Molecular phylogeny of three desert truffles from Iran based on ribosomal genome

فیلوژنی مولکولی سه گونه دنبلان کوهی از ایران براساس ژنوم ریبوزومی

R. Mostowfizadeh-Ghalamfarsa✉**:** Assistant Prof., Department of Plant Protection, School of Agriculture, Shiraz University, Bajgah, Shiraz 7144133111, Iran
(E-mail: rmostofi@shirazu.ac.ir)

S. Jamali: Ph.D. student,, Department of Plant Protection, School of Agriculture, Shiraz University, Bajgah, Shiraz 7144133111, Iran

Z. Banihashemi: Prof., Department of Plant Protection, School of Agriculture, Shiraz University, Bajgah, Shiraz 7144133111, Iran

Abstract

The ITS region including the 5.8S gene of rDNA of three desert truffle species were amplified using ITS4 and ITS1 primers. The ITS sequences were compared to those of other related authentic sequences obtained from GenBank. Among 12 specimens studied, seven isolates corresponded to *Terfezia claveryi* reported by other authors. Iranian *T. claveryi* specimens had an average similarity of 99.4% (range 98.7–100%) among themselves, while all *T. claveryi* sequences analyzed had an average of 95.2% (range 87.2–100%) similarity. Four specimens corresponded to *Trimania pinoyi*, being a sister taxon to *Tirmania nivea*, of which only one specimen could be studied. Iranian *T. pinoyi* specimens had an average of 99.9% similarity (range 99.8–100%) among themselves, and 97.2% (range 93.1–100%) between all *T. pinoyi* sequences compared. All *T. nivea* sequences, including Iranian specimen, had an average of 96.6% similarity (range 92.2–99.8%). *T. claveryi* and *T. pinoyi* are widespread in the country and are mostly associated with *Helianthemum salicifolium* and *Carex* sp. Iranian truffles flora resembles those of Mediterranean region and South coast of the Persian Gulf.

Keywords: *Ascomycota*, Phylogenetics, ectomycorrhizal fungi, internal transcribed spacer of rDNA

رضا مستوفی‌زاده قلمفرسا✉: استادیار بخش گیاه‌پزشکی، دانشکده کشاورزی، دانشگاه شیراز، باجگاه، شیراز، کد پستی ۷۱۴۴۱۳۳۱۱۱.
(E-mail: rmostofi@shirazu.ac.ir)

صمد جمالی: دانشجوی دکتری بخش گیاه‌پزشکی، دانشکده کشاورزی، دانشگاه شیراز، باجگاه، شیراز، کد پستی ۷۱۴۴۱۳۳۱۱۱.

ضیاءالدین بنی‌هاشمی: استاد بخش گیاه‌پزشکی، دانشکده کشاورزی، دانشگاه شیراز، باجگاه، شیراز، کد پستی ۷۱۴۴۱۳۳۱۱۱.

چکیده

نمونه‌هایی از سه گونه دنبلان کوهی از مناطق مختلف جغرافیایی ایران جمع‌آوری شد. منطقه ITS شامل ژن ۵/۸S از rDNA با استفاده از آغازگرهای عمومی ITS4 و ITS1 تکثیر و توالی‌یابی شد. توالی‌های به دست آمده با سایر آرایه‌های معتبرِ مستخرج از بانک ژن مقایسه شد. در میان ۱۲ نمونه مطالعه شده، هفت جدایه با نمونه‌های *Terfezia claveryi* گزارش شده توسط سایر محققان، مطابقت داشت. نمونه‌های *T. claveryi* ایرانی به طور میانگین ۹۹/۴٪ (با دامنه ۹۸/۷-۱۰۰٪) با یکدیگر تشابه داشتند، در حالی که میانگین تشابه کلیه *T. claveryi* های مورد مطالعه ۹۵/۲٪ (با دامنه ۸۷/۲-۱۰۰٪) بود. چهار نمونه مطابق با *Trimania pinoyi*، به صورت آرایه خواهری *Tirmania nivea* خود را نشان دادند که از گونه اخیر تنها یک نمونه یافت شد. نمونه‌های *T. pinoyi* ایران دارای ۹۹/۹٪ میانگین تشابه (با دامنه ۹۹/۸-۱۰۰٪) در بین خود و ۹۷/۲٪ (با دامنه ۹۳/۱-۱۰۰٪) میانگین تشابه در بین کلیه توالی‌های مورد مقایسه بودند. کلیه توالی‌های *T. nivea*، از جمله نمونه ایران دارای میانگین تشابه ۹۶/۶٪ (با دامنه ۹۲/۲-۹۹/۸٪) بودند. *T. claveryi* و *T. pinoyi* در قسمت‌های مختلف کشور یافت شده، اغلب همراه با *Helianthemum salicifolium* و *Carex* sp. بودند. فلور دنبلان‌های ایرانی شبیه به دنبلان‌های مدیترانه و کرانه‌های جنوبی خلیج فارس است.

