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Original Article

Species diversity of indigenous *Trichoderma* from alkaline pistachio soils in Iran

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Abstract: The diversity of *Trichoderma* spp. was investigated in alkaline soils of pistachio orchards at different geographic areas in Kerman province, Iran. A total of 161 *Trichoderma* isolates were obtained and identified at the species level by their morphological characters and sequence analysis of their internal transcribed spacer (ITS) and *tef1-α* genomic regions. Totally, five species of *Trichoderma* were identified, including *T. harzianum*, *Trichoderma* sp., *T. virens*, *T. brevicompactum* and *T. longibrachiatum*. ITS nucleotide sequences could not find molecular differences between *T. harzianum* and *Trichoderma* sp. To identify and differentiate these two species, the sequences of the translation-elongation factor 1-α (*tef1-int 4* (large)) were determined for five representative isolates of *T. harzianum* and *Trichoderma* sp. The TrichoBLAST similarity search using the *tef1-α* sequences of the *T. harzianum* and *Trichoderma* sp. isolates determined in this study, revealed as *T. harzianum*. However, there was 34 nucleotides difference in *tef1-α* sequences, between the two groups of isolates. According to the results, more than 80% of the isolates belonged to two species *T. harzianum* and *Trichoderma* sp. *T. harzianum* was introduced as the dominant species in soil of pistachio orchards. Logistic regression analysis showed no relationship between the soil properties (pH, EC) and presence of *Trichoderma* spp. (R^2 = 0.26, Pr = 0.74, 0.26 > 0.05).

Key words: Alkaline soil, morphology, phylogeny, rDNA, *tef1-α*.

INTRODUCTION

The fungal genus *Trichoderma* (Hypocreales, Ascomycetes) includes cosmopolitan soil-borne species that are frequently found in decaying wood, compost and other organic matters (Harman *et al*. 2004, Samuels 2006). Several *Trichoderma* species are significant biocontrol agents against fungal plant pathogens, either through direct parasitism, competition with pathogens for nutrients, stimulation of plant health, or inducing plant systemic resistance to pathogens (Bailey et al. 2006, Harman et al. 2004, Hjeljord & Tronsmo 1998). The ability for mycoparasitism in some species also has a negative economic impact on the commercial production of *Agaricus bisporus* and *Pleurotus ostreatus* Rolland mushrooms (Hatvani et al. 2007, Krupke et al. 2003, Samuels et al. 2002). On the other hand, *Trichoderma* species produce a wide diversity of metabolites, most notably commercially important cellulase and hemicellulases, antibiotics, peptaibiotics, as well as toxins, such as trichodermamides and trichothecenes that display in vitro cytotoxicity (Degenkolb et al. 2008, Kubicek & Penttila 1998, Liu et al. 2005). Because of the great effect of *Trichoderma* species on human activity, there is a great need for their accurate identification.

The initial approach to understand the diversity and relationship between *Trichoderma* species based on morphological observation was made by Rifai (1969) and later by other researchers (Bissett 1984, 1991a, 1991b, 1992, Hoyos-Carvajal et al. 2009). However, accurate species identification only based on morphology is difficult, because of the paucity and similarity of useful morphological characters (De Respinis et al. 2010, Druzhinina et al. 2005). Bissett et al. (2003) stated that precise resolution of *Trichoderma* species is possible only through combination of morphological and molecular methods, and increasing number of morphologically cryptic species that can be distinguished through their DNA characters are being described (Samuels et al. 2010). This has already resulted in incorrect identification and the propagation of errors for strains associated with the production of secondary metabolites (Humphris et al. 2002), human diseases (Gautheret et al. 1995), and biological control (Kullnig et al. 2001). However, with the advent of molecular methods and identification tools which are based on sequence analysis of multiple genes, including rDNA (the nuclear ribosomal internal transcribed spacers (ITS) and 28S rDNA gene (LSU)), and genes encoding actin, calmodulin, endochitinase, RNA polymerase II and translation-elongation factor 1-alpha (*tef1-α*), it is now possible to identify every *Trichoderma* isolate and/or recognize it as a putative new species (Druzhinina & Kubicek 2005, Kubicek et al. 2008, Samuels 2006).

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For the identification of new species in the genus *Trichoderma*, most authors have used the combination of ITS and *tef1-α* (Bissett et al. 2003, Kraus et al. 2004, Kubicek *et al*. 2003, Lu et al. 2004). At present, the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (ISTH) lists more than 100 species, all of which have been characterized at the molecular level, using *Trich*OKey program as a molecular identification tool for *Trichoderma* species (http://www.isth.info). Many studies have been carried out on the taxonomy of this genus in the world. Nevertheless, the information about the diversity of *Trichoderma/Hypocrea* in Iran is scarce. A preliminary checklist of micromycetes in Iran reported 25 *Trichoderma* species (Ershad 2009).

However, all of these species were identified based on morphological and in some cases molecular characters through amplification of rDNA-ITS region. Due to the mentioned reasons, the establishment of biocontrol agents seems to be the first and the most important step in their use in biological control. Thus, identifying the effective native biocontrol agents to be used against plant pathogens in an area have received considerable interest. As the latter application implies the introduction of *Trichoderma* into the rhizosphere of a given ecosystem, knowledge of the indigenous *Trichoderma* taxa in different soils and climates will contribute to criteria influencing the choice of strains to be applied. The occurrence and species diversity of *Trichoderma* in soil and many other different substrata has been the subject of several investigations in different areas, including South-East Asia (Kubicek et al. 2003), Austria (Wuczkowski et al. 2003), on alkaline agricultural soil in the Nile valley, Egypt (Gherbawy et al. 2004), South America (Druzhinina et al. 2005), China (Zhang et al. 2005, Sun et al. 2012), Sardinia (Migheli et al. 2009), neotropical regions such as Colombia, Mexico, Guatemala, Panama, Peru, Ecuador and Brazil (Hoyos-Carvajal et al. 2009) and Poland (Blaszczyk et al. 2011).

There are no reports for biodiversity of *Trichoderma* species on saline and alkaline soils of pistachio orchards in Iran and the world. Because of the alkaline and saline nature of Iranian pistachio soils and the importance and applications of *Trichoderma* species in biological control of plant pathogens, the objective of the present study was to evaluate the occurrence and species diversity of *Trichoderma* isolates recovered from alkaline soils of pistachio orchards based on morphological and molecular analyses.

MATERIALS AND METHODS

Sampling and isolation of *Trichoderma* isolates

Soil samples were collected from a depth of 0 – 60 cm of alkaline pistachio soils and the rhizosphere of pistachio trees in Kerman province, Iran, during 2010 – 2012. The soil samples were then transferred into sterile polyethylene bags and transported to the laboratory. *Trichoderma* isolates were isolated from soil samples using dilution plate technique (Johnson 1959), on a selective *Trichoderma* medium (Elad & Chet 1983). The plates were incubated at most for 10 days in dark at room temperature (25˚C ± 2). Regularly, after 48 hours, the colonies were counted and transferred to Petri dishes containing PDA (potato dextrose agar, Merck, Germany)-in case of suitable growth- for strain purification, using single spore method. Pure cultures were transferred to the tubes containing PDA and stored at 4˚C for further studies. All of the *Trichoderma* isolates are available from mycological collections of Department of Plant Protection, Faculty of Agriculture, Vali-e-Asr University of Rafsanjan. Representative isolates of each of the identified species have also been deposited at Iranian Fungal Culture Collection in Iranian Research Institute of Plant Protection, Tehran, Iran.

