Shahid Bahonar University of Kerman
Journal of Advances in Plant Protection
JAPP 2023
Association of Iranian Plant Protection Societies

Genetic diversity of *Trichoderma* spp. isolated from alkaline pistachio soils using morphological and molecular characteristics

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Article Info.

Article type:
Original article

Article history:
Received 13 Nov 2024
Received in revised form 22 Dec 2024
Accepted 24 Dec 2024
Available Online 27 Dec 2024

Keywords:
Alkaline Soil,
Random Primer,
RAPD-PCR,
Ribosomal RNA.

Abstract

The genetic diversity of *Trichoderma* species isolated from pistachio