واژه‌های کلیدی: آسکومیکوتا، فیلوژنی، قارچ‌ریشه‌های برونی، توالی‌های جداکننده نسخه‌برداری شده داخلی

Introduction

Desert truffles are hypogeous ascocarps of Ascomycetes occurring in arid and semi-arid ecosystems of Mediterranean region and Middle Eastern countries (Díez *et al.* 2002). They belong to *Pezizales* (Trappe 1979) and mostly the genera *Terfezia* and *Tirmania* (Morte & Honrubia 1995, Moawad *et al.* 1997, Shavita & Volk 2007). However, there are other truffle genera in *Pezizales* such as *Picoa* and *Mattirolomyces* which occasionally could be found in these regions (Alsheikh & Trappe 1983, Moreno *et al.* 2000, 2001). Apart from these genera, some other desert truffles inhabit in semi-arid ecosystems of other continents. For instance, *Mycoclelandia* with two species is a part of Australian mycoflora (Trappe & Beaton 1984) and the genera *Kalaharituber* and *Eremiomyces* were reported from South Africa (Ferdman *et al.* 2005).

These fungi are economically and ecologically valuable (Halasz *et al.* 2005). They are considered to be one of the oldest human foods in their origin countries, appreciated for their nutritional value and as flavouring (Hussain & Al-Ruqaie 1999), and also antimicrobial effects, especially in treating skin and eye diseases (Janakat *et al.* 2004). Additionally, these ectomycorrhizas promote plants growth and productivity through different mechanisms such as enhancement of nutrient uptake, production of growth hormones and protection of plants rhizosphere against soil-borne diseases (Hayman 1983, Smith & Read 1997, Buscot *et al.* 2000). Desert truffles establish ectomycorrhizal symbioses with members of the *Cistaceae*, mainly *Helianthemum* species (Awameh 1981, Morte & Honrubia 1995, Morte *et al.* 2000, Kovacs *et al.* 2003, Dikie *et al.* 2004).

Morphological characters have been used to describe different species of truffles including spore size and ornamentation, peridium morphology, gleba colour, and organoleptic characters (Trappe 1979, Moawad *et al.* 1997). Due to limited morphological characters, these fungi are difficult to identify at species level. Molecular phylogenetic studies have recently demonstrated that morphological characters of hypogeous Ascomycetes can be unreliable (Ferdman *et al.* 2005). Other molecular studies on truffles have led to the reclassification of some genera or species (O'Donnell *et al.* 1997, Hansen *et al.* 2001).

Almost all molecular studies on truffle identification were based on comparison of sequences or restriction fragment length polymorphisms (RFLPs) of the internal transcribed spacers (ITS) of rDNA (e.g. Henrion *et al.* 1994, Paolocci *et al.* 1995, 1999). The major phylogenetic studies on *Pezizales* (Spatafora 1995, Landvik *et al.* 1997, Harrington *et al.* 1999), specially truffles (O'Donnell *et al.* 1997, Roux *et al.* 1999, Díez *et al.* 2002, Ferdman *et al.* 2005) were also rooted in these regions. Different parts of the rRNA genes present different levels of conservation which can be exploited to analyze any desired phylogenetic level (Hillis & Dixon 1991). For example, the monophyletic origins of the genera *Terfezia* and *Tirmania* are illustrated through analysis of ITS region (Díez *et al.* 2002).

Many researchers have studied various aspects of desert truffles in the Mediterranean region and the Middle East (Morte & Honrubia 1995, Moawad *et al.* 1997, Hussain & Al-Ruqaie 1999) but no such studies have been carried out in Iran. Few older studies, report the presence of truffles in various parts of Iran (Chatin 1897, Petrak & Esfandiari 1941). A recent morphological study reported the collection of *Tirmania pinoyi* and *Terfezia leonis* in Fars, Hormozgan and E Azerbaijan provinces adjacent to *Artemisia* and *Helianthemum* species (Daneshpazhuh 1991). Unfortunately the locations of the collected samples were not specified. *Terfezia boudieri* also has been reported from Tarom in Zanjan province mostly associated with *Carex* species (Ammarellou *et al.* 2006) but also *Kobresia bellardii* (Ammarellou & Saremi 2008). However, the accuracy of the species identification merely on the basis of morphological features is questionable.

