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

S. Jamali[1] and Z. Banihashemi[1*]

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 $CaCO_3$ 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. claveryi* 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.*

[1] Department of Plant Pathology, College of Agriculture, Shiraz University, Shiraz, Islamic Republic of Iran.
[*] Corresponding author; e-mail:

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*. (l999), 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. 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 cm^3 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.

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-amonium bromide (CTAB) protocol (Gardes *et al*., 1991). The *ITS* regions of nuclear rDNA were amplified with *ITS*1 and *ITS*4 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 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´-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 $MgCl_2$ 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
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	AF276670
Terfezia claveryi	Alc03	France	AF276671
Terfezia claveryi	STDA1	Iran	GQ337859
Terfezia claveryi	STSH1	Iran	GQ228096
Terfezia claveryi	STKH1	Iran	GQ228093
Terfezia claveryi	STTA	Iran	HM352540
Terfezia claveryi	STSI	Iran	HM352541
Terfezia claveryi	STSH2	Iran	HM352542
Terfezia claveryi	STKH2	Iran	HM352543
Terfezia claveryi	STFA	Iran	HM352544
Terfezia claveryi	STDR	Iran	HM352545
Terfezia claveryi	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	La1	Iran	GQ231540
Tirmania pinoyi	STLA1	Iran	GQ228094
Tirmania pinoyi	STLA2	Iran	HM352547
Tirmania pinoyi	STKH3	Iran	HM352548
Tirmania pinoyi	STBD1	Iran	HM352549
Tirmania pinoyi	STKH4	Iran	HM352550
Picoa lefebvrei	87PJ	Spain	AF387654
Picoa lefebvrei	84PJ	Spain	AF387653
Picoa lefebvrei	102PJ	Spain	AF387652
Picoa Lefebvrei	IRA-MBA sb11	Tunisia	GU391570
Picoa lefebvrei	KM162949	-	GQ981519
Picoa lefebvrei	KM162948	-	GQ981518
Picoa lefebvrei	STAB1	Iran	GQ228092
Picoa lefebvrei	STBL1	Iran	GQ228095
Picoa juniperi	IRA-MBA sb12	Tunis	Gu391569
Picoa juniperi	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

Restriction Reactions

The mycorrhiza *ITS* region restriction was performed with the enzyme *Alu* I. In a total volume of either 15 or 25 µl, either 10 or 20 µl of the PCR products were combined with $1/10^{th}$ 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 µm diam. The asci are club-shaped, 90-150×40-70 µm, 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 µm, 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

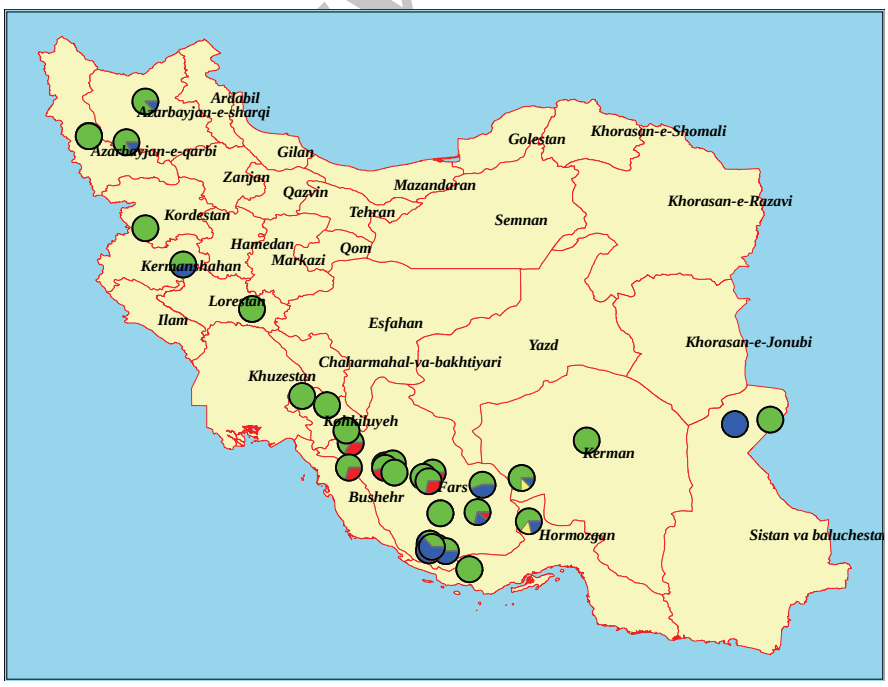

Figure 1. Distribution of desert truffles in different areas of Iran (GIS Map), Green (*T. claveryi*), Blue (*T. pinoyi*), Yellow (*T. nivea*) and Red (*P. lefebvrei* and *P. juniperi*).

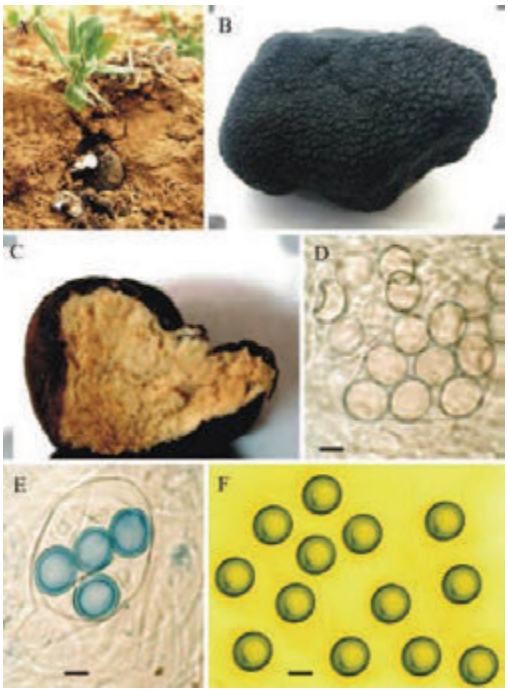

Figure 2. ***Picoa juniper***: (A) *P. juniperi* and its mycorrhizal host plant *Helianthemum ledifolium*; (B) Ascocarps; (C) Section of ascocarp; (D) Eight-spored ascus, (E,F) Smooth spores. Bars= 16.7 µm (Figures D-F).

