

Terfezia claveryi truffles at Hafr Al Batin, Saudi Arabia: A study based on morphological and molecular characterizations

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ABSTRACT

Desert truffles have been harvested in a wild area in Saudi Arabia for both food and medicinal purposes for centuries. Limited information exists on the host plant and desert truffle diversity found in Hafr Al Batin, Saudi Arabia. *Terfezia claveryi* isolates from Hafr Al Batin, have been identified based on their morphology and Internal Transcribed Spacer (ITS)-rDNA sequencing data. Pictures, macro- and micro-descriptions of the two isolates, along with ITS phylogenetic data, revealed how they relate to other members of the genus and their host plants. According to the results, the herbaceous *Helianthemum lippii* was the dominant host plant at all different locations in Hafr Al Batin. In the investigated locations, two different strains of *T. claveryi* were identified. *T. claveryi* 49–621 was the most widely distributed strain across all surveyed sites, with a mean relative abundance of 63.4%, followed by *T. claveryi* 1–564, whose incidence was 11.7%. According to the findings of this study, host plants and the local environment influence the distribution of desert truffles throughout the Hafr Al Batin region.

1. INTRODUCTION

Desert truffles are tuber-shaped hypogeous ascocarps of mycorrhizal Ascomycetes. They have a distinct geographical distribution restricted to semi-arid and dry portions of Saudi Arabian desert regions [1]. In the Mediterranean Basin and the Middle East, various kinds of desert truffles have been discovered [2]; however, certain uncommon species can be found in Asia [3]. The fruiting bodies of several edible hypogean fungi belonging to the phylum Ascomycota, which were categorized in the order of *Pezizales* after phylogenetic studies, are generally known as desert truffles [4]. The *Terfezia*, *Eremiomyces*, *Elderia*, *Kalahari Mycoleandria*, *Mattiolomyces*, *Phalangium*, *Picoa*, *Reddelomyces*, and *Ulurua* genera are among those included in this category [5]. However, the only Basidiomycetes that are regarded as a desert truffle are *Horakiella watarrkana* [6]. The word “truffle” originates from the Latin term “tuber,” which means “swelling” in various regions of the world. “Al-Kamah” signifies “covered” in Arabic, whereas “Al-Faaga’a” pertains to the cracking of the soil above the fruiting body that occurs after it has swollen [1]. The diverse morphological keys proposed over the years are crucial [7,8]. It has been possible to establish a convincing delineation between species and taxa through molecular (gene sequencing studies) and morphological methods [9-11].

Desert truffles were discovered more than a quarter century ago and grow naturally in many parts of Saudi Arabia [12-14]. For a long time, desert truffles have been a staple food and a source of income. In addition, truffles are utilized as an edible food source because they contain a considerable number of high-quality proteins, fiber, amino acids, carbohydrates, and fatty acids [15]. Moreover, they have been used in traditional medicine for their antioxidant, anti-inflammatory, antibacterial, antifungal, antiviral, and anticancer properties [3,16]. Recently, they have been used for treating eye pathogens [17]. Saudi Arabians have a long history of collecting desert truffles in natural conditions. As a result of the ongoing overexploitation of this valuable natural resource, this crop is at risk, underscoring the need for its protection. Two types of Saudi Arabian truffles are also present. They can be distinguished by their harvesting area, size, color, and host. “Terfess red of Tafilalet,” “Terfess white of Tafilalet,” “Ezzebdi,” or “Terfess rose of Mamora” are examples of these terms. Each type appears in the rainfall season in spring, although some can emerge as early as November and December. Hafr Al Batin region’s desert truffle has received very little research, so determining its diversity must be considered in the context of Saudi Arabia’s fungal diversity.