In the present study we attempted to identify desert truffles from various geographical regions of Iran,

extending from Sistan & Baloochestan (adjacent to Pakistan) to E Azerbaijan provinces (adjacent to Turkey), using morphological observation and molecular assays. This study also reports the phylogenetic relationships of different species of the Iranian desert truffles based on ribosomal RNA genome. This phylogenetic study could provide a basis for designing molecular detection and identification tools, which in turn could be used in laboratory, soil, and symbiont rhizosphere of these truffles for biological studies.

Materials and Methods

- Organisms and specimen's conditions

The origins of fruiting bodies studied are listed in Table 1. Several visits were made to the truffle-bearing areas of Iran during the months from February to May 2007–2009. Voucher specimens deposited in fungal collection of the Department of Plant Protection, Shiraz University, Shiraz, Iran. Fresh samples were used for cultivation and the rest were kept at –20° C for further studies.

Table 1. Origins of Iranian specimens studied and their GenBank sequence accession numbers for ITS sequences

Specimen	Year	Province	Locality	Species	GenBank
Sa1	2007	Fars	Sarvestan	*Terfezia claveryi*	EU519461
Si2	2007	Kerman	Sirjan	*T. claveryi*	FJ197819
Sh1	2006	Fars	Shiraz	*T. claveryi*	GQ888691
Fa1	2006	Fars	Fasa, Mianjangal	*T. claveryi*	GQ888693
Da1	2006	Fars	Darab, Ghale-Biaban	*T. claveryi*	GQ888694
Kh1	2009	Sistan & Baloochestan	Khash	*T. claveryi*	GQ888692
Tb1	2009	E Azerbaijan	Tabriz	*T. claveryi*	GQ888690
La1	2007	Fars	Lar	*Tirmania pinoyi*	GQ231540
Kh2	2009	Sistan & Baloochestan	Khash	*T. pinoyi*	GQ888695
Kh3	2009	Sistan & Baloochestan	Khash	*T. pinoyi*	GQ888696
Bd1	2009	Hormozgan	Bishederaz	*T. pinoyi*	GQ888697
Si1	2007	Kerman	Sirjan	*T. nivea*	FJ197820

- Cultivation

Attempts were made to cultivate truffles from fruiting body tissues on modified Melin-Norkrans (MMN) agar medium (Marx 1969, Kovacs *et al.* 2003) (in mg l^{-1} CaCl$_2$ 50, NaCl 25, (NH$_4$)$_2$PO$_4$ 250, KH$_2$ PO$_4$ 500, MgSO$_4$.7H$_2$O 150, Fe Cl$_3$ 12, thiamine-HCl 100, glucose 10 g l^{-1}, solidified with 17 g l^{-1} of agar; adjusted to pH 8.0). Fungal tissues were surface-sterilized in 0.5% sodium hypochlorite for 1–2 min and rinsed with sterile distilled water, dried on paper towel, and small pieces of about 3–5 mm were plated on the medium and incubated at 25° C in the dark and checked periodically for growth.

- Morphological study

Fresh truffles were brought to the laboratory, washed with tap water and cut transversely and small sections were made from the inner glebal tissues and observed by light microscopy. Periderm colour, glebal texture and colour, shape of asci, number of ascospores per ascus and ascospore ornamentation were checked. Glebal sections were stained with Melzer's reagent (approximately 2.5–3.75% potassium iodide, 0.75–1.25% iodine, in 50% water and 50% chloral hydrate). At least 50 ascospores were measured. Thin sections mounted in Melzer's reagent were used to record spore morphology and ornamentation for identification (Moawad *et al.* 1997). Specimens identified based on available taxonomic keys (Trappe 1979, Alsheikh & Trappe 1983, Moawad *et al.* 1997).

- DNA extraction

DNA was extracted from tissue cut out from the inner part of the ascocarp to avoid contamination by other micro-organisms. Tissue was freeze-dried for 24 h. Approximately 5 mg of freeze-dried tissue was used for each DNA extraction. DNA was extracted from

specimens using a genomic DNA purification kit, (Fermentas, UK) according to the manufacturer's instructions. The amount of DNA obtained was estimated by a NanoDrop spectrophotometer (NanoDrop Technologies, USA).

