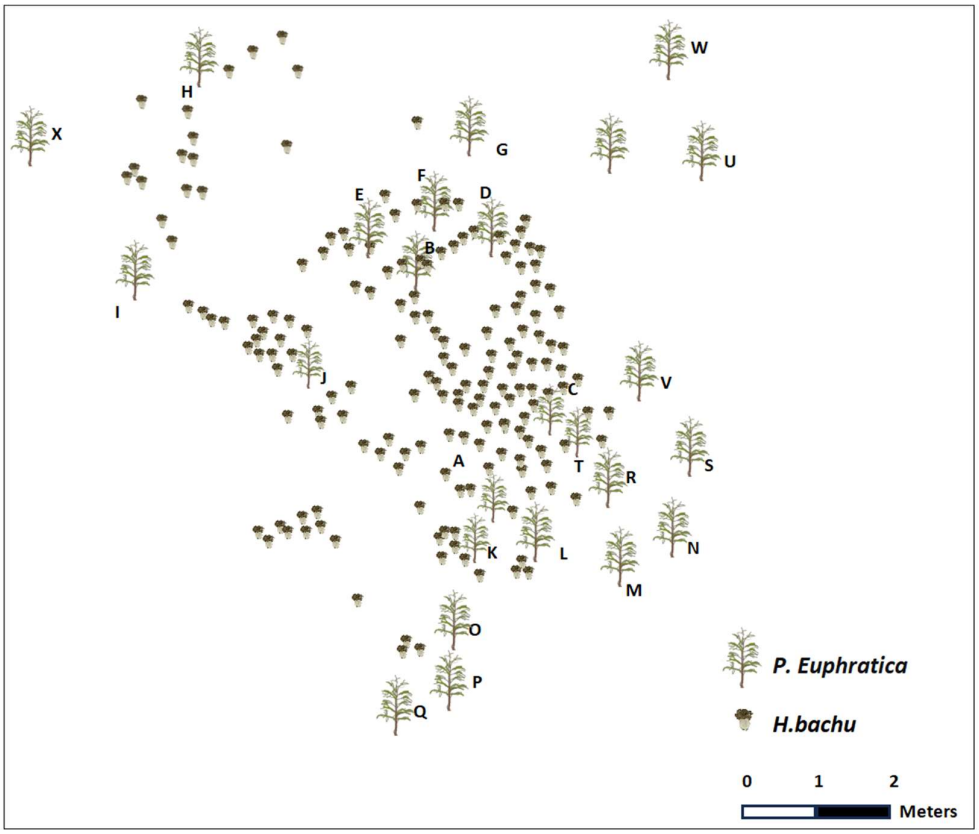


Figure 2. The distribution of *Helvella bachu* and *Populus euphratica* in the quadrat in 2022. Different letters indicated the different *P. euphratica*.

3.2. Ectomycorrhiza Identification through Analysis of ITS Sequences

Following the harvest of fruiting body of *H. bachu*, several mycorrhizal samples were carefully selected under a stereo microscope. A total of 20 mycorrhizal samples were collected from the location where the fruiting bodies occurred in the aforementioned *P. euphratica* forest quadrat and were blended for DNA extraction. PCR amplification was performed using the specific primers ITS1F and ITS4 for ectomycorrhiza [18], which resulted in a target band of approximately 780 bp. The amplified DNA was subsequently sequenced and the sequence has been deposited in GenBank under the accession number PP082046. Phylogenetic analysis conclusively identified the mycorrhizal samples as *H. bachu* (Figure 3).

Additionally, mycorrhizal samples were collected from windbreak poplars in Yahumudan village, Bazhaziemi town, Makti county, and Kashi City, where the fruiting bodies of *H. bachu* were found. Twenty mycorrhizal samples with different morphologies were selected, and three of them displaying similar morphology were identified as *H. bachu* (Figure 3) with GenBank entry numbers PP082043, PP082044, and PP082045. Based on these findings, it is highly probable that *H. bachu* is a type of ectomycorrhizal fungi.

