J. Agr. Sci. Tech. (2013) Vol. 15: 377-387

Nested-PCR for Detecting *Terfezia claveryi* in Roots of *Helianthemum* Species in Field and Greenhouse Conditions

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ABSTRACT

Roots of *Helianthemum* species were collected from various rangeland sites in Fars, and other provinces in Iran. The partial small subunits of ribosomal DNA genes were amplified with the genomic DNA extracted from their roots by nested polymerase chain reaction (PCR) using the universal fungal primer pair ITS1/ITS4 and specific primer pair FTC/RTC, which was designed based on internal transcribed spacer 1, 2 and 5.8S gene of rDNA sequences of *Terfezia claveryi.* The nested-PCR was sensitive enough to re-amplify the direct-PCR product, resulting in a DNA fragment of 500 bp. The efficacy of the nested-PCR showed that it could re-amplify the direct-PCR product and detect 2fg genomic DNA. Restriction fragment length polymorphism (RFLP) was analyzed using the two restriction enzymes *Hinf* I and *Alu* I. Nucleotides sequence analysis revealed that the sequences from infected *Helianthemum* species were close to those of *T. claveryi*. With the nested PCR method, *H. lipii* and *H. salicifolium* were confirmed as host plants of *T. claveryi* in greenhouse inoculated plants and also in the rangelands of different areas in Fars and other provinces in Iran.

Keywords: Desert truffles, *Helianthemum* species, Iran, ITS-RFLP, Nested PCR.

INTRODUCTION

Truffles are usually produced through mycorrhizal seedlings grown under controlled nursery conditions and transplanting under field conditions. The mycorrhizal status of these plants is a crucial step. The majority of ectomycorrhizal plants are trees and woody shrubs. Small numbers of herbaceous plants are known to form ectomycorrhizas, including members of the Cistaceae such as *Helianthemum salicifolium, H. ledifolium* and *H. lipii* (Leduc *et al*., 1986; Hussain and Al-Ruqaie, 1999; Gutierrez *et al*., 2003; Slama *et al*., 2006). *Terfezia* species have been observed to form different mycorrhizal types, which have been synthesized with Cistaceae family under laboratory (Roth-Bejerano *et al*. 1990; Fortas and Chevalier 1992; Morte *et al*., 1994; Morte and Honrubia, 1995; Morte *et al*., 2000) and field conditions (Morte and Honrubia, 1995). *T. leptoderma* produces a Hartig net without mantle, essentially an underdeveloped ectomycorrhiza in roots of different Cistaceae species (Chevalier *et al*., 1984; Dexheimer *et al*., 1985) and *T. arenaria* (*leonis*) (Roth-Bejerano *et al*., 1990). *T. boudieri* were reported to form endomycorrhizae lacking Hartig net and mantle but displaying undifferentiated intracellular hyphae (Awameh, 1981; Alsheikh, 1984) and *T. claveryi* (Alsheikh, 1984; Dexheimer *et al*., 1985). Fortas and Chevalier (1992) studied *Helianthemum guttatum* colonized by *T. arenaria*, *T. claveryi*, or *Tirmania pinoyi* and found a shift from ectomycorrhizae at high phosphate concentration to endomycorrhizae at low concentration. Moreover, Gutierrez *et al*. (2003), working with *Helianthemum almeriense* colonized by *T. claveryi* and

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[DOR: 20.1001.1.16807073.2013.15.2.19.8]