The majority of *Terfezia* species form mycorrhizal symbioses with *Cistaceae* plants, primarily with perennial and annual *Helianthemum* species [18-20]. This mycorrhizal association is extremely well adapted to semi-arid environments by exploiting a physiological mechanism to resist drought [21-23]. The significant variation in the soils found in desert truffles reflects the environmental settings in which they grow. There is evidence that *Terfezia* species can adapt to a wide range of

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soil pH, edaphic conditions, and textures [24]. The discovery of new species is made possible using cutting-edge molecular techniques on the hypogeous fungus group, which is home to desert truffles. The number of new species discovered within the genera of desert truffles will rise [25]. Two *Terfezia* isolates were the primary focus of this study's description. To achieve this, we conducted conventional morphological studies along with phylogenetic analysis using Internal Transcribed Spacer (ITS)-rDNA sequences from *Terfezia* samples collected from Hafr Al Batin, Saudi Arabia.

2. MATERIALS AND METHODS

2.1. Study Area

Hafr Al Batin (28°26'3"N 45°57'49"E), located in the eastern region of Saudi Arabia, has an arid climate, and the annual rainfall is 63 mm [26]. The average temperature from December to February ranges from 20–23°C as a maximum to 6–8°C (as a minimum) and the soil type is arid soil, sand, and gravel [27].

2.2. Choice of Sampling Sites

During February to May 2022, a total of 60 fresh fruiting truffle bodies that were in full bloom were harvested. They were gathered in accordance with the recommendations and guidelines provided by individuals responsible for information gathering in the study regions. It is most common to find the Saudi Arabian truffle in the vicinity of *Helianthemum* herbaceous plants, with mycorrhizal associations are formed by them [20,28]. Truffles were discovered in situ near the roots in the species *Helianthemum* [29] by examining fractures and swellings in soil surfaces induced by ascocarp development.

2.3. Samples Preparation

Samples were divided into two groups (white and brown). Eight gleba cubes of each group were sampled and stored in a 1.5 mL Eppendorf tube containing 70% ethanol (v/v) to preserve the DNA for further analysis. At the same time, a portion of fresh gleba was scraped with a scalpel from each truffle and mounted on a microscopic slide with glycerol to perform spore morphological analysis. The morphological characterization of truffle spores was conducted using a light microscope (Euromex iScope), and photographs were taken with an OLYMPUS DP74 digital camera. Genetic analysis was employed to compare molecular information with the other available data.

2.4. Morphological and Microscopic Characteristics

The morphology of macroscopic features, particularly shape and color, was detailed for each portion of the truffle, namely the ascocarp and the gleba. Samples were sliced using a sharp blade, immersed in 5% KOH, and examined with an optical microscope (×100). Various properties such as ascospore form, quantity per ascus, and position were assessed for species identification [7,30,31]. All this information was compared to that previously described by various authors [9,10,32–35].

2.5. Analysis of Soil Samples

Soil samples were collected at a depth of approximately 10–25 cm at each truffle harvesting location. At a temperature of 25°C in the open space, soil samples were air-dried in the laboratory. To evaluate the samples' physical and chemical properties, they were sieved through a 2 mm sieve and then subjected to standard analysis techniques. As part of the analysis, soil parameters such as particle diameter, electrical properties, pH, and total CaCO₃ were assessed. Salinization

was determined using the following equations: 640 conductivities in dS/m [36]; pH was measured with a pH-meter on a soil: water ratio of 1:5; and total CaCO₃ was assessed by Bernard calcimeter. Phosphorus, Ca⁺⁺, Mg⁺⁺, and K⁺ were measured by an atomic absorption spectrometer on a soil: water extract ratio of 1:5 [36]. HCO₃⁻ was titrated with H₂SO₄ and SO₄ by the gravimetric analysis following precipitation as barium chloride. Using sterile water and 1 g of dry weight, the soil sample was processed into a suspension for microbiological examination. To estimate aerobic heterotrophic fungal populations, the usual spread-plate dilution method published by Seeley and VanDemark [37] was employed. One mL of the soil suspension was diluted serially (10 times). To isolate the fungus, potato dextrose agar was supplemented with 0.05% (w/v) chloramphenicol (to prevent bacterial growth) and incubated at room temperature for 7 days.

