# Specific Primers for Identification and Detection of Three Major Desert Truffle Species in Iran with Special Reference to *Terfezia claveryi*

# R. MOSTOWFIZADEH-GHALAMFARSA[1][**], Z. BANIHASHEMI[1][*] and S. JAMALI[1][*]

[1]Department of Plant Protection, College of Agriculture, Shiraz University, Shiraz, I. R. Iran

ABSTRACT- Desert truffles are hypogeous ascomyceteous ectomycorrhizal fungi, occurring in arid and semi-arid ecosystems. A PCR-based method was developed for the identification of 3 major desert truffles of Iran: *Terfezia claveryi*, *Tirmania pinoyi* and *Tirmania nivea* based on internal transcribed spacers of rDNA. Two specific PCR primers were designed for *T. claveryi*, 4 for *T. pinoyi*, and 2 for *T. nivea*. All primers were specific to their homologous species and neither set amplified purified DNA from other truffle species or soil-borne micromycetes. In conventional PCR, the limits of detection were 10-500 pg DNA. ITS-Tfc1 primers detected the species in host tissues using a nested-PCR method by ITS1 and ITS4 universal primers as external set.

## INTRODUCTION

Desert truffles are the hypogeous fruiting bodies of some ascomycetes belonging to *Pezizaceae*, *Pezizales* (8). They always establish ectomycorrhizal symbiosis with a range of plants, mainly members of the *Cistaceae*. These ascomycetes occur in arid and semi-arid ecosystems of the Middle East and Mediterranean Regions (5, 6, 9, 20, 21, 23). They mainly belong to the genera *Terfezia* and *Tirmania* which have high ecologic and economic importance (1, 23, 26). Phylogenetic analyses based on rDNA show that there are different species of *Terfezia*, *Tirmania* and *Picoa* in Iran (6, 27). Ascomata of desert truffles are not only appreciated as delicacies, but also considered as alternative medicines (17, 18, 28).

In order to describe different species of truffles, characters such as spore, ascus, and ascoma morphology as well as the odor of their fruit bodies have been traditionally

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*Associate Professor, Professor and Former Ph. D. Student, Respectively.
** Corresponding Author

used (37). These morphologic and organoleptic features, however, are not sufficient for the identification of desert truffles at the species level (6). In recent years, molecular phylogenetic analysis of ascomycetes has revealed that the morphology of hypogeous fungi could be misleading due to convergent reductions in many morphological characters (8). For example, O'Donnell et al. (29) and Hansen et al. (10) demonstrated that certain hypogeous members of the *Pezizales* have more resemblance to epigeous rather than other hypogeous members.

Ribosomal DNA contains different levels of conservation which can be exploited to analyze any desired phylogenetic level (14, 41). By way of illustration, analysis of ITS regions of rDNA clearly demonstrated the monophyletic origins of *Terfezia* and *Termania* (6). Hence, most phylogenetic studies on *Pezizales* in general (11, 22 and 34) and truffles in particular (6, 8, 33) have been based directly on the internal transcribed spacer (ITS) of rDNA or a combination of ITS and subunits of the rRNA genes (*e.g.* 32). Moreover, the identification of truffle species mostly relayed on the comparison of sequences in this region or the restriction fragment polymorphism of ITS (2, 12, 15, 30, 31). These kinds of typing studies, however, have mainly aimed at truffles producing ectomycorrhiza with forest trees (e. g. *Tuber* and related taxa) rather than desert truffles.

The absence of characteristic macro and micromorphological elements for the identification of desert truffles contributes to the shortage of suitable morphological identification techniques. Hence, the invention of a simple, fast and accurate method of identification seems necessary. Such methods would also provide appropriate tools for the identification of these species in studies related to the ecology and competitive abilities of desert truffles.

The present paper reports a molecular approach for the identification and detection of Iranian major desert truffles (*Terfezia claveryi*, *Tirmania pinoyi*, and *Tirmania nivea*) based on simple and nested polymerase chain reaction using species-specific ITS-based PCR primers with special reference to *T. claveryi*.