- DNA amplification and sequencing

DNA of the internal transcribed spacer regions (ITS) were amplified using the universal primers ITS1: 5'- TCC GTA GGT GAA CCT GCG G -3' and ITS4: 5'- TCC TCC GCT TAT TGA TAT GC -3' (White *et al.* 1990). Amplifications were performed in a CG1-96 thermocycler (Corbett Research, Australia). The PCR mixture contained: 10–20 ng of template DNA, 1 µM of each primer, 100 µM of dNTPs, 0.4 U *Taq* DNA polymerase (CinnaGen, Iran), 1.5 mM of $MgCl_2$, 2.5 µl of 10× PCR buffer, 100 mM BSA, in a reaction volume of 25 µl. All PCRs consisted of 1 cycle of 94° C for 3 min; 30 cycles of 95° C for 30 s, 50° C for 30 s, 72° C for 60 s; and a final cycle of 72° C for 10 min. Successful amplification was confirmed by gel electrophoresis (1 h at 80 Volts) on 1.0% agarose gels in 1× TBE buffer. Gels were stained using ethidium bromide and DNA fragments were visualised under UV light.

- Sequencing of amplified product

The amplification products of all specimens were purified through GenJET PCR purification kit (Fermentas, UK) to remove excess primers and nucleotides. PCR products were sequenced (Tech Dragon, Hong Kong) in forward and reverse orientation using the primers used for amplification and a dye terminator cycle sequencing kit (BigDye sequencing kit, Applied Biosystems, USA) on an ABI377-96 automated sequencer (Applied Biosystems, USA) according to the manufacturer's instruction. All sequences are submitted to GenBank (NCBI, http://www3.ncbi.nlm.nih.gov/Entrez) (Bethesda, MD, USA).

- Phylogenetic analysis

Sequences of the internal transcribed spacer regions including the 5.8S gene of rDNA were used to study phylogenetic relationships of the studied taxa. The internal transcribed spacers sequences of rDNA generated in this study (Table 1) were compared to those of other taxa obtained from GenBank (Table 2). A preliminary alignment of sequences was made using ClustalX (Thompson *et al.* 1997) with subsequent visual adjustment. The alignment was analyzed by both distance-based and maximum likelihood methods in PHYLIP (Felsenstein 1993). The transition/transversion parameter was estimated using the PUZZLE program (Strimmer & von Haeseler 1996). This parameter was used in the PHYLIP DNAML (Felsenstein & Churchill 1996) and DNADIST (Felsenstein 1993) programs. The robustness of the DNAML tree was tested using 500 bootstrap trials. The trees were drawn using Treeview (Page 1996). The final tree and matrix of sequences are submitted to TreeBASE (University at Buffalo, USA; http://www. treebase.org).

Results

- Distribution of truffles in different regions

Samples from Khash in Sistan & Baloochestan province (near Pakistan border) were harvested in late winter 2009 and samples from E Azerbaijan province (east of Turkey) were harvested during April–May 2009. In Fars province with different climatic conditions, samples were collected from the southern parts during March and from the northern parts usually in April (Table 1). The finding of truffles depended on the degree of rainfall during the previous season (November and December). Positive soils had a loamy texture, a pH about 8, and *Helianthemum* or *Carex* species were present. All *in vitro* attempts for cultivation of truffles failed.

- Morphological identification

During this study three species were identified which are described as follows:

Table 2. GenBank accession numbers of internal transcribed spacers sequences of rDNA of truffle species used for phylogenetic studies and origin of these species

Species	Accession[*]	Origin	Species	Accession	Origin
Choiromyces echinulatus	AF435829[b]	Botswana	*Terfezia leptoderma*	AF276678[a]	Spain
				AF276679[a]	Spain
Mattirolomyces terfezioides	AF276680[a]	Hungary		AF396862[a]	Spain
	AF276681[a]	Hungary		AF396863[a]	Spain
				AF396864[a]	France
Terfezia arenaria	AF276674[a]	Spain			
	AF276675[a]	Spain	*Terfezia olbiensis*	AF387656[c]	NS
				AF387657[c]	NS
Terfezia boudieri	AF276672[a]	Kuwait			
	AF276673[a]	Algeria	*Terfezia pfeilii*	AF301420[b]	Botswana
	AF301418[b]	Israel		AF301422[b]	Botswana
	AF092096[b]	Israel			
	AF092097[b]	Israel	*Tirmania nivea*	AF276665[a]	Tunisia
	AF092098[b]	Israel		AF276666[a]	Spain
				AF276667[a]	Tunisia
Terfezia claveryi	AF276670[a]	Morocco		AF276668[a]	Kuwait
	AF276671[a]	Spain			
	AF301421[b]	Morocco	*Tirmania pinoyi*	AF276669[a]	Algeria
	AF387648[c]	NS			
			Tuber melanosporum	AF167097[b]	NS