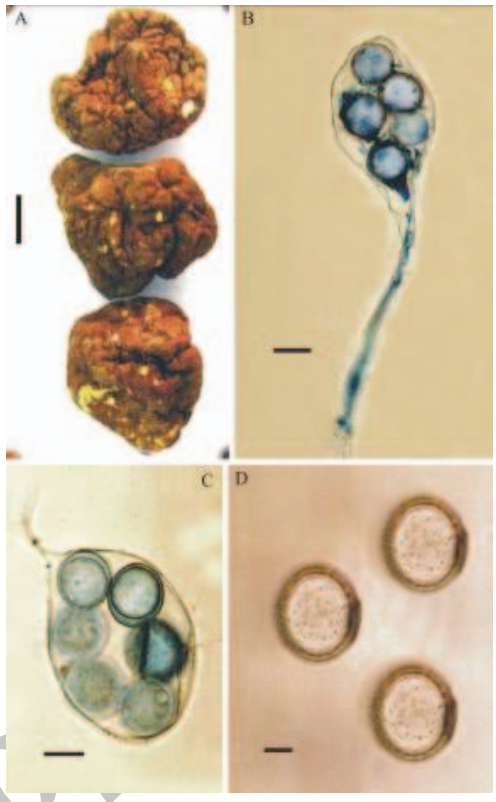

Figure 3. *Picoa lefebvrei*: (A) Ascocarps; (B) Ascus with ascospores; (C) Pedicellate ascus, (E) Ellipsoidal spores bearing small warts. Bars= 1 cm (Figure A); 25.6 µm (Figure B); 16.7 µm (Figure C), 10 µm (Figure D).

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 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 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 µm in diameter, hyaline and double layered (Figure 5). These observations are similar to those

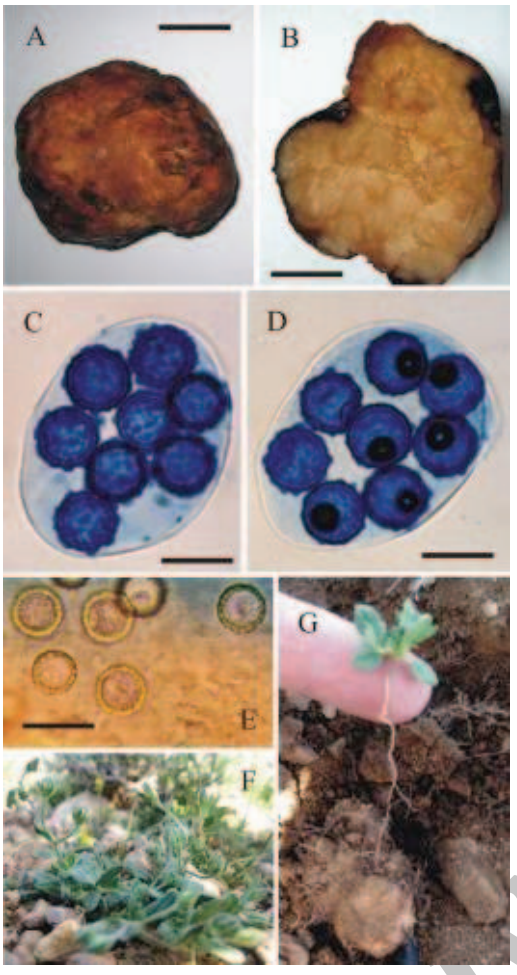

Figure 4. *Terfezia claveryi*: (A) Ascocarp; (B) Section of ascocarp; (C) Eight-spored ascus; (D) Ascospores with germpore; (E) Double layer ascospores, (F, G) *T. claveryi* and its mycorrhizal host plant *Helianthemum salicifolium*, D) Eight-spored ascus, E), Bars= 1 cm (Figure A, B); 25.6 µm (Figures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

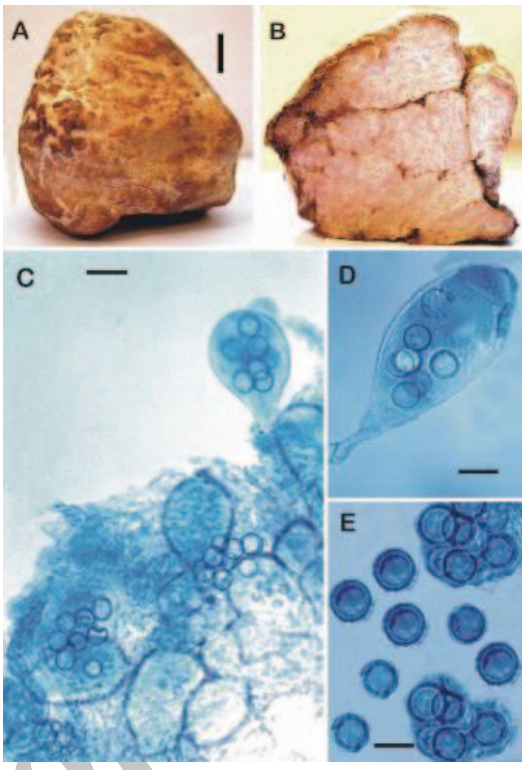

Figure 5. *Tirmania pinoyi*: (A) Ascocarp; (B) Section of ascocarp; (C) Asci with ascospores; (D) Ascus with pedicle, (E) Double layer spores. Bars= 1 cm (Figure A); 25.6 µm (Figure C); 16.7 µm (Figures D and E).