## MATERIALS AND METHODS

### Organisms and Specimens' Conditions

Details of the truffle specimens examined in this study as well as those of other fungi are listed in Table 1. All isolates were either collected directly from the xerophilus shrub lands or provided from local markets by authors. Voucher specimens were deposited in the fungal collection of the Department of Plant Protection, Shiraz University, Iran. The specimens were stored at -20°C.

### DNA Extraction

DNA was extracted from the tissue cut out from the inner part of the ascocarp to avoid contamination by other microorganisms. The tissue was freeze-dried for 24 h. Approximately 5 mg of freeze-dried tissue was used for each DNA extraction which was carried out using a DNG$^{TM}$-PLUS kit (Sinagen, Iran) according to the manufacturer's instructions. The DNA pellet was dissolved in 50 µl of sterile distilled water and

incubated at 37 ºC for 1 h, after adding 5 µl of RNase A (Fermentase, Britain) solution (5 mg µl⁻¹). The resulting DNA was stored at -20º C. The amount of DNA obtained was estimated by a NanoDrop spectrophotometer (NanoDrop Technologies, USA).

**Table 1. Origins of studied species and their species-specific amplification of DNA sequences by different primer sets**

| species | Specimen code | Year collected | Province, Location | GenBank accession | Amplification using | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | ITS-Tfc1[a] | ITS-Tmn1[b] | ITS-Tmp1[c] | ITS-Tmp2[d] | ITS-Tmp3[e] |
| *Terfezia claveryi* | Sa1 | 2007 | Fars, Sarvestan | EU519461 | + | - | - | - | - |
| *T. claveryi* | Si2 | 2007 | Kerman, Sirjan | FJ197819 | + | - | - | - | - |
| *T. claveryi* | Sh1 | 2006 | Fars, Shiraz | GQ888691 | + | - | - | - | - |
| *T. claveryi* | Fa1 | 2006 | Fars, Fasa, Mianjangal | GQ888693 | + | - | - | - | - |
| *T. claveryi* | Da1 | 2006 | Fars, Darab,Ghale-Biaban | GQ888694 | + | - | - | - | - |
| *T. claveryi* | Kh1 | 2009 | Sistan & Baloochestan, Khash | GQ888692 | + | - | - | - | - |
| *T. claveryi* | Tb1 | 2009 | East Azerbaijan, Tabriz | GQ888690 | + | - | - | - | - |
| *T. claveryi* | Lo1 | 2009 | Lorestan, Khoramabad | | + | - | - | - | - |
| *T. claveryi* | Sp1 | 2009 | Spain, ? | | + | - | - | - | - |
| *Tirmania pinoyi* | La1 | 2007 | Fars, Lar | GQ231540 | - | - | + | + | + |
| *T. pinoyi* | Kh2 | 2009 | Sistan & Baloochestan, Khash | GQ888695 | - | - | + | + | + |
| *T. pinoyi* | Kh3 | 2009 | Sistan & Baloochestan, Khash | GQ888696 | - | - | + | + | + |
| *T. pinoyi* | Bd1 | 2009 | Hormozgan, Bishederaz | GQ888697 | - | - | + | + | + |
| *Tirmania nivea* | Si1 | 2007 | Kerman, Sirjan | FJ197820 | - | + | - | - | - |
| *Aspergillus niger* | An1 | 2009 | Fars, Shiraz | | - | - | - | - | - |
| *Curvelaria* sp. | Cru. | 2009 | Fars, Shiraz | | - | - | - | - | - |
| *Fusarium oxysporum* | FO22 | 2009 | Fars, Shiraz | | - | - | - | - | - |
| *Fusarium solani* | Fs12 | 2009 | Fars, Shiraz | | - | - | - | - | - |
| *Rhizoctonia solani* | Rs6 | 2009 | Fars, Shiraz | | - | - | - | - | - |
| *Penicillium digitatum* | P142 | 2009 | Fars, Shiraz | | - | - | - | - | - |
| *Phytophthora drechsleri* | SUAh4 | 2002 | Fars, Shiraz | | - | - | - | - | - |
| *Pythium aphanidermatum* | PA1 | 2009 | Fars, Shiraz | | - | - | - | - | - |
| *Trichoderma harzianum* | Th1 | 2009 | Fars, Shiraz | | - | - | - | - | - |