2.6. Molecular Characterization

2.6.1. DNA extraction

Genomic DNA was extracted from the two isolates following the methods of Hassan *et al.* 2022 [38]. The samples were ground with liquid nitrogen and homogenized in a sterilized mortar with 1.5 mL of CTAB extraction buffer, 20 µL of Triton x-100, and 100 µL of 10% SDS. The sample mixture was transferred to a microcentrifuge tube and incubated at 60°C for 60 min. The supernatant from the samples was transferred to a new Eppendorf tube after being centrifuged at 15,000 rpm for 10 min. The supernatant was treated with an equal volume of chloroform-isoamyl alcohol before being centrifuged at 10,000 rpm for 10 min. The aqueous phase was transferred gently to a new Eppendorf tube. To precipitate in nucleic acids, 0.45 volumes of ice isopropanol were added and mixed thoroughly by inversion before being incubated at 25°C for 1 h. Following incubation, samples underwent a 15-min, 15,000 rpm centrifugation. After discarding the supernatant, the pellet was washed with 1 mL of 70% ethanol and centrifuged at 12,000 g for 5 min. The pellet underwent a 5-min, room-temperature air drying process. Then, the elution step was performed using 50 µL of TE buffer. The concentration and purity of purified DNA were measured by BioTek Epoch2 Microplate reader (Thermo Scientific, Waltham, MA, USA). DNA purity was $>1.8 \pm 0.20$ under the absorbance ratio A_{260}/A_{280} .

2.6.2. Polymerase chain reaction (PCR) assay

Based on fungal genomic DNA, PCR amplification was carried out utilizing the ITS region of the rDNA, which contains the 5.8S ribosomal gene. The universal primer pairs ITS 1 (TCCGTAGGTGAACCTTGCGG) and ITS 4 (TCCTCCGCTTATTGATATGC) were used for PCR amplification [39]. PCR reaction was performed in a 25 µL mixture containing 0.2 µM of each primer at a concentration of 10 pM, 200 µM of dNTPs mix, 2.5 µL of ×10 PCR reaction buffer, 1.5 µM MgCl₂, 1.25 units of TAKARA Taq DNA polymerase (Cat. #: R001AM), 2 µL of template DNA, and the final volume was adjusted with sterilized double-distilled water. The reactions were amplified using a PCR thermocycler (AriaMx), which had the following settings: 95°C for 3 min, 35 cycles at 95°C for 50 s, 52°C as the annealing temperature for 1 min, an extension of 72°C for 1 min, and a final extension temperature of 72°C for 10 min. For further purification and downstream use, amplified PCR products were stored at 20°C.

2.6.3. DNA sequencing

PCR amplicons were purified according to the manufacturer's QIAquick Gel Extraction Kit (Cat. #: 28704). The purified PCR fragments were

sequenced by Macrogen Company, South Korea, using the automated Sanger Sequencing method. The obtained sequences for the *ITS-rDNA* gene were analyzed using the Standard Nucleotide BLAST tool and registered in the NCBI database under accession numbers ON909007 and ON909008 for *Terfezia claveryi* isolate Hafr Al Batin and Hafr Al Batin2, respectively. (<http://www.ncbi.nlm.nih.gov>).

2.6.4. Pairwise alignment and phylogenetic construction

Jalview software [40] was utilized to display single-nucleotide polymorphisms (SNPs) and the consensus generated from the pairwise sequence alignment of the two obtained isolates, as well as their alignment with the nearest strains in the NCBI database. (<http://www.jalview.org/>).

DNASTAR (version 5.05) was employed in this analysis to generate contiguous sequences of the *Terfezia* isolates. Sequences from the most closely related species within the *Terfezia* genus were retrieved from GenBank. The *Peziza montirivicola* strain M 0274465 (NR_148194; type material) was used as the outgroup for the phylogenetic tree in this study. MAFFT (Kato and Standley, 2013) [41] was used to align all sequences, while BMGE (Criscuolo and Gribaldo, 2010) [42] was used to refine the alignment by addressing gaps and removing weak, uninformative characters. Phylogenetic analyses utilizing maximum-likelihood (ML) and maximum-parsimony (MP) methodologies were performed using MEGA X (version 10.2.6) (Kumar *et al.*, 2018) [43]. The robustness of the most parsimonious trees was evaluated through 1,000 replications (Felsenstein, 1985) [44]. The optimal nucleotide substitution model for the ML analysis was determined using the Akaike Information Criterion in ModelTest 3.7 (Posada and Crandall, 1998) [45].