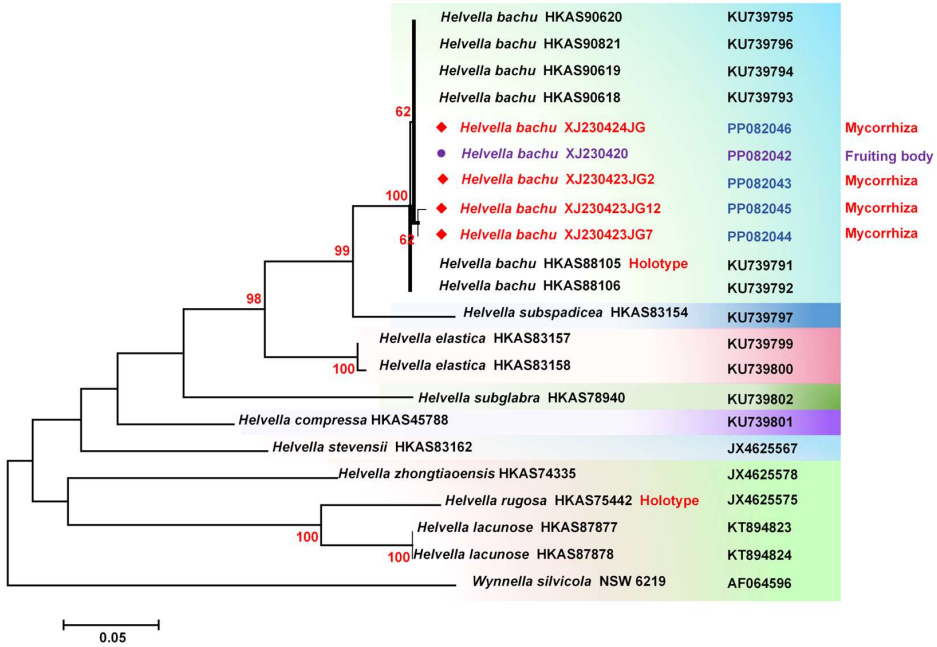


Figure 3. Molecular phylogenetic analysis of *Helvella bachu* and mycorrhiza by maximum likelihood method. The species in red and purple indicated the sequences from fruiting body and mycorrhiza respectively which were obtained from this study, while the others were downloaded from GenBank.

3.3. Macro- and Micro-Morphological Characteristics of Mycorrhiza of Helvella bachu

The root tips displayed a light earth color (Figure 4A–D). The mycorrhiza formed a coral-like structure without branches. The diameter of the mycorrhiza ranged from 200 to 500 µm, while the length varied from 1 to 2 mm. A thin sheath was observed wrapping around the root tip, with some hyphae present surrounding the root system (Figure 4B). The mycorrhizae appeared with a light earth color and had a curved smooth cylindrical shape. Paraffin sectioning and trypan blue staining revealed that the intercellular space in the transverse section was stained blue, indicating that the mycelia had penetrated the intercellular space and formed the characteristic structure known as the Hartig net (Figure 4E,F). Epitaxial hyphae were observed around the mycorrhizal structure (Figure 4D). Additionally, the mycorrhizae were encircled by a dense network of white hyphae on the surface of the root system, referred to as the mantle (Figure 4F).

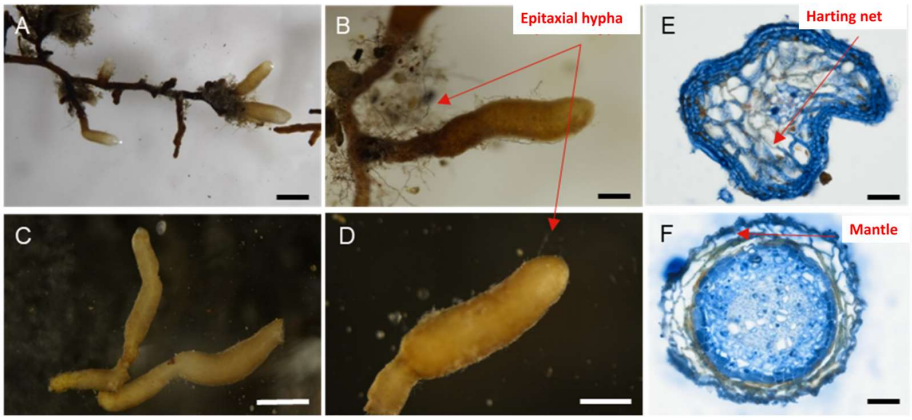


Figure 4. Macro- and micro-morphological characteristics of the mycorrhiza of *Helvella bachu*. (**A**–**D**) Macrograph of *H. bachu* mycorrhizae; (**E**,**F**) transverse structure of *H. bachu* mycorrhizae with Hartig net (**E**) and mantle (**F**). Scale: (**A**): 1 mm; (**B**–**D**): 500 µm; (**E**,**F**): 30 µm.

