## 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 pezizalean hypogeous fungi in Iran. Among the specimens, Terfezia claveryi, 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 stenophylum have ectomycorrhizal associations with five species in the studied areas. The results of molecular analysis showed that C. stenophylum roots form mycorrhizal associations with T. claveryi. 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 CaCO<sub>3</sub> and silt contents than the T. claveryi, 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. clavervi 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. claveryi 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., Later. Alsheikh (1984) presented 1979). 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 ectoendomycorrhizae 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 exhaustively Jakucs (2001)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 epiphylogenetic relations among 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 (Esfandiariand and Petrak 1950). Esfandiari and Petrak mentioned the presence of T. hafizi in 1897 (Petrak, 1949). Daneshpajuh reported the

presence of two genera of Terfeziacea, T. leonis and T. pinovi (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 Picoa and deals with the main and 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 cm<sup>3</sup> 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 mycorhizal 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.

#### Jamali and Banihashemi

## **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, using the Cetyl-trimethylperformed amonium 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 *ITS*1 or *ITS*4 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 2parameter (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-cctattgcttccactggacagg-3) in position 58-79 of the *T. claveryi ITS*1

region sequences, and the reverse primer *ITS4TC* (5-ctacctgatctgaggtcacccaa-3) that

corresponds to the complement of positions 551-573 in the *ITS*4 region. PCR conditions, including primer and MgCl<sub>2</sub> 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.

Species	Sample no.	Origin	Genbank accession
	1	6	
Terfezia claveryi	83TC	Spain	AF387648
Terfezia claveryi	94 TC	Spain	AF387647
Terfezia claveryi	20 TC	Spain	AF387646
Terfezia claveryi	110 TC	Spain	AF387645
Terfezia claveryi	SKtc4	Tunisia	GU474810
Terfezia claveryi	SKtc3	Tunisia	GU474805
Terfezia claveryi	SKtc2	Tunisia	GU474804
Terfezia claveryi	SKtc1	Tunisia	GQ474801
Terfezia claveryi	-	Israel	AF301421
Terfezia claveryi	Cly06	France	AF2/66/0
Terfezia claveryi	AICU3	France	AF2/66/1
Terfezia claveryi Terfezia elaveryi	SIDAI	Iran	GQ337859
Terfezia claveryi	515H1 STVU1	Iran	GQ228090
Terfezia elaveryi	SIKII	Iran	UQ228095
Terfezia claveryi	STIA	Iran	HM352541
Terfezia claveryi	STSH2	Iran	HM352542
Terfezia claveryi	STKH2	Iran	HM352543
Terfezia claveryi	STEA	Iran	HM352544
Terfezia clavervi	STDR	Iran	HM352545
Terfezia clavervi	STSA	Iran Iran	HM352546
Terfezia arenaria	are20	France	AF276675
Terfezia arenaria	are19	France	AF276674
Terfezia boudieri	DDtb1	Tunisia	GU474809
Terfezia boudieri	ABtb4	Tunisia	GU474808
Terfezia boudieri	5-2008/HHL	Slovenia	FN395016
Terfezia boudieri	SMtb4	Tunisia	GU474797
Tirmania nivea	Tun08	France	AF276665
Tirmania nivea	Tun06	France	AF276667
Tirmania nivea	Tab04	France	AF276666
Tirmania nivea	4-2008/HHU	Slovenia	FN395015
Tirmania nivea	Niv05	France	AF276668
Tirmania nivea	Si2	Iran	FJ197820
Tirmania pinoyi		France	AF276669
Tirmania pinoyi	1-2008/HHU	Slovenia	FN395012
Tirmania pinoyi	2-2008/HHU	Slovenia	FN395013
Tirmania pinoyi	3-2008/HHM	Slovenia	FN395014
Tirmania pinoyi	Bd1	Iran	GQ888697
Tirmania pinoyi	Lal	Iran	GQ231540
Tirmania pinoyi	STLA1	Iran	GQ228094
Tirmania pinoyi	STLA2	Iran	HM352547
Tirmania pinoyi	STKH3	Iran	HM352548
Tirmania pinoyi	SIBDI	Iran	HM352549
Bissa lefebunsi	97DI	lian Secie	AE297654
Picoa lefeburei	0/PJ 9/DI	Spain	AF387034 AF287652
Picoa lefeburei	102DI	Spain	AF387652
Picoa Lafebyrai	ID21J IRA-MRA sh11	Tunisia	GU391570
Picoa lefeburei	KM1620/0	-	GO981519
Picoa lefebvrei	KM162948		GO981518
Picoa lefebvrei	STAR1	Iran	GO228092
Picoa lefebvrei	STBL1	Iran	GO228095
Picoa juniperi	IRA-MBA sh12	Tunis	Gu391569
Picoa iuniperi	IRA-MBA sb10	-	Gu391568
Picoa juniperi	IRA-MBA sb7	-	Gu391565
Picoa juniperi	IRA-MBA sb8	-	Gu391566
Picoa juniperi	STBL3	Iran	JF970607
Picoa juniperi	STBL4	-	JF970606
Geopora cervina	TAA117898	Estonia	FM206419
Geopora cervina	TAA188517	-	FM206418
Geopora cervina	TAA188304	-	FM206417
Geopora arenicola	TAA179462	Estonia	FM206463
Geopora arenicola	TAA116784	-	FM206462