[a] Combination of ITS-TfcF1 and ITS-TfcR1 primers. [b] Combination of ITS-TmnF1 and ITS-TmnR1 primers. [c] Combination of ITS-TmpF1 and ITS-TmpR1 primers. [d] Combination of ITS-TmpF2 and ITS-TmpR2 primers. [e] Combination of ITS-TmpF1 and ITS-TmpR2 primers. + = Positive PCR product. - = Negative PCR product.

## DNA Amplification and Sequencing

The DNA of the internal transcribed spacer regions (ITS) was amplified and sequenced using the protocol mentioned in Mostowfizadeh-Ghalamfarsa et al. (27).

## Primer Design

Sequenced regions of the internal transcribed spacers 1, 2, and 5.8S gene of the rDNA (ITS) from 11 desert truffle species along with a collection of desert truffle specimens from different parts of Iran previously studied by the authors (27) or other researchers (6, 8) were recovered from GenBank using the Nucleotide Sequence Search Program provided by the National Center for Biotechnology Information (NCBI, http://www3.ncbi.nlm.nih.gov/Entrez) (Bethesda, MD, USA). Multiple sequence alignments of each set of genes were made using ClustalX (36) with subsequent visual adjustments. Sequences were examined for the conserved regions unique to either *Terfezia claveryi*, *Tirmania pinoyi*, or *Tirmania nivea*. Selected primers were analyzed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast) against the sequence they were originally based on. Primers were then evaluated for criteria such as melting temperature ($T_m$), self-dimerization, self-annealing, potential hairpin formation and G-C content using Oligo Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html) (19).

For nested-PCR, universal primers ITS1 (5'- TCC GTA GGT GAA CCT GCG G -3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3') (41) were applied as external primers.

## PCR Protocol

PCR was carried out in 25 µl reactions containing 2.5 µl 10× PCR buffer (Fermentas, UK), 2.5 µl 10 mg ml$^{-1}$ BSA, 2.5 µl 1 mM dNTPs, 1.5 µl 25 mM MgCl$_2$, 0.5 µl 100 µM forward primer, 0.5 µl 100 µM reverse primer, 0.2 µl 5 U/µl *Taq* DNA polymerase (Fermentas, UK) and 50 ng target DNA.

Amplifications were performed in a CG1-96 thermocycler (Corbett Research, Australia). PCR was originally carried out with a program of 94 °C for 3 min (initial denaturation) followed by 30 cycles of 94 °C for 30 s, a gradient of annealing temperature from 58-70 °C for 30 s, 72 °C for 2 min, and a final extension of 72 °C for 10 min for both simple and nested-PCR. Successful amplification was confirmed by gel electrophoresis (1 h at 80 V) on 1.0% agarose gels in 1× TBE buffer. Gels were stained using ethidium bromide, and DNA fragments were visualized under UV light.

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.

## Specific Identification and Detection of Desert Truffles

### Primers specificity

To determine the specificity of the primers, PCR was conducted on high quality genomic DNAs of various morphologically and molcoularly characterized desert truffles species and certain other soil-borne micromycetes (Table 1) using specific primer sets.

**Primer sensitivity.** To resolve the sensitivity of the primers, spectrophotometrically quantified DNA was serially diluted with HPLC water over 10 orders (100 ng- 10 fg) of magnitude (Table 4). Sensitivity of detection was then determined by each specific primer set.

**Detection of *T. Claveryi.*** In order to detect *T. claveryi* in symbiont plants, tissues of *Carex* sp. were collected from infested fields. The roots were sectioned, stained with malachite green (1% in DW) and visualized under a light microscope for mycelial strands between the plant's cells. DNA was extracted from freeze-dried infested tissues using the above mentioned method and amplified using ITS-TfcF1 and ITS-TfcR1 primers both directly and as internal primers for nested-PCR. Other primer sets were also tested as controls. The final amplicon was sequenced and compared with the original *T. claveryi* ITS fragment.