Ascospores ellipsoid, 15-18×11-14 µm 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 $CaCO_3$ 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 $CaCO_3$. 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.

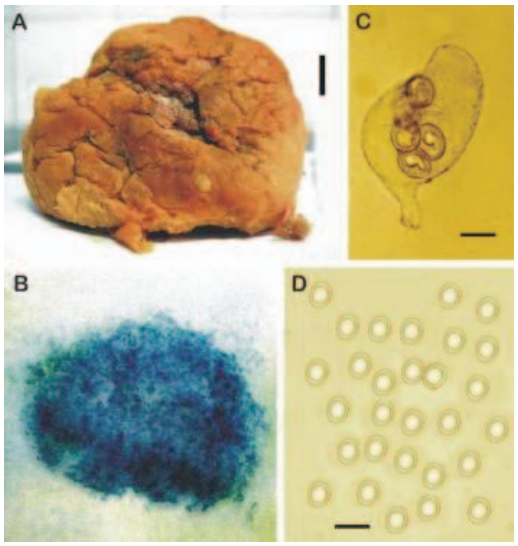

Figure 6. *Tirmania nivea*: (A) Ascocarpe; (B) Reaction with Melzer reagent; (C) Ascus with short pedicel, (D) Ellipsoidal, smooth spores. Bars= 1 cm (Figure A); 16.7 µm (Figures C and D).

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 $CaCO_3$ 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).

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

parameters	Shiraz (Beiza)	Larestan (Aghosh)	Larestan (Arad)	Larestan (Ahmadan)	Shiraz (Guyom)	Shiraz (Ciakhdarengun)	Sarvestan (Tangemahdi)	Fasa (Garbaigan)	Darab (Ghalebiaban)	Larestan (Khonj)	Shiraz (Bajgah)	Fasa (Emamzad ebesmaeil)
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 ($P< 0.05$).

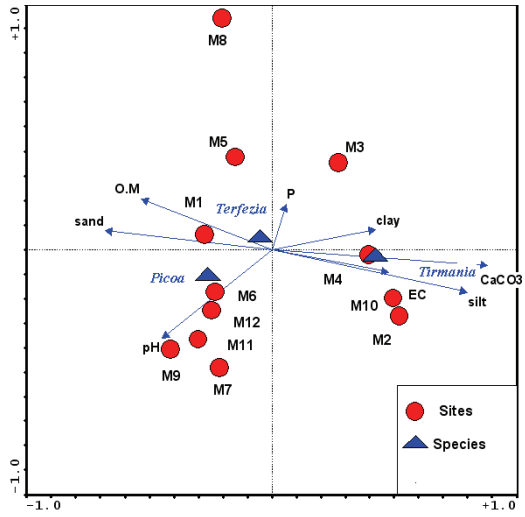

Figure 7. Correspondence analysis (CA) of the desert truffles communities found in different parts of Fars Province. The eigenvalues of the first and second axes in the two-dimensional ordination diagrams are as: CA1= 0.368 and CA2 =0.029. Sum of all eigenvalues of CA is 0.418. (Abbreviations on arrows: 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 *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

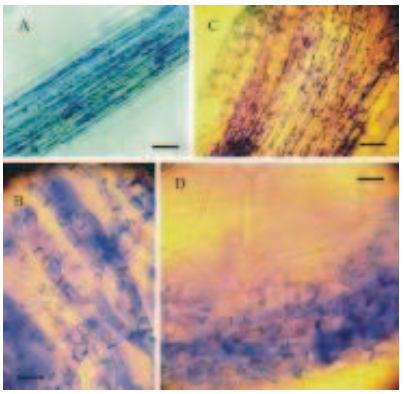

Figure 8. Cleared anilin blue-stained *H. salicifolium* (A, B) and *H. ledifolium* (C, D) roots colonized with hyphae of *T. claveryi*. Bars= 61.9 µm (Figures A and C), 16.7 µm (Figures B and D).

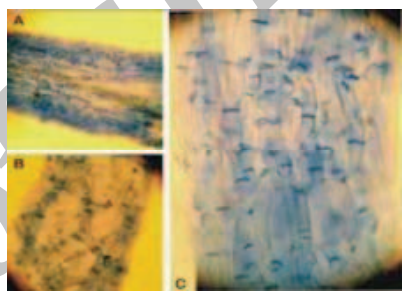

Figure 9. Cleared anilin blue-stained *C. stenophylum* roots clonized with hyphae of *T. claveryi*: (A) The attached mycorrhizal hyphae to *C. stenophylum* roots, (B, C) with large magnification. Bars= 61.9 µm (Figure A); 16.7 µm (Figures B and C).

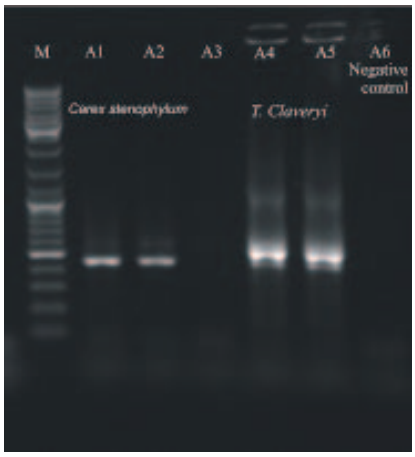

Figure 10. Amplification products of *Carex stenophylum* and *Terfezia claveryi*: M: Marker; Lanes A1 and A2: Specific primer *ITS1TC/ITS4TC* amplified DNA of *C. stenophylum* roots; A3: Negative control (roots without mycorrhizae); A4 and A5: Specific primer *ITS1TC/ITS4TC* amplified DNA of *T. claveryi*, 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 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 *ITS*2 sequence.