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



The mycorrhiza *ITS* region restriction was performed with the enzyme *Alu* I. In a total volume of either 15 or 25  $\mu$ l, either 10 or 20  $\mu$ l of the PCR products were combined with 1/10<sup>th</sup> 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 claveryi*, *Tirmania pinoyi*, *T. nivea*, *Picoa lefebvrei* and *P. juniper*. *T. claveryi* was present in different parts of Iran. The distribution of desert truffle samples and their incidence are shown in Figure 1. *T. claveryi* was mentioned for the first time in Iran by Malençon in 1973 (Malençon, 1973). *P. lefebrei* 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  $\mu$ m diam. The asci are club-shaped, 90-150×40-70  $\mu$ m, with a pedicle very variable in length, non amyloid and mostly 8 spores per ascus. The ascospores globose to ellipsoidal, hyaline, 21-24.5×20-23.5  $\mu$ 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. claveryi*), Blue (*T. pinovi*), 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) Eightspored ascus, (E,F) Smooth spores. Bars= 16.7  $\mu$ m (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 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  $\mu$ m (Figure B); 16.7  $\mu$ m (Figure C), 10  $\mu$ m (Figure D).

or subglobose, hyaline and non amyloid. Ascospores are globose reticulate (see Figure 4) hyaline in youth, at maturity light brown, 16-20  $\mu$ m 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 ascocarpes 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  $\mu$ m in diameter, hyaline and double layered (Figure 5). These observations are similar to those



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

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

## 5- T. nivea

The ascocarpes 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  $\mu$ m (Figure C); 16.7  $\mu$ m (Figures D and E).

Ascospores ellipsoid,  $15-18 \times 11-14 \ \mu m$  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 CaCO<sub>3</sub> among sites. Soils collected from different parts of Fars Province, where an abundance of desert truffles was found, were mostly sandy and loamy sand, alkalin pH, low in organic matter and high CaCO<sub>3</sub>. 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.

A		c
B		
	, o	0.00

Terfezia claveryi in Roots of Helianthemum species -

**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);  $16.7 \mu m$  (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 genusenvironment 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 CaCO<sub>3</sub> 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 stenophylum. Mycorrhization of the desert truffles species and host plants were observed in the root samples of H. salicifolium, H. ledifilium and Carex stenophylum. The field, laboratory and anatomical studies showed that H. salicifolium, H. ledifolium and C. stenophylum 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).

parameters	Shiraz (Beiza)	Larestan (Aghosch)	Larestan (Arad)	Larestan (Ahmadan)	Shiraz (Guyom)	Shiraz (Ciakhdarengun)	Sarvestan (Tangemahdi)	Fasa (Garbaigan)	Darab (Ghalebiaban)	(Khonj)	Shiraz (Bajgah)	Fasa (Emamzad ehesmacil)
Clay (%)	2.6a	0.14a	0.14a	24b	76	3.2a	la	13b	3.96a	21b	7b	69
Silt (%)	17.68a	32.14b	30.14b	49.4b	21.28a	19.08a	18.28a	10.4a	19.9a	500	19a	17a
Sand (%)	79.72b	67.72c	69.72c	26.6d	71.72a	77.726	80.72a	76.6b	76.14b	296	74b	776
EC(dS m <sup>-1</sup> )	0.43a	0.53a	0.47a	0.4a	0.42a	0.42a	0.43a	0.41a	0.4a	0.43a	0.41a	0.42a
Hd	7.86a	8.1a	7.83a	7.69a	8.1a	8.5a	8.24a	8a	8.4a	8.2a	8.1a	8.la
CaCO <sub>3</sub> (%)	29d	44	45b	58a	31c	31c	33c	31c	33c	47b	31c	32c
(%) WO	2.68a	1.9a	2.35a	0.44a	2.68a	2.3a	2.37a	1.7a	1.3a	2.3a	2.2a	2. la
P (Available mg Kg <sup>1</sup> )	2.4a	3.1a	2.7a	2.2a	2.9a	3.1a	2.5a	2.8a	2.9a	2.7a	2.2a	2.3a



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 twodimensional 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); Larestan M3 =(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 *Helianthemun* and *C. stenophylum* 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. stenophylum* roots directly from under zone (bottom) of *T. claveryi* (Figure 4-B). Specific primer *ITS1TC/ITS4TC* amplified DNA from these mycorrhized *C. stenophylum* roots (Figure 10, lanes A1, A2). The *ITS* fragment amplified from *C. stenophylum* root DNA was comparable in size to *ITS* fragments of *T. claveryi* fruit-bodies (Figure 10). DNA extracted from *C. stenophylum* 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. claveryi*. Bars= 61.9  $\mu$ m (Figures A and C), 16.7  $\mu$ m (Figures B and D).