Picoa lefebvrei, encountered only endomycorrhizae under field conditions, only ectomycorrhizae in vitro (with a mantle, the only report of a mantle in a *Terfezia* mycorrhiza), and mostly ectomycorrhizae lacking a mantle in pots under greenhouse conditions. Since the morphological features of mycorrhizal roots in early stages of infection are not sufficient for positive differentiation of truffles species during their symbiotic phase, a range of PCR protocols, mostly based on amplification of the internal transcribed spacer (ITS) region of nuclear ribosomal RNA genes, have been developed (Henrion *et al*., 1994; Chevalier *et al*., 1995; Paolocci *et al*., 1995, 1997; Mello *et al*., 1999; Gandeboeuf *et al*., 1997; Amicucci *et al*., 1998; Bertini *et al*., 1998). Even in forest truffles, fungal identification from morphological analyses of ectomycorrhiza is more problematic than fruit body identification (Sejalon-Delmas *et al*., 2000). Primers designed for the amplification of species-specific ITS regions have been used to develop sensitive techniques for the detection of truffles species (Amicucci *et al*., 1998; Henrion *et al*., 1994; Longato and Bonfante, 1997; Paolocci *et al*., 1995, 1997; Rubini *et al*., 1998). Other PCR-based methods that have also been used include RAPD (Gandeboeuf *et al*., 1997), microsatellites (Lanfranco *et al*., 1993), and the specifically designed corresponding primers. One challenge of working with root tips is that low amounts of DNA or DNA of low quality are obtained after DNA extraction, leading to weak or non amplifications. Furthermore, plant DNA is usually co-extracted with fungal DNA in root tip samples. Only few studies have been focused on the direct identification of truffles in actively colonized root using nested PCR. Zampieri *et al*. (2009) set up a nested PCR protocol to detect *Tuber* DNA in soil. Amicucci *et al*. (2002) used nested PCR for highly degraded food products, which could not be amplified by direct PCR. Mello *et al*. (1999) used nested PCR as a reliable tool for the detection of white truffles in mycorrhizal roots. Kovacs *et al*. (2007) used nested PCR for identification of host plants and description of sclerotia of the truffle *Mattirolomyces terfezioides*. Most of the previous studies aimed at truffles producing ectomycorrhiza with forest trees (e.g. *Tuber*) rather than those of desert truffles. Therefore, the objective of this study was to apply nesting polymerase chain reaction for the identification of host plants of *T. claveryi* for future survey.

MATERIALS AND METHODS

Sampling and Seed Collection

Most specimens and ripe fruits containing mature seeds of *Helianthemum* species used in this work were obtained from different parts of Fars and other provinces of Iran including Lorestan, Kerman, Hormozgan, and Sistan and Baluchestan, during 2007-2010. (Table 1). The seeds were surface sterilized (33% H_2O_2, 15 minutes) and washed three times in sterilized tap water for 40 minutes. Mechanical scarification was achieved by vigorously rubbing the seeds between two sheets of fine-grained sand paper to remove the testa without injuring the embryo (Perez-Garcia and Gonzalez-Benito, 2005). After seeds germination, the well-developed seedlings were transferred to pots containing a mixture of peat-vermiculate-sand (1:1:1 v/v) in a greenhouse and fertilized weekly with Fontana nutrient solution (Figure 1). Control plants without mycorrhizal inoculums were grown under the same condition.

Inoculation

The spore suspension from well matured ascocarps consisted of 10 g of scratched ascocarps per liter of distilled water. The spore solution was shaken overnight before inoculating the plants. Each plant was inoculated with five milliliters of the spore suspension (10^5-10^6 spores per plant).

[DOR: 20.1001.1.16807073.2013.15.2.19.8]

Table 1. Collection of *Terfezia claveryi* and host plants in Iran.