3. RESULTS AND DISCUSSION

3.1. Truffle Presence and Distribution

Desert truffles are only available in a few locations, such as Turkey, Portugal, Spain, France, and other nations in the Mediterranean basin [46-48]. *Terfezia* and *Tirmania* are the two genera of the harvested Terfez. Four species make up the genus *Terfezia*: *Terfezia olbiensis*, *T. leptoderma*, *T. boudieri*, and *T. claveryi*. One species, *Tirmania nivea*, belongs to the genus *Tirmania*. From February to May 2022, the author sampled various locations in the Hafr Al Batin region and collected a total of 60 fresh fruiting truffle bodies. The harvested

truffle ascocarps typically have a spherical appearance, a lobed shape, and a foot (pedicel) at the base. The surface of the peridium is quite smooth and covered with tiny scales, as observed under a binocular magnification for collected types. Small pale yellowish veins, cream color, and white contribute to the distinctive appearance of the gleba.

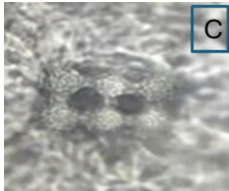
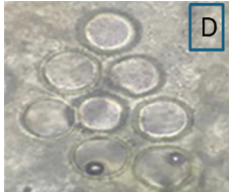
The first isolate, *T. claveryi* 49–621, was the most widely distributed species across all surveyed sites, with a mean relative abundance of 63.4%, followed by the second isolate, *T. claveryi* 1–564, whose incidence varied at 11.7%. Harvested species were associated with *H. lipii* in the Hafr Al Batin region. Our results indicated that the desert truffles grow in the sand, near *Helianthemum* roots. This result agrees with Bermaki *et al.*, 2017 [28].

3.2. Study of the Morphology of the Examined Truffles

T. claveryi 1–564 - harvested February 2021. Up to 11.6 × 4.7 cm in size, ascocarps that are frequently subglobose in shape, occasionally flattened, and possess a short, smooth, or wrinkled pedicel that is white to cream in color before turning yellow [Table 1A]. The peridium is between 0.5 and 2 mm thick and has a light cream tint that turns white when exposed to sunlight. The gleba is whitish or yellow, squishy, and dotted with tiny veins. When examined under a microscope, *T. claveryi*'s gleba reveals numerous hyphae containing ascospores. Asci [Table 1C] are pedicelled, ellipsoid to ovoid in shape, and range in size from 62 to 78 × 33 to 42 μm. They typically feature 6 amyloid ascospores. Their pedicle is short and has a thin wall.

T. claveryi 49–621 was harvested in April 2021. The peduncle at the base of subglobose to tuber-form ascocarps measures 5.9 × 12.4 cm in diameter [Table 1B]. Its surface ranges from dark brown to brown and is cracked. Peridium: Brownish, 0.75–2 mm thick, with a smooth, often fractured surface. The gelatinous skin has obvious veins that irregularly define fertile patches, reddish to pinkish in color. Asci: 62–78 × 71–79 μm in diameter, subglobose or ovoid, randomly distributed in fungal tissues. It contains eight ascospores when fully mature. The spherical yellowish-hyaline ascospores measure 19–22 × 21–24 μm, floating freely inside asci, with thick double-walled structures and a germ pore [Table 1D]. The morphological and anatomical characteristics of *T. claveryi* relevant to this study perfectly align with previous characterizations of the same truffle species (Morocco [49]; Tunis [2]; Kuwait [50]; Saudi Arabia [1]; Iraq [51]; and Egypt [52]).

