

Hosts and Distribution of Desert Truffles in Iran, Based on Morphological and Molecular Criteria

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ABSTRACT

Terfezia, *Picoa* and *Tirmania*, so called desert truffles, are mycorrhizal fungi mostly endemic to arid and semi-arid areas of the Mediterranean Region, where they are associated with *Helianthemum* and *Carex* species. The aim of this work was to study the identification, molecular analysis, distribution and hosts of these peizizalean hypogeous fungi in Iran. Among the specimens, *Terfezia clavaryi*, *Tirmania pinoyi*, *T. nivea*, *Picoa lefebvrei* and *P. juniperi* were identified. Field, laboratory and anatomical studies showed that *Helianthemum ledifolium*, *H. salicifolium*, *H. lippi* and *Carex stenophyllum* have ectomycorrhizal associations with five species in the studied areas. The results of molecular analysis showed that *C. stenophyllum* roots form mycorrhizal associations with *T. clavaryi*. Hyphal mantle was absent from sections of the mycorrhizas. The results of physico-chemical analyses on soil samples from different parts of the Fars Province in Iran showed that the genus *Tirmania* was more prevalent in soils with high CaCO₃ and silt contents than the *T. clavaryi*, *P. lefebvrei* and *P. juniperi*. The Canonical Correspondence Analysis (CCA) indicated that soil structure were an important environmental parameter influencing desert truffles distribution.

Keywords: *Carex*, Canonical correspondence analysis, Deseret truffles, Distribution, Ectomycorrhiza, GPS, *Helianthemum*.

INTRODUCTION

Desert truffles are the hypogeous ascocarps produced by some ascomycetous mycorrhizal fungi, which can be found in semi-arid ranges of North Africa, South of Europe and Middle East, including Iran. Desert truffles in Iran are locally known as "Donbal" and usually appear after the rainy season in the months of early March to late April. Several species of the genus *Terfezia* and *Tirmania* form mycorrhizae mainly on roots of members of the Cistaceae family, including different species of the genus *Cistus*, *Tuberaria* and *Helianthemum*. These plants and their associated mycota may play a major role in the maintenance of Mediterranean shrublands and xerophytic grasslands, and thus in preventing erosion and desertification (Honrubia *et al.*, 1992). Because of the market value of the ascocarps of some of these hypogeous species,

their mycorrhizal state has been widely studied in the field and by *in vitro* experiments (Morte and Honrubia 1994). *T. leptoderma* Tul. forms mycorrhizae with *H. salicifolium* (L.) Mill. showing a well developed Hartig-net but no sheath (Dexheimer *et al.*, 1985; Leduc *et al.*, 1986). The association formed by *T. clavaryi* Chatin and *H. salicifolium* was considered as endomycorrhiza, but it showed both ecto- and endomycorrhizal characteristics (Dexheimer *et al.*, 1985). Intracellular hyphae adhering to the inner surface of the colonized root cells were more abundant than intercellular hyphae (Dexheimer *et al.*, 1985). The mycorrhizae obtained from *T. clavaryi* and *H. almeriense* Pau were reported as "both the extramatrical and intercellular hyphae were moniliform or beadlike" and "the intracellular hyphae formed coils which filled the whole lumen" (Morte and Honrubia, 1994). The anatomical results of the mycorrhization of *H. ledifolium* and *H.*

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salicifolium by *T. boudieri* and *T. claveryi* (and with two other *Tirmania* species) were reported as intracellular arbuscules (Awamah *et al.*, 1979). Later, Alsheikh (1984) presented anatomical characterization of the same two *Helianthemum* and the same four fungi species collected in natural habitats. The hyphae were always observed to colonize the cortical cells forming “many short curved and densely interwoven extensions”. Epidermal cells were also colonized, but they often collapsed. *H. almeriense*, which is of great interest for reafforestation, establishes ecto-endomycorrhizae with ascomycetes such as *Terfezia* sp. and *Balsamia* sp. (Morte and Honrubia, 1994). Mycorrhizae synthesized by the *Helianthemum* genus were studied by Read *et al.* (1977) and Cano *et al.* (1991). According to these authors, the mycorrhizae were light to dark brown colored whereas nonmycorrhizal roots were white. More recently, Kovacs and Jakucs (2001) exhaustively described “*Helianthemirhiza hirsuta*” ectomycorrhiza, from *H. ovatum* (Viv.) Dun., which is characterized by ochre to brown cottony, simple mycorrhizal systems with straight, slightly bent or tortuous ends. Mycorrhizae of *H. ledifolium* (L.) Mill. and *H. salicifolium* (L.) Mill. with different *Terfezia* and *Tirmania* species were described by Awameh *et al.* (1979) and Awameh and Alsheikh (1980). In mycorrhiza formed by *H. almeriense* with *T. claveryi* and *P. lefebvrei*, four different mycorrhizal systems are described. For both fungal species, *H. almeriense* formed an endomycorrhiza in natural field conditions, an ecto- and ectendomycorrhiza without a sheath in pot cultures, and an ectomycorrhiza with a characteristic sheath and Hartig net in in-vitro cultures (Gutierrez, 2001).

Leduc *et al.* (1986) and Fortas and Chevalier (1992) demonstrated the existence of mycorrhizae formed by truffles (e.g. *T. arenaria*, *T. claveryi*, *T. pinoyi*) on *Helianthemum* and *Cistus*. Four species, namely *T. nivea*, *T. pinoyi*, *T. boudieri*, and *P. lefebvrei* have been reported in association with *H. lippi*, in Saudi Arabia and other Middle Eastern countries (Hussain and Al-Ruqaie, 1999). The early reports on mycorrhizal incidence in sedges were reviewed by Harley and Harley (1987), Tester *et al.* (1987) and Newman and Reddell (1987).

Morphological characters such as spore and peridium morphology, gleba color, and sporocarp odor have been used to differentiate desert truffles, but they can be difficult to identify to the species level because convergent evolution has decreased the number of available characters (Diez *et al.*, 2002). In recent years, however, molecular phylogenetic research on sequestrate fungi has repeatedly demonstrated that morphology of hypogeous fungi can be misleading. Evolution of hypogeous species typically involves a convergent reduction in macromorphological characters, often entailing loss of features otherwise useful to distinguish related epigeous taxa. More specifically, molecular analyses of the *Pezizales* and the phylogenetic relations among epi- and hypogeous species have been conducted by O'Donnell *et al.* (1997), Norman and Egger (1999), Percudani *et al.* (1999), Roux *et al.* (1999), Hansen *et al.*, (2001), and Diez *et al.*, (2002). Moreover, analysis of the 18S rDNA sequences has revealed a close relationship between *Terfezia* and the *Pezizaceae* (Percudani *et al.* 1999, Norman and Egger 1999). Further, *Choiromyces* was moved from the *Terfeziaceae* to the *Tuberaceae* (O'Donnell *et al.*, 1997, Percudani *et al.*, 1999), and *Terfezia terfezioides* was removed from *Terfezia* and reinstated as *Mattirolomyces terfezioides* (Percudani *et al.*, 1999, Diez *et al.*, 2002). *Picoa* is not closely related to the *Pezizaceae* (O'Donnell *et al.*, 1997), where *Picoa carthusiana* Tul. & Tul. is referred to by the synonym *Leucangium carthusianum* (Tul.) Paol. *P. juniperi* and *P. lefebvrei* were reassigned to the genus *Picoa* based on large subunit sequence rDNA and internal transcribed spacer rDNA data (Sbissi *et al.*, 2010). The phylogenetic concept of species requires that species represent a monophyletic set of organisms. In the case of desert truffles, species delimitation by morphological characters seems to be consistent. However, molecular phylogenetic analyses are needed, in order to verify whether morphological species of desert truffles also represent phylogenetic species. The first scientific reference to the presence of desert truffle in Iran was made by Chatin who recorded *T. aphroditis* and *T. hanotauxii* (Esfandiari and Petrak 1950). Esfandiari and Petrak mentioned the presence of *T. hafizi* in 1897 (Petrak, 1949). Daneshpajuh reported the

presence of two genera of *Terfeziaceae*, *T. leonis* and *T. pinoyi* (Daneshpajuh, 1991). One species, namely *T. boudieri* has been reported in association with *Kobresia bellardii*, in Iran (Ammarellou, *et al.*, 2007). The first ascomycete genus (*Picoa* sp.) record for the fungi flora of Iran was reported by Ammarellou and Trappe (Ershad, 2009). Molecular phylogeny of three desert truffles from Iran based on ribosomal genome has been studied by Mostofizadeh *et al.*, (2010). The present work focuses on three major genera of hypogeous fungi, *Terfezia*, *Tirmania* and *Picoa* and deals with the main morphological species distribution and hosts of these pezizalean hypogeous fungi in Iran. A portion of the present work has been reported earlier (Jamali and Banihashemi, 2010).