Determination of chemical properties of soil

To determine the relationship between the abundance of *Trichoderma* population and values of electrical conductivity (EC) and pH, the soil samples were dried at room temperature and processed according to the applicable protocol (Ehyaie & Behbahanizade 1993). Five hundred mg of soil samples were passed through 2 mm sieve and sterile distilled water was added to prepare a saturated paste. The samples were stirred briefly and allowed to equilibrate for 24h at room temperature. The pH of each saturated soil sample was then measured using a digital pH meter (744 Metrohm, Sweden). The EC of each saturated paste extract was measured using a digital EC TDS analyzer (WTW 3310 model, Germany).

Morphological identification of *Trichoderma* isolates

The preliminary identification of *Trichoderma* isolates was done based on morphological observation and comparison with morphological identification keys from Gams & Bissett (1998) as well as Samuels et al. (2009). Colony appearance was described on PDA at 25˚C. The growth rate of colony, formation and shape of tufts or pustules, occurrence of diffusing pigment in agar plate and sporulation model were used for macroscopic observation. For microscopic criteria structure, morphology, size and shape of conidiophores, phialides, conidia and chlamydospores were measured on PDA and CMD media (corn meal agar, Merck, Germany, with 0.5% w/v dextrose) at 25˚C under ambient daylight conditions during approximately one week. The following characters were measured: phialide width at the widest point, phialide length and length/width ratio (L/W), conidium length, width and length/width ratio (L/W), presence of chlamydospores and

chlamydospore width. Fifty units of each character were measured for each isolate.

DNA extraction and RAPD PCR amplification

Each isolate was grown on 100 ml of liquid potato dextrose medium. Cultures were maintained at 25±1˚C with shaking (150 rpm) for three days. Mycelial mats were harvested by filtration, washed three times with sterile distilled water and powdered with liquid nitrogen using a mortar and pestle. The powdered mycelia were kept at -20˚C. Genomic DNA was extracted from the pulverized mycelium, using a modification of the cetyltrimethylammonium bromide (CTAB) extraction procedure, described by Alaei et al. (2009). A volume of 400µl of extraction buffer (1.4 M NaCl, 50mM Tris-Hcl pH 8, 0.01 M Na-EDTA, 1% β-mercaptoethanol and 2% CTAB) was added to 100 mg of each sample. The reaction mixture was briefly vortexed and incubated at 65˚C in a water bath for 30 min. Then, 400 µl of chloroform: isoamyl alcohol (24:1 (vol:vol)) was added to the sample. The mixture was emulsified using a vortex and subsequently centrifuged at 7378 g for 15 min. The clear supernatant was transferred to a new tube and the nucleic acids were precipitated with 200 µl isopropanol and centrifuged at 10625 g for 5 min. The pellet was washed in 70% ethanol and re-centrifuged. Finally, the pellets were dried at room temperature, suspended in 50 µl of Tris-EDTA buffer (10mM Tris-HCl, 1mM EDTA), and stored at -20˚C. The DNA concentrations were determined using Nanodrop (BioRad). The DNA was amplified with the random amplified polymorphic (RAPD) technique, using the primer A-5 (5'-AGGGGTCTTG-3') (Chakraborty et al. 2010). Amplification was carried out in a C-1000 Touch™ thermal cycler (Bio Rad, USA) in a volume of 25 µl, containing 75 ng of template genomic DNA, PCR reaction buffer (10 mM Tris-HCl, 50 mM of KCl; pH 9.0), 2.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.2 µM of oligonucleotide primers and 1.25 unit *Taq* DNA polymerase (Bioflux biotech). The amplification conditions were as described by Chakraborty et al. (2010) with some modification: an initial denaturation step of 5 min at 94˚C, followed by 40 cycles of 1 min at 94˚C, 1 min at 36˚C and 90 s at 72˚C, and a final extension step of 10 min at 72˚C. Twenty microliters of RAPD-PCR products were separated in a 1.5% agarose gel for electrophoresis with 1X TAE buffer, followed by staining with ethidium bromide and the bands were visualized under UV light. The image of the gel electrophoresis was documented through Bio-Profile Bio-1D gel documentation system. All reproducible polymorphic bands were scored and RAPD profile patterns of different isolates of *Trichoderma* were obtained by primer A-5.

Representative isolates of *Trichoderma* and DNA sequencing

To confirm the morphological identification of *Trichoderma* species, molecular identification of *Trichoderma* isolates was carried out based on DNA sequencing of the ITS (ITS1-5.8S-ITS2). In cases where ITS1 and ITS2 did not provide unambiguous identification, a 0.3 kb fragment of *tef1-α*, containing the large intron was amplified using the primer pair EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') and EF1-986R (5'-TACTTGAAGGAACCCTTACC-3') (Druzhinina et al. 2004). *Trichoderma* isolates were grouped in preliminary experiments based on morphological characteristics and the data from RAPD molecular marker using the primer A-5 (Chakraborty et al. 2010). PCR amplification of the representative isolates of each group was achieved with a C-1000 Touch™ thermocycler (Bio Rad, USA). The ITS region was amplified, using the primer pair ITS1F (5'-CTTGGTCATTTAGAGGAA GTAA-3') (Gardes & Bruns 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990), in a final volume of 50 µl by mixing 5 µl of diluted (1:10) DNA extract (containing 85-300 ng/µl of template genomic DNA) with 0.2 µM of each of the primers, 0.2 mM of dNTPs, 2.5 mM of $MgCl_2$, PCR reaction buffer (10 mM Tris-HCl, 50 mM KCl; pH 9.0) and 1.25 unit *Taq* DNA polymerase. The cycle parameters were as follows: an initial denaturation for 5 min at 95˚C, followed by 40 cycles of 1 min denaturation at 94˚C, 1 min primer annealing at 45˚C, 1 min extension at 72˚C and a final extension of 10 min at 72˚C. PCR amplification of a 0.3 kb fragment of *tef1-α*, containing the large intron was done in a final volume of 25 µl under the same PCR conditions as described above with the exception of the annealing temperature of 49˚C. Aliquots of 5 µl of PCR products were analyzed by electrophoresis in a 1.5% agarose gel and stained with ethidium bromide for visualization under UV light. The PCR products were cut from agarose gel and purified using a commercial Axyprep PCR Cleanup Kit according to the manufacturer's protocol and sequenced in both directions by Bioneer Company (Daejeon, South Korea). Sequences were analyzed using BioEdit ver. 7.1.3 (Thomas Hall, Ibis Biosciences an Abbott Company) and Chromas Pro ver. 1.7.1 (Technelysium, Australia) and verified manually. The resulting consensus sequences were submitted to the BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) against the GenBank database of the National Center for Biotechnology Information (NCBI) and the *Tricho*BLAST interface (http://www.isth.info/tools/blast/index.php) (Druzhinina *et al.* 2005).The ITS and *tef1* gene nucleotide sequences determined in this study have been submitted to the GenBank database.

Phylogenetic analysis

DNA sequences were aligned using the multiple sequence alignment program Clustal X 1.81 (Thompson et al. 1997) and then visually adjusted. The alignment of sequence data was performed comprising complete ITS1, 5.8S and ITS2 sequences of 21 representative isolates along with the sequences of the identified

species of sections *Pachybasium* B, *Hypocreanum*, *Lone Lineage* and *Longibrachiatum* obtained from Genebank, including *T. harzianum* (HQ259312, AF19 4009, AF194008, AF194011, AF194014, AF194019, AY605733), *T. aureoviride* (AF362108, AF362109, JQ040329, JQ040330), *T. virens* (EU280073, AF099 006, AF099008, HQ608079), *T. brevicompactum* (EU280087, EU280088, JQ040334, JQ040333) and *T. longibrachiatum* (JQ040374, JQ040373, JQ040376, EU280095). Single gaps were treated as missing data. Phylogenetic analysis was performed in MEGA 4.0 (Tamura *et al*. 2004). *Trichoderma viride* AY665699 (section *Trichoderma*) and *T. longibrachiatum* AY93 7420 were used as outgroups for ITS and *tef1-α* analyses, respectively. Phylogenic trees for both rDNA ITS and *tef1-α* sequences were generated using Maximum Parsimony (MP) and Neighbor Joining (NJ), respectively. The NJ tree was constructed using the Kimura2 parameter model. The MP analysis was performed using a heuristic search, with a starting tree obtained via step-wise addition. Stability of the clades was assessed with 1000 bootstrap replications.