Considering the molecular identification, morphology, and micro-anatomical structure, it is evident that *H. bachu* is closely associated with the host plant and functions as an ectomycorrhiza through symbiosis.

3.4. Identification of the Host Plant of Helvella bachu

We collected leaves from the 10 locations where the *H. bachu* mushrooms were harvested (Table 1) to determine the specific species of the *Populus* trees that serve as hosts for *H. bachu*. DNA was extracted, amplified, and sequenced with the nuclear gene fragment ITS2, targeting a nuclear gene specific to *Poplus* (S2F and S3R) for identification. The sequences have been deposited in NCBI with accession numbers PP188354 to PP188366. Phylogenetic analyses revealed the presence of four kinds of *Populus* trees, namely *P. euphratica*, *P. pruinosa*, *P. nigra*, and *P. alba* var. *pyramidalis* (synonym *Populus bolleana*) (Figure 5). Notably, *P. alba* var. *pyramidalis* and *P. tremula* (Specimen H1, 2, 8, and 10) were grouped together and could not be distinguished. Upon detailed comparison and distinction of specimens from the China Digital Herbarium (CVH) (https://www.cvh.ac.cn/, accessed on 1 March 2024), it was observed that the morphological characteristics of *P. tremula* leaves differed from those of our Leuce tree. The margin of *P. tremula* leaves exhibited shallow and dense serrations, whereas the margin of our Leuce samples' leaves showed deeply and sparsely serrated features, mostly resembling a palmate shape. Consequently, the Leuce samples were consistent with the characteristics of *P. alba* var. *pyramidalis*, leading to the classification of the host as *P. alba* var. *pyramidalis*.