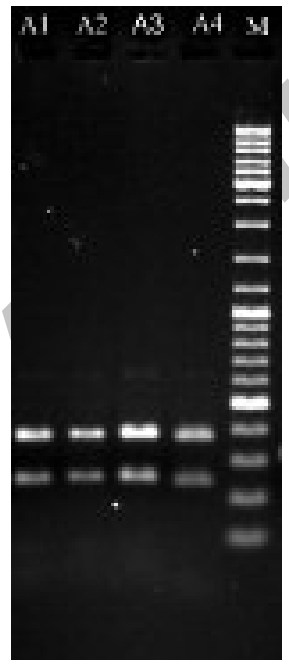

Figure 11. *Alu* I digested *ITS* fragments of *T. claveryi* fruit-bodies and mycorrhized *C. stenophylum* roots. 1% agarose gel. Lanes A1 and A2: Roots of *C. stenophylum*, Lanes A3 and A4: *T. claveryi* fruit-bodies.

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

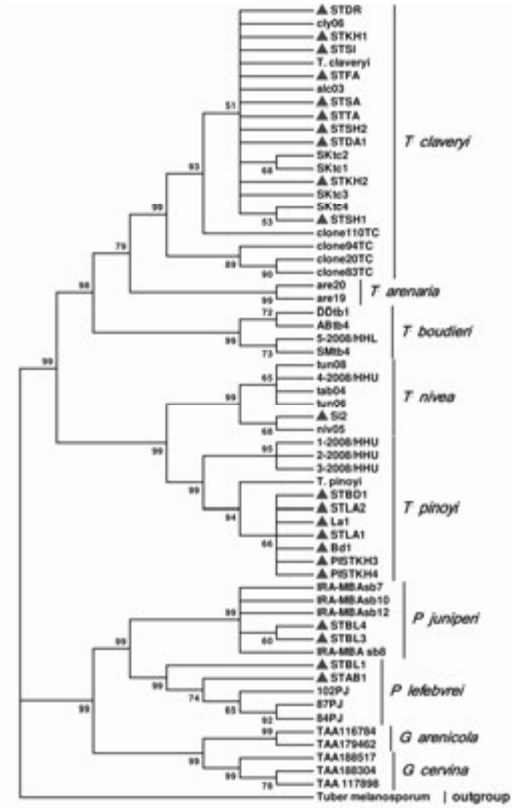

Figure 12. Neighbor joining phylogram of 61 *ITS* rDNA sequences of pezizalean desert truffles based on distance analysis (Kimura's 2 parameter). Number in branches are the bootstrap values as percentage bootstrap replication from a 1,000 replicate analysis. The shapes refer to desert truffles in Iran.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