**Restriction Fragment Identification.** To resolve any probable unspecific ITS-Tfc1 set PCR products from those of symbiont tissues, restriction site maps were constructed using sequences of the ITS-Tfc1 set PCR product (NEBcutter ver.2, New England Biolabs, UK) (http://tools.neb.com/NEBcutter2/index.php) (39). Maps were compared with closely related species for differential unique restriction sites. Appropriate enzymes were selected according to the fragments' differences in length. PCR products were digested according to the manufacturer's instructions. Digested bands were visualized by electrophoresis in 1% (w/v) agarose gels in 1× TBE buffer.

## RESULTS

Specific PCR primers, two for *T. claveryi* and *T. nivea*, and four for *T. pinoyi*, were designed based on the unique regions of the internal transcribed spacers of rDNA (Table 2). ITS-TfcF1 located on the ITS1 region was designed to be used in concert with the reverse primer ITS-TfcR1, a part of the ITS2 region of *T. claveryi*. ITS-TmnF1 was situated on the ITS1 region, whereas ITS-TmnR1 was designed based on the ITS2 region. Both ITS-TmpF1 and ITS-TmpR1, as well as ITS-TmpF2, were part of the ITS, while ITS-TmpR2 was located on the ITS2 region of the rDNA. The optimized PCR conditions for each putative species-specific primer pairs of desert truffles is summerized in Table 3.

**Specificity and Sensitivity of the Designed Primers**

The expected size of amplification product for each set of species-specific primer is shown in Table 3. When each designed primer set was used, an amplicon of the expected size was obtained from the DNA of all morphologically and molecularly well-characterized species tested (Fig. 1). Neither set amplified purified DNA from other non-correspondent truffle species or soil-borne micromycetes (Table 1).

The sensitivity of simple PCR by different primer pairs ranged from 500 pg purified DNA per µl sample (for all primer sets except ITS-Tfc1) to 10 pg for the ITS-Tfc1 set (Table 4).

**Table 2. Putative specific primers designed for detection of *Terfezia claveryi*, *Tirmania nivea* and *Tirmania pinoyi***

| Primer | Target Species | Target DNA | Primer sequence | Accession[a] | Length | Location[b] | $Tm^c$ (°C) | GC[d] (%) | MW[e] (g/mol) |
|---|---|---|---|---|---|---|---|---|---|
| ITS-TfcF1[f] | *Terfezia claveryi* | ITS [h] | 5′ TTC ACC TTG TGT GGA ACC YC 3′ | EU519461 | 20 | 101-120 | 52 | 50 | 6059 |
| ITS-TfcR1[g] | | | 5′ GAG GCA AGT ACA ATC AAT C 3′ | | 19 | 551-533 | 46 | 42 | 5784.8 |
| ITS-TmnF1 | *Tirmania nivea* | ITS | 5′ CAG TGG TAA GAC CAA TCT ATC 3′ | FJ197820 | 21 | 160-180 | 47 | 43 | 6414.2 |
| ITS-TmnR1 | | | 5′ CCA AAG TTA TCC AAG TAT TTG C 3′ | | 22 | 432-411 | 48 | 36 | 6773.5 |
| ITS-TmpF1 | *Tirmania pinoyi* | ITS | 5′ CAT GTT TTA CCT TTG TGT AAG G 3′ | GQ888696 | 22 | 99-120 | 48 | 36 | 6746.5 |
| ITS-TmpR1 | | | 5′ GAC AAT TTG TTT TCA ATC AAG G 3′ | | 22 | 205-184 | 47 | 32 | 6717.5 |
| ITS-TmpF2 | *Tirmania pinoyi* | ITS | 5′ CCA TCT WCC AAA ACC TTG ATT G 3′ | GQ888696 | 20 | 171-192 | 50 | 41 | 6629.4 |
| ITS-TmpR2 | | | 5′ GAA TTT CTG AAG GAC GAC TCT G 3′ | | 22 | 470-449 | 51 | 45 | 6694.4 |
| ITS-TmpF 1 | *Tirmania pinoyi* | ITS | | | | | | | |
| ITS-TmpR2 | | | | | | | | | |

[a] Reference to the GenBank accession containing the DNA sequence, on which the primer is based. [b] Reference to the location of the primer within the original DNA sequence. [C] Melting temperature of the primer. [d] GC-content of the primer. [e] Molecular weight of primer. [f] Forward. [g] Reverse. [h] Internal transcribed spacers 1, 2 and 5.8S gene of rDNA.