[*] GenBank accession number for the internal transcribed spacers sequences of rDNA. [a] Díez *et al.* 2002.
[b] Ferdman *et al.* 2005. [c] Gutierrez, Honrubia & Morte (direct submission). NS = Not specified.

Terfezia claveryi Chatin

Based on the Melzer's reaction and spore morphology, the species was tentatively identified as *T. claveryi*. This species was found in various provinces such as Fars, Sistan & Baloochestan, and E Azerbaijan. Ascocarps were hypogeous, subglobose to napiform with different sizes (up to 12 cm in diam.). Peridium was smooth, often with grooves as a result of substratum condition, dark brown at maturity. Asci were hyaline, globose to ellipsoid, readily separated from glebal hyphae. Ascospores were one-celled, globose, light brown at maturity, measuring 19–22 μm in diam. (average 20.5 μm), with reticulate ornamentation.

Tirmania pinoyi (Maire) Malençon

This species was collected in southern Fars, Sistan & Baloochestan and Hormozgan provinces. It accounted for the early crop of truffles. Ascocarps were hypogeous, globose to subsglobose, dark brown, with age usually up to 200–300 grams (12 cm diam.). Peridium was smooth with inflated cells. Gleba was white to yellow, fleshy, with fertile pockets. A drop of Melzer's reagent on a cross section of fresh mature truffle gives a green to blue colour reaction. Asci were amyloid, with short stipe. Ascospores were one-celled, globose, 16–20 μm in diam. (av. 19.5 μm) with smooth surface.

Tirmania nivea (Desf.) Trappe

This species was found only once in a sample from Sirjan in Kerman province. Ascocarps are hypogeous, light-coloured. Gleba was fleshy, soft and yellowish. Peridium was smooth, becoming pink with age and positive reaction with Melzer's reagent. Asci were containing eight one-celled, hyaline ascospores and broadly ellipsoid measuring 14.5–17 × 13–15.5 μm (average 16 × 14 μm).

- Molecular study

Among 12 specimens of truffles collected from different parts of Iran, seven were identified as *T. claveryi* by comparison with other sequences reported earlier (Table 2). Three specimens were positively identified as *T. pinoyi*, and one as *T. nivea*.
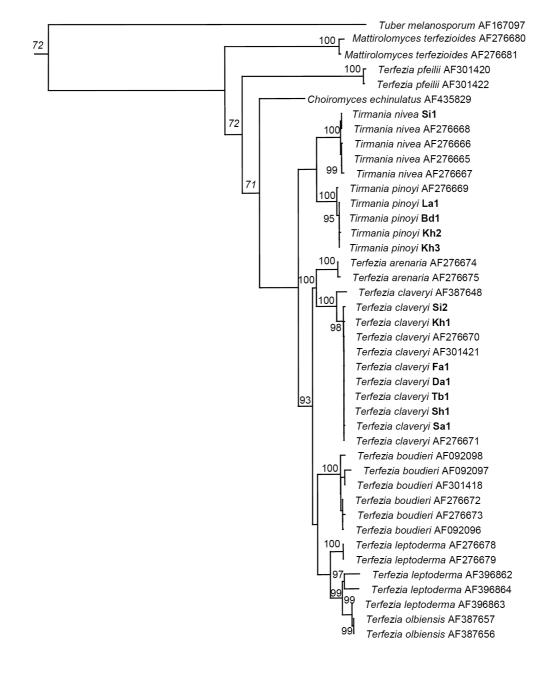
- Sequence data and phylogenetic analysis