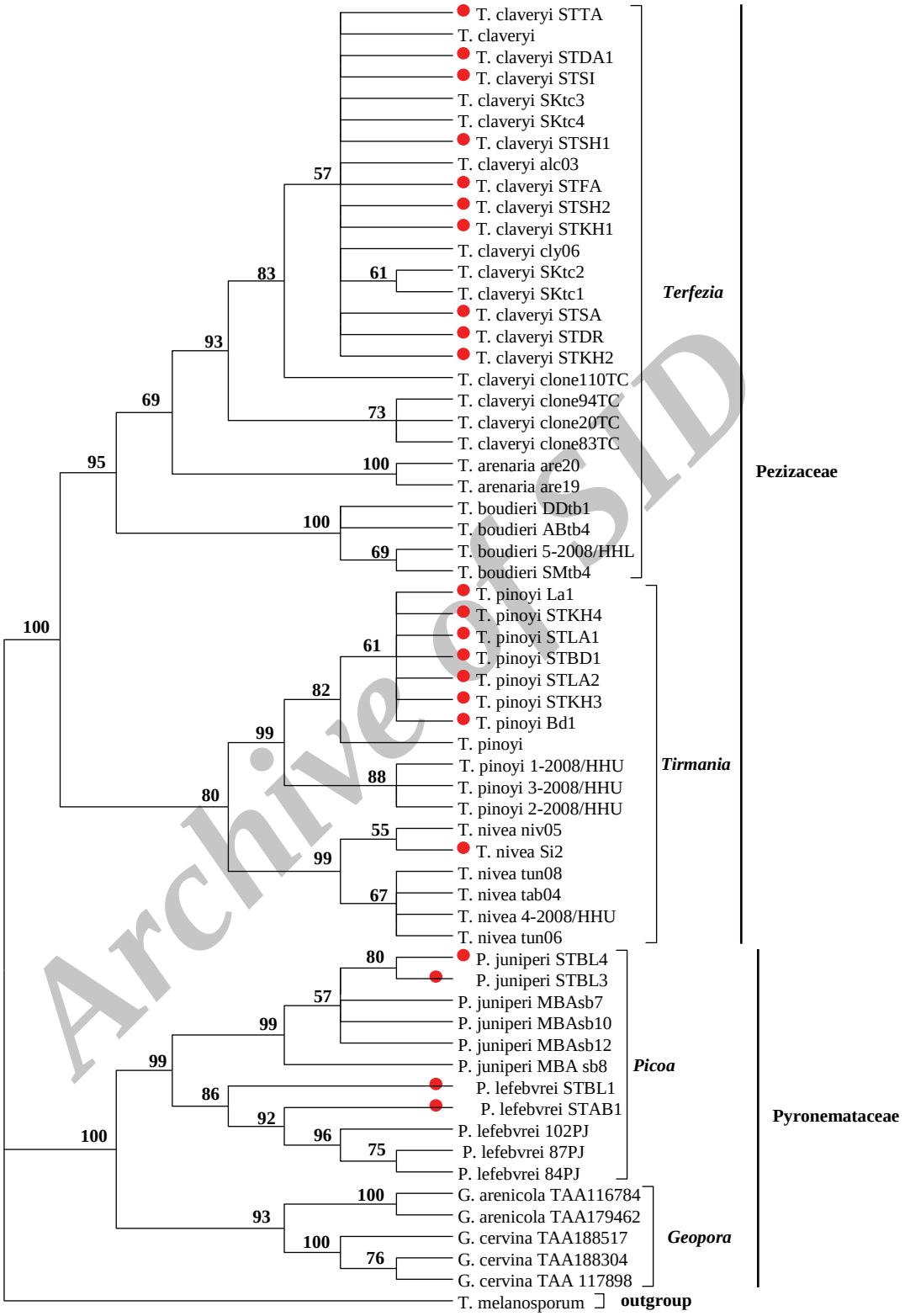

Figure 13. Rooted 50% majority rule consensus tree resulting from 1000 bootstrap replications of the parsimony analysis of the *ITS* rDNA sequences (Consistency index: CI= 0.82; Retention index: RI= 0.91, Rescaled consistency index: RCI= 0.72). Analysis was conducted using the heuristic search algorithm. 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 *ITS*1 and *ITS*4 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. claveryi* 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 *ITS*1 and *ITS*4 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 *Alu*I 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. 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).

REFERENCES

1. Abd-Allah, S. K., Iissa, A. H., Ewaz, J. O. and Al-Bader, S. M. 1989. Taxonomy of Edible Hypogeous Ascomycotina of Iraqi. *Int. J. Mycol. Lichenol.*, **4(1-2)**: 9-21.
2. Allen, E. B., Chambers, J. C., Connor, K. F., Allen, M. F. and Brown, R. W. 1987. Natural Reestablishment of Mycorrhizae in Disturbed Ecosystems. *Arctic Alpine Res.*, **19**: 11–20.
3. Alsheikh, A. 1984. Mycorrhizae of Annual *Helianthemum* Species Formed with Desert Truffles. *Proc. 6th North Amer. Conf. Mycorrhizae*, Oregon State University, Corvallis (USA), OR; no.445.
4. Alsheikh, M. and Trappe, J. M. 1983a. Desert Truffles: The Genus *Tirmania*. *Trans. Brit. Mycol. Soc.*, **81**: 83-90.
5. Alsheikh, M. and Trappe, J. M. 1983b. Taxonomy of *Phaeangium lefebvrei*: A Desert Truffle Eaten by Birds. *Can. J. Bot.*, **61**: 1919-1925.
6. Altschuli, S. F., Madden, T. L., Scha Ffer, A. A., Zhang, J. H., Zhang, Z., Miller, W. and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs. *Nucl. Aci. Res.*, **25**: 3389–3402.
7. Ammarellou, A. Saremi, H. and Gucin, F. 2007. Evaluation of Morphology, Cytology and Mycorrhizal Relationships of Desert Truffles in Iran. *Paki. J. Bio. Sci.*, **10**: 1486-1490.
8. Ammarellou, A., Smith, M. E., Tajick, M. A. and Trappe, J. M. 2011. The Phylogenetic Placement of *Picoa*, with a First Report on *Picoa lefebvrei* (Pat.) Maire (= *Phaeangium lefebvrei*) from Iran. *Int. J. Environ. Res.*, **5(2)**: 509-514.
9. Awameh, M. S. and Alsheikh, A., 1979. Laboratory and Field Study of Four Kinds of Truffle (Kamah), *Terfezia* and *Tirmania* Species, for Cultivation. *Mush. Sci.,* **10**: 507–517.
10. Awameh, M. S., Alsheikh, A. and Al-Ghawas, S. 1979. Mycorrhizal Synthesis between *Helianthemum ledifolium*, *H. salicifolium* and Four Species of the Genera *Terfezia* and *Tirmania* Using Ascospores and Mycelial Cultures Obtained from Ascospore Germination. *Proc. 4th North Amer. Conf. Mycorrhizae*, Colorado State University, Forth Collins (USA).
11. Awameh, M. S. and Alsheikh, A. 1980. Features and Analysis of Spore Germination in the Brown Kamah *Terfezia claveryi*. *Mycologia,* **72**: 494-499.
12. Bellgard, S. E. 1991. Mycorrhizal Associations of Plant Species in Hawkesbury Sandstone Vegetation. *Aust. J. Bot*., **39**: 357–364.
13. Bokhary, H. A. 1987. Desert truffle Al-Kamah of the Kingdom of Saudi Arabia. I. Occurrence, Identification and Distribution. *Arab Gulf. J. Sc. Res.*, **B5**: 245-255.
14. Bokhary, M. A. and Parvez, S. 1988. Desert Truffle Al-Kamah of Kingdom of Saudi Arabia. 2. Additional Contribution. *Arab Gulf J. Sci. Res. Agric. Biol. Sci.,* **B6**: 103-112.
15. Bokhary, M. A. and Parvez, S. 1992. Soil Mycoflora from Truffle Native Areas of Saudi Arabia. *Mycopathologia,* **118**: 103-107.
16. Calonge, F. D., Garcõ!a, F, Santos, J. C. and Juste, P. 1995. Contribucio!n al Estudio de los Hongos de Valladolid y Provincias Limõ!trofes. III. *Algunas* Sspecies Hipogeas Interesantes. *Boletin de la Sociedad Micologic de Madrid,***20**: 291-299.
17. Cano, A., Honrubia, M. and Molina-Ninrola, C. 1991. Mycorrhizae in Seminarid Ecosystems: Synthesis of Mycorrhizae between *Terfezia claveryi* Chat., *Picoa juniperi* Vit. and *Helianthemum almeriense* (*Cistaceae*). In: *Proceedings of the Third*