**Table 3. Optimized PCR conditions for putative species-specific primer pairs of desert truffles and their amplicon length**

| Primer set | Initial denaturation | Number of cycles | Denaturation | Annealing | Extension | Final Extension | Length[*] |
|---|---|---|---|---|---|---|---|
| ITS-Tfc1[a] | 94(180) [**] | 30 | 94(30) | 66(30) | 72(120) | 72(600) | 451 |
| ITS-Tmn1[b] | 94(180) | 30 | 94(30) | 63(30) | 72(120) | 72(600) | 273 |
| ITS-Tmp1[c] | 94(180) | 30 | 94(30) | 64(30) | 72(120) | 72(600) | 107 |
| ITS-Tmp2[d] | 94(180) | 30 | 94(30) | 67(30) | 72(120) | 72(600) | 300 |
| ITS-Tmp3[e] | 94(180) | 30 | 94(30) | 66(30) | 72(120) | 72(600) | 372 |

[a] Combination of ITS-TfcF1 and ITS-TfcR1 primers. [b] Combination of ITS-TmnF1 and ITS-TmnR1 primers. [c] Combination of ITS-TmpF1 and ITS-TmpR1 primers. [d] Combination of ITS-TmpF2 and ITS-TmpR2 primers. [e] Combination of ITS-TmpF1 and ITS-TmpR2 primers. [*] Amplicon length (bp). [**] Temperature 'ºC' (time 's').
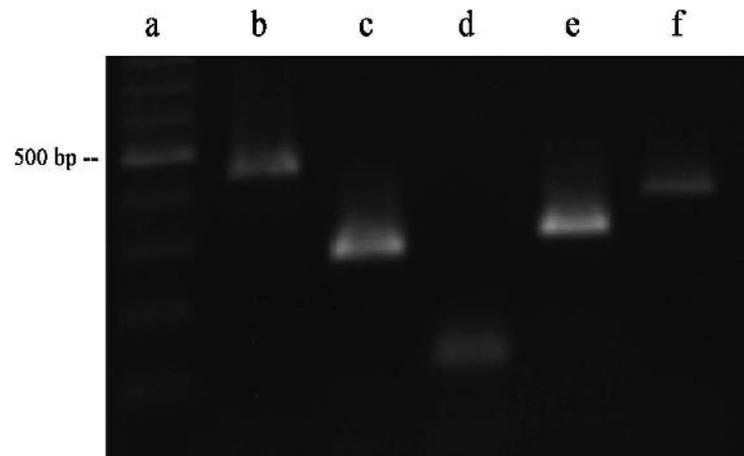
**Fig. 1. Amplicon size of products of the various species-specific primer sets after PCR with their homolog truffle species. (a) 100 bp DNA ladder, (b) ITS-Tfc1 primer set (*Terfezia claveryi*, isolate Sh1), (c) ITS-Tmn1 primer set (*Tirmania nivea*, isolate Si1), (d) ITS-Tmp1 primer set (*Tirmania pinoyi*, isolate La1), (e) ITS-Tmp2 primer set (*T. pinoyi*, isolate La1), (f) ITS-Tmp3 primer set (*T. pinoyi*, isolate La1)**

**Table 4. Effect of DNA quantity (per μl sample) on PCR product band density of putative species-specific primer sets for desert truffles**

| Primer sets | DNA Quantity | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 100 ng | 10 ng | 1 ng | 500 pg | 100 pg | 50 pg | 10 pg | 1 pg | 100 fg | 10 fg |
| ITS-Tfc1[a] | +++ | +++ | +++ | +++ | ++ | ++ | + | - | - | - |
| ITS-Tmn1[b] | +++ | +++ | ++ | ++ | - | - | - | - | - | - |
| ITS-Tmp1[c] | +++ | +++ | ++ | ++ | - | - | - | - | - | - |
| ITS-Tmp2[d] | +++ | +++ | ++ | + | - | - | - | - | - | - |
| ITS-Tmp3[e] | +++ | ++ | + | + | - | - | - | - | - | - |