All specimens were amplified using ITS4 and ITS1 universal primers. From 12 specimens that were studied with the ITS sequences, seven isolates (Fig. 1) were clustered in a distinct monophyletic group related to *T. claveryi* from other authors (Díez *et al.* 2002, Ferdman *et al.* 2005, Gutierrez, Honrubia & Morte (direct submission, Table 2). Our *T. claveryi* specimens had an average of 99.4% similarity with a range of 98.7–100% similarity between themselves and only an average of 95.2 with a range of 87.2–100% similarity between all *T. claveryi* sequences analyzed. Re-examination of the data showed that the reduction of the lower range could be due to poor sequencing data at both ends of the sequences compared. Additionally the rRNA gene ITS sequences of four specimens were clustered with a *T. pinoyi* specimen (Díez *et al.* 2002) all of which appeared as a sister taxon to *T. nivea* clade containing only one of the specimens studied (Fig. 1, TreeBASE accession URL:http://purl.org/phylo/treebase/phylows/study/ TB2:S10469, Table 1). Our *T. pinoyi* specimens had an average of 99.9% similarity with a range of 99.8–100% similarity between themselves and an average of 97.2 with a range of 93.1–100% similarity between all *T. pinoyi* sequences studied. All *T. nivea* specimens including our only one had an average of 96.6% similarity with a range of 92.2–99.8% similarity over a *ca* 590 bp sequence run.

The present study is the first comprehensive attempt to survey desert truffles in various geographical regions of Iran. The presence of at least two genera and three species is confirmed. Identification of truffles only on the basis of their morphology is sometimes difficult or misleading (Moreno *et al.* 2000, 2001). Evolution of hypogeous Ascomycetes, especially truffles, typically involves a convergent reduction in macromorphological characters (Ferdman *et al.* 2005). Therefore, application of molecular phylogenetics for accurate identification of these species would be inevitable.

In order to study phylogenetic relationships of the desert truffles of Iran, present study conducted on the basis of ITS 1, 2 and 5.8S gene of rDNA. Samples produced a *ca* 590 bp fragment of DNA which was lengthwise consistent with those from GenBank (Table 2).

Discussion

Analysis of sequence alignments shows that, in 41 taxa compared (in addition to *Tuber melanosporum* as an outgroup) there are 421 potentially phylogenetic informative sites (about 58.72% of nucleotide sites) which were mainly comprised of substitutions, deletions and insertions. The final expected transition/transversion ratio was 1.16. This amount of information could be suitable for phylogenetic analysis and clearly resolved species boundaries in constructed phylogram (Fig. 1). We applied both distance-based and maximum likelihood (data not shown) methods. Although, the comparison of two types of trees illustrates concordance between them and results were broadly similar, neighbor-joining tree shows better separation and more intuitive grouping of taxa.

Tuber melanosporum AF167097
Mattirolomyces terfezioides AF276680
Mattirolomyces terfezioides AF276681
Terfezia pfeilii AF301420
Terfezia pfeilii AF301422
Choiromyces echinulatus AF435829
Tirmania nivea **Si1**
Tirmania nivea AF276668
Tirmania nivea AF276666
Tirmania nivea AF276665
Tirmania nivea AF276667
Tirmania pinoyi AF276669
Tirmania pinoyi **La1**
Tirmania pinoyi **Bd1**
Tirmania pinoyi **Kh2**
Tirmania pinoyi **Kh3**
Terfezia arenaria AF276674
Terfezia arenaria AF276675
Terfezia claveryi AF387648
Terfezia claveryi **Si2**
Terfezia claveryi **Kh1**
Terfezia claveryi AF276670
Terfezia claveryi AF301421
Terfezia claveryi **Fa1**
Terfezia claveryi **Da1**
Terfezia claveryi **Tb1**
Terfezia claveryi **Sh1**
Terfezia claveryi **Sa1**
Terfezia claveryi AF276671
Terfezia boudieri AF092098
Terfezia boudieri AF092097
Terfezia boudieri AF301418
Terfezia boudieri AF276672
Terfezia boudieri AF276673
Terfezia boudieri AF092096
Terfezia leptoderma AF276678
Terfezia leptoderma AF276679
Terfezia leptoderma AF396862
Terfezia leptoderma AF396864
Terfezia leptoderma AF396863
Terfezia olbiensis AF387657
Terfezia olbiensis AF387656

substitution/site

0.1

Fig. 1. Detailed phylogram of Iranian desert truffle specimens together with 10 desert truffle species. Numbers followed by specimens indicate GenBank accession numbers for ITS sequences. The phylogram was constructed by DNA distance-based analysis of the combined ITS1, 5.8S subunit, and ITS2 regions of the genomic ribosomal RNA. The numbers at the branch points indicate the percentages of bootstrap values ≥50%.