MATERIALS AND METHODS

Collection and Soil Sampling

GPS information for each area was recorded with a GPSMAP device model 76CSx. The GPS methodology was used for the surveys because of the long period of record and the necessity to obtain a large quantity of high quality data. In order to process the data sets collected, a GIS was applied to import the GPS data. The software used for the project was ArcView 3.0, produced by ESRI. Soil samples for analysis were collected from different areas in which desert truffles were most abundant. Canonical correspondence analysis (CCA) with presence/absence data for the sequence types at different parts of Fars Province was performed and the results were summarized in an ordination diagram using CANOCO software 4.5 (Microcomputer Power, Ithaca, NY, USA). Correspondence analysis is a multivariate statistical method that allows comparisons of community composition between all sites. Soil parameters were tested for significant differences between the sites using Tukey's post hoc test.

Morphological Studies

Fresh and dried specimens were hand sectioned with a razor blade and placed in KOH 5%, and stained with Melzer's reagent

and cotton blue in lactic acid. Various characteristics such as shape, size, color of ascocarp, and asci, number of ascospores per ascus, color and ornamentation of ascospores, reaction to Meltzer's reagent (Ferdman *et al.*, 2005), and potassium hydroxide, shape, size and arrangement of ascospores at maturity were employed using available keys (Trappe, 1979).

Vegetation and Mycorrhizal Relationships

For the purpose of detecting the symbiosis relationship of the plant species with the fungal species, at first a soil sample with the size of about 15 cm³ was gently cast aside and after thorough washing the soil profile, the soil was carefully removed from roots, and the connection between the roots of the adjacent plants and the lower part of the desert truffle was studied. The samples were transferred to the lab and after washing, examined first using light microscope in search of external structures and presence of mycorrhiza. Subsequently, parts of the root were immersed in 10% potassium hydroxide and left overnight at 60°C. The remaining root parts were kept in water at 4°C. If clearing in KOH at 90°C was not sufficient, the samples were left at 90°C until they were discolored. Afterwards, the samples were soaked and washed in distilled water for a minimum of 2 hours while water was being changed several times. The last washing water was acidified (pH 3.5-3, approximately) with a few drops of lactic acid and roots were left in the solution for a minimum of 30 minutes. Cleared roots were stained with lactic acid-anilin blue and then put back into the acidic washing solution to eliminate superfluous stain (Grace and Stribley, 1991). Colonization by the desert truffles was seen in cryosections of unstained samples or those stained with aniline-blue, by light microscopy. For the examination of the mycorrhizal structure, the sectioned roots were fixed in FAA, dehydrated in butyl alcohol series and after being embedded in paraffin wax, sections of about 15 µm were obtained using a Cambridge rotary microtome.



Molecular Studies

DNA Extraction and ITS Amplification

DNA was extracted from 22 samples of desert truffles which had been identified morphologically as *T. claveryi* (ten samples), *Tirmania pinoyi* (seven samples), *T. nivea* (one sample), *Picoa lefebvrei* (two samples) and *P. juniperi* (two samples). Samples for DNA extraction were excised from the inner part of the ascocarp to avoid contamination by other microorganisms. Fifty milligrams of tissue was used for each DNA extraction, performed using the Cetyl-trimethyl-ammonium bromide (CTAB) protocol (Gardes *et al.*, 1991). The ITS regions of nuclear rDNA were amplified with ITS1 and ITS4 primers (White *et al.*, 1990) on a CORBETT RESEARCH model CG1-96 thermocycler. For ITS amplifications the samples were prepared as follows: a reaction volume of 25 µl containing 12.5 µl of a diluted DNA sample (1:10 dilutions of the original extract), 2.5 µl reaction buffer, 20 pmoles of each primer, 1.25 nmoles of each deoxynucleotide, and 0.5 U of *Taq* DNA polymerase. The thermocycle was run: 3 minutes at 94°C, then 30 cycles as follows: 30 seconds at 94°C, 30 seconds at 50°C and 2 minutes at 72°C. An elongation period of ten minutes was allowed at 72° before cooling or removing the tubes. Amplified fragments were visualized under UV light after electrophoresis on 1% agarose gels stained with ethidium bromide and run in 1×TBE buffer.

Sequencing of the Amplified ITS Regions

Amplified DNA was purified with GeneJET PCR purification kit. Sequencing reactions were performed on purified PCR products with ITS1 or ITS4 primers. The sequence was determined with an ABI prism 377 DNA sequencer. DNA sequences of the ITS regions were deposited at the National Center for Biotechnology Information (NCBI) GenBank (Table 1).

Phylogenetic and Statistical Analyses

Closest matches to each sequence were determined using the BLASTN sequence similarity search tool in GenBank (Altschul *et al.*, 1997). Multiple alignments were performed with CLUSTALW (Thompson *et al.*, 1994) using default settings and were manually optimized with BIOEDIT v.7.0.9 (Hall, 1999). Phylogenetic analyses were performed with MEGA4 using maximum parsimony (MP) (Kumar *et al.*, 2004) and neighbor-joining (NJ) with the Kimura 2-parameter (K2P) model. Insertions and deletions were taken into account. The complete deletion method was employed in gap handling for all alignment sites. All sites containing alignment gaps were removed from the analysis before calculations and then treated as missing data. The confidence of branching was assessed by computing 1,000 bootstrap re-samplings (Felsenstein, 1985).

Specific Primers for Detection of *T. claveryi* in Hosts

Oligonucleotide sequences to specifically detect *T. claveryi* in hosts were designed based on all available sequences (GenBank[NCBI]) of the ITS regions. Alignment was performed with MEGA4 software, using the Clustal W algorithm. Two primers were designed: the forward primer ITS1TC (5'-cctattgcttcactggacagg-3') in position 58-79 of the *T. claveryi* ITS1 region sequences, and the reverse primer ITS4TC (5'-ctacctgatctgaggtcacccaa-3') that corresponds to the complement of positions 551-573 in the ITS4 region. PCR conditions, including primer and MgCl₂ concentrations, annealing temperature, time of annealing and extension steps, and the number of PCR cycles were optimized to maximize the yield of the desired amplification product while minimizing levels of non-specific products.

Table 1. Sequences obtained from the National Center for Biotechnology Information (NCBI).