[a] Combination of ITS-TfcF1 and ITS-TfcR1 primers. [b] Combination of ITS-TmnF1 and ITS-TmnR1 primers. [c] Combination of ITS-TmpF1 and ITS-TmpR1 primers. [d] Combination of ITS-TmpF2 and ITS-TmpR2 primers. [e] Combination of ITS-TmpF1 and ITS-TmpR2 primers.
+++ = Very good. ++ = Good. + = Reasonable. - = No band.
ng = nano $(10^{-9})$ gram. pg = pico $(10^{-12})$ gram. fg = femto $(10^{-15})$ gram.

## Detection of *T. claveryi* in Infected Symbiont Plants

*T. claveryi* mycorrhiza failed to be amplified directly by the ITS-Tfc1 set; however, nested-PCR amplified the fungus in roots selectively. The fragment size turned out to be exactly as predicted. The sequenced fragment confirmed the identity of the specific amplicon, which was exactly the expected fragment of *T. claveryi* rDNA ITS.

Restriction site map analysis of the 451 bp fragment of *T. claveryi* amplified by the ITS-Tfc1 set together with the comparison of the correspondent regions in other closely related species revealed that there were three restriction enzymes uniquely capable of cutting this fragment. The restriction enzymes consisted of two one cutter *Bpm*I (GsuI) and *Bci*VI (BfuI) which could cut the amplicon at nucleotide 344 and 312 (Fig. 2), respectively, and one double cutter *Bae*I which could cut the fragment at nucleotides 284

and 317. *In vitro* application of *Bpm*I and *Bci*VI produced the exact expected band size combination which was unique to the *T. claveryi* 451 bp fragment (Fig. 3)



**Fig. 2. Restriction digest map of 451 bp fragment of ITS rDNA in *Terfezia claveryi* amplified by ITS-Tfc1 primer set, followed by *Bpm*I and *Bci*VI restriction enzymes digestion**
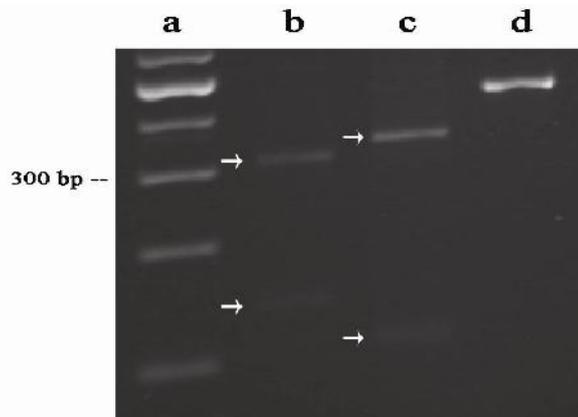


**Fig. 3. Amplicon size of products of the ITS-Tfc1 primer set (d) after digested by (b) *Bci* VI, and (c) *Bpm* I restriction enzymes. (a) 100 bp DNA ladder. Arrows show the locations of digested fragments**

## DISCUSSION

In order to find a molecular approach for the identification and detection of three Iranian major desert truffles (*Terfezia claveryi*, *Tirmania pinoyi*, and *Tirmania nivea*) specific primers were designed based on internal transcribed spacers of ribosomal DNA. Primers were tested on *T. claveryi*, *T. pinoyi* and *T. nivea* fruitbodies and found to be effective for the selective amplification of all three species. *T. claveryi* primers could also detect *T. claveryi* mycorrhiza by nested-PCR using the external universal primers ITS1 and ITS4. Identities of the original *T. claveryi* fragments generated by the species-specific ITS-Tfc1 primer set were also confirmed by single cutter restriction enzymes *Bpm*I and *Bci*VI in gel.