RESULTS

Morphological identification

A total of 200 alkaline soil samples were collected from pistachio orchards at different geographic areas in Kerman province, Iran. Measurement of pH and EC are shown in Table1. The soils in the present study were near neutral to alkaline with a pH range of 7.0 to 8.3. The EC ranged from 1.5 to12.3 dSm^{-1}. One hundred and sixty one isolates of *Trichoderma* were obtained and purified. Isolates were identified to the species level by a combination of morphological and genotypic characters. Before molecular identification, the *Trichoderma* isolates were grouped based on the examination of their morphology on PDA and CMD, using macroscopic as well as microscopic characteristics. Identification and origin of the 161 isolates are listed in Table 1. Five species were identified in this study: *T. harzianum* Rifai, *Trichoderma* sp., *T. virens* (J.H. Miller, Giddens & A.A. Foster) von Arx, *T. brevicompactum* (G.F. Kraus, C.P. Kubicek & W. Gams) and *T. longibrachiatum* Rifai. However, based on macroscopic and microscopic morphological criteria, 87 and 44 isolates were identified as *T. harzianum* and *Trichoderma* sp., respectively. Isolates of *Trichoderma* sp. were differentiated from isolates of *T. harzianum* with clavate to ellipsoidal or subglobose conidia, and phialides with 2 or 3–verticillate, narrow ampulliform or lageniform, dirty yellow to brownish yellow pigmentation with the development of needle shape, golden yellow crystals in agar plate and their colonies typically gave an olive green to brownish green color (Fig. 1 and Fig. 2). Nineteen isolates were identified as *T. virens.* Only two isolates were identified as *T. longibrachiatum.* Nine isolates were identified as *T. brevicompactum* according to description of Kraus et al. (2004).

RAPD PCR analysis

Trichoderma species were grouped based on the morphological characteristics. All of the isolates of each group were analyzed by RAPD molecular marker using the primer A-5 (Chakraborty *et al*. 2010). Detailed analyses of the RAPD-PCR profiles were revealed no intra-specific variability and genetic diversity among *Trichoderma* isolates obtained from one soil. Even in some cases, the isolates obtained from various soil samples that were similar in morphology, had genetic similarity. The RAPD-PCR profiles analysis showed 21 different band patterns. The representative isolates of each profile were selected for DNA sequencing.

DNA sequencing

PCR amplification of the rDNA ITS sequences of 21 representative isolates of *Trichoderma* (Table 2), using ITS1F-ITS4 primer pair was successful. Direct sequencing of the gel-purified PCR fragments from the isolates was successful and consistently produced good sequencing reads. All sequences were submitted to GenBank. Their accession numbers are listed in Table 2. The similarity search using the sequences of the isolates determined in this study showed that the majority of the sequenced strains (12 isolates of *T. harzianum* out of 21 repre sentative isolates) were closely similar to *T. harzianum* isolates in *Trich*OKey and GeneBank (Table 2). The ITS sequence of five isolates out of 21 representative isolates in which were identified as *Trichoderma* sp. based on morphological studies were closely similar to *T. harzianum* (CPK2656) and *Trichoderma aureviridea* (JQ040330) in *Trich*OKey and GeneBank respectively (Table 2). In fact, the rDNA ITS genomic region of *T. harzianum* as well as *Trichoderma* sp. isolates showed high sequence similarity and were identified as *T. harzianum*. Detailed analyses of the rDNA ITS sequences revealed no intra-isolates variability among *T. harzianum* and *Trichoderma* sp. obtained from one alkaline soil sample. All of the *T. harzianum* rDNA ITS fragments had only 4 bp difference in ITS1 and 7 bp difference in ITS2 (Fig. 3).On the other hand, RAPD-PCR analysis with A-5 primer confirmed that no genetic diversity was found within isolates obtained from one soil. Due to the identical ITS sequences for isolates of *T. harzianum* and *Trichoderma* sp. isolates, the final identification of these isolates were done by sequenceing of *tef1* for representative isolates (Table 2). However, 34 nucleotide differences were observed between the two groups of isolates of *T. harzianum* and *Trichoderma* sp. The BLAST similarity search in *Trich*oBLAST using the sequences of the *T. harzianum* (*tef1-α*) revealed the similarity to *H. Lixii/T.harzianum* entries CBS227.95 (E-value's: e – 146) and for *Trichoderma*

sp. (*tef1-α*) was similar to *H. Lixii/T.harzianum* entries GJS97.264 and E-value's of e – 152.

Phylogenetic analysis

Phylogenetic analyses aimed to determine the phylogenetic position and relationship of the obtained isolates among the identified species of *Trichoderma*. The phylogenetic analyses were done based on Maximum Parsimony (MP) analyses. In the ITS tree, the Harzianum clade (Clade A), including *T. harzianum* and *Trichoderma* sp., the Virens clade with *T. virens* (Clade B), the Longibrachiatum clade with *T. longibrachiatum* (Clade C) and the Lutea clade with *T. brevicompactum* (Clade D) were distinguished with bootstrap support of 75%. Among the 161 isolates, 93.2% (150 isolates), 5.6% (9 isolates) and 1.2% (2 isolates) were located in *Trichoderma* sections *Pachybasium*, *Lone Lineages* and *Longibrachiatum*, respectively. The separation of these sections was supported by bootstrap values of 94 and 99%, respectively. Seventeen isolates located within *Trichoderma* sect. *Pachybasium* were grouped in a complex including *T. harzianum* and *Trichoderma* sp. (Fig. 4). Five isolates out of these isolates (Ta3-90, Ta9-117, Ta1-41, Ta2-43 and Ta7-116) formed a separate subclade with a bootstrap value 79%. Seven (Th22-45, Th55-147, Th26-62, Th27-65, Th38-127, Th24-61, Th52-134) and five isolates (Th19-43, Th1-1, Th33-113, Th23-53, Th4-11) were grouped in two distinct subclades with poor resolution.