Phylogenetic analysis of specimens based on internal transcribed spacers of rRNA genome put them into three monophyletic lineages. The first group is comprised of specimens from Fars, Kerman, Sistan & Baloochestan, and E Azerbaijan provinces which together with authentic specimens of *T. claveryi* constitute a monophyletic clade. The reference specimens were from Spain and Morocco (Díez *et al.* 2002, Ferdman *et al.* 2005), and also a specimen with unknown origin (Gutierrez, Honrubia & Morte; direct submission in GenBank) (Table 2). Average convergence ratio between Iranian taxa was low and estimated 2.8%, while it was 4.8% between all sequences with a maximum of 12.8%. Examining the sequence alignments more closely, it was revealed that the maximum of convergence was not real and was mainly due to low sequencing quality at both ends of accession AF387648 (clone 83TC) (Table 2) of *T. claveryi* from GenBank. By excluding the clone 83TC, the average convergence ratio would become 3.6% (range 0–7.6). This level of similarity is also demonstrated by Díez *et al.* (2002). In spite of the different geographical origin of their specimens, the genetic similarity among *T. claveryi* specimens was high. Our *T. claveryi* robustly diverged from its sister taxon, *Terfezia arenaria*, with a bootstrap value of 100%.

The second clade is comprised of specimens from Fars, Sistan & Baloochestan and Hormozgan provinces together with a sequence of *Tirmania pinoyi* from Algeria (Díez *et al.* 2002), which form another monophyletic group. Despite the overall distribution of Iranian specimens, only 0.2% of average convergence ratio was observed which was about 2.8% between all specimens. In neighbor-joining tree, the specimens of this species and its sister clade, *T. nivea*, diverged from each other with a 99% supporting value for 500 bootstrap runs.

Only one specimen collected form Sirjan in Kerman province grouped in a clade of *Tirmania nivea* containing specimens from Spain, Tunisia and Kuwait. This group diverged from monophyletic *T. pinoyi* lineage with a high supporting value. The maximum convergence ratio in this group was 7.8, whereas the average was 3.4 among studied sequences which is a result of high genetic similarity.

The high level of genetic similarity among specimens of studied species and other sequences with different geographical origins is a result of some kind of special evolution in ITS region. rDNA contains a mosaic of highly conserved and variable regions that enable inter- and intra-specific comparisons (Hibbett 1992). These genes occur in multiple arrays, and mutations in their noncoding region occur at a rate that approximates the rate of species emergence. Over time, such mutations become fixed through unequal crossing over and gene conversion, a process that is commonly termed concerted evolution (Brasier *et al.* 1999). Within a species ITS tend to be distinct and monomorphic. They, therefore, are suitable for species discrimination across a wide range of organisms including fungi (Bruns *et al.* 1991). Based on nuclear ITS region and mitochondrial large ribosomal RNA (mt LrRNA) Wang *et al.* (2007) could discriminate closely related species of *Tuber*, a forest inhabitant relative of desert truffles. Díez *et al.* (2002) also illustrate the monophyletic origin of the genera *Terfezia* and *Tirmania* based on ribosomal genome. Moreover, phylogenetic analysis of *Terfezia boudieri* isolates have corroborated the discovery of three internal transcribed spacer types in this morphological species and revealed the existence of cryptic species within the isolates (Ferdman *et al.* 2009). However, it is revealed that some parts of rDNA, such as 18S rDNA, have a limited capacity for studying the intra-species relationships in hypogeous *Ascomycota* (Henrion *et al.* 1994).

Regardless of the high level of similarity of ITS region in specimens, which has led to discrimination of monophyletic taxa, comparison of aligned sequences showed that there are some single nucleotide polymorphisms (SNPs) among some regions of sequences. These SNPs could be suitable for analysis of the population genetics of these truffles and at the same time it is an applicable tool for studying the evolution of these taxa (Nicod & Largiader 2003, Riccioni *et al.* 2008, Ferdman *et al.* 2009).