Only one set of primers was found to be specific to *T. claveryi* and *T. nivea*. Neither *in silico* or *in vitro* analysis showed matches in the ITS regions of other truffles for ITS-TfcF1, ITS-TfcR1, ITS-TmnF1 and ITS-TmnR1. Simple PCR using ITS-Tfc1 and ITS-Tmn1 sets was successful, with the limit of detection for ITS-Tfc1 being at least 50 times higher than that of any other designed set. ITS-Tfc1 also detected the single available strain from Spain easily. In case of *T. pinoyi*, two forward and two reverse primers were designed and three combinations were tested for their applicability to

detect the species. Although the ITS-TmpF2 forward primer was designed to have a degenerated site at nucleotide number 7, there seems to be no obvious difference between these three sets in terms of sensitivity and specificity.Yet, the specific fragment obtained from ITS-Tmp1 set at 500 pg DNA, was a bit sharper than the other two. ITS sequences of all three desert truffle species, whether retrieved from direct sequencing or from GenBank, exhibited highly genetic uniformity, emphasizing the potential capability of tested primers to detect and identify strains from other geographical regions.

In all three species, frozen samples were identified as good as fresh ones. It seems that the application of  BSA in PCR reaction mixtures prevented false-negative reactions due to inhibitors of *Taq* DNA polymerase activities existing in the truffles' fruiting bodies. Previous studies also confirm such observation (15, 21, 24, 31).

*T. claveryi* mycorrhiza, obtained from the field, had no apparent morphological features to clearly differentiate them from non-colonized hosts, stressing the importance of molecular tools for their identification. Even forest truffles have been reported to have more problematic fungal identification from morphological analyses of ectomycorrhiza as compared to fruit body identification (35). The ITS-Tfc1 primer set employed in simple PCR failed to recognize *T. claveryi* in any DNA samples from mycorrhiza. To facilitate the identification of *T. claveryi* mycorrhiza, nested-PCR with universal ITS1 and ITS4 primers efficiently detected the fungus in its associated plant roots. Our field observations revealed that this procedure is able to detect the truffle wherever mycorrhized shrublands exist, in contrast to non infected plant samples from greenhouses, which did not produce any corresponding fragment. Such molecular approach towards the sensitive identification and detection of *T. claveryi* opens up new pathways to monitoring the mycorrhizal distribution of these economically important fungi, especially in Iranian fields as major habitats of desert truffles. It could also facilitate host recognition as well as inoculation studies under greenhouse conditions.

It has been shown by Urbanelli et al. (38) that a given host plant can be mycorrhized by several taxa on a very small and precise root segment. Although sequencing has shown that the 451 bp amplified fragment is the exact match of *T. claveryi* ITS rDNA, there were some concerns about the amplification of a false fragment by universal ITS1 and ITS4 universal primers during nested-PCR with the same size or even amplification of non target mycorrhiza of closely related species by ITS-Tfc1 primer sets. To resolve this hypothetical problem, restriction map analyses with *Bpm*I and *Bci*VI restriction enzymes produced 344 / 107, and 312 / 139 bp bands, respectively; furthermore, the combination of two enzymes produced 312 /107 /32 bp bands (data not shown). The banding pattern generated by the combination of these enzymes is unique for *T. claveri* and could be applied instead of time consuming and costly sequencing analyses.

The evolution of hypogeous ascomycetes, especially truffles, typically involves a convergent reduction in macromorphological characters (8). Therefore, the identification of truffles merely on the basis of their morphology is sometimes difficult or misleading (26). Classical identification based on morphological or physiological characters is also time-consuming and labor-intensive, and requires considerable knowledge of the species involved. Consequently, false determinations are frequently made. With advances in molecular biology, methods utilizing DNA for the identification of species have been targeted for development. Almost all molecular studies on truffle identification were

based on the comparison of sequences or restriction fragment length polymorphism (RFLP) of the internal transcribed spacers (ITS) of rDNA (e.g. 12, 30, 31).