European Symposium on Mycorrhizas, Sheffield, UK.
18. Chevalier, G., Dupre, C., Riousset, L. and Dexhemier, J. 1984. Synthese Mycorhizienne entre *Terfezia leptoderma* Tul et Diverses Cistaces. *Agronomie*, **4:**210–211.
19. Daneshpajuh, B. 1991. Two New Species of Hypogeous Ascomycetes in Iran. *10th Iran. Plant Prot.Cong*. Kerman. 123.
20. Dexheimer J., Gerard J., Leduc J. P., and Chevalier G. 1985. Etude Ultrastructurale Compare´e des Associations Symbiotiques Mycorhiziennes *Helianthemum salicifolium–Terfezia claveryi* et *Helianthemum salicifolium–Terfezia leptoderma*. *Can. J. Bot.*, **63**:582–591.
21. Diez, J., Manjon, J. L. and Martin, M. F. 2002. Molecular Phylogeny of the Mycorrhizal Desert Truffles (*Terfezia* and *Tirmania*), Host Specificity and Edaphic Tolerance. *Mycologia,* **94(2)**: 247–59.
22. El-Kholy, H. 1989. Genetical and Physiological Studies on Truffles. PhD. Thesis, Fac, of Agric, Cairo University, Egypt.
23. Ershad, D. 2009. *Fungi of Iran.* Ministry of Jihad-e-Agriculture, 531 PP.
24. Esfandiari, E. and Petrak, F. 1950. Pilze aus Iran-Sydowia. *Horn,* **4:** 11-38.
25. Felsenstein, J. 1985. Confidence Limits on Phylogenies: an Approach Using the Bootstrap. *Evolution*, **39**: 783–791.
26. Ferdman, Y., Aviram, S., Roth-Bejerano, N., Trappe, J. M. and Kagan-Zur, V. 2005. Phylogenetic Studies of *Terfezia pfeilii* and *Choiromyces echinulatus* (*Pezizales*) Support New Genera for Southern African Truffles: *Kalaharituber* and *Eremiomyces*. *Mycol. Res.*, **109**: 237-245.
27. Fernald, M. L. 1980. *Grays Manual of Botany*. D. Van Nostrand Company, 1585 PP.
28. Fortas, Z. and Chevalier, G. 1992. Effet des Conditions de Culture sur la Mycorhization de l'*Helianthemum guttatum* par Trois Espèces de Terfez des Genres *Terfezia* et *Tirmania* d'Algérie. *Can. J. Bot.*, **70**: 2453–2460.
29. Gardes, M., White, T. J., Fortin, J. A., Bruns, T. D. and Taylor, J. W. 1991. Identification of Indigenous and Introduced Symbiotic Fungi in Ectomycorrhizae by Amplification of Nuclear and Mitochondria1 Ribosomal DNA. *Can. J. Bot.*, **69**: 180-190.
30. Gay, P. E., Grubb, P. J. and Hudson, H. J. 1982. Seasonal Changes in the Concentrations of Nitrogen, Phosphorus and Potassium, and in the Density of Mycorrhiza, in Biennial and Matrix-forming Perennial Species of Closed Chalkland Turf. *J. Ecol.,* **70**: 571–593.
31. Ghahraman, A. 1996. The Families and Genera of the Flora of Iran. Research Institute of Forest Rangelands, Tehran, Iran. PP.1031.
32. Grace, C. and Stribley, D. P. 1991. A Safer Procedure for Routine Staining of Vesicular Arbuscular Mycorrhizal Fungi. *Mycol. Res.*, **95**: 1160-1162.
33. Gutierrez, A. 2001. Caracterizacion, Identificacion y Cultivo en Campo de las Trufas de Desierto. Doctoral Thesis, University of Murcia, Murcia.
34. Hall, T. A. 1999. BioEdit: A User-friendly Biological Sequence Alignment Editor and Analysis Program for Windows95/98/NT. *Nucleic Acid Symp*. Ser.**, 41**: 95–98.
35. Halwagy, R. and Halwagy, M. 1974. Ecological Studies on the Desert of Kuwait. 1. The Physical Environment, *J. Univ. Kuwait (Sci.)*, **1**: 75-86.
36. Hansen, K., Lassoe, T. and Pfister, D. H. 2001. Phylogenetics of the *Pezizaceae*, with Emphasis on *Peziza*. *Mycologia*, **93**: 958-990.
37. Harley, J. L. and Harley, E. L. 1987. A Check-list of Mycorrhiza in the British Flora. *New Phytol*., **105**: 1-102.
38. Hartnett, D. C., Samunus, R. J., Fischer, L. E. and Hetrick, B. A. D. 1994. Plant Demographic Responses to Mycorrhizal Symbiosis in Tallgrass Prairie. *Oecologia*, **99**: 21–26.
39. Haselwandter, K. and Read, D. J. 1980. Fungal Associations of Roots of Dominant and Sub-dominant Plants in High-alpine Vegetation Systems with Special Reference to Mycorrhiza. *Oecologia* **45**: 57–62.
40. Honrubia M, Cano A. and Molina-Nin˜irola, C. 1992. Hypogeous Fungi from Southern Spanish Semi-arid Lands. *Persoonia,* **14**: 647–653.
41. Hussain, G. and Al-Ruqaie, I. M. 1999. Occurrence, Chemical Composition, and Nutritional Value of Truffles: An Overview. *Paki. J. Biol.Sci*., **2(2):** 510-514.
42. Jamali, S. and Banihashemi, Z. 2010. Two New Species of Hypogeous Ascomycetes, *Tirmania nivea* and *Phaeangium lefebvrei* from Iran. *Proc.19th Iran. Plant Prot. Cong.* Tehran. 67.
43. Koske, R. E., Gemma, J. N. and Flynn, T. 1992. Mycorrhizae in Hawaiian Angiosperms: A Survey with Implications for the Origin of the Native Flora. *Ame. J. Bot.*,**79**: 853–862.