Location	Province	Latitude	Longitude	Elevation (m)	Species	Host plant	Year collected
Sarvestan	Fars	29^0 14´.949 N	53^0 14´.319 E	1788	*Terfezia claveryi*	*Helianthemum salicifolium*	2006,2009
Fasa, Mianjangal	Fars	29^0 10´.520 N	53^0 19´.643 E	1769	*T. claveryi*	*H. lipii, H. salicifolium*	2007,2009
Shiraz, Guyom	Fars	29^0 51´.244 N	52^0 25´.318 E	1913	*T. claveryi*	*H. salicifolium*	2009
Shiraz, Kharameh	Fars	29^0 31´.244 N	52^0 45´.318 E	-	*T. claveryi*	*H. salicifolium*	2007
Shiraz, Sadra	Fars	29^0 47´.041 N	52^0 31´.849 E	1830	*T. claveryi*	*H. salicifolium*	2009
Shiraz,Beiza	Fars	30^0 00´.361 N	52^0 23´.679 E	1937	*T. claveryi*	*H. salicifolium*	2009
Shiraz, Dareh keli	Fars	29^0 51´.135 N	52 25´.194 E	1912	*T. claveryi*	*H. salicifolium*	2010
Shiraz, Dareh gale	Fars	29^0 51´.364 N	52^0 24´.205 E	1861	*T. claveryi*	*H. salicifolium*	2010
Fasa, Emamzadeh esmaeil	Fars	29^0 08´.246 N	53^0 24´.967 E	1737	*T. claveryi*	*H. salicifolium*	2010
Shiraz ,Sadra	Fars	29^0 47´.041 N	52^0 31´.849 E	1724	*T. claveryi*	*H. salicifolium*	2010
Shiraz,Tol akhondi	Fars	29^0 51´.000 N	52^0 23´.259 E	1855	*T. claveryi*	*H. salicifolium*	2010
Kazerun	Fars	29^0 52´.00 N	51^0 41´.00 E	-	*T. claveryi*	*H. salicifolium*	2010
Fasa, Garbaigan	Fars	28^0 43´.00 N	53^0 58´.00 E	1174	*T. claveryi*	*H. lipii, H. salicifolium*	2008
Darab	Fars	28^0 35´.66 N	54^0 41´.313 E	1450	*T. claveryi*	*H. lipii, H. salicifolium*	2009
Larestan, Evaz	Fars	27^0 47´.905 N	53^0 49´.068 E	943	*T. claveryi*	*H. lipii, H. salicifolium*	2010
Bastak	Hormozgan	27^0 21´.00 N	54^0 27´.00 E	998	*T. claveryi*	*H. salicifolium*	2010
Cirjan, Baghat	Kerman	29^0 25´.124 N	55^0 40´.674 E	1735	*T. claveryi*	*H. salicifolium*	2010

Figure 1. (A) Seedlings of *Helianthemum salicifolium*; (B) Seeds of *Helianthemum salicifolium*; (C) Seedlings of *Helianthemum lipii*, (D) Seeds of *H. lipii*.

Ascospores germination rate was 55% (Figure 2).

Microscopic Analysis of Roots

Mycorrhizal roots sampled from plants obtained from semi-sterile systems and from the field were washed and examined, using light microscope, in search of external structures and presence of mycorrhiza. Subsequently, parts of the roots were immersed in 10% potassium hydroxide and left overnight at 60°C. Afterwards, the samples were soaked and washed in distilled water for a minimum of 2 hours and water was changed several times. The last washing water was acidified with 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 transferred to acidic washing solution to eliminate superfluous stain (Grace and Stribley, 1991).

Extraction of DNA

Total genomic DNA was extracted from freeze-dried infested roots from fields and greenhouse inoculated plants using ascospore of *T. claveryi* at various intervals

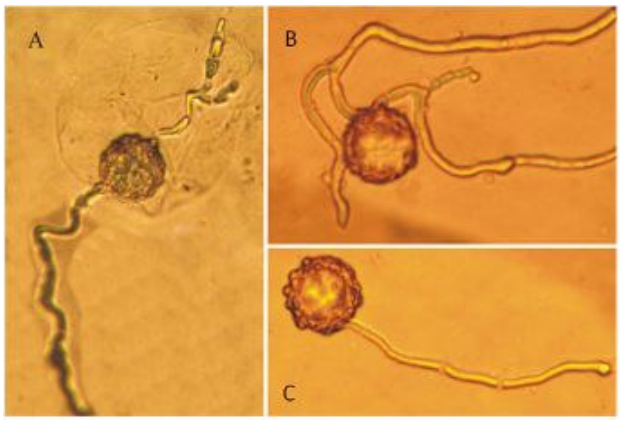

Figure 2. *Terfezia claveryi*: (A) Germinated spore in ascus, 400×, (B, C) Branched hyphae originating from spore 400×.