Species	Sample no.	Origin	Genbank accession
<i>Terfezia claveryi</i>	83TC	Spain	AF387648
<i>Terfezia claveryi</i>	94 TC	Spain	AF387647
<i>Terfezia claveryi</i>	20 TC	Spain	AF387646
<i>Terfezia claveryi</i>	110 TC	Spain	AF387645
<i>Terfezia claveryi</i>	SKtc4	Tunisia	GU474810
<i>Terfezia claveryi</i>	SKtc3	Tunisia	GU474805
<i>Terfezia claveryi</i>	SKtc2	Tunisia	GU474804
<i>Terfezia claveryi</i>	SKtc1	Tunisia	GQ474801
<i>Terfezia claveryi</i>	-	Israel	AF301421
<i>Terfezia claveryi</i>	Cly06	France	AF276670
<i>Terfezia claveryi</i>	Alc03	France	AF276671
<i>Terfezia claveryi</i>	STDA1	Iran	GQ337859
<i>Terfezia claveryi</i>	STSH1	Iran	GQ228096
<i>Terfezia claveryi</i>	STKH1	Iran	GQ228093
<i>Terfezia claveryi</i>	STTA	Iran	HM352540
<i>Terfezia claveryi</i>	STSI	Iran	HM352541
<i>Terfezia claveryi</i>	STSH2	Iran	HM352542
<i>Terfezia claveryi</i>	STKH2	Iran	HM352543
<i>Terfezia claveryi</i>	STFA	Iran	HM352544
<i>Terfezia claveryi</i>	STDR	Iran	HM352545
<i>Terfezia claveryi</i>	STSA	Iran Iran	HM352546
<i>Terfezia arenaria</i>	are20	France	AF276675
<i>Terfezia arenaria</i>	are19	France	AF276674
<i>Terfezia boudieri</i>	DDtb1	Tunisia	GU474809
<i>Terfezia boudieri</i>	ABtb4	Tunisia	GU474808
<i>Terfezia boudieri</i>	5-2008/HHL	Slovenia	FN395016
<i>Terfezia boudieri</i>	SMtb4	Tunisia	GU474797
<i>Tirmania nivea</i>	Tun08	France	AF276665
<i>Tirmania nivea</i>	Tun06	France	AF276667
<i>Tirmania nivea</i>	Tab04	France	AF276666
<i>Tirmania nivea</i>	4-2008/HHU	Slovenia	FN395015
<i>Tirmania nivea</i>	Niv05	France	AF276668
<i>Tirmania nivea</i>	Si2	Iran	FJ197820
<i>Tirmania pinoyi</i>	-	France	AF276669
<i>Tirmania pinoyi</i>	1-2008/HHU	Slovenia	FN395012
<i>Tirmania pinoyi</i>	2-2008/HHU	Slovenia	FN395013
<i>Tirmania pinoyi</i>	3-2008/HHM	Slovenia	FN395014
<i>Tirmania pinoyi</i>	Bd1	Iran	GQ888697
<i>Tirmania pinoyi</i>	La1	Iran	GQ231540
<i>Tirmania pinoyi</i>	STLA1	Iran	GQ228094
<i>Tirmania pinoyi</i>	STLA2	Iran	HM352547
<i>Tirmania pinoyi</i>	STKH3	Iran	HM352548
<i>Tirmania pinoyi</i>	STBD1	Iran	HM352549
<i>Tirmania pinoyi</i>	STKH4	Iran	HM352550
<i>Picoa lefebvrei</i>	87PJ	Spain	AF387654
<i>Picoa lefebvrei</i>	84PJ	Spain	AF387653
<i>Picoa lefebvrei</i>	102PJ	Spain	AF387652
<i>Picoa Lefebvrei</i>	IRA-MBA sb11	Tunisia	GU391570
<i>Picoa lefebvrei</i>	KM162949	-	GQ981519
<i>Picoa lefebvrei</i>	KM162948	-	GQ981518
<i>Picoa lefebvrei</i>	STAB1	Iran	GQ228092
<i>Picoa lefebvrei</i>	STBL1	Iran	GQ228095
<i>Picoa juniperi</i>	IRA-MBA sb12	Tunis	Gu391569
<i>Picoa juniperi</i>	IRA-MBA sb10	-	Gu391568
<i>Picoa juniperi</i>	IRA-MBA sb7	-	Gu391565
<i>Picoa juniperi</i>	IRA-MBA sb8	-	Gu391566
<i>Picoa juniperi</i>	STBL3	Iran	JF970607
<i>Picoa juniperi</i>	STBL4	-	JF970606
<i>Geopora cervina</i>	TAA117898	Estonia	FM206419
<i>Geopora cervina</i>	TAA188517	-	FM206418
<i>Geopora cervina</i>	TAA188304	-	FM206417
<i>Geopora arenicola</i>	TAA179462	Estonia	FM206463
<i>Geopora arenicola</i>	TAA116784	-	FM206462



Restriction Reactions

The mycorrhiza *ITS* region restriction was performed with the enzyme *Alu* I. In a total volume of either 15 or 25 μ l, either 10 or 20 μ l of the PCR products were combined with 1/10th vol. of the buffer provided by the manufacture and 1-3 U of *Alu* I. Reaction tubes were placed in a 37°C bath for 4-6 hours and reaction was stopped by adding 1/10 vol. of gel loading buffer loaded directly on a gel.

RESULTS

Desert truffles were detected by the presence of common associated plants, soil topography and by observing the cracks and the curvature of the soil over the ascocarps. Based on morphological and molecular characteristics, five hypogeous fungi were identified as *Terfezia clavervyi*, *Tirmania pinoyi*, *T. nivea*, *Picoa lefebvrei* and *P. juniper*. *T. clavervyi* was present in different parts of Iran. The distribution of desert truffle samples and their incidence are shown in Figure 1. *T. clavervyi* was mentioned for the first time in Iran by Malençon in 1973 (Malençon, 1973). *P. lefebvrei* and *P. juniperi*

were detected in most parts of Fars Province. *T. nivea* was detected only in the specimens collected from Baghaat in Sirjan and Haji-Abad in Hormozgan Province. *T. pinoyi* was present in Sistan and Baluchestan, Tabriz, and Fars (Larestan) Provinces (Figure 1). Based on morphological characters, *T. pinoyi* had been reported earlier from Iran (Daneshpajuh, 1991).

Description of Iranian Desert Truffles

1-*Picoa juniper*

The ascocarps are hypogeous, gregarious 0.5-7 cm diam., roughened, subglobose, dark. Peridium is black without reddish tones, with or without a sparse mycelium, hyphae 8-11 μ m diam. The asci are club-shaped, 90-150 \times 40-70 μ m, with a pedicle very variable in length, non amyloid and mostly 8 spores per ascus. The ascospores globose to ellipsoidal, hyaline, 21-24.5 \times 20-23.5 μ m, with a large lipid guttule and smooth (Figure 2). The description of *P. juniperi* resembled that of Moreno *et al.*, (2000) and Sbissi *et al.* (2010).

2- *Picoa lefebvrei*

The ascocarps are hypogeous, gregarious 0.5-5

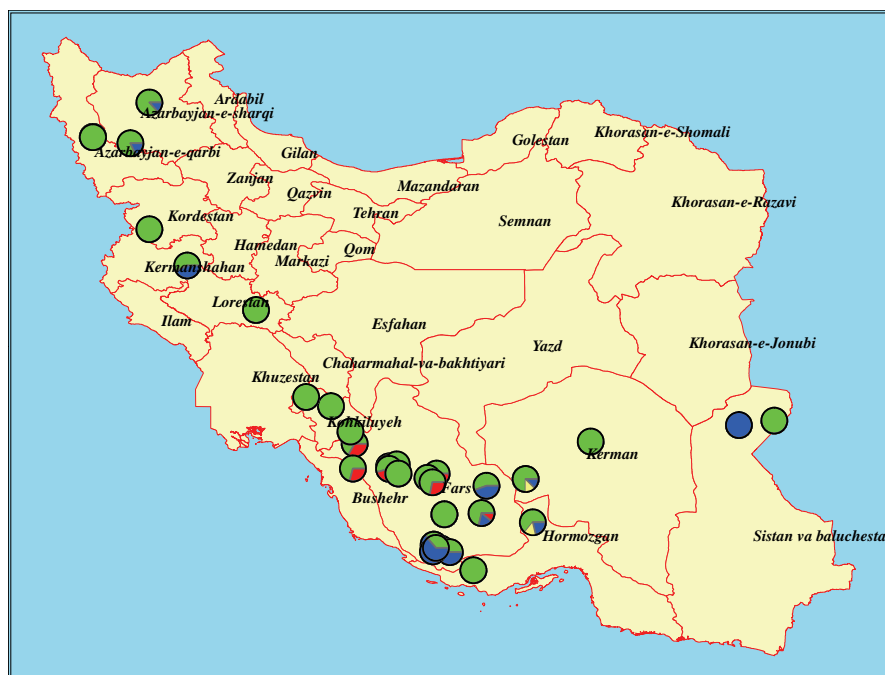


Figure 1. Distribution of desert truffles in different areas of Iran (GIS Map), Green (*T. clavervyi*), Blue (*T. pinoyi*), Yellow (*T. nivea*) and Red (*P. lefebvrei* and *P. juniperi*).