Table 1: Some morphological and anatomical features of fruiting bodies of *Terfezia claveryi* 1–564 (A: Ascocarp, C: Ascus with six ascospores) and *Terfezia claveryi* 49–621 (B: Ascocarp, D: Ascus with eight ascospores), collected from the Hafr Al Batin region.

Sample ID/Query Accession Number	Recognized Species	Collection Date/site/ Host plant	Ascocarps	Ascus and Spore Morphology	Consensus Taxa/GenBank Accession Number
1–564/ON909007	<i>Terfezia claveryi</i>	February 2022/Hafr Al Batin/ <i>H. lippii</i>	White 		MK967454.1/Iraq
49–621/ON909008	<i>Terfezia claveryi</i>	April 2022/Hafr Al Batin/ <i>H. lippii</i>	Brown 		GQ888692.1/Iran

3.3. Soil Analysis

Table 2 presents the results of the microbiological and physicochemical studies conducted on soil samples collected from various locations in the Hafr Al Batin region. Soil investigations of desert truffle-producing locations revealed significant homogeneity in pedological properties across the two truffle isolates. A study of the soils indicated a sand-like texture, extremely low amounts of organic matter, an alkaline pH, and very low levels of nitrogen and phosphate. Conversely, the amounts of potassium and calcium were high. According to a study on grain size based on the American system of classifying soil textures, all the habitats for truffles in the Hafr Al Batin have soils with a sandy texture and single-grained structure, exhibiting high rates of sand grains (72.7–73.2%). This sample had a sand-like texture, minimal organic matter, an alkaline pH, and exceptionally low nitrogen and phosphorus contents. Low levels of organic matter are closely associated with low soil P availability [24]. It was found that the pH range of desert truffle habitats was between 8.1 and 8.3. According to the 1:5 aqueous extract’s scale of categorization for salinity, the electrical conductivity values were low and ranged between 0.82 and 0.85 dS/m, indicating non-saline soils. Organic materials constituted up 0.71–0.83% of the total substance. These results demonstrate a

Table 2: Microbiological and physiochemical soil analyses at desert truffle harvesting sites in the Hafr Al Batin region.

Soil Parameter	<i>Terfezia claveryi</i> 49–621 (n=16)	<i>Terfezia claveryi</i> 1–564 (n=7)
Chemical composition		
Organic matter (%)	0.83	0.71
K ⁺ (cmol ⁺ /kg)	0.16	0.16
Phosphorus (ppm)	19.18	19.95
Ca ⁺⁺ (cmol ⁺ /kg)	8.11	8.38
Mg ⁺⁺ (cmol ⁺ /kg)	2.05	1.99
HCO ₃ ⁻ (cmol ⁺ /kg)	0.46	0.32
SO ₄ ⁻ (cmol ⁺ /kg)	0.32	0.33
Texture (%)		
Clay	1.9	1.7
Silt	9.5	9.2
Sand	73.2	72.7
Ec (dS/m)	0.85	0.82
pH	8.3	8.1
Microbiological analysis (cfu/gm soil)		
<i>Mucor circinelloides</i> van Tiegham	8.6×10 ⁵	7.3×10 ⁵

significant deficiency in soil organic matter, as measured by Morond’s scale of organic matter [53,54]. Researchers have found that desert truffles form symbiotic relationships with *Cistaceae* roots, particularly *Helianthemum lippii*. The genus *Helianthemum* is well-known in literature for establishing connections with truffles in many parts of the world, such as Algeria [33] and Morocco [28].