[DOR: 20.1001.1.16807073.2013.15.2.19.8]

after planting. Fifty milligrams of root were used for each DNA extraction, using the Cetyl-trimethyl-amonium bromide (CTAB) protocol (Gardes *et al*., 1991). The resulting DNA extracts were quantified by a NanoDrop spectrophotometer (NanoDrop Technologies, USA). Contamination by proteins and humic acids in the DNA was evaluated by $OD_{260/280\ nm}$ and $OD_{260/230\ nm}$ ratios.

Primer Design

Oligonucleotide sequences were designed based on all available sequences (NCBI, http://www3.ncbi.nlm.nih.gov/Entrez) (Bethesda, MD, USA) of the ITS regions. Alignment was performed with MEGA4 software, using the Clustal W algorithm (Thompson *et al*., 1994). Two primers were designed: the forward primer FTC, in position 58-79 of the *T. claveryi* ITS1 region sequences, and the reverse primer RTC, that corresponds to the complement of positions 551-573 in the ITS2 region. The primers were analyzed using Primer-Blast (http://www.ncbi.nlm.nih. gov/tools/primer-blast) against the sequence they originally were based. Primers were then evaluated for criteria such as melting temperature, self-dimerization, self annealing, potential hairpin formation and G-C content using OligoAnalyzer (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/). 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. To determine specificity of the primers, PCR was conducted on the high quality genomic DNA of various morphologically and moleculary characterized desert truffles species and some other soil-borne fungi (*Fusarium oxysporum*, *Clonostachys rosea*, *Penicillium digitatum*, *Eupenicillium lanosum*, *Phaeoacremonium rubrigenum*, *Gliomastix* sp.) using the FTC/RTC specific primer.

PCR Analysis

Two sets of primers were used to amplify DNA in PCR that comprised two steps, i.e. direct-PCR followed by nested-PCR. Oligonucleotide primer pair 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) was used in direct PCR to amplify rDNA intergenic regions from the infected roots. Different dilutions of total DNA were prepared before using them as templates in direct PCR. A final volume of 25 µl PCR mixture contained 5 µl of a diluted DNA sample (1:10 dilutions of the original extract), 2.5 µl of 10× PCR reaction buffer, 0.75 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTPs (10 mM each), 0.75 µl of each primer (100 pmol μl^{-1}), 0.25 µl of *Taq* DNA polymerase and 14.5 µl of RNase free sterile water. Controls with no DNA were included in every set of amplifications to test for DNA contamination in reagents and reaction buffers. The amplifications were performed on a CORBETT RESEARCH model CG1-96 thermocycler programmed as follows: 3 min at 94°C, then 30 cycles as follows: 30 seconds at 94°C , 30 s at 50°C and 2 minutes at 72°C . An elongation period of 10 minutes was allowed at 72° C 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. One µl each of direct-PCR product (obtained from total DNA of various dilutions) was re-amplified by nested-PCR. Those components of nested-PCR mixtures were the same as the ones described for direct-PCR, except the primers. The nested-PCRs were primed using the second set of primers, FTC and RTC. The thermocycler was programmed as above. An aliquot of 5 µl was analyzed in 1% agarose gel as mentioned in the direct-PCR and photographed on gel documentation system.

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Sensitivity of the nested-PCR was tested using different concentrations of direct-PCR products at 200 ng, 20 ng, 2 ng, 200 pg, 20 pg, 2 pg, 200 fg, 20 fg and 2 fg per microliter in a 50 µl PCR reaction. One µl of each sample was re-amplified in nested-PCR. The PCR conditions and parameters remained the same as described above.