The high level of genetic similarity among specimens of the studied species and other sequences with different geographical origins is a result of special evolution in their ITS region. The rDNA contains a mosaic of highly conserved and variable regions that enable inter- and intra-specific comparisons (13). These genes occur in multiple arrays, and mutations in their noncoding region occur at a rate that approximates that of species emergence. Over time, such mutations become fixed through the process of unequal crossing over and gene conversion, commonly known as concerted evolution (3). Within a species, ITS tends to be distinct and monomorphic and hence, suitable for species discrimination across a wide range of organisms including fungi (4). Based on nuclear ITS region and mitochondrial large ribosomal RNA (mt LrRNA) Wang et al. (40) discriminated closely related species of *Tuber*, a forest inhabitant relative of desert truffles. Many other studies that provide enough information for species-specific primers to be designed for white or black truffles have been conducted on single-base polymorphisms in the ITS rDNA of forest truffles (e.g. 15, 24, 25, 31, 35). It seems that the ITS region of rDNA could also provide a reliable tool for truffle oligonucleotide bar codes (7).

Present findings could be a basis for precise and rapid species-specific detection and identification of frequent Iranian desert truffles, especially *T. claveryi* and their symbiont counterparts. In addition, they could also be used to study their biology in detail and to monitor the mycorrhizal distribution. Any attempt to cultivate these economically important mycorrhiza demands a useful tool for tracking dynamics of truffles in natural and cultivated soils. The specific primers described here could satisfy this need and provide researchers with useful feedback regarding the detection of their inoculation and cultivation practices. The tools could also be applied for specimens of the studied species from other parts of the world, especially Mediterranean areas and Southern coasts of the Persian Gulf, due to the high level of genetic similarity in the ITS regions of different strains of these species (27).

## ACKNOWLEDGMENTS

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# آغازگرهای اختصاصی برای شناسایی و ردیابی سه گونه دنبلان کوهی غالب ایران با تأکید بر *Terfezia claveryi*

رضا مستوفی‌زاده قلمفرسا**¹ ضیاءالدین بنی‌هاشمی*¹ و صمد جمالی*¹

¹بخش گیاه‌پزشکی، دانشکده کشاورزی، دانشگاه شیراز، شیراز، ج. ا. ایران

**چکیده‌ـ** دنبلان‌های کوهی، آسکوکارپ زیرزمینی برخی آسکومیست‌های قارچریشه‌ای هستند که در زیست‌بوم‌های نیمه خشک یافت می‌شوند برای شناسایی سه دنبلان کوهی عمده‌ی ایرانی: *Terfezia claveryi*، *Tirmania pinoyi* و *Tirmania nivea* روشی مبتنی بر واکنش زنجیره‌ای پلیمراز بر اساس توالی‌های جداکننده‌ی نسخه‌برداری شده‌ی داخلی در دی‌ان‌ای ریبوزومی ابداع گردید. دو آغازگر اختصاصی برای *Terfezia claveryi*، چهار آغازگر اختصاصی برای *Tirmania pinoyi* و دو آغازگر اختصاصی برای *Tirmania nivea* طراحی شد. کلیه‌ی آغازگرها نسبت به گونه‌ی خودی‌شان اختصاصی بودند و هیچ‌کدام دی‌ان‌ای خالص شده از سایر دنبلان‌ها و از برخی از ریزقارچ‌های خاک‌برد را تکثیر نکردند. در واکنش زنجیره‌ای پلیمراز سنتی، حد ردیابی دی‌ان‌ای ۱۰ تا۵۰۰ پیکوگرم اندازه‌گیری شد. مجموعه آغازگر ITS-Tfc1 با استفاده از واکنش زنجیره‌ای پلیمراز تودرتو و به کمک آغازگرهای عمومی ITS1 و ITS4 قادر به ردیابی گونه‌ی مربوطه در بافت گیاه میزبان بود.

**واژه های کلیدی:** آغازگرهای اختصاصی گونه، توالی‌های جداکننده‌ی نسخه‌برداری شده داخلی دی‌ان‌ای ریبـوزومی، دنبلان کوهی، قارچ‌ریشه‌های برونی

*به ترتیب دانشیار، استاد و دانشجوی پیشین دکتری
**مکاتبه کننده