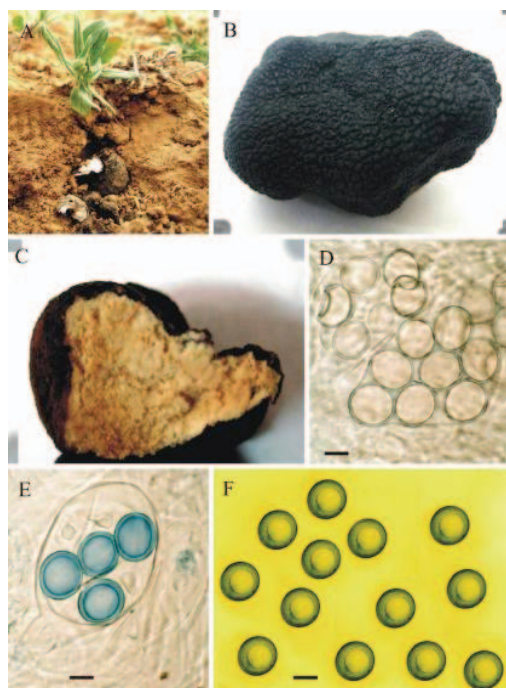


Figure 2. *Picoa juniper*: (A) *P. juniperi* and its mycorrhizal host plant *Helianthemum ledifolium*; (B) Ascocarps; (C) Section of ascocarp; (D) Eight-spored ascus, (E,F) Smooth spores. Bars= 16.7 µm (Figures D-F).

cm diam., roughened to verrucose, subglobose, brown to dark. The peridium is yellow to pale brown, hyphae 7-11 µm diam., with granules on its surface. Gleba solid, of fertile pockets separated by sterile but otherwise undifferentiated veins, the fertile pockets gray to olive. The asci are club-shaped, 90-140×30-70 µm, with a pedicle very variable in length, non amyloid and 8 spores per ascus. The ascospores globose to ellipsoidal, hyaline, 28-34×24-32 µm, with a large lipid guttule, at maturity uniformly warty (Figure 3). The description of *P. lefebvrei* resembled those of Alsheikh and Trappe (1983a), Patouillard (1894), Pacioni and El-Kholy (1994) Moreno *et al.* (2000) and Sbissi *et al.* (2010).

3-Terfezia claveryi

Ascoma hypogeous, subglobose to turbinate, fresh weight ranges from 20 to 350g. Peridium is dull yellow to pale yellow with orange brown in youth, at maturity brownish black. Gleba is fleshy and solid. Drop of iodine on cross section of fresh mature ascocarpe gives an orange color or no reaction. Asci are globose to ellipsoidal

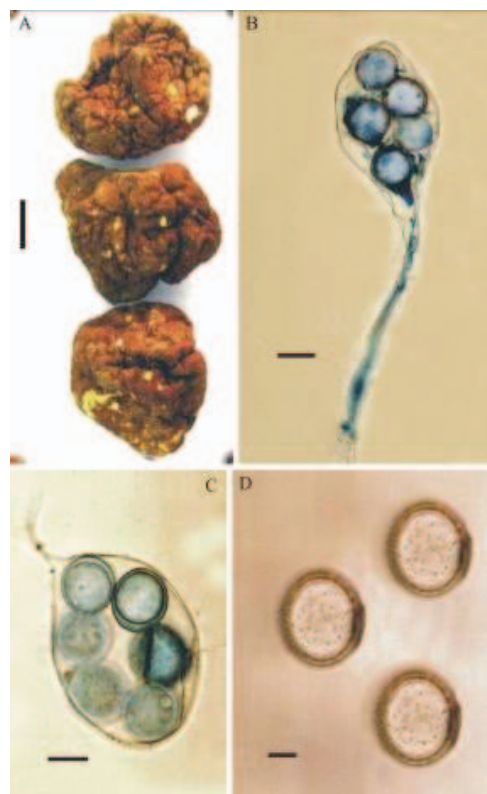


Figure 3. *Picoa lefebvrei*: (A) Ascocarps; (B) Ascus with ascospores; (C) Pedicellate ascus, (E) Ellipsoidal spores bearing small warts. Bars= 1 cm (Figure A); 25.6 µm (Figure B); 16.7 µm (Figure C), 10 µm (Figure D).

or subglobose, hyaline and non amyloid. Ascospores are globose reticulate (see Figure 4) hyaline in youth, at maturity light brown, 16-20 µm in diameter, mostly 7-8 spores in asci. Spores are dissimilar in size and double layered (Figure 3). The description of *T. claveryi* resembled those of Awameh and Al-Sheikh (1980), Bokhary and Parvez (1988), Malençon (1973) and Trappe (1979).

4-Tirmania pinoyi

The ascocarps are hypogeous, subglobose to turbinate, glabrous, wrinkled or cracked, with a basal mycelial attachment, yellow to brown or reddish brown. Peridium with large hyphae and many inflated cells. Gleba is white to pale pink veins. A drop of iodine on cross section of fresh mature ascocarp gives a green to blue color. Asci are clavate to obovoid, with 8 spores and amyloid. Ascospores spherical, 16-18 µm in diameter, hyaline and double layered (Figure 5). These observations are similar to those

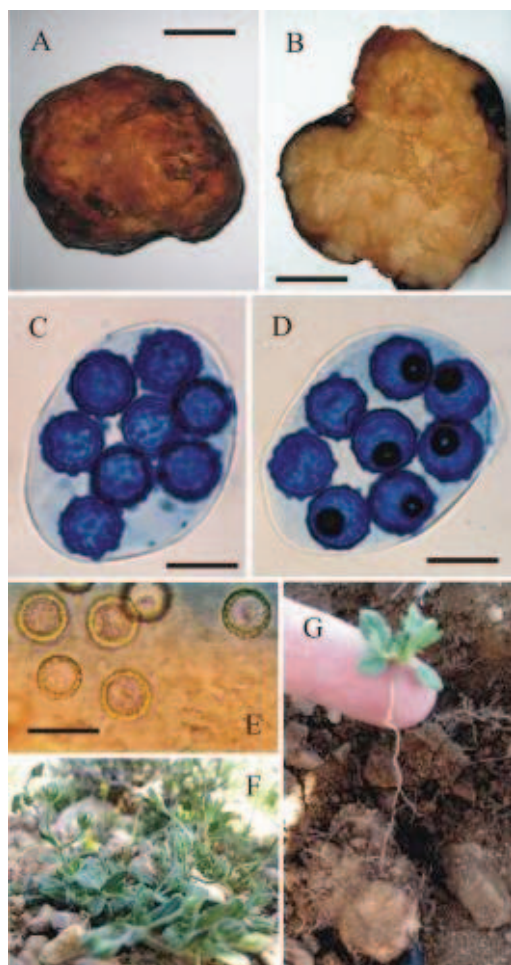


Figure 4. *Terfezia clavaryi*: (A) Ascocarp; (B) Section of ascocarp; (C) Eight-spored ascus; (D) Ascospores with germ pore; (E) Double layer ascospores, (F, G) *T. clavaryi* and its mycorrhizal host plant *Helianthemum salicifolium*, (D) Eight-spored ascus, (E), Bars= 1 cm (Figure A, B); 25.6 μ m (Figures C and D); 16.7 μ m (Figure D).

mentioned by Al-Sheikh and Trappe (1983a), Bokhary (1987) and Abd-Allah *et al.* (1989).

5- *T. nivea*

The ascocarps are hypogeous, subglobose or pyriform, glabrous, with a basal mycelial attachment, yellowish white becoming yellowish brown with age. The peridium is pseudoparenchymatous, smooth, consisting of large hyphae and inflated cells. Gleba is white becoming yellowish white with age. A drop of iodine on cross section of fresh mature ascocarp gives a green to blue color. Asci are ellipsoid to obovoid, with mostly 8 spores and amyloid.