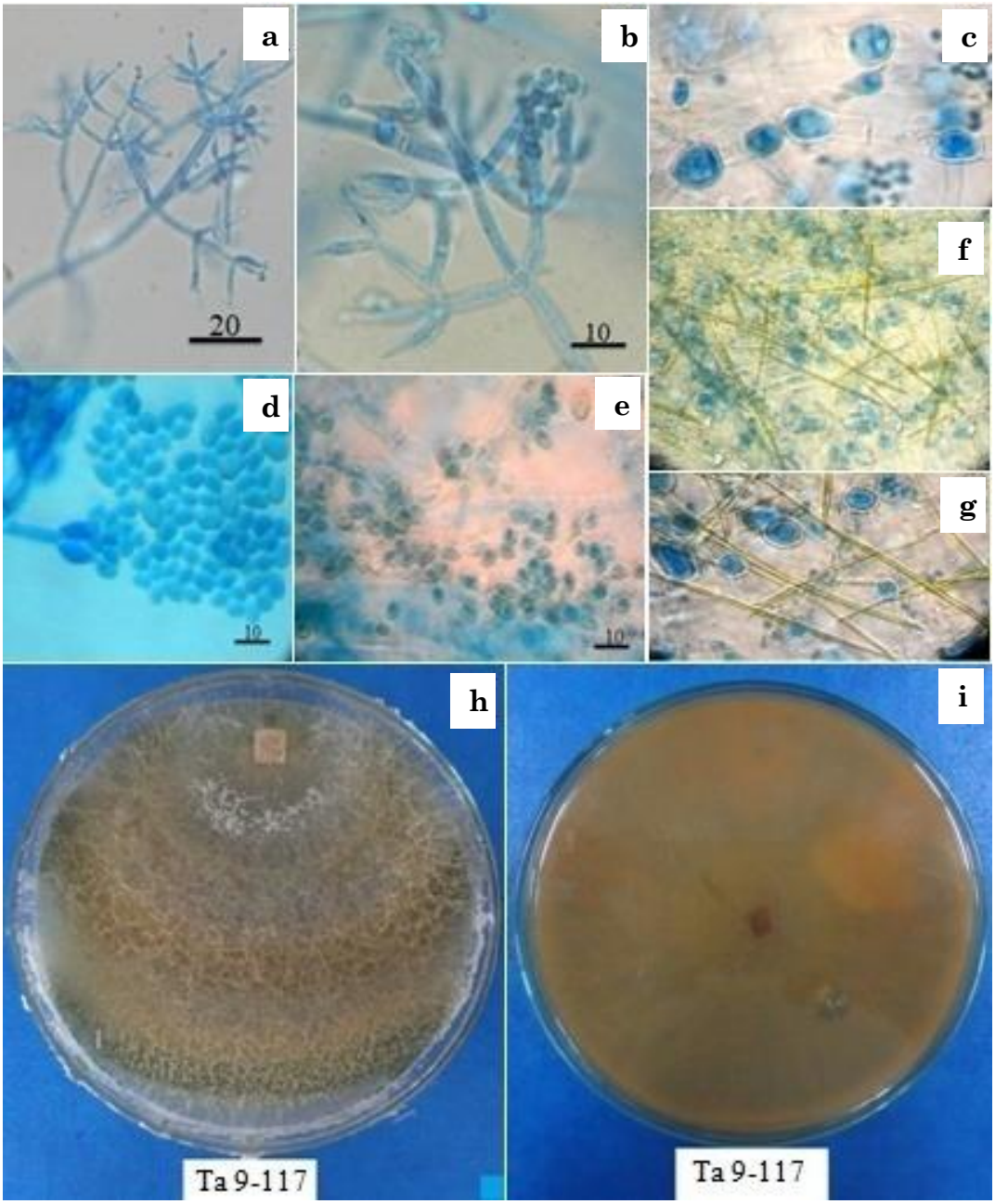


Fig. 1. *Trichoderma* sp.: **a-b.** Phialides, **C.** Chlamydospore, **d-e.** Conidia, **f-G.** Needle shaped and yellow crystals, **h.** Colony on PDA after 10 days, **i.** Reverse colony on PDA after 10 days.

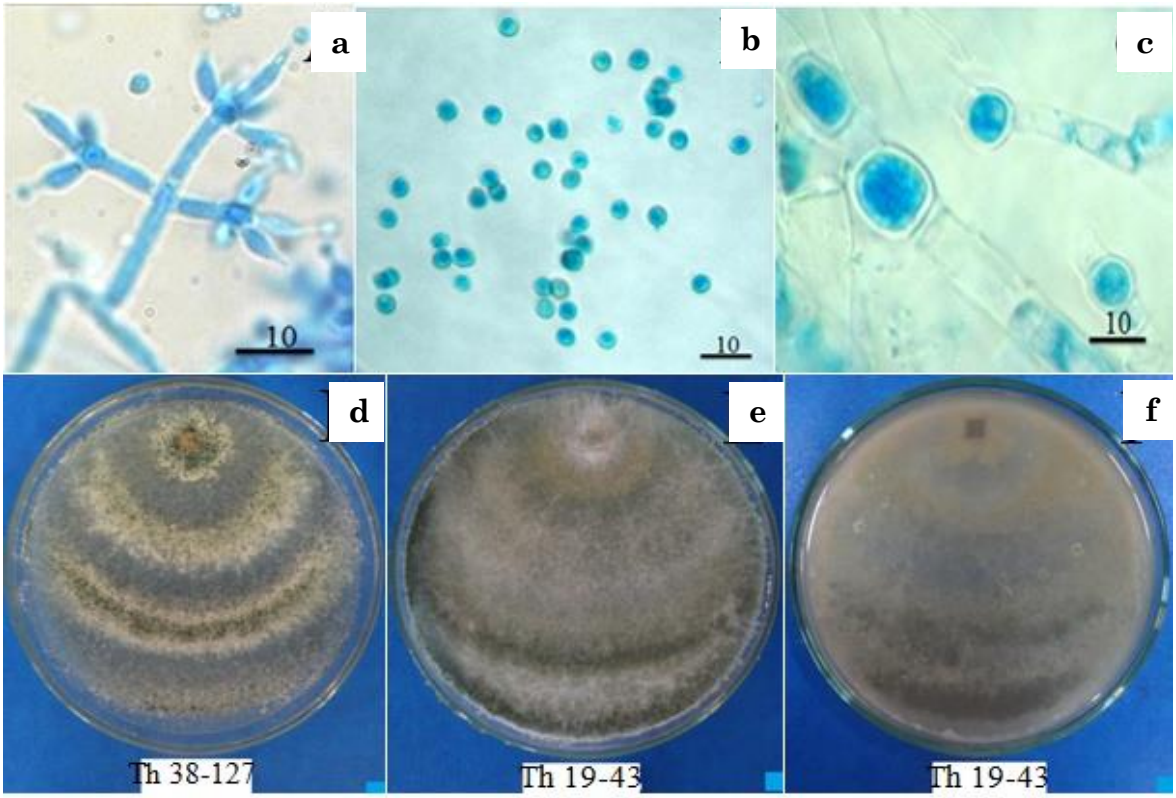
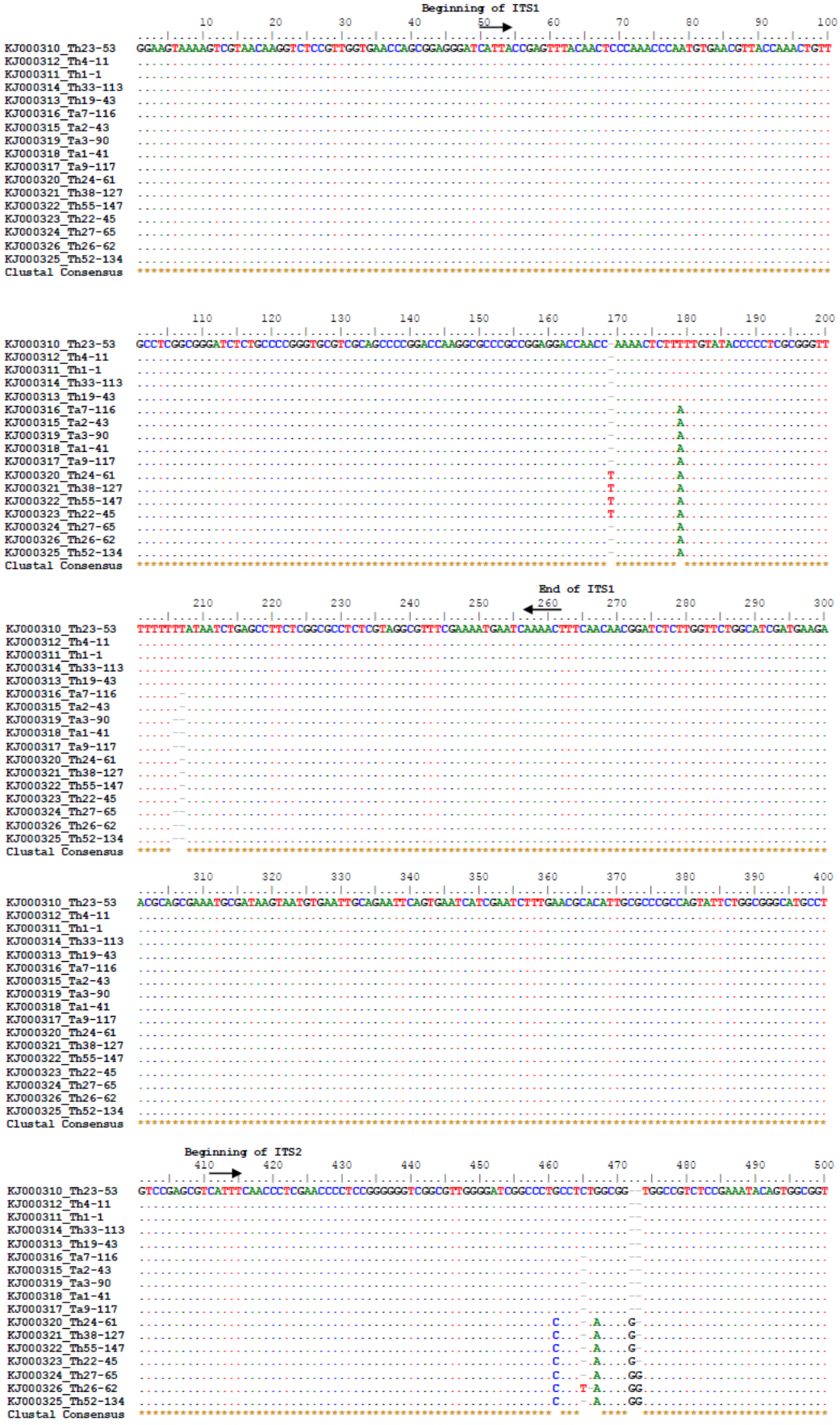