44. Kovacs, G. and Jakucs, E. 2001. "*Helianthemirhiza hirsuta*" *Helianthemum ovatum* (Viv.) Dun. In: "*Description of Ectomycorrhizae*", (Eds.): Agerer, R., Danielson, R. M., Egli, S., Ingleby, K., Luoma, D. and Treu, R.. Munich, **5:** 49–53.
45. Kovacs, G., Bagi, I., Vagvolgy, Cs., Kottke, I. and Oberwinkler, F. 2002. Studies on the Root Associations of the Truffle *Terfezia terfezioides*. *Acta Microbio. Imm. H.*, **49(2–3)**: 207–213.
46. Kumar, S., Tamura, K., Jakobsen, I. B. and Nei, M. 2004. MEGA4: Molecular Evolutionary Genetics Analysis Software. *Bioinformatics*, **17**: 1244-1245.
47. Leduc, J. P., Dexheimer, J. and Chevalier, G. 1986. Etude Ultrastructurale Comparee des Associations de Terfezia leptoderma avec Helianthemum salicifolium, Cistus albidus et Cistus salviaefolius. In Proc. 1st Eur. Symp. Mycorrhizae, Physiological and Genetical Aspects of Mycorrhizae, (Eds): Gianinazzi-Pearson, V. and Gianinazzi, S. INRA, Paris, PP. 291-295.
48. Lovera, M. and Cuencea, G. 1996. Arbuscular Mycorrhizal Infection in *Cyperaceae* and *Gramineae* from Natural, Disturbed, and Restored Savannas in La Gran Sabana, Venezuela. *Mycorrhiza,* **6**: 111–118.
49. Maire, R. 1906. Notes Mycologiques. *Annal. Mycol.*, **4**: 329-399.
50. Malençon, G. 1973. Champignons Hypoges du Nord de IAfrique. I. *Ascomycetes. Persoonia*, **7**: 261-288.
51. Mejstrik, V. K. 1972. Vesicular-arbuscular Mycorrhizas of the Species of a *Molinietum coeruleae* L. I. Association: The Ecology. *New Phytol.*, **71**: 883–890.
52. Meney, K. A., Dixon, K. W., Scheltema, M. and Pate, J. S. 1993. Occurrence of Vesicular Mycorrhizal Fungi in Dryland Species of *Restionaceae* and *Cyperaceae* from South-west Western Australia. *Aust. J. Bot.,* **41**: 733–737.
53. Miller, R. M., Smith, C. I., Jastrow, J. D. and Bever, J. D. 1999. Mycorrhizal Status of the Genus *Carex* (Cypraceae). *Am. J. Bot.*, **86(4)**: 547–553.
54. Moreno, G., Diez, J. and Manjon, J. L. 2000. *Phaeangium lefebvrei* and *Tirmania nivea*, Two Rare Hypogeneous Fungi from Spain. *Mycol. Res.*, **104(3)**: 378-381.
55. Morte, A. and Honrubia, M. 1994. Metodo para la Micorrizaci_n *In vitro* de Plantas Micropropagadas de *Helianthemum* con *Terfezia claveryi*: Patent P9402430. University of Murcia, Oficina Espanola de Patentes y Marcas.
56. Mostofizadeh, G. R., Jamali, S. and Banihashemi, Z. 2010. Molecular Phylogeny of Three Desert Truffles from Iran Based on Ribosomal Genome. *Rostaniha,* **11**:151-162.
57. Newman, E. L. and Reddell, P. 1987. The Distribution of Mycorrhiza among Families of Vascular Plants. *New Phytol.* **106**: 745-751.
58. Norman, J. E. and Egger, K. N. 1999. Molecular Phylogenetic Analysis of *Peziza* and Related Genera. *Mycologia,* **91**: 820-829.
59. O'Donnell K., Cigelnik E., Weber N. S. and Trappe, J. M. 1997. Phylogenetic Relationships among Ascomycetous Truffles and True and False Morels Inferred from 18S and 28S Ribosomal DNA Sequence Analysis. *Mycologia*, **89**:48–65.
60. Pacioni, G. and El-Kholy, H. 1994. Tartufi del Deserto Egiziano. *Micol. Veget. Medit.,* **9**: 69-84.
61. Patouillard, N. 1894. Les Terfez de la Tunisie. *J. de Botanique*, **8**: 153-156.
62. Pendleton, R. L. and Smith, B. N. 1983. Vesicular-arbuscular Mycorrhizae of Weedy and Colonizer Plant Species at Disturbed Sites in Utah. *Oecologia,* **59**: 296–301.
63. Percudani, R., Trevisi, A., Zambonelli, A. and Ottanello, S. 1999. Molecular Phylogeny of Truffles *(Pezizales: Terfeziaceae, Tuberuceae)* Derived from Nuclear rDNA Sequence Analysis. *Mole. Phylog. Evol.,* **13**: 169-180.
64. Petrak, F. 1949. Beitrage zur Pilzflora Irans–Sydowia. **3**: 268-332.
65. Read, D. J., Koucheki, H. K. and Hodgson, J. 1976. Vesicular-arbuscular Mycorrhiza in Natural Vegetation Systems. *New Phytol.,* **77**: 641–653.
66. Read, D. J., Kianmehr, H. and Malibari, A. 1977. The Biology of Mycorrhiza in Helianthemum Mill. *New Phytol.*, **78**: 305–312.
67. Read, D. J. and Haselwanter, K. 1981. Observation of the Mycorrhizal Status of Some Alpine Plant Communities. *New Phytol.*, **88**: 341-352.
68. Riousset, L., Riousset, G., Jalade, M. and Chevalier, G. 1989. Prima Raccolta in Francia di *Phaeangium lefebvrei* Patouillard 1894. *Fungo* **II**: 9-11.
69. Riousset, L., Riousset, G., Jalade, M. and Chevalier, G. 1996. Prima Raccolta in Francia di *Picoa lefebvrei* (Patouillard) Maire 1906. *Il Fungo suppl.*, **3**: 15-17.