Nowadays, both genera identified in this study are considered members of *Pezizaceae*, *Pezizales* (Læssøe & Hansen 2007). Hibbett *et al.* (2007) in their higher-level phylogenetic classification of the fungi classified *Pezizales* as an independent order in subphylum *Pezizomycotina*. Studies of O'Donnell *et al.* (1997) demonstrated the independent lines of epigeous/hypogeous fruit body evolution in the *Pezizales*. Their findings have significantly affected the classification of desert truffles. Although all fungi traditionally classify as *Pezizaceae* exhibit amyloid reaction in their asci, analysis of the 18S rDNA sequences has revealed a close relationship between non-amyloid *Terfezia* and the *Pezizaceae* (Norman & Egger 1999, Percudani *et al.* 1999). Thus the amyloid reaction is of limited value as a diagnostic criterion at family level. Studies of Díez *et al.* (2002) showed that the *Tirmania* clade comprises species with smooth spores and amyloid asci, which were found in deserts. While, the *Terfezia* clade grouped species found in semi-arid habitats having ornamented and spherical spores, with non-amyloid asci. The present work confirms these findings, because most of *Tirmania* specimens were collected from arid provinces such as Sistan & Baloochestan, Kerman, and Hormozgan, or in case of specimen La1 collected from Lar, and arid region of Fars province (Table 1).

In 1897, Chatin reported *Terfezia aphroditis* Chatin from E Azerbaijan (Tabriz) and *Terfezia hanotauxii* Chatin from Tehran province of Iran for the first time. Subsequent works confirmed specimens of *Terfezia hafizii* Chatin from Fars and Tehran provinces (Petrak & Esfandiari 1941, Esfandiari 1946, Petrak 1949). More recently, other reports corroborated *Terfezia boudieri* Chatin from Zanjan province (Ammarellou *et al.* 2006), *Terfezia leonis* (Tul.) Tul. from Fars and E Azerbaijan provinces (Daneshpazhuh 1991), and *T. pinoyi* (Daneshpazhuh 1991) from Hormozgan province. Apart form *T. pinoyi* report from Hormozgan (Daneshpazhuh 1991) which is consistent with the specimens from Bishederaz, Hormozgan in the present work, findings based on molecular works did not confirm

other reports from Iran. All the above mentioned reports were exclusively based on morphological features of ascoma, therefore it is possible that reduced convergent characteristics of fruit bodies be the source of flowed identification or, in some cases, description of new species (e.g. *T. hafizii*). This is the first report of the presence of *T. claveryi* and *T. nivea* from Iran. However, it seems that Iranian truffles flora resemble those of Mediterranean region and South coast of the Persian Gulf.

Terfezia and *Tirmania* are reported from the same habitats, associated with *Helianthemum salicifolium* on heavy, alkaline soils. *H. salicifolium* is reported to be a host of *T. nivea*, *T. pinoyi* and *T. claveryi* in Tunisia, Kuwait and Spain (Díez *et al.* 2002). The distribution of the genus in Iran seems to depend on environmental factors, especially temperature. *Tirmania* species are collected in late winter in southern parts of Fars province or southern Iran like Sistan & Baloochestan and Hormozgan provinces.

More surveys are needed before these observations can be generalized. In Fars province, annual *H. salicifolium* and perennial *Carex* spp. were found to be associated with truffles. Daneshpazhuh (1991) reported *T. pinoyi* and *T. leonis* from Hormozgan, Fars and E Azerbaijan provinces, associated with *Artemisia* and *Helianthemum.* The association of *T. boudieri* with *Carex* species (Ammarellou *et al.* 2006) and *Kobresia belardii* a herbaceous monocotyledonous hosts was also reported in Zanjan, a cold climate region in Iran (Ammarellou & Saremi 2008). Other potential perennial truffle hosts will have to be examined in different parts of Iran as well. The prevalence of truffles in Iran is dependent on the precipitation, especially during November or December. Apparently rain in late fall is more important than that in late winter or early spring. Due to low rate of ascospore germination, we cultivated truffles from fruiting body tissues, hence either this or the nutritional composition of Melin-Norkrans agar (Marx 1969, Kovacs *et al.* 2003) which originally prepared for other species could be the source of cultivation failure.

Unavailability of specimens from other parts of Iran due to successive drought, other researchers' reluctance to provide us with their herbarium specimens, and most importantly the vast area under investigation (> 1.6 M Km^2) contributes to a delimitative phylogenetic analysis of the Iranian desert truffles. Present findings, however, could be a basis for species-specific primer design followed by detection and precise identification of frequent Iranian desert truffles, their symbiont counterparts, and detailed studies on their biology and distribution. We are trying to develop specific primers for rapid identification at the species level, which could facilitate host recognition. Inoculation studies under greenhouse conditions are also underway.

Acknowledgments

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