Abstract

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

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

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

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

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

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

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

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

**Materials and Methods**

- **Organisms and specimen's conditions**

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

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Year</th>
<th>Province</th>
<th>Locality</th>
<th>Species</th>
<th>GenBank</th>
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<td>T. nivea</td>
<td>FJ197820</td>
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</table>

- **Cultivation**

  Attempts were made to cultivate truffles from fruiting body tissues on modified Melin-Norkrans (MMN) agar medium (Marx 1969, Kovacs et al. 2003) (in mg l⁻¹ CaCl₂ 50, NaCl 25, (NH₄)₂PO₄ 250, KH₂PO₄ 500, MgSO₄.7H₂O 150, FeCl₃ 12, thiamine-HCl 100, glucose 10 g l⁻¹, solidified with 17 g l⁻¹ of agar; adjusted to pH 8.0). Fungal tissues were surface-sterilized in 0.5% sodium hypochlorite for 1–2 min and rinsed with sterile distilled water, dried on paper towel, and small pieces of about 3–5 mm were plated on the medium and incubated at 25°C in the dark and checked periodically for growth.

- **Morphological study**

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

- **DNA extraction**

  DNA was extracted from tissue cut out from the inner part of the ascocarp to avoid contamination by other micro-organisms. Tissue was freeze-dried for 24 h. Approximately 5 mg of freeze-dried tissue was used for each DNA extraction. DNA was extracted from
specimens using a genomic DNA purification kit, (Fermentas, UK) according to the manufacturer’s instructions. The amount of DNA obtained was estimated by a NanoDrop spectrophotometer (NanoDrop Technologies, USA).

- DNA amplification and sequencing

DNA of the internal transcribed spacer regions (ITS) were amplified using the universal primers ITS1: 5’- TCC GTA GGT GAA CCT GCG G -3’ and ITS4: 5’- TCC TCC GCT TAT TGA TAT GC -3’ (White et al. 1990). Amplifications were performed in a CG1-96 thermocycler (Corbett Research, Australia). The PCR mixture contained: 10–20 ng of template DNA, 1 µM of each primer, 100 µM of dNTPs, 0.4 U Taq DNA polymerase (CinnaGen, Iran), 1.5 mM of MgCl2, 2.5 µl of 10× PCR buffer, 100 mM BSA, in a reaction volume of 25 µl. All PCRs consisted of 1 cycle of 94° C for 3 min; 30 cycles of 95° C for 30 s, 50° C for 30 s, 72° C for 60 s; and a final cycle of 72° C for 10 min. Successful amplification was confirmed by gel electrophoresis (1 h at 80 Volts) on 1.0% agarose gels in 1× TBE buffer. Gels were stained using ethidium bromide and DNA fragments were visualised under UV light.

- Sequencing of amplified product

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

- Phylogenetic analysis

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

Results

- Distribution of truffles in different regions

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

- Morphological identification

During this study three species were identified which are described as follows:
Table 2. GenBank accession numbers of internal transcribed spacers sequences of rDNA of truffle species used for phylogenetic studies and origin of these species

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession</th>
<th>Origin</th>
<th>Species</th>
<th>Accession</th>
<th>Origin</th>
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<td>AF396863</td>
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<td>AF396864</td>
<td>France</td>
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* GenBank accession number for the internal transcribed spacers sequences of rDNA.  
  
  a Diez et al. 2002.  
  b Ferdman et al. 2005.  
  c Gutierrez, Honrubia & Morte (direct submission).  
  
**Terfezia claveryi** Chatin

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

**Tirmania nivea** (Desf.) Trappe

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

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

- Sequence data and phylogenetic analysis

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

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

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

**Discussion**

Analysis of sequence alignments shows that, in 41 taxa compared (in addition to *Tuber melanosporum* as an outgroup) there are 421 potentially phylogenetic informative sites (about 58.72% of nucleotide sites) which were mainly comprised of substitutions, deletions and insertions. The final expected transition/transversion ratio was 1.16. This amount of information could be suitable for phylogenetic analysis and clearly resolved species boundaries in constructed phylogram (Fig. 1). We applied both distance-based and maximum likelihood (data not shown) methods. Although, the comparison of two types of trees illustrates concordance between them and results were broadly similar, neighbor-joining tree shows better separation and more intuitive grouping of taxa.
Fig. 1. Detailed phylogram of Iranian desert truffle specimens together with 10 desert truffle species. Numbers followed by specimens indicate GenBank accession numbers for ITS sequences. The phylogram was constructed by DNA distance-based analysis of the combined ITS1, 5.8S subunit, and ITS2 regions of the genomic ribosomal RNA. The numbers at the branch points indicate the percentages of bootstrap values $\geq 50\%$. 

substitution/site

0.1
Phylogenetic analysis of specimens based on internal transcribed spacers of rRNA genome put them into three monophyletic lineages. The first group is comprised of specimens from Fars, Kerman, Sistan & Balouchestan, and E Azerbaijan provinces which together with authentic specimens of *T. claveryi* constitute a monophyletic clade. The reference specimens were from Spain and Morocco (Díez et al. 2002, Ferdman et al. 2005), and also a specimen with unknown origin (Gutiérrez, Honrubia & Morte; direct submission in GenBank) (Table 2). Average convergence ratio between Iranian taxa was low and estimated 2.8%, while it was 4.8% between all sequences with a maximum of 12.8%. Examining the sequence alignments more closely, it was revealed that the maximum of convergence was not real and was mainly due to low sequencing quality at both ends of accession AF387648 (clone 83TC) (Table 2) of *T. claveryi* from GenBank. By excluding the clone 83TC, the average convergence ratio would become 3.6% (range 0–7.6). This level of similarity is also demonstrated by Díez et al. (2002). In spite of the different geographical origin of their specimens, the genetic similarity among *T. claveryi* specimens was high. Our *T. claveryi* robustly diverged from its sister taxon, *Terfezia arenaria*, with a bootstrap value of 100%.