Fig. 2. *Trichoderma harzianum*: **a.** Phialide, **b.** Conidia, **c.** Chlamydospore, **d-e.** Colony on PDA after 10 days, **f.** Reverse colony on PDA after 10 days.

Table 1. Soil properties and morphological identification of strains of *Trichoderma* isolated at different geographical areas in Kerman province, Iran.

location	soil sample code	number of isolates	isolate code(s)	soil properties		species
				pH	EC(dS.m^{-1})	
Rafsanjan-Nogh	R 01	3	Th01:Th03	7.91	6.1	*T. harzianum*
Bardsir	B 11	2	Th04:Th05	7.72	7.99	*T. harzianum*
Rafsanjan-HematAbad	R 41	1	Ts01	-	-	*Trichoderma* sp.
Rafsanjan-HematAbad	R 43	15	Th06:Th20	8.07	9.1	*T. harzianum*
Rafsanjan-HematAbad	R 43	1	Ts02	8.07	9.1	*Trichoderma* sp.
Rafsanjan-RaeesAbad	R 45	2	Th 21:Th 22	7.87	4.02	*T. harzianum*
Rafsanjan	R 53	1	Th 23	-	-	*T. harzianum*
Rafsanjan-AliAbad	R 61	2	Th 24:Th 25	-	-	*T. harzianum*
Rafsanjan-EsmaeelAbad	R 62	1	Th 26-62	7.65	5.57	*T. harzianum*
Rafsanjan-EsmaeelAbad	R 65	1	Th 27-65	7.68	4.99	*T. harzianum*
Rafsanjan-EsmaeelAbad	R 67	3	Th28:Th30	7.99	6.72	*T. harzianum*
Rafsanjan-GhaderAbad	R 69	1	Th31	7.72	12.25	*T. harzianum*
Rafsanjan-Ahmadieh	R 77	4	Tb01:Tb04			*T. brevicompactum*
Anar	A90	1	Ts03	7.5	4.39	*Trichoderma* sp.
Sirjan	S 111	1	Th 32	8.2	2.38	*T. harzianum*
Sirjan	S113	2	Th 33:Th 34	8.24	3.73	*T. harzianum*
Sirjan	S 115	1	Ts04	7.94	12.26	*Trichoderma* sp.
Sirjan	S 116	3	Ts05:Ts7	8.13	2.77	*Trichoderma* sp.
Sirjan	S117	30	Ts08:Ts39	7.61	7.2	*Trichoderma* sp.
Sirjan	S 118	5	Ts38:Ts42	8.28	3.97	*Trichoderma* sp.
Rafsanjan-JalalAbad	R127	17	Th35:Th51	7.97	4.05	*T. harzianum*
Sirjan-FakhrAbad	S 132	6	Tv01:Tv06	7.83	3.59	*T. virens*
Sirjan-Khafriz	S 133	1	Tv07	8.3	2.41	*T. virens*
Sirjan- Khafriz	S 133	1	Tb05	8.3	2.41	*T. brevicompactum*
Sirjan-JafarAbad	S134	2	Th52: Th53	8.12	2.31	*T. harzianum*
Sirjan-AliAbad	S 136	2	Tb06:Tb07	7.67	2.22	*T. brevicompactum*
Sirjan-AliAbad	S 136	5	Tv08:Tv12	7.67	2.22	*T. virens*
Rafsanjan-GhavamAbad	R142	2	Ts43:Ts44	8	6.8	*Trichoderma* sp.
Sirjan- Khafriz	S 145	7	Tv13:Tv19	7.96	1.82	*T. virens*
Sirjan	S 146	2	Tb08:Tb09	8.12	1.48	*T. brevicompactum*
Sirjan-NosratAbad	S147	4	Th54:Th57	7.92	4.05	*T. harzianum*
Anar	A189	29	Th58: Th87	7.97	1.86	*T. harzianum*
Rafsanjan	R54	2	Tl01: Tl02	-	-	*T. longibrachiatum*


Beginning of ITS1
End of ITS1
Beginning of ITS2
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KJ000319_Ta3-90
KJ000318_Ta1-41
KJ000317_Ta9-117
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Clustal Consensus

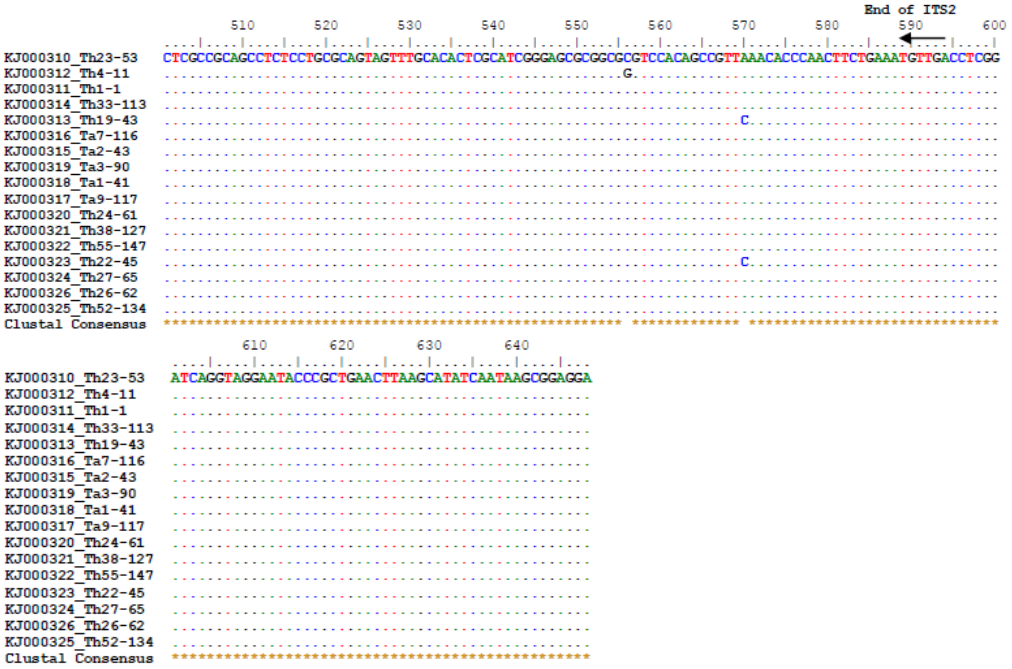

Fig. 3. Alignment of the complete nucleotide sequence of the internal transcribed spacer (ITS1 and ITS2) region of the nuclear ribosomal RNA genes of *Trichoderma harzianum* and *Trichoderma* sp. isolates, including the 5.8S subunit. The sequences are written 5' to 3'. Identical nucleotides are indicated by dots. The ITS1 and ITS2 regions are marked with arrows.