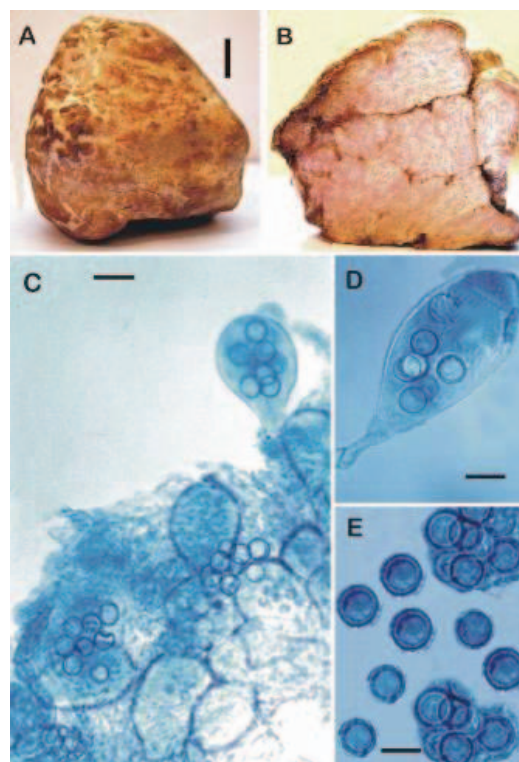


Figure 5. *Tirmania pinoyi*: (A) Ascocarp; (B) Section of ascocarp; (C) Asci with ascospores; (D) Ascus with pedicle, (E) Double layer spores. Bars= 1 cm (Figure A); 25.6 μ m (Figure C); 16.7 μ m (Figures D and E).

Ascospores ellipsoid, 15-18 \times 11-14 μ m in diameter, hyaline and double layered (Figure 6). This description is in complete harmony with those given by Trappe (1979), Al-Sheikh and Trappe (1983a), Bokhary (1987), Abd-Allah *et al.* (1989) and El-Kholy (1989).

Soil Analysis

The results of physico-chemical analyses on soil samples from different parts of Fars Province are shown in Table 2. Statistical analysis using Tukey's post hoc test showed only slight differences in soil structure parameters and amount of CaCO₃ among sites. Soils collected from different parts of Fars Province, where an abundance of desert truffles was found, were mostly sandy and loamy sand, alkaline pH, low in organic matter and high CaCO₃. These soils are considered as basic and non saline soils. The results of CCA based on the absolute numbers of each genus at the sites are shown in Figure 6.

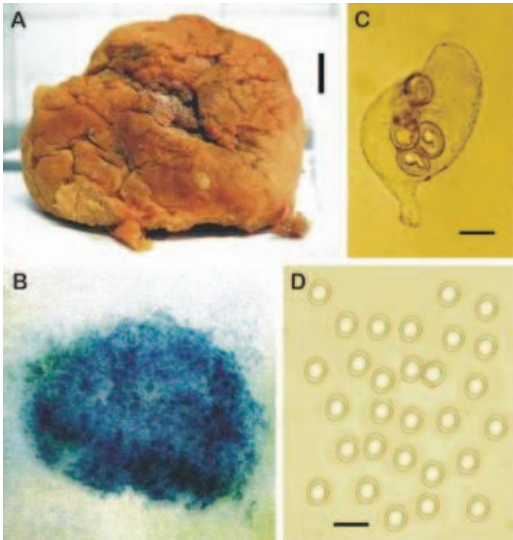


Figure 6. *Tirmania nivea*: (A) Ascocarpe; (B) Reaction with Melzer reagent; (C) Ascus with short pedicel, (D) Ellipsoidal, smooth spores. Bars= 1 cm (Figure A); 16.7 µm (Figures C and D).

The environmental parameters were superimposed on the genera distribution (Figure 7). The relative position of the arrows reflects the relationship of the axes with the environmental parameters. Eigenvalues for axes 1 and 2 were 0.29 and 0.045, respectively. The genus–environment correlations were 0.991 for axis 1 and 0.815 for axis 2. *T. claveryi*, *P.lefebvrei* and *P. juniperi* occur in sandy soils. The genus *Tirmania* was more prevalent in soils with high CaCO₃ and silt percentage than the *T. claveryi*, *P.lefebvrei* and *P. juniperi* (Figure 7).

Vegetation and Mycorrhizal Relationships

Different species of *Helianthemum* (Cistaceae) and sedges (Cyperaceae) are the most abundant plant species occurring in the natural vegetation of the studied areas. According to the available key (Ghahreman, 1996; Fernald, 1970), plant species were identified as *Helianthemum salicifolium*, *H. ledifilium*, *H. lippii* and *Carex stenophyllum*. Mycorrhization of the desert truffles species and host plants were observed in the root samples of *H. salicifolium*, *H. ledifilium* and *Carex stenophyllum*. The field, laboratory and anatomical studies showed that *H. salicifolium*, *H. ledifolium* and *C. stenophyllum* have ectomycorrhizal associations with four species of truffles in the studied areas (all data not shown). Hyphal mantle was absent from sections of the mycorrhizas (Figures 8 and 9).

Table 1. Physico-chemical parameters of the root zone soil samples collected from different parts of Fars Province.

parameters	Shiraz (Beiza)	Larestan (Aghoseh)	Larestan (Arad)	Larestan (Ahmadan)	Shiraz (Guyom)	Shiraz (Ciakhdarengun)	Sarvestan (Tangemahdi)	Fasa (Garbagun)	Darab (Ghalebiaban)	Larestan (Khoni)	Shiraz (Bajgah)	Fasa (Emanzad ehesmaeil)
Clay (%)	2.6a	0.14a	0.14a	24b	7b	3.2a	1a	13b	3.96a	21b	7b	6b
Silt (%)	17.68a	32.14b	30.14b	49.4b	21.28a	19.08a	18.28a	10.4a	19.9a	50c	19a	17a
Sand (%)	79.72b	67.72c	69.72c	26.6d	71.72a	77.72b	80.72a	76.6b	76.14b	29b	74b	77b
EC(dS m ⁻¹)	0.43a	0.53a	0.47a	0.4a	0.42a	0.42a	0.43a	0.41a	0.4a	0.43a	0.41a	0.42a
pH	7.86a	8.1a	7.83a	7.69a	8.1a	8.5a	8.24a	8a	8.4a	8.2a	8.1a	8.1a
CaCO ₃ (%)	29d	44b	45b	58a	31c	31c	33c	31c	33c	47b	31c	32c
OM (%)	2.68a	1.9a	2.35a	0.44a	2.68a	2.3a	2.37a	1.7a	1.3a	2.3a	2.2a	2.1a
P (Available mg Kg ⁻¹)	2.4a	3.1a	2.7a	2.2a	2.9a	3.1a	2.5a	2.8a	2.9a	2.7a	2.2a	2.3a

Data were determined using the methods described by Page *et al.* (1982). Data are presented as means from three replicate samples; letters show significant differences between sites according to Tukey's post hoc test (P<0.05).

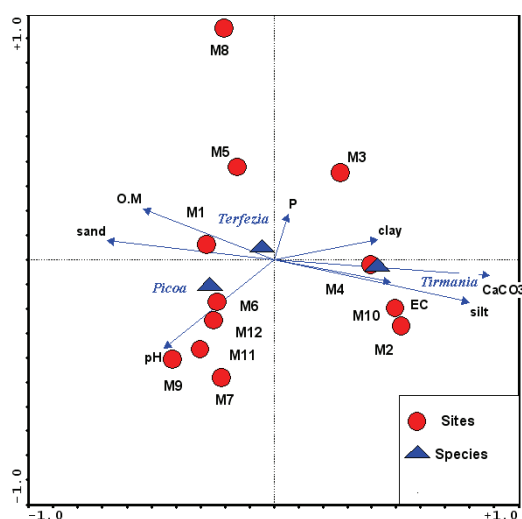


Figure 7. Correspondence analysis (CA) of the desert truffles communities found in different parts of Fars Province. The eigenvalues of the first and second axes in the two-dimensional ordination diagrams are as: CA1= 0.368 and CA2 =0.029. Sum of all eigenvalues of CA is 0.418. (Abbreviations on arrows: EC: Electrical conductivity, OM: Organic matter). [M1= Shiraz (Beiza); M2= Larestan (Aghoseh); M3= Larestan (Arad); M4= Larestan (Ahmadan); M5= Shiraz (Guyom); M6= Shiraz (Ciakhdarengun); M7= Sarvestan (Tangemahdi); M8= Fasa (Garbaigan); M9= Darab (Ghalebiaban); M10= Larestan (Khonj); M11= Shiraz (Bajgah); M12= Fasa (Emamzadehesmaeil)].