Restriction Fragment Length Polymorphisms and Sequencing

The restriction pattern of the amplification products obtained from the root samples was examined with *Alu* I and *Hinf* I to confirm their identity. Amplified DNA of all specimens was purified with GenJET PCR purification Kit (Fermentas, UK) to remove excess primers and nucleotides. Sequencing reaction was performed on purified PCR products with ITS1 or ITS4 primers. The sequence was determined with an ABI prism 377 DNA sequencer (Applied Biosystems, USA) according to the manufacturer's instruction. Digested bands were visualized by electrophoresis in 1% (w/v) agarose gels in 1× TBE buffer.

RESULTS

Specificity and Sensitivity of the PCR Aamplification

All isolates studied gave strong amplification of a single PCR product of about 630 bp using primers ITS1/ITS4 (Figure 3). One set of specific primers for *T. claveryi*, FTC/RTC, designed to target ITS1 and ITS2 region, had the following sequences: 5´-*CCTATTGCTTCCACTGGACAGG-3´*and *5´-CTACCTGATCTGAGGTCACCCAA-3,´* respectively. The primers of the developed species-specific PCR designed amplified a 500 bp long region (Figure 4) including 5.8S rRNA gene. All of the 50 *T. claveryi* isolates from Fars and other provinces in Iran had single amplified PCR products using *T. claveryi*-specific primers. No amplification occurred when the specific primers were tested with other fungal isolates. Sensitivity of the primer set FTC\RTC was 20 pg from fruiting body of *T. claveryi* total genomic DNA (Figure 5).

Detection of *T. claveryi* in *Helianthemum* Roots Collected in Fields

Terfezia claveryi mycorrhiza failed to be amplified by specific primers directly. The PCR with the designed species-specific primers could be used as a second step of a nested PCR reaction after the amplification with ITS-1 and ITS-4, which makes the reaction very sensitive. At least ten samples from every plant species were checked with PCR, and two plant species were found to be partners of *T. claveryi*. Usually, after the

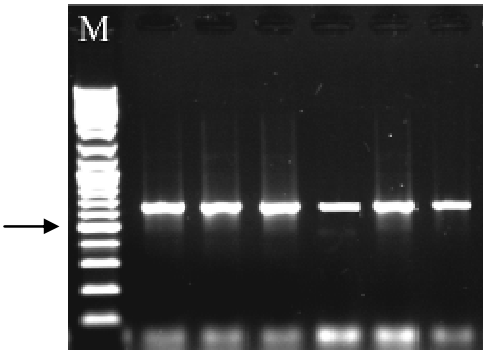

Figure 3. Gel electrophoresis of PCR-amplified products using primers ITS1/ITS4. Lane M: 100 bp DNA ladder; Other lanes: *Terfezia claveryi* isolates, Arrow: 500 bp.

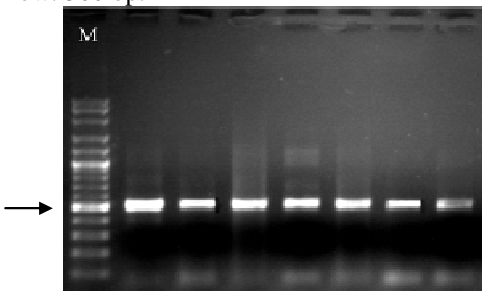

Figure 4. Gel electrophoresis of PCR-amplified products using *Terfezia claveryi*-specific primers FTC/RTC. Lane M: 100 bp DNA ladder; Other lanes: *Terfezia claveryi* isolates, Arrow: 500 bp.