Table 2. Representative *Trichoderma* isolates included in molecular study and their highest similarities with *Trich*OKey and NCBI GenBank species (for ITS rDNA and *tef1*)

species	strain code	accession number		GenBank strains identification/ Identities		
		ITS rDNA	*tef1*	(ITS-TrichoKEY)	(ITS-NCBI)	(*tef1*-TrichoBLAST)
Trichoderma sp.	Ts 1-41	KJ000318	-	*H. lixii* CPK2656 (100%)	*T.aureoviride* JQ040330	
Trichoderma sp.	Ts 2-43	KJ000315	-	*H. lixii* CPK1085 (99%)	*T.aureoviride* JQ040329	
Trichoderma sp.	Ts 3-90	KJ000319	-	*H. lixii* CPK2656 (100%)	*T.aureoviride* JQ040330	
Trichoderma sp.	Ts 7-116	KJ000316	KJ206536	*H. lixii* CPK1085 (99%)	*T.aureoviride* JQ040330	*T.harzianum*AY605845
Trichoderma sp.	Ts 9-117	KJ000317	-	*H. lixii* CPK2656 (100%)	*T.aureoviride* JQ040330	
Trichoderma sp.	Ts39-118	-	KJ206537			*T.harzianum*AY605845
T. brevicompactum	Tb 2-77	KJ000306	-	CPK723 (99%)	EU280087	
T. harzianum	Th 1-1	KJ000311	KJ206538	CPK2649 (99%)	*T.harzianum* HQ259312	*T.harzianum*EU918165
T. harzianum	Th 4-11	KJ000312	-	CPK2649 (99%)	*T.harzianum* HQ259312	
T. harzianum	Th 19-43	KJ000313	-	CPK2649 (99%)	*T.harzianum* HQ259312	
T. harzianum	Th 22-45	KJ000323	-	CPK2660 (99%)	*T.harzianum* AY605733	
T. harzianum	Th 23-53	KJ000310	-	CPK2649 (99%)	*T.harzianum* HQ259312	
T. harzianum	Th 24-61	KJ000320	-	CPK2660 (100%)	*T.harzianum* AY605733	
T. harzianum	Th 26-62	KJ000326	-	CPK1102 (100%)	*T.harzianum* AY605733	
T. harzianum	Th 27-65	KJ000324	-	CPK1102 (100%)	*T.harzianum* AY605733	
T. harzianum	Th 33-113	KJ000314	-	CPK2649 (99%)	*T.harzianum* HQ259312	
T. harzianum	Th 38-127	KJ000321	KJ206535	CPK2660 (100%)	*T.harzianum* AY605733	*T.harzianum*AY605832
T. harzianum	Th 52-134	KJ000325	-	CPK1102 (100%)	*T.harzianum* AY605733	
T. harzianum	Th 55-147	KJ000322	KJ206534	CPK2660 (100%)	*T.harzianum* AY605733	*T.harzianum*AY605832
T. longibrachiatum	Tl 1-54	KJ000309	-	CPK1692 (99%)	JQ040374	
T. virens	Tv 3-132	KJ000307	-	CPK2941 (100%)	*T.virens* AF099008	
T. virens	Tv 17-142	KJ000308	-	CPK2939 (100%)	*T.virens* AF099008	

131 *Trichoderma* isolates were identified as *T. harzianum* (*H. lixii*), but this species is known to include several ITS alleles (Hermosa *et al*. 2004, Migheli *et al.* 2009, Karimian et al. 2014) and is considered to be a species complex (Chaverri *et al.* 2003, Karimian et al. 2014). Two out of 20 strains (Tv3-132 and Tv17-142) included within *Trichoderma* sect. *Pachybasium* were grouped with *T. virens* which formed a separate clade with a bootstrap value of 98%, isolate Tb2-77 was grouped with *T. brevicompactum* with a bootstrap value of 99% and Tl1-54 was the only isolate in this study which belonged to section *Longibrachiatum* and was grouped with *T. longibrachiatum* with a bootstrap value of 99% (Fig. 4). A phylogenetic analysis based on *tef1* sequences was also performed for the representative isolates of *T. harzianum* and *Trichoderma* sp. (Fig. 5).