3.4. DNA Sequences and Phylogenetic Analyses

The amplified PCR products of the *ITS-rDNA* gene of the isolates were sequenced and deposited in the NCBI database under accession numbers ON909007 and ON909008 for *T. claveryi* isolate Hafr Al Batin and Hafr Al Batin 2, respectively. BlastN and Jalview alignment results revealed that the two isolates, Hafr Al Batin and Hafr Al Batin 2 were closely like *T. claveryi* strain MZR12 (MK967454) and *T. claveryi* isolate Kh1 (GQ888692) with identity ratios of 97.34% and 98.74%, respectively. Pairwise alignment analysis of *T. claveryi* isolate Hafr Al Batin (ON909007) and the nearest sequence (MK967454) exhibited 8 SNPs and 2 GAPs [Figure 1]. Meanwhile, the alignment between *T. claveryi* isolates Hafr Al Batin2 (ON909008) and the nearest sequence (GQ888692) revealed 5 SNPs and one gap [Figure 2]. Interestingly, the pairwise sequence alignment between the two obtained isolates indicated a 97.1% identity ratio. This divergence resulted from 9 SNPs and 3 gaps, reflecting possible different features among the two isolates [Figure 3].

In phylogenetic analysis, thirteen sequences produced a total of 614 characters in the final ITS dataset, of which 442 were accurately aligned, 144 were designated as variable, and 57 were identified as relevant. Tamura’s 3-parameter model, utilizing a discrete gamma distribution (T92 + G), effectively illustrated the relationships among taxa. Eight trees were generated using the MP method. The ML tree [Figure 4] exhibits the following attributes: a tree length of 337, a maximum log likelihood of −2359.15, a consistency index of 0.748634, a retention value of 0.799127, and a composite index of 0.598253. The strains in this study are positioned within the same clade as *T. claveryi* strains RUF-TC2, J0297, MA: FU 8240, and MUB: Fung-0922, which is supported by a robust bootstrap value of 81% ML/87% MP. As a result, the isolates in this study were identified as *T. claveryi* [Figure 4]. The ITS sequences of *T. claveryi* strains in this investigation were submitted to GenBank as ON909007 and ON909008.

The morphological features of *T. claveryi* [Table 1] and the molecular study results [Figure 4] provide compelling evidence that these two species are one and the same. We identified these isolates as belonging to the same species by comparing them to all other taxa that were morphologically and phylogenetically comparable to them. Host specialization and edaphic tolerances (fungal and/or host tolerances)

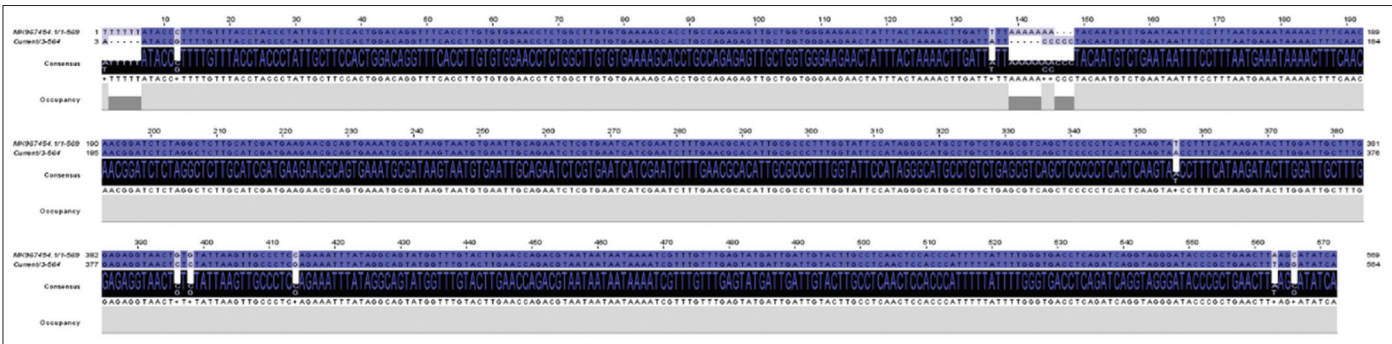


Figure 1: The image shows single-nucleotide polymorphisms and GAPs between *Terfezia claveryi* isolate Hafr Al Batin (ON909007) and the nearest sequence (MK967454) deposited in GenBank.