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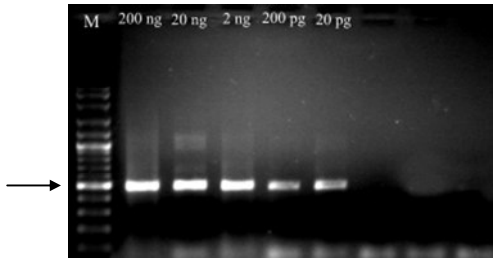

Figure 5. Sensitivity of PCR with primers FTC/RTC using different concentrations of fruit body of *Terfezia claveryi* DNA. Lane M: 100 bp DNA ladder, Arrow: 500 bp.

first PCR, no band corresponding to *T. claveryi* rDNA was detected in *Helianthemum*-infected roots and healthy *Helianthemum* species in the rangelands, but the specific fungal amplification products were often detected after the second amplification. The inability of direct PCR to detect *T. claveryi* necessitated nesting amplified products with *T. claveryi* specific primer pair. Nesting of ITS1/ITS4 amplified products with FTC/RTC primers resulted in amplification of 500-bp products in *Helianthemum*-infected roots from the fields.

Detection of *T. claveryi* in Artificially Infected *Helianthemum* Roots in Greenhouse

The *Helianthemum* plants directly grown from seed under nursery conditions took at least three months to produce plants suitable for fungal inoculation. All of the samples of *Helianthemum*-infected roots in greenhouse were positive for the presence of the 500-bp product (Figures 7 and 8). No PCR products were amplified in any of the DNA samples from healthy plants assayed by the nested PCR primer pairs. Direct detection of *T. claveryi* in roots of inoculated seedlings failed in greenhouse using regular PCR, as PCR products were not detectable. Using a nested PCR approach with primers ITS1/ITS4 for the first round, and the specific primers FTC/RTC for the second round amplification, *T. claveryi* was detected in root samples from inoculated seedlings. Two random root samples that had 500 bp PCR product amplified by FTC/RTC had 100 DNA sequences identical with previously published DNA sequences of *T. claveryi* isolates. This confirmed that the FTC/RTC-amplified PCR products in root DNA were from *T. claveryi* isolates. Nested PCR increased the sensitivity of primers to 2 fg for primers FTC\RTC (Figure 6). All sequenced amplicons were identical with the ITS sequence of *T. claveryi*. *Alu* I and *Hin*f I restriction of the *Helianthemum* root ITS fraction revealed a profile identical to ITS RFLP profiles of *T. claveryi* (data not shown).

DISCUSSION

Prior to this work, molecular detection of *T. claveryi* in host plants had not been accomplished, although the hosts were diagnosed almost 20 years ago using

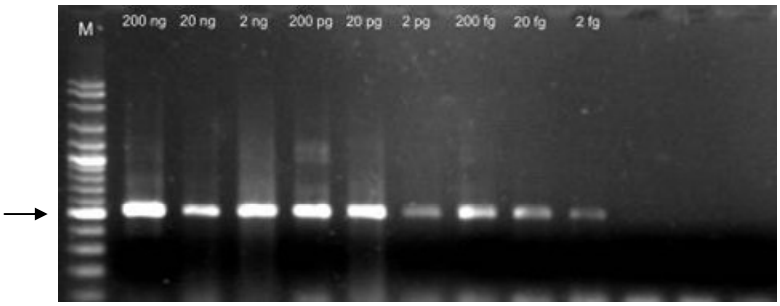

Figure 6. Nested PCR using primers ITS1/ITS4 for the first round of amplification and primers FTC/RTC for the second amplification from roots. Lane M: 100 bp DNA ladder, Arrow: 500 bp.