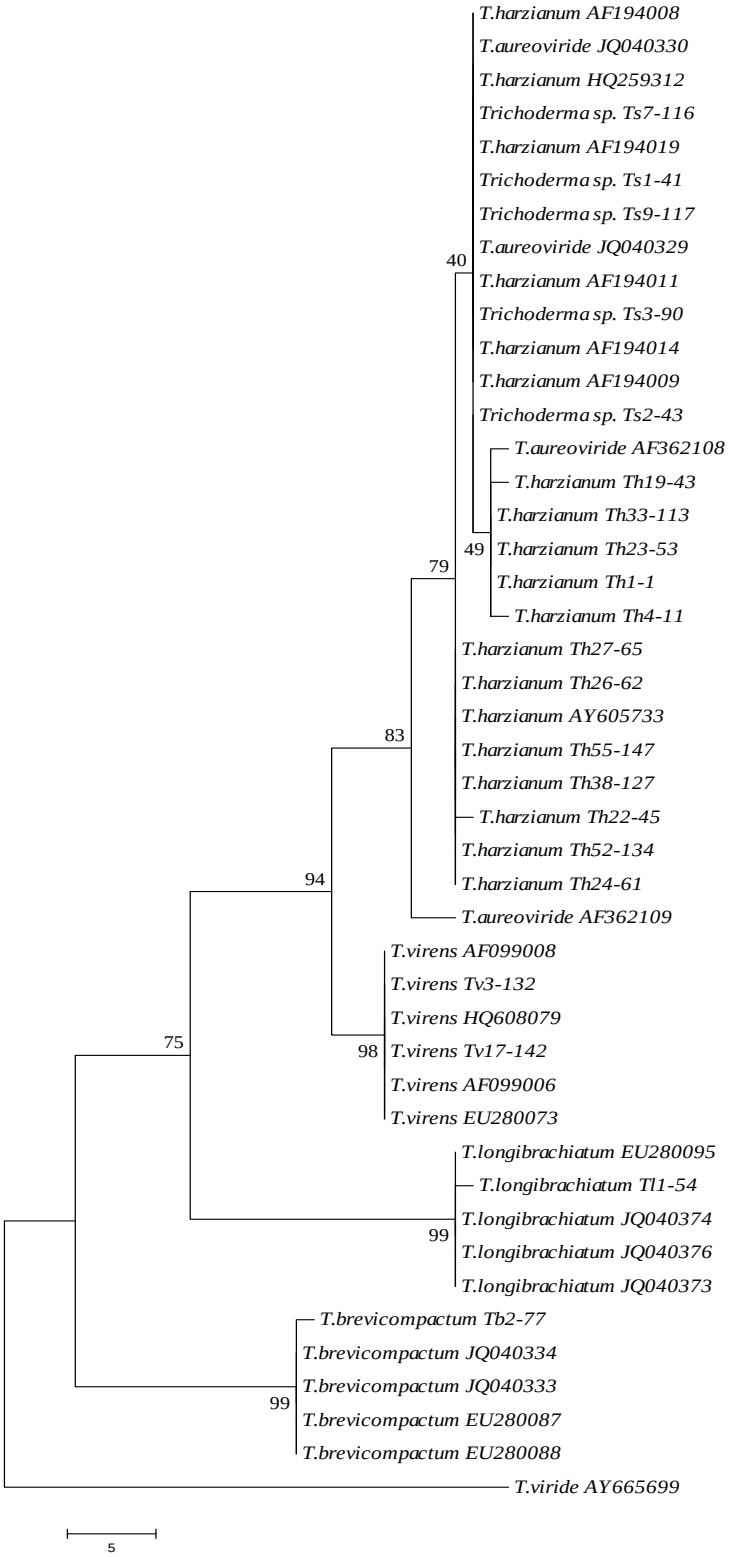

Fig. 4. Phylogenetic tree of representative isolates of *Trichoderma* belonging to sections *Pachybasium, Lone Lineages* and *Longibrachiatum*, inferred by Maximum Parsimony analysis of ITS1, 5.8s and ITS2 sequences in MEGA 4.0. *T. viride* (Accession No. AY665699) was used as outgroup. The bootstrap support from 1000 replication is indicated on the branches. Isolates of related to *Trichoderma* sp.: Ts1-41, Ts2-43, Ts3-90, Ts7-116, Ts9-117; *T. harzianum*: Th19-43, Th23-53, Th1-1, Th4-11, Th33-113, Th26-62, Th52-134, Th27-65, Th22-45, Th55-147, Th38-127 and Th24-61; *T. virens*: Tv3-132, Tv17-142, *T. brevicompactum*: Tb2-77 and *T. longibrachiatum*: Tl1-54.

As a result, the isolates related to two species *T. harzianum* and *Trichoderma* sp. were located within three separate clades. Two isolates (Ts116 and Ts118) were identified as *Trichoderma* sp. based on morphological characteristics, forming a separate clade with a bootstrap value of 94%. Isolate Th1 was grouped in a distinct clade with a bootstrap value of 82% and two isolates (Th127 and Th147 which were identified as *T. harzianum*) were located in separate clades that were resolved with high bootstrap support 100% (Fig 5).

The phylogenetic position of species based on *tef1-α* had correlation with their morphological characteristics. In fact, the isolates related to two species *T. harzianum* and *Trichoderma* sp. were differentiated based on morphological characteristics, as well as RAPD-PCR analysis using four random primers OPA4, OPA3, A-5, Pr3. Based on the results, isolates are grouped into two major clusters which shared 0.47% similarity (Mirkhani et al. unpublished).

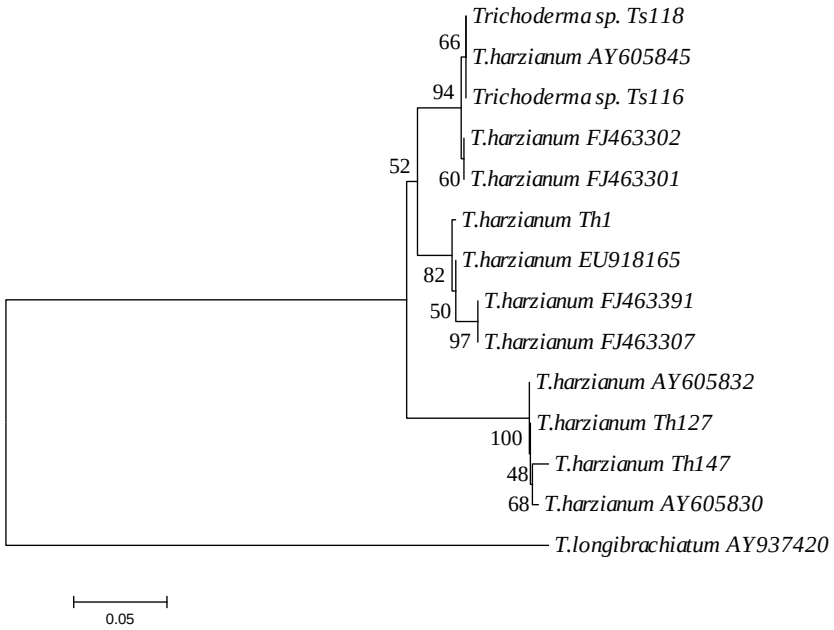

Fig. 5. Phylogenetic tree of the *Trichoderma* isolates inferred by Nieghbour Joining analysis of *tef1-α* sequences in MEGA 4.0. The bootstrap support from 1000 replication is indicated on the branches.

DISCUSSION

Trichoderma strains have biotechnological potential as biological agents for the control of soil-borne plant pathogens and for their ability to increase root growth and development, crop productivity, resistance to abiotic stresses, and uptake and use of nutrients. However, the choice of active *Trichoderma* strains is important in designing the effective and safe biocontrol strategies. In fact, acidity and alkaline conditions are factors that can affect *Trichoderma* population, such as its presence, density, longevity, as well as production of enzymes (Kredics *et al.* 2003, Michel-Aceves *et al.* 2001, Samaniego 2008).

Regarding the above-mentioned points, we aimed to isolate and identify indigenous *Trichoderma* strains from alkaline soils which can be potentially used as biocontrol agents to control many plant pathogenic fungi and nematodes. The present study is a preliminary assessment of *Trichoderma* diversity in alkaline soils of pistachio orchards in Iran. *Trichoderma* isolates were identified at the species level by analysis of their morphological characters and sequence analysis of their ITS and *tef1-α* genomic regions as the phylogenetic markers. A low degree of biodiversity of *Trichoderma* isolates was found and five species were identified, including: *T. harzianum*, *Trichoderma* sp., *T. virens*, *T. brevicompactum* and *T. longibrachiatum*. Molecular identification of *Trichoderma* species confirmed their morphological identification except for *Trichoderma* sp. that its rDNA ITS sequences was closely resembled those described for isolates of *T. harzianum*. Isolates related to this two species were not differentiated by their ITS sequences, because sequences of the two species are very similar. Although the results of genetic diversity and phylogeny studies of *T. harzianum*, even using ITS rDNA gene sequence analysis, was shown a complex speciation within *H. lixii*/ *T. harzianum* species group (Druzhinina et al., 2010, Karimian *et al*., 2014) but our data based on morphological criteria on some strains was different, so we identified them as *Trichoderma* sp. and need to do further investigation of gene sequences.

Based on the morphological criteria, production of the brownish yellow colony with the development of needle shape, golden yellow crystals are observed as the characteristics of *Trichoderma* sp. isolates, when

incubated at 25°C on PDA, whereas a pale yellow colony without crystals is associated with all *T. harzianum* isolates. All of the isolates assigned to *T. harzianum* and *Trichoderma* sp. grow fast at 25, 30 and 35°C on PDA. Jaklitsch (2009) reported that the anamorph strains related to *H. lixii* obtained from Europe grew at 35°C and had optimum growth at 30°C on all media. They had often pigment appearing like yellow crystals in the colony, but often dissolving again and unstable as well as not having a clear shape. In our study, any of the *T. harzianum* isolates did not produce crystals, whereas all *Trichoderma* sp. isolates produced needle shaped golden yellow crystal bodies which were stable in the medium.

Studies on biodiversity of *Trichoderma* were carried out in Russia, Siberia and Himalaya (Kullnig-Gradinger *et al*. 2000), South-East Asia (Kubicek et al. 2003), Austria (Wuczkowski et al. 2003), on alkaline agricultural soil in the Nile valley, Egypt (Gherbawy et al. 2004), South America (Druzhinina et al. 2005), China (Zhang et al. 2005, Sun et al. 2012), Sardinia (Migheli et al. 2009), neotropical regions such as Colombia, Mexico, Guatemala, Panama, Peru, Ecuador and Brazil (Hoyos-Carvajal et al. 2009) and Poland (Blaszczyk et al. 2011).