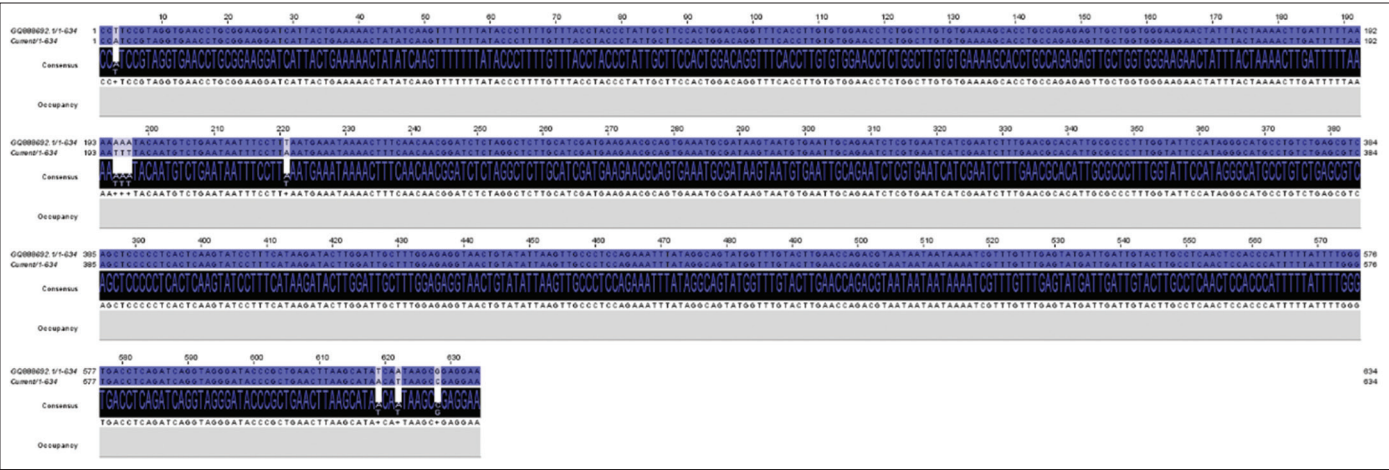


Figure 2: The image shows single-nucleotide polymorphisms and GAPs between *Terfezia claveryi* isolate Hafr Al Batin2 (ON909008) and the nearest sequence (GQ888692) deposited in GenBank.

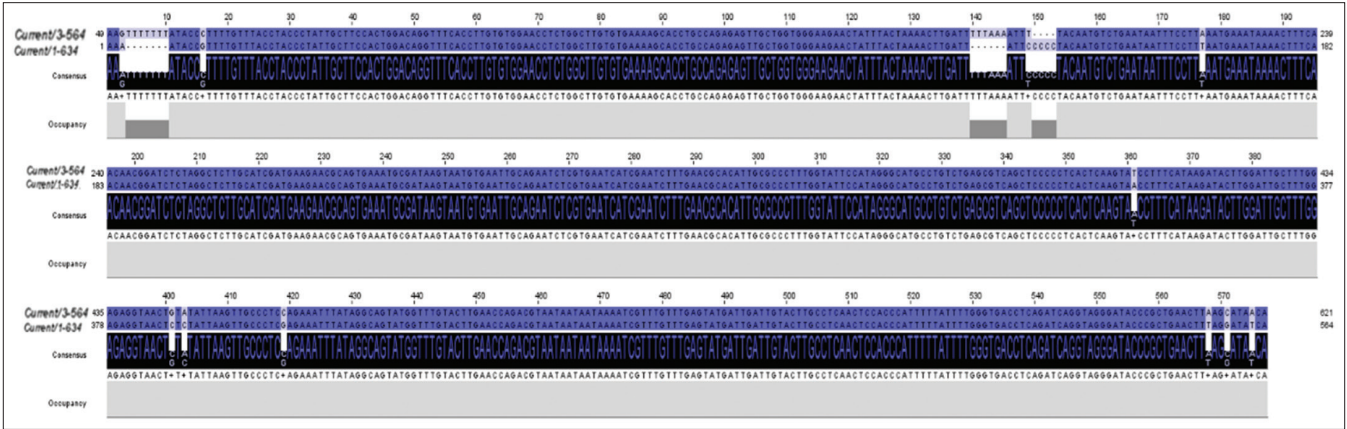


Figure 3: The image shows single-nucleotide polymorphisms and GAPs between the two obtained isolates Hafr Al Batin (ON909007) and Hafr Al Batin2 (ON909008).