70. Roux, C., Sejalon-Delrnas, N., Martins, M., Parguey-Leduc, A., Dargent, R. and Becard, G. 1999. Phylogenetic Relationships between European and Chinese Truffles Based on Parsimony and Distance Analysis of *ITS* Sequences. *FEMS Microbiol. Lett.*, **180**: 147-155.
71. Sbissi, I., Neffati, M., Murat, C., and Gtari, M. 2010. Phylogenetic Affiliation of the Desert Truffles *Picoa juniperi* and *Picoa lefebvrei*. Antonie Van Leeuwenhoec. **98(4):** 429-436.
72. Singer, R. 1961. *Mushrooms and Truffles. XXIII: 272*. Leonard Hill Publisher, Ltd., London. PP.189.
73. Tedersoo, L., May, T. M. and Smith, M. E. 2010. Ectomycorrhizal Lifestyle in Fungi: Global Diversity, Distribution, and Evolution of Phylogenetic Lineages. *Mycorrhiza*, **20**: 217-263.
74. Tester, M., Smith, S. E. and Smith, F.A. 1987. The Phenomenon of Nonmycorrhizal Plants. *Can. J. Bot.*, **65**: 419-431.
75. Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Position-specific Gap Penalties and Weight Matrix Choice. *Nucl. Aci. Res.*, **22**: 4673–4680.
76. Trappe, J. M. 1971. A Synopsis of the *Carbomycetaceae* and *Terfeziaceae* (Tuberales). *Trans. Brit. Mycol. Soc.*, **57**: 85-92.
77. Trappe, J. M. 1979. The Orders, Families, and Genera of Hypogeous Ascomycotina (Truffles and Their Relatives). Mycotaxon, **IX:** 297-340.
78. Urbanelli, S., Sallicandro, P., Devito, E. and Bullini, L. 1998. Identification of Tuber Mycorrhizae Using Multilocus Electrophoresis. *Mycologia,* **90**: 389-395.
79. White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: "*PCR Protocols: A Guide to Methods and Applications",* (Eds.): Gelfand, M. A., Sninsky, D. H. and White, T. J.. Academic Press, San Diego, CA, PP. 315-322.
80. Wetzel, P. R. and Van Der Valk, A. G. 1995. Vesicular-arbuscular Mycorrhizae in Prairie Pothole Wetland Vegetation in Iowa and North Dakota. *Can. J. Bot.*, **74**: 883–890.

میزبان ها و پراکنش دنبل‌های بیابانی در ایران بر اساس ریخت شناسی و روش‌های مولکولی

ص. جمالی و ض. بنی هاشمی

چکیده

جنس های *Terfezia, Picoa* و *Tirmania* دنبل های بیابانی نامیده می شوند که در زیست‌بوم‌های خشک و نیمه خشک مناطق مدیترانه‌ای با گونه‌های گیاهی *Helianthemum* و *Carex* ایجاد قارچ ریشه می‌کنند. اهداف مورد مطالعه در این مقاله بررسی میزبان‌ها و پراکنش دنبل‌های بیابانی در ایران بر اساس ریخت شناسی و روش‌های مولکولی است. در بین نمونه‌ها پنج گونه *Terfezia claveryi*, *Tirmania nivea, T. pinoyi, Picoa lefebvrei* و *P. juniperi* تشخیص داده شدند. بررسی‌های مزرعه‌ای، آزمایشگاهی و آناتومیکی نشان دادند که که گونه‌های *Helianthemum salicifolium, H. ledifolium, H. lippi* و *Carex stenophylum* رابطه برون ریشه‌ای با گونه‌های قارچی تشخیص داده شده دارند. از روش‌های مولکولی مبتنی بر واکنش زنجیره‌ای پلی‌مراز نیز برای تائید رابطه قارچ-ریشه‌ای گونه گیاهی *C. stenophylum* با *T. claveryi* استفاده شد. حباب‌های هیفی در برش‌های گرفته شده از برون ریشه‌ها مشاهده نشدند. نتایج آنالیز فیزیکی –شیمیایی روی نمونه‌های خاک در مناطق مختلف استان فارس نشان دادند که جنس *Tirmania* در خاک‌هایی با درصد سیلت و آهک بالا، نسبت به گونه‌های *T. claveryi*, *P. juniperi* و *P. lefebvrei* شیوع بیشتری دارد. آنالیز تطبیق متعارفی نشان داد که ساختار خاک، پارامتر مهم محیطی است که در پراکنش دنبل‌های بیابانی تاثیرگذار است.