Only some hyphae growing along the surface of the roots were observed. The infection was intracellular, affecting the outer layers of cortical cells. Different species of *Helianthemum* and *C. stenophyllum* occurred only where desert truffles were found and developed at the same time. Mycorrhizal association between species of *Helianthemum* and desert truffles has been reported by many authors.

We isolated the *C. stenophyllum* roots directly from under zone (bottom) of *T. clavaryi* (Figure 4-B). Specific primer *ITS1TC/ITS4TC* amplified DNA from these mycorrhized *C. stenophyllum* roots (Figure 10, lanes A1, A2). The *ITS* fragment amplified from *C. stenophyllum* root DNA was comparable in size to *ITS* fragments of *T. clavaryi* fruit-bodies (Figure 10). DNA extracted from *C. stenophyllum* roots without mycorrhiza was not amplified with specific

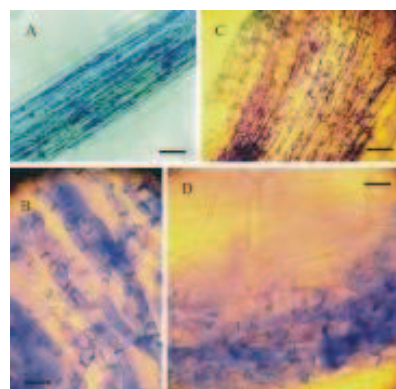


Figure 8. Cleared anilin blue-stained *H. salicifolium* (A, B) and *H. ledifolium* (C, D) roots colonized with hyphae of *T. clavaryi*. Bars= 61.9 μ m (Figures A and C), 16.7 μ m (Figures B and D).

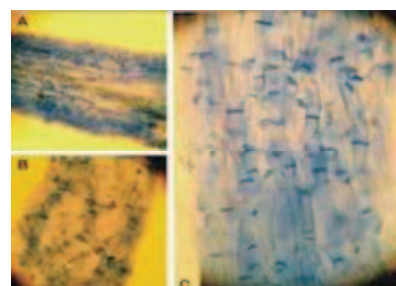


Figure 9. Cleared anilin blue-stained *C. stenophyllum* roots colonized with hyphae of *T. clavaryi*: (A) The attached mycorrhizal hyphae to *C. stenophyllum* roots, (B, C) with large magnification. Bars= 61.9 μ m (Figure A); 16.7 μ m (Figures B and C).

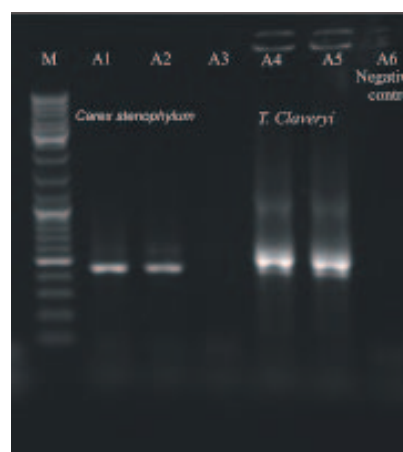


Figure 10. Amplification products of *Carex stenophyllum* and *Terfezia clavaryi*: M: Marker; Lanes A1 and A2: Specific primer *ITS1TC/ITS4TC* amplified DNA of *C. stenophyllum* roots; A3: Negative control (roots without mycorrhizae); A4 and A5: Specific primer *ITS1TC/ITS4TC* amplified DNA of *T. clavaryi*, A6: Negative control.

primers (Figure 10, lane A3). *Alu* I restriction of the *C. stenophyllum* root *ITS* fraction revealed a profile identical to *ITS* RFLP profiles of *T. claveryi* (Figure 11, lanes 1, 2). Restriction with *Alu* I gives two fragments 400 and 275 bp for *T. claveryi* (Figure 11, lanes A3, A4).

Sequence Comparisons

Search for similar sequences in the GenBank DNA database using Blast program (<http://blast.ncbi.nlm.nih.gov/blast.cgi>) produced significant alignments with the *ITS* sequences of *Terfezia claveryi* (GQ337859, GQ228093, GQ228096, HM352540-HM352546), *Tirmania nivea* (FJ197820), *T. pinoyi* (GQ228094, GQ231540, GQ888697, HM352547-HM352550), *Picoa lefebvrei* (GQ228092, GQ228095) and *P. juniperi* (JF970606, JF970607). Among 22 specimens studied based on *ITS* sequencing, ten isolates corresponded to *T. claveryi* reported by other authors. Our *T. claveryi* specimens had an average identity of 99.4% (range 98.7-100%) among themselves, while all *T. claveryi* sequences analyzed had an

average of 95.2% (range 93.2-100%). Seven specimens corresponded to *T. pinoyi*, being a sister taxon of *T. nivea*, of which only one specimen could be studied. Our *T. pinoyi* specimens had an average of 99.9% identity (range 99.8-100%) among themselves, and 97.2% (range 93.1-100%) between all *T. pinoyi* sequences compared. All *T. nivea* sequences, including our specimen, had an average of 96.6% identity (range 92.2-99.8%). Two specimens corresponded to *P. lefebvrei* that had an average of 99.5% (range 98-100%) identity among themselves, and 98% (95-100%) between all *P. lefebvrei* sequences compared. Two specimens corresponded to *P. juniperi* that had an average of 99 % (range 98-100%) identity among themselves, and 98% (95-100%) between all *P. juniperi* sequences compared. The variations in the length of the *ITS* sequences were often attributable to deletions and insertions. Gaps were therefore introduced in order to align the sequences. The total length of the alignment comprised a small portion of the flanking 18S and 28S rDNA genes, the *ITS* region, the 5.8S rDNA, and the *ITS2* sequence.

Phylogenetic Inferred Trees

The *ITS* phylogenetic trees inferred by both distance-based (Figure 12) and cladistic methods (Figure 13) showed the same topology, although there were differences in percent bootstrapping. Trees were branched into two main clades, which were well supported by bootstrap values: one including *Picoa/Geopora* (99% NJ and 100% MP) and the other *Terfezia/Tirmania* clade (99% NJ and 100% MP). In this tree, some of the *Terfezia* group nodes, notably separation of *T. claveryi* and *T. arenaria* (79% NJ, 69% MP) as a distinct clade and grouping of the latter with the *T. boudieri* clade (98% NJ, 95% MP), were strongly supported by distance and cladistic method. The bootstrap percentages to separate *T. arenaria* from *T. claveryi* with two methods are low. Most of the *Terfezia* spp. group together to constitute a monophyletic *Terfezia* clade, the closest neighbor of which is the *Tirmania* lineage. *T. boudieri* forms a distinct group as sister to *T. claveryi* and *T. arenaria*. The *Terfezia claveryi* clade (99% NJ, 93% MP) was composed of a collection with ornamented and

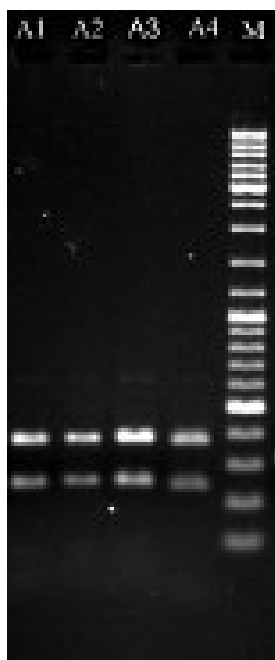


Figure 11. *Alu* I digested *ITS* fragments of *T. claveryi* fruit-bodies and mycorrhized *C. stenophyllum* roots. 1% agarose gel. Lanes A1 and A2: Roots of *C. stenophyllum*, Lanes A3 and A4: *T. claveryi* fruit-bodies.