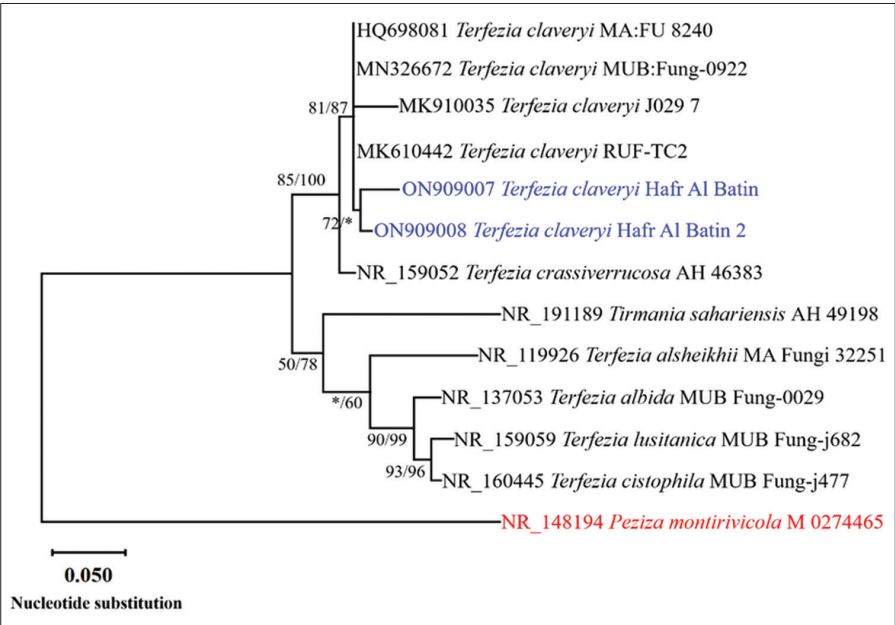


Figure 4: The maximum likelihood evolutionary tree obtained from a heuristic search (1000 replications) using maximum-likelihood (ML)/maximum-parsimony (MP) analysis of Internal Transcribed Spacer sequences of *Terfezia claveryi* strains in this study (in blue) compared to the most similar species of the genus *Terfezia* in GenBank. Bootstrap support values for ML/MP $\geq 50\%$ are indicated near the respective nodes. The tree is rooted to *Peziza montirivicola* M 0274465 (in red).

may also be important contributors to the *Terfezia* genus's high species diversity, although other variables may also be at play [55]. In this regard, *T. claveryi* and *T. albida* are similar in that they both prefer alkaline soils and *Helianthemum* species as hosts, although the latter's gleba is less grayish in contrast to that of *T. claveryi*.

4. CONCLUSION

In this taxonomic and bioecological investigation of desert truffles, which was originally conducted in Hafr Al Batin region, 2 isolates of *T. claveryi* have been identified. These isolates are somewhat limited, but they exhibit remarkable adaptations to their environment, characterized by severe aridity and rudimentary soil properties, which are associated with *H. lippii* (*Cistaceae*). Harvested truffles grow ecologically in sandy, slightly alkaline, somewhat calcareous soils that are low in organic and mineral content. As far as climatic conditions are concerned, these fungi occur in hot arid environments, with heavy rains, followed thereafter by periods of dryness. The ascospore sizes produced by the two isolates studied here vary. It is believed that they represent separate species since the first isolate generates spiny ascospores while the other hyaline ascospores. Our rigorous comparisons of ascocarps and ascospores, along with thorough ITS-rDNA sequence analysis, support the identification of just one species.

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6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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8. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

11. PUBLISHER'S NOTE

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The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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