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

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

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

Regardless of the high level of similarity of ITS region in specimens, which has led to discrimination of monophyletic taxa, comparison of aligned sequences showed that there are some single nucleotide polymorphisms (SNPs) among some regions of sequences. These SNPs could be suitable for analysis of the population genetics of these truffles and at the same time it is an applicable tool for studying the evolution of these taxa (Nicod & Largiader 2003, Riccioni et al. 2008, Ferdman et al. 2009).
Nowadays, both genera identified in this study are considered members of *Pezizaceae*, *Pezizales* (Læssøe & Hansen 2007). Hibbett et al. (2007) in their higher-level phylogenetic classification of the fungi classified *Pezizales* as an independent order in subphylum *Pezizomycotina*. Studies of O’Donnell et al. (1997) demonstrated the independent lines of epigeous/hypogeous fruit body evolution in the *Pezizales*. Their findings have significantly affected the classification of desert truffles. Although all fungi traditionally classify as *Pezizaceae* exhibit amyloid reaction in their asci, analysis of the 18S rDNA sequences has revealed a close relationship between non-amyloid *Terfezia* and the *Pezizaceae* (Norman & Egger 1999, Percudani et al. 1999). Thus the amyloid reaction is of limited value as a diagnostic criterion at family level. Studies of Díez et al. (2002) showed that the *Tirmania* clade comprises species with smooth spores and amyloid asci, which were found in deserts. While, the *Terfezia* clade grouped species found in semi-arid habitats having ornamented and spherical spores, with non-amyloid asci. The present work confirms these findings, because most of *Tirmania* specimens were collected from arid provinces such as Sistan & Baloochestan, Kerman, and Hormozgan, or in case of specimen La1 collected from Lar, and arid region of Fars province (Table 1).

In 1897, Chatin reported *Terfezia aphroditis* Chatin from E Azerbaijan (Tabriz) and *Terfezia hanotauxii* Chatin from Tehran province of Iran for the first time. Subsequent works confirmed specimens of *Terfezia hafizii* Chatin from Fars and Tehran provinces (Petrak & Esfandiari 1941, Esfandiari 1946, Petrak 1949). More recently, other reports corroborated *Terfezia boudieri* Chatin from Zanjan province (Ammarellou et al. 2006), *Terfezia leonis* (Tul.) Tul. from Fars and E Azerbaijan provinces (Daneshpazhuh 1991), and *T. pinoyi* (Daneshpazhuh 1991) from Hormozgan province. Apart from *T. pinoyi* report from Hormozgan (Daneshpazhuh 1991) which is consistent with the specimens from Bishederaz, Hormozgan in the present work, findings based on molecular works did not confirm other reports from Iran. All the above mentioned reports were exclusively based on morphological features of ascoma, therefore it is possible that reduced convergent characteristics of fruit bodies be the source of flawed identification or, in some cases, description of new species (e.g. *T. hafizii*). This is the first report of the presence of *T. claveryi* and *T. nivea* from Iran. However, it seems that Iranian truffles flora resemble those of Mediterranean region and South coast of the Persian Gulf.

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

More surveys are needed before these observations can be generalized. In Fars province, annual *H. salicifolium* and perennial Carex spp. were found to be associated with truffles. Daneshpazhuh (1991) reported *T. pinoyi* and *T. leonis* from Hormozgan, Fars and E Azerbaijan provinces, associated with *Artemisia* and *Helianthemum*. The association of *T. boudieri* with Carex species (Ammarellou et al. 2006) and *Kobresia belardi* a herbaceous monocotyledonous hosts was also reported in Zanjan, a cold climate region in Iran (Ammarellou & Saremi 2008). Other potential perennial truffle hosts will have to be examined in different parts of Iran as well. The prevalence of truffles in Iran is dependent on the precipitation, especially during November or December. Apparently rain in late fall is more important than that in late winter or early spring. Due to low rate of ascospore germination, we cultivated truffles from fruiting body tissues, hence either this or the nutritional composition of Melin-Norkrans agar (Marx 1969, Kovacs et al. 2003) which originally prepared for other species could be the source of cultivation failure.
Unavailability of specimens from other parts of Iran due to successive drought, other researchers’ reluctance to provide us with their herbarium specimens, and most importantly the vast area under investigation (>1.6 M Km²) contributes to a delimitative phylogenetic analysis of the Iranian desert truffles. Present findings, however, could be a basis for species-specific primer design followed by detection and precise identification of frequent Iranian desert truffles, their symbiont counterparts, and detailed studies on their biology and distribution. We are trying to develop specific primers for rapid identification at the species level, which could facilitate host recognition. Inoculation studies under greenhouse conditions are also underway.

Acknowledgments

This study was funded by the Iran National Science Foundation (INSF), award number 85035/14.

References


concepts of the small, white, European group of *Tuber* spp. based on morphology and rDNA ITS sequences with special reference to *Tuber rapaeodorum*. Mycol. Prog. 4: 281–290.