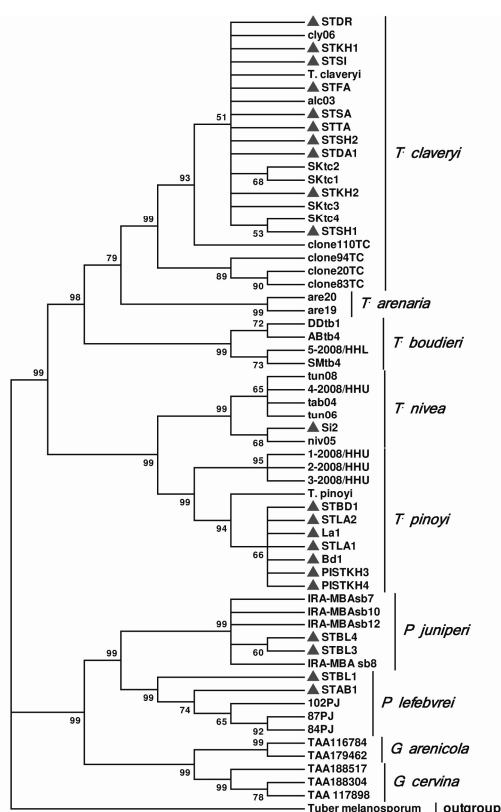


Figure 12. Neighbor joining phylogram of 61 ITS rDNA sequences of pezizalean desert truffles based on distance analysis (Kimura's 2 parameter). Number in branches are the bootstrap values as percentage bootstrap replication from a 1,000 replicate analysis. The shapes of *Tuber* desert truffles in Iran. Sequence of *Tuber melanosporum* were used as outgroup.

spherical spores, and establishes mycorrhizal symbiosis with several species of the *Helianthemum* including *H. salicifolium*, *H. ledifolium*, *H. lippii* and *Carex stenophyllum*. The *Tirmania* clade comprised desert truffles with smooth spores and amyloid asci, which were found in rangeland. *T. pinoyi* was a sister taxon of *T. nivea*. *P. lefebvrei* and *P. juniperi* form a distinct group (99% NJ, 99% MP) as sister to *G. arenicola* and *G. cervina* (99% NJ, 100% MP). Two trees presented here provide strong support for a close relationship between *P. lefebvrei* and *P. juniperi*. The *Picoa* clade (99% NJ, 99% MP) was composed of collections with globose to ellipsoidal spores and non amyloid asci which were found in rangelands and establishes mycorrhizal symbiosis with several species of

the *Helianthemum* including *H. salicifolium* and *H. ledifolium*. Sequences fall into two main groups, one including *Terfezia* spp. and *Tirmania* spp., and the other *Picoa* spp. and *Geopora* spp. Both show relatively small differences within but relatively large differences between the groups.

DISCUSSION

The present surveys from different regions showed that desert truffles exist in different parts of Iran. Based on the morphological and molecular characters, *Terfezia claveryi*, *Tirmania nivea*, *T. pinoyi*, *Picoa lefebvrei* and *P. juniperi* were identified. The morphological features of five species resembled those of Awameh and Al-Sheikh (1980), Bokhary and Parvez (1988), Al-Sheikh and Trappe (1983b), El-Kholy (1989), Abd-Allah *et al.* (1989), Moreno *et al.* (2000) and Sbissi *et al.* (2010). *T. claveryi* was mentioned for the first time in Iran by Malencon in 1973 (Malencon, 1973). Based on morphological characters, *T. pinoyi* had been reported earlier from Iran (Daneshpajuh, 1991). *T. nivea* and *P. juniperi* are newly recorded for Iran. *T. nivea* and *P. lefebvrei* had been previously reported from Iran (Jamali and Banihashemi, 2010). *P. lefebvrei* was reported in the Arabian Peninsula by Alsheikh and Trappe (1983b) from Kuwait. They also examined samples from Tunisia, Algeria, Libya and Iraq. They were the first to report that the spores were ornamented at maturity. Alsheikh and Trappe (1983b), based on spore ornamentation and the hyphal tomentum, placed *Picoa lefebvrei* in *Phaeangium*, erected by Patouillard (1894). Moreno *et al.*, (2000) disagree with the separation of *Phaeangium* genus from *Picoa*. The spores of *P. lefebvrei* were described as smooth by Patouillard (1894) and Maire (1906). Patouillard (1894), however, noticed strong similarities between *P. lefebvrei* and *P. juniperi*, confirming that both species belong in *Picoa*. In Europe, it has been reported from France (Civry-sur-Serein, Yonne), by Rioussset *et al.* (1989, 1996) associated with *H. nummularium* (L.) Miller and from Spain, by Calonge *et al.* (1995) among grass and calcareous soil. *P.*

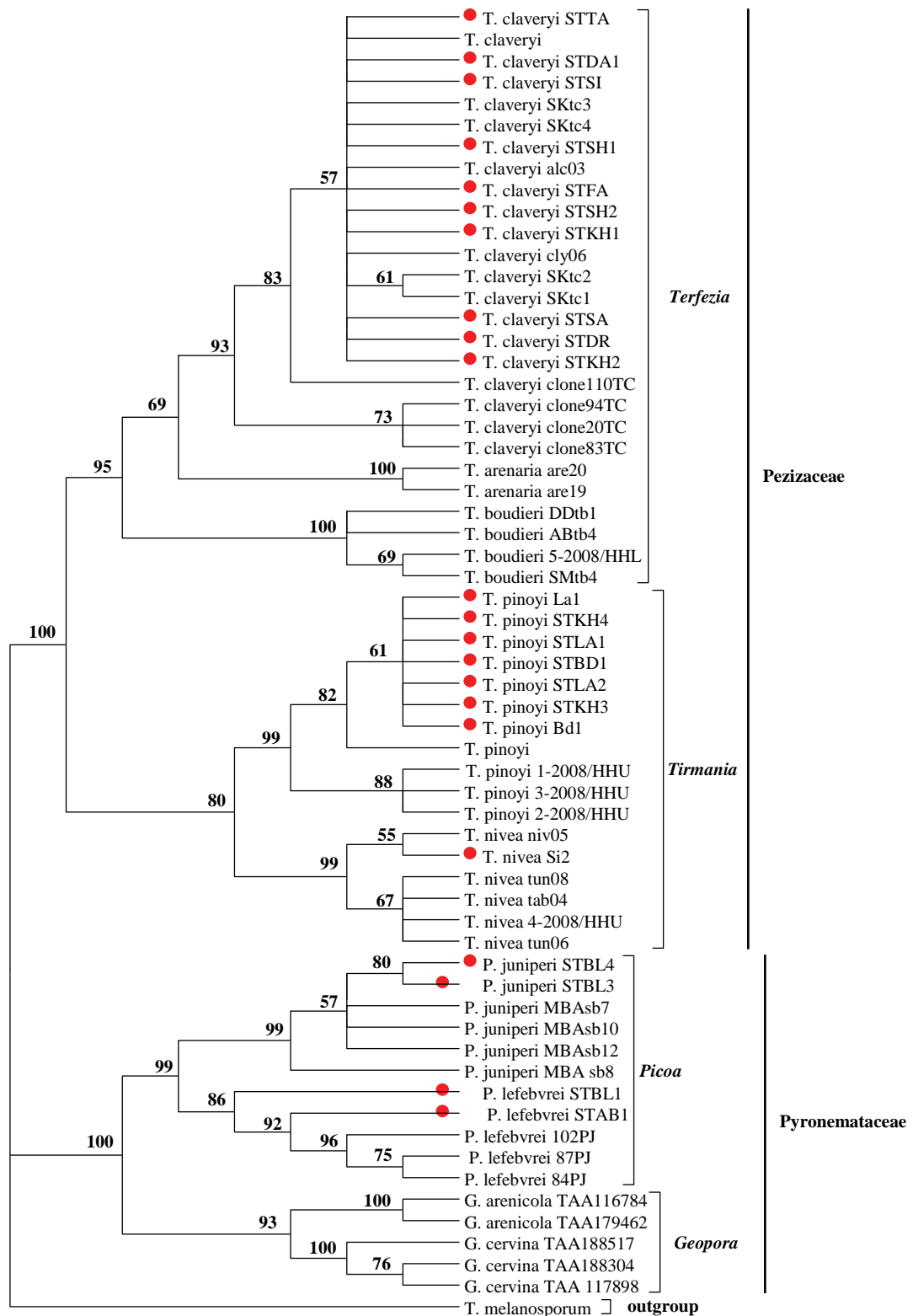


Figure 13. Rooted 50% majority rule consensus tree resulting from 1000 bootstrap replications of the parsimony analysis of the *ITS* rDNA sequences (Consistency index: CI= 0.82; Retention index: RI= 0.91, Rescaled consistency index: RCI= 0.72). Analysis was conducted using the heuristic search algorithm. Numbers on the branched are the bootstrap values (%). The red dots refer to desert truffles in Iran.