**Figure 9.** Cleared anilin blue-stained *C. stenophylum* roots clonized with hyphae of *T. claveryi*: (A) The attached mycorrhizal hyphae to *C. stenophylum* roots, (B, C) with large magnification. Bars= 61.9  $\mu$ m (Figure A); 16.7  $\mu$ m (Figures B and C).



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

primers (Figure 10, lane A3). *Alu* I restriction of the *C. stenophylum* 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 lefebvreyi (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



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

average of 95.2% (range 93.2-100%). Seven specimens corresponded to T. pinovi, 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. pinovi 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





T pinoy

G arenicol

G cervine

TLAI

APJ 4411678

TAA18851

showed that desert truffles exist in different parts of Iran. Based on the morphological and molecular characters, Terfezia claveryi. Tirmania nivea, T. pinovi, 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 1973). Based on in 1973 (Malencon. 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 (Civrysur-Serein, Yonne), by Riousset 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 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 refer to 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 stenophylum. The Tirmania clade comprised desert truffles with smooth spores and amyloid asci, which were found in rangeland. T. pinovi was a sister taxon of T. nivea. P. lefebvrei and P. juniper 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



**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 Pyronemataceae 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 Pyronemataceae. Based on the results of our study, we conclude that the genus Picoa is a close relative of Geopora within the family Pyronemataceae (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. stenophylum 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; Wetzel 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. stenophylum 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. stenophylum ITS restriction profile (Figure 9) with an ITS profile obtained from the fruit-bodies indicates derivation from T. clavervi DNA. It seems, from anatomical and molecular evidence, that C. stenophylum 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. pinovi 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. stenophylum* (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 دنبلهای بیابانی در ایران بر اساس ریخت شناسی و روشهای مولکولی



جنس های Terfezia, Picoa و Tirmania دنبل های بیابانی نامیده می شوند که در زیست بومهای خشک و نیمه خشک مناطق مدیترانهای با گونههای گیاهی Helianthemum و Carex ایجاد قارچ ریشه می کنند. اهداف مورد مطالعه در این مقاله بررسی میزبانها و پراکنش دنبل های بیابانی در ایران بر اساس ریخت شناسی و روش های مولکولی است. در بین نمونه ها پنج گونه بررسی میزبانها و پراکنش دنبل های بیابانی در ایران بر اساس ریخت شناسی و روش های مولکولی است. در بین نمونه ها پنج گونه بررسی میزبانها و پراکنش دنبل های بیابانی در ایران بر اساس ریخت شناسی و روش های مولکولی است. در بین نمونه ها پنج گونه بررسی میزبانها و پراکنش دنبل های بیابانی در ایران بر اساس ریخت شناسی و روش های مولکولی است. در بین نمونه ها پنج گونه بررسی میزبانها و پراکنش دنبل های بیابانی در ایران بر اساس ریخت شناسی و روش های مولکولی است. در بین نمونه ها پنج گونه بررسی های مزرعه ای آزمایشگاهی و آناتومیکی نشان دادند که که گونه های با گونه های قارچی تشخیص داده شد. از بررسی های مزرعه ای آزمایشگاهی و آناتومیکی نشان دادند که که گونه های با گونه های قارچی تشخیص داده شده دارند. از روش های مولکولی مبتنی بر واکنش زنجیره ای پلی مراز نیز برای تائید رابطه قارچ -ریشه ای با گونه های قارچی تشخیص داده شده دارند. از دوش های مولکولی مبتنی بر واکنش زنجیره ای پلی مراز نیز برای تائید رابطه قارچ -ریشه ای گونه گیاهی Mum میلی نوی - شوش های مودی از میروی اوش های مولکولی مبتنی بر واکنش زنجیره ای پلی مراز نیز برای تائید رابطه قارچ -ریشه ای گونه گیاهی Mum ماده شده دارند. از دوش های مولکولی مبتی بر واکنش زنجیره ای پلی مراز نیز برای تائید رابطه قارچ -ریشه مشاهده نشدند. نتایج آنالیز فیزیکی - شیمیایی روی اوش های مونه های خالی میند. نتایج آنالیز فیزیکی - شیمیایی روی انه های مانهای منهای مشاهده نشدند. نتایج آنایز فیز یکی - شیمیایی روی انه های ماده در مند. نتایج آنایز فیزیکی - شیمیایی روی انه ولمای خالی مای ماده داند که جنس ماه هاهده نشدند. در خاک هایی با درصد سیلت و آهک بالا، نسبت داد در مانه ماهای با مانهای تائیر گذار است.