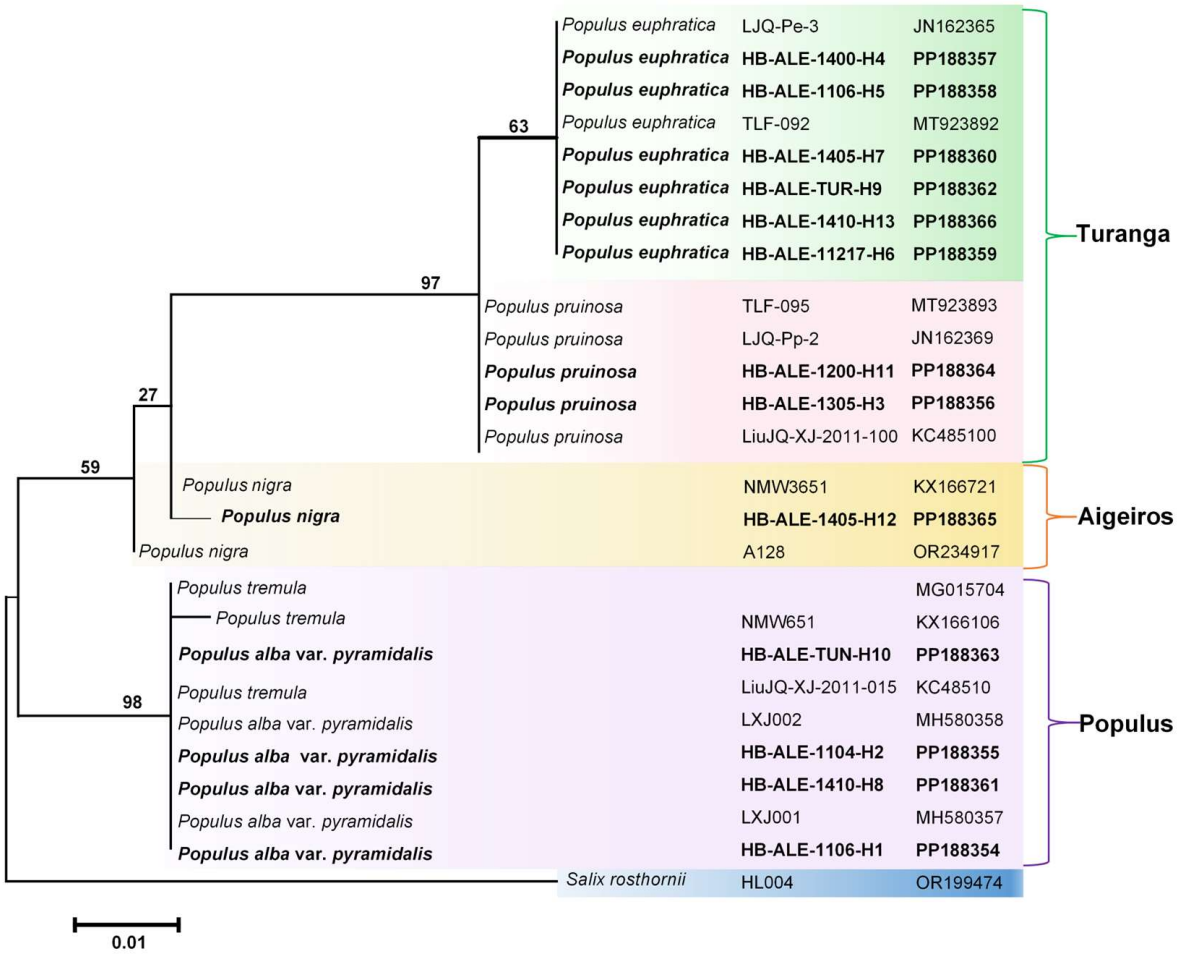


Figure 5. Molecular phylogenetic analysis of host plants of *Helvella bachu* by maximum likelihood method.

This information suggests that *H. bachu* is associated with specific species of *Populus* trees, providing further insight into its ecological preferences and distribution.

The species in bold indicate the sequences were obtained from this study, while the others were downloaded from GenBank. The right column lists the GenBank accession, and the middle column shows the specimen number as indicated in Table 1 or obtained from GenBank. The species in blue is the outgroup.

4. Discussion

While certain species within the *Helvella* genus have been confirmed to be ectomycorrhizal fungi, the ecological roles of many other species have become a subject of controversy. These particular species have alternatively been considered as either saprotrophic or mycorrhizal [10]. Here, by combining the field habitat of *H. bachu*, along with macro- and micro-anatomical characteristics of the plant root tip, and molecular information of the fruiting body, mycorrhiza, and host plant, we have confirmed that *H. bachu* forms an ectomycorrhizal relationship with *Populus*.

4.1. Characterization of Helvella bachu Mycorrhiza

It has been reported that Pezizalean ECMs are primarily characterized by a pseudoparenchymatous mantle, a well-developed Hartig net, and infrequent, thick, stout, and thick-walled emanating hyphae [9]. In this study, mycorrhizal samples of *H. bachu* collected in Maigeti County were analyzed, with 20 mycorrhizal samples representing approximately 10 different forms selected for molecular identification. This process confirmed that the *H. bachu* mycorrhiza exhibited a light earth color and featured a curved smooth cylindrical shape. Additionally, a thick mycelial mantle was observed, with the presence of a Hartig net and a small amount of epitaxy mycelia. Apart from the mycelium of ascomycetes *H. bachu* lacking a clamp connection, the mycorrhiza of *H. bachu* lacked rhizomorphs and sclerotia. These findings were consistent with the morphological structure of ectomycorrhiza from the Pezizomycetes of Ascomycotina, as reported by Tedersoo et al. [9] and the insights presented by Brundrett et al. [22]. It was reported that the mycorrhiza of Pezizaceae has no branching, dichotomous, or coralliform branches [9], which is consistent with our results. However, mycorrhizae of some *Lactarius* species predominantly exhibited a bifurcated structure [23,24].

4.2. Morphologic Observation of Mycorrhiza

When observing the morphology of mycorrhiza, sample pretreatment and sectioning techniques play a crucial role. The mycorrhiza was found to detach easily from the root tips, making detection challenging. Therefore, it is essential to handle the root tips with great care and gentleness. Various sectioning techniques were attempted, including frozen, paraffin, and freehand methods. The freehand section proved to be the most unstable, while frozen sectioning for plant tissue required exploration of corresponding fixatives due to the tissue's tendency to break. Ultimately, the paraffin section technique yielded remarkable results. During the slicing process, efforts were made to cut the mycorrhizal slice as thinly as possible, targeting slices within 4 μm.

Out of twenty mycorrhizal samples collected from windbreak poplars of Maigeti county and Kashi City, only three were identified as *H. bachu* through ITS sequence analysis. Despite the scarcity of other mushrooms during the *H. bachu* season, diverse mycorrhizae, including *Inocybe vulpinella* and species of Pyronemataceae, were found while identifying those root tips. Undoubtedly, this situation significantly complicates the task of identifying the specific mycorrhiza associated with *H. bachu*.