lefebvrei and *P. juniperi* were reassigned to the genus *Picoa* based on large subunit sequences rDNA and internal transcribed spacer rDNA data. Morphological studies of spores, asci, peridia, and gleba revealed high similarities between *P. lefebvrei* and *P. juniperi*, thereby confirming the membership of both species in the genus *Picoa* (Sbissi *et al.*, 2010). These two species were primarily distinguishable based on ascospore ornamentation. Ribosomal DNA analysis has enabled the genus *Picoa* to be assigned to the Pyrenomataceae and to confirm that *Picoa* is closely related to *Geopora* (Tedersoo *et al.*, 2010). *Picoa* is clearly a lineage sister to *Geopora*, and is thus phylogenetically distant from *Tirmania* and *Terfezia*. Ammarellou *et al.* (2011) document the presence of *Picoa lefebvrei* in Iran and, using the phylogenetic analysis of the *ITS* and 28S rDNA, showed that this species belongs to the *Geopora-Tricharina* clade of the Pyrenomataceae. Based on the results of our study, we conclude that the genus *Picoa* is a close relative of *Geopora* within the family Pyrenomataceae (Figure 13). According to our results including morphological and molecular studies, we disagree with the separation of this genus from *Picoa*.

Four plant species including, *H. salicifolium*, *H. ledifolium*, *H. lippi* and *Carex stenophyllum* were detected in conjunction with the above species. Mycorrhizal association between species of *Helianthemum* and desert truffles has been reported by many authors (Trappe, 1971; Awameh and Al-Sheikh, 1979; Al-Sheikh and Trappe, 1983a, Dexheimer *et al.*, 1985; Bokhary, 1987; Fortas and Chevalier, 1992 and Bokhary and Parvez, 1992). *C. stenophyllum* was reported for the first time as a partner of *T. claveryi*. In spite of the general consensus that the Cyperaceae are nonmycorrhizal, there have been numerous reports of mycorrhizal infection in certain species (Mejstrik, 1972; Read, *et al.*, 1976; Haselwandter and Read, 1980; Read and Haselwandter, 1981; Gay, *et al.*, 1982; Pendleton and Smith, 1983; Allen *et al.*, 1987; Bellgard, 1991; Koske, *et al.*, 1992; Meney *et al.*, 1993; Hartnett *et al.*, 1994; Wetzal and van der Valk, 1995; Lovera and Cuenca, 1996). *Carex*, a genus of about 2000 species distributed world-wide, belongs to the subfamily *Caricoideae* of the family Cyperaceae (Mejstrik, 1972; Read, *et al.*, 1976). Although the *ITS1* and *ITS4* primers were

designed for the amplification of fungal sequences (White *et al.*, 1999), it has been reported that they may amplify *ITS* in certain plants as well. Specific primers *ITS1TC/ITS4TC* amplified DNA of mycorrhized *C. stenophyllum* roots, but did not amplify DNA from negative control (roots without mycorrhizae), that strongly suggests that the amplified *ITS* fragment observed in the root DNA extract is derived from fungal DNA. The identity of the *C. stenophyllum ITS* restriction profile (Figure 9) with an *ITS* profile obtained from the fruit-bodies indicates derivation from *T. claveryi* DNA. It seems, from anatomical and molecular evidence, that *C. stenophyllum* roots form mycorrhizal association with *T. claveryi*. It has been shown by Urbanelli *et al.*, (1998) that a given host plant can be mycorrhized by several taxa on very small and precise root segment. Although it has been shown by sequencing that 510 bp amplified fragment is the exact match of *T. claveryi ITS* rDNA, there were some worries about amplification of a falls fragment by *ITS1* and *ITS4* universal primers with the same size or even amplification of non target mycorrhiza of closely related species by the specific primer. To resolve this hypothetical problem, restriction map analysis with *AluI* restriction enzyme produced 400/275 bp bands. The banding pattern generated by the combination of this enzyme is unique for *T. claveryi* and could be applied instead of time consuming and costly sequencing analysis.

The soil analysis showed that soils are alkaline and non-saline. *T. claveryi*, *P. lefebvrei* and *P. juniperi* occur in sandy soils. In contrast, *T. pinoyi* and *T. nivea* live in siliceous sands and gypsum soils (Figure 7). It has been previously reported that *T. claveryi*, *T. nivea* and *T. pinoyi* occur in saline desert and gypsiferous-saline soils (Singer, 1961; Halwagy and Halwagy, 1974; Al-Sheikh and Trappe, 1983a). In the case of *Tirmania* spp., *T. nivea* collections analyzed were found in basic soils and were associated with the basophilous plant *H. salicifolium*. Also *T. pinoyi* was collected under the basophilous plant *H. salicifolium*. It has been previously reported that *T. pinoyi* was collected under the acidophilous plant *H. guttatum* (Diez *et al.*, 2002). The distribution pattern of the desert truffles species seems to correlate with soil texture, soil pH and EC and host, which might

have played a key role in their speciation. Although *H. salicifolium*, *H. ledifolium* (Diez *et al.*, 2002) and *C. stenophyllum* (Miller, *et al.*, 1999) occur in basic soils, other species of *Helianthemum* e.g., *H. guttatum*, occur only in acid soils. Soil features therefore have an impact on the distribution of the host plants.

Analysis of the *ITS* sequences showed the separation of the *Picoa* from the *Terfezia-Tirmania* clade. They seem to represent two different evolutionary pezizalean lineages. Most of the *Terfezia* spp. group together and constitute a monophyletic *Terfezia* clade, the closest neighbour of which is the *Tirmania* lineage. *T. claveryi* and *T. arenaria* form a distinct group as sister to *T. boudieri*. Isolates of the genus *Picoa* are distant from *Terfezia-Tirmania* clade and form a separate lineage. Two trees presented here provide strong support for a close relationship between *Terfezia* and *Tirmania* clade. The present phylogenetic analyses confirm that these morphological species are also phylogenetic species. This is in agreement with the analyses of the *ITS* sequences by Diez *et al.* (2002).

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میزبان ها و پراکنش دنبل های ییابانی در ایران بر اساس ریخت شناسی و روش های مولکولی

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چکیده

جنس های *Tirmania* و *Terfezia*, *Picoa* دنبل های ییابانی نامیده می شوند که در زیست بوم های خشک و نیمه خشک مناطق مدیترانه ای با گونه های گیاهی *Helianthemum* و *Carex* ایجاد قارچ ریشه می کنند. اهداف مورد مطالعه در این مقاله بررسی میزبان ها و پراکنش دنبل های ییابانی در ایران بر اساس ریخت شناسی و روش های مولکولی است. در بین نمونه ها پنج گونه *P. juniperi* و *Terfezia clavervyi*, *Tirmania nivea*, *T. pinoyi*, *Picoa lefebvrei* تشخیص داده شدند. بررسی های مزرعه ای، آزمایشگاهی و آناتومیکی نشان دادند که گونه های *Helianthemum salicifolium*, *H. ledifolium*, *Carex stenophyllum* و *C. stenophyllum* با گونه های قارچی تشخیص داده شده دارند. از روش های مولکولی مبتنی بر واکنش زنجیره ای پلی مرز نیز برای تأیید رابطه قارچ-ریشه ای گونه گیاهی *C. stenophyllum* با *T. clavervyi* استفاده شد. حباب های هیفی در برش های گرفته شده از برون ریشه ها مشاهده نشدند. نتایج آنالیز فیزیکی-شیمیایی روی نمونه های خاک در مناطق مختلف استان فارس نشان دادند که جنس *Tirmania* در خاک هایی با درصد سیلت و آهک بالا، نسبت به گونه های *P. lefebvrei* و *T. clavervyi*, *P. juniperi* شیوع بیشتری دارد. آنالیز تطبیق متعارفی نشان داد که ساختار خاک، پارامتر مهم محیطی است که در پراکنش دنبل های ییابانی تأثیر گذار است.