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anatomical methods (Kovacs *et al*., 2007). Of these techniques, PCR testing was found to be the most sensitive and reliable. Nested PCR using species-specific primers for *T. claveryi* is a highly sensitive method that allows detection of fungal hyphae present in the root of host plants. Any attempt to cultivate these economically important mycorrhiza demands a useful tool for tracking dynamics of truffles in natural and cultivated soils. Detection of host plants of *T. claveryi* may enable the cultivation of this economically important mycorrhiza in greenhouse condition. Nested PCR with species-specific primers carried out on trypan blue-stained roots has already been successfully used in pot cultures where the fungal species were known (Tuinen *et al*., 1998). A nested PCR approach is often needed for detection of host plants (Schneider and Gibb, 1997) when they occur at low levels or are distributed unevenly in their host plants (Goodwin *et al*., 1994; Andersen *et al*., 1998). Poor amplification of target DNA by direct PCR is sometimes attributed to inhibitors present in the roots of the host plant (Cheung *et al*., 1993; Schneider and Gibb, 1997). PCR sensitivity is a concern in any mycorrhiza-detection method using field samples. One approach to increasing sensitivity is to use nested PCR. In this study, nested PCR included two rounds of amplification using universal primers for the first round to increase the target DNA templates, then, using the internal specific primers for the second round. Detection of *T. claveryi* in the root of the host plants in rangelands, using a single round of PCR with the primer pair ITS1/ITS4, was not consistent, probably because of the low concentration of the target DNA. By contrast, the nested PCR approach presented here produced consistent and reproducible results. Nested PCR has been reported to increase PCR sensitivity by 10-1,000 times (Zampieri *et al*., 2009). In the present experiments, nested PCR increased the sensitivity of *T. claveryi*-specific primers by 1,000 fold to 2 fg. The nested PCR assay has potential as a diagnostic tool for detecting *T. claveryi* in the host plant roots. Detection of *T. claveryi* using the described PCR assay was positive for inoculated seedlings in the greenhouse. In this study, DNA was extracted from the root samples of *Helianthemum* species from different areas of Fars and other provinces in Iran. *T. claveryi* was detected in all of the root samples. Nested PCR using ITS1/ITS4 and FTC/RTC has proven to be a reliable molecular tool for identification of host plants of *T. claveryi*. Moreover, nested PCR permits rapid identification of the fragmented sample and does not require sequencing. The advantages of this strategy are its high sensitivity and the possibility of applying it to highly degraded samples, yielding good results. The disadvantage caused by this high sensitivity, which can give rise to false 'positive' results, can be easily overcome by using additional steps to sterilize all the materials used and re-amplifying the control blank sample. Monitoring the mycorrhizae is very important in truffle growing since it allows us to know if the plantations are developing well or if truffle mycorrhizae have been replaced by other more competitive fungi.

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استفاده از واکنش زنجیره ای پلی مراز تو در تو برای تشخیص *Terfezia claveryi* در ریشه گونه های گیاهی گل آفتاب (*Helianthemum* spp.) در شرایط مزرعه و گلخانه

ص. جمالی و ض. بنی هاشمی

چکیده

به منظور تشخیص مولکولی میزبان های گیاهی *Terfezia claveryi*، ریشه گونه های مختلف گل آفتاب از مراتع مختلف استان فارس و دیگر استان های ایران جمع آوری شد. زیر واحد کوچک ریبوزومی دی. ان. ای. ژنومی استخراج شده از ریشه گیاهان، به روش واکنش زنجیره ای پلی مراز تو در تو با استفاده از آغازگرهای عمومی و اختصاصی طراحی شده بر اساس توالی های جدا کننده ی نسخه برداری شده ی داخلی ۱، ۲ و ژن ۵/۸ اس دی ان ای ریبوزومی *T. claveryi* تکثیر شدند. کارایی واکنش زنجیره ای پلی مراز تو در تو نشان داد که این روش قادر به تکثیر مجدد محصول واکنش زنجیره ای پلی مراز و ردیابی دی. ان. ای. ژنومی تا دو فمتو گرم می باشد. چند شکلی طولی قطعات برشی با استفاده از دو آنزیم برشی *Hinf* I و *Alu* I صورت گرفت. نتایج توالی نوکلئوتیدی نشان داد که توالی ها از گیاهان آلوده گل آفتاب همولوژی بسیار بالا با توالی *T. claveryi* دارند. با واکنش زنجیره ای پلی مراز تو در تو دو گونه *Helianthemum lippii* و *H. salicifolium* به عنوان میزبان های این گونه قارچی در گیاهان مایه زنی شده در شرائط گلخانه و مراتع مختلف استان فارس و دیگر استان ها تشخیص داده شدند.

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