4.3. Identification of Helvella bachu Host Plants

H. bachu mushrooms were confirmed to form ectomycorrhizal associations with species of *Populus*, a genus of trees with economic and ecological importance. Traditionally, species in the genus *Populus* have been classified into the following six sections based on their

morphological traits and crossability: Abaso, Leuce (*Populus*), Aigeiros, Tacamahaca, Leucoides, and Turanga [25]. In the present study, the hosts of *H. bachu* were initially identified as three factions, namely section Turanga (*Populus euphratica* and *P. pruinosa*), section Aigeiros (*P. nigra*), and section Leuce (*Populus alba* var. *pyramidalis*) using nuclear gene ITS2 sequence (Figure 5).

Populus alba, commonly known as the white poplar, is an ecologically and economically important species widely distributed and cultivated in Xinjiang. *Populus alba* var. *pyramidalis* represents one variety of this species. Moreover, natural populations of the *P. alba* species frequently hybridize with other closely related species, resulting in numerous natural hybrids [26]. It remains to be determined whether *P. alba*, its other varieties, and the natural hybrids serve as hosts for *H. bachu*. During our field investigation, a local farmer informed us that *H. bachu* was observed in a grafting forest of *P. alba* on *P. euphratica*, although we did not find any mushrooms. While we have confirmed four kinds of host plants, it is plausible that the host of *H. bachu* includes more than just these four species, necessitating broader sampling for a comprehensive understanding.

In 2018, Cristina has synthesized *H.* cf. *lacunosa* mycorrhizae on *Pinus ayacahuite* [12]. Based on this results, we can conduct in-depth research on mycorrhizal seedling synthesis of *H. bachu* on *Populus*. However, this process involves infection using pure culture mycelia or sterile spore solution, which could take from 3–5 months to as long as 2–5 years.

4.4. Establishment of a Conservation Area and In Situ Propagation of Helvella bachu

The life cycle of *H. bachu* depends on the involvement of plants. Due to the challenging nature of pure culture and artificial cultivation, establishing a conservation area and in situ propagation of *H. bachu* appears to be a practical strategy.

We have established a conservation area within the young *P. euphratica* forest at Tarim University. After two years of management, there was a 14.75% increase in the number of fruiting bodies during the second year. Other researchers attempted to inoculate with soil containing the mycelia, ascospore suspension, and tissues of *Helvella* in the field. Their efforts resulted in a 100% survival rate, with the fruiting body density reaching 3.4 times higher than that observed in the natural area [27].

Furthermore, promising outcomes have been achieved in the semi-artificial simulated cultivation of highly desirable ectomycorrhizal fungi such as *Tuber*, *Tricholoma*, *Lactarius*, *Chanterellus*, *and Rhizopogon* [28–33]. These successes provide a strong foundation for similar technologies for *H. bachu*.

The Food and Agriculture Organization of the United Nations' Forest Tree Genetic Resources Panel confirmed *P. euphratica* as a core protected tree in arid and semi-arid areas [8]. Regenerating and protecting the damaged desert riparian ecosystem, which features *P. euphratica* as a significant component, has become a paramount concern for the Chinese government. Given that *H. bachu* mushrooms form ECM and mutually beneficial symbioses with *Populus* tree species, they are crucial organisms in nutrient and carbon cycles in forest ecosystems, particularly in the *P. euphratica* forest. *H. bachu* may potentially promote the growth of *Populus*, enhance the drought tolerance of *Populus*, and aid *Populus* in withstanding the arid desert climate, akin to other ectomycorrhizal fungi. Implementing appropriate mycorrhizal propagation technology to expand *Populus* planting in desert hinterlands could not only reduce desertification but also increase the occurrence of *H. bachu*, thereby fostering economic development and ecological environment protection in southern Xinjiang under mutually beneficial conditions.

5. Conclusions

In summary, we have identified the mycorrhiza of *H. bachu* and confirmed it to be an ectomycorrhizal fungus. This determination was based on research into the occurrence, ITS sequences, and macro- and micro-anatomical characteristics of both the fruiting bodies and mycorrhizae of *H. bachu*. The host plants consisted of four *Populus* species, including *P. euphratica*, *P. pruinosa*, *P. nigra*, and *P. alba* var. *pyramidalis* (synonym *P. bolleana*). The

establishment of a conservation area and in situ propagation of *H. bachu* hold significant economic and ecological importance.

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