In Iran, more than 25 species of *Trichoderma* have been identified from different substrates such as soil, wood and plant material samples across the country, including Southern coast of the Caspian Sea (Nazmi Rodsari et al. 2007, Zafari et al. 2002, 2004, Naeimi et al. 2014). In comparison with these studies, the regions with alkaline soils appear to be with a relatively low biodiversity of *Trichoderma*, in which *T. harzianum* and *Trichoderma* sp. are the predominant taxa. In this study, *T. harzianum* was the most frequently isolated species (54.4%) and *Trichoderma* sp. (27.3%) was the second most common species. *T. harzianum* was the predominant taxon in many locations and habitats (Druzhinina et al. 2005, Druzhinina et al. 2010, Kubicek et al. 2003, Migheli et al. 2009, Wuczkowski et al. 2003, Zhang et al. 2005).

T. harzianum is the most commonly reported species in the genus, occurring in diverse ecosystems and ecological niches. *T. harzianum* sensu stricto is also a species with a broad north temperate distribution, including at least North America, Europe and Asia (Zhang et al. 2005). *T. harzianum*, which is commonly associated with the rhizosphere of cultivated plants, is frequently used as a biocontrol agent against phytopathogenic fungi. The predominance of *T. harzianum* in many different environments might be explained by its ability to assimilate a comparatively wide array of carbon substrates (Zhang et al. 2005). The concept of *T. harzianum* as a genetically variable complex, comprised by one morphological species and possibly several phylogenetic species (Chaverri et al. 2003, Druzhinina et al. 2010, Gams & Bissett 1998, Karimian et al. 2014) is coherent with the adaptive range of this taxon. Another striking result from this study was that only one soil sample yielded two isolates as *T. longibrachiatum*. According to its phylogenetic position, it is unlikely that this is due to the use of biased cultivation conditions, because this method readily isolated *T. longibrachiatum* and *T. citrinoviride* from soils of India, eastern USA and Iran (Kullnig-Gradinger et al. 2000, Zafari et al. 2002). Furthermore, the basic medium used in these studies can be used to grow virtually all recognized *Trichoderma* species. It is, therefore, concluded that members of this section are in low abundance or almost absent in the soils investigated.

Trichoderma strains were expected to be found in more than 50% of the sampled plots, because of their cosmopolitan character and the fact that it is a natural inhabitant of soils, but *Trichoderma* strains were found in just 15% of the plots (30 samples from 200 soil samples). These results correspond to those reported by Campos et al. (2012), in which *Trichoderma* strains were found in just 22% of the sampled sites. However, they differ from the results reported by Michel-Aceves et al. (2001), that native strains of *Trichoderma* were found in 88% of the sampled sites. Moreover, in the research conducted by Kubicek et al. (2003), strains of *Trichoderma* from all soil samples were separated. The results show that soils of pistachio orchards of Kerman province are not rich in terms of number of isolates and species diversity of *Trichoderma*. Furthermore, strains of this important genus are not compatible with soil ecological conditions of pistachio orchards. In fact, the environmental parameters, such as soil temperature, moisture, pH, organic matter (OM), nutrient content which affect the growth and proliferation of fungal genus and plant types are key factors affecting soil colonization by *Trichoderma* species (Gherbawy et al. 2004).

Soil acidity is one of the important factors for the establishment of *Trichoderma* species. Thus, the reason for this low degree of biodiversity of *Trichoderma* in the soil of pistachio orchards may be related to its alkaline pH value. Danielson & Davey (1973) stressed that the soil pH is one of the most critical parameters for *Trichoderma* propagation. Kredics et al. (2003) reported that species of *Trichoderma* grow optimally at around pH 4.0-5.0, and exhibit little or no growth below pH 2.0 and above 6.0. Acidity is a factor which affects presence, density and longevity of this fungal genus (Michel-Aceves et al. 2001). Okoth et al. (2007) and Campus et al. (2012) reported that *Trichoderma* is abundant in acid soils. Our results therefore showed that *Trichoderma* could also be isolated from such adverse

habitats, but with a lower frequency than the other soils, while it is possible that the five species found have a general tolerance to high pH and EC. On the other hand, we could not detect any correlation between pH and EC of the soil and the *Trichoderma* species recovered. Correlation between any of these characters and the five taxa were essentially random, and it is thus believed that the populations of *Trichoderma* species detected in this study are generally indigenous components of the soil in pistachio orchards in Iran. The results of the present study correspond to the findings reported by Gherbawy *et al*. (2004), that only *T. harzianum* and the anamorph of *Hypocrea orientalis* were found in the soils of Nile valley in Egypt.

Present results confirm the ecological plasticity of genus *Trichoderma*, as pointed out by Samuels (2006) and Infante *et al.* (2009). Although *Trichoderma* species could be found in all altitudes and all types of soils, but geographic distribution of *Trichoderma* species are quite different. Some species such as *T. pseudokoningii* and *T. harzianum* are broadly spread, while others like *T. viride* have a limited geographic distribution and are not commonly found in the colder northern regions. Another example is *T. aureoviride*, whose distribution is limited to the United Kingdom and northern Europe (Samuels 2006). Thus, the set of factors such as soil moisture, temperature, texture and structure, organic matter (OM), nutrient content, plants type, specie type and the existence of other organisms in soil will affect the adaptation of a species in a region. In fact, the soil is a very complex environment and it is generally the interaction of several factors which can affect the number of soil microorganisms, diversity or activity (Bourguignon 2008).

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تنوع گونه ای *Trichoderma* در خاک‌های قلیایی باغ‌های پسته در ایران

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چکیده: در این تحقیق، تنوع گونه‌های تریکودرما در خاک باغ‌های پسته در مناطق مختلف استان کرمان بررسی شد. در مجموع تعداد ۱۶۱ جدایه‌ی تریکودرما جداسازی و در سطح گونه براساس ویژگی های مورفولوژیکی و تکثیر و ترادف نوکلئوتیدی ناحیه‌ی ITS (ITS1-5.8S-ITS2) از DNA ریبوزومی و ژن *tef1-α* شناسایی شدند. براساس ویژگی‌های مورفولوژی و مولکولی جدایه متعلق به پنج گونه‌ی *Trichoderma harzianum*، *Trichoderma* sp.، *T. virens*، *T. brevicompactum* و *T. longibrachiatum* می‌باشند. ناحیه ITS تفاوت‌های نولکئوتیدی بین دو گونه *T. harzianum* و *Trichoderma* sp. را نشان نداد. بنابراین، برای شناسایی و تمایز دو گونه، توالی چهارمین اینترون بزرگ ژن *tef1-α* پنج جدایه‌ی نماینده *T. harzianum* و *Trichoderma* sp. تعیین توالی شد. بررسی تشابه توالی ها با استفاده از برنامه‌ی TrichoBLAST، توالی‌های *tef1-α* جدایه‌های *T. harzianum* و *Trichoderma* sp. به‌عنوان *T. harzianum* تعیین شدند، درحالی‌که ۳۴ تفاوت نوکلئوتیدی در توالی چهارمین اینترون بزرگ *tef1-α* دو گروه از جدایه‌ها وجود داشت. نتایج نشان داد که بیش از ۸۰ درصد جدایه‌های تریکودرما، به گونه‌ی*T. harzianum* و *Trichoderma* sp. تعلق دارند. گونه *T. harzianum* به‌عنوان گونه‌ی غالب خاک باغ‌های پسته‌ی استان کرمان معرفی گردید. براساس آنالیز رگرسیونی، رابطه‌ی معنی‌داری بین فراوانی جمعیت تریکودرما و مقادیر pH و EC در خاک باغ‌های پسته‌ی این منطقه وجود ندارد ($R^2 = 0.26$, $Pr = 0.74, 0.26 > 0.05$).

کلمات کلیدی: خاک قلیایی، مورفولوژی، فیلوژنی، rDNA، *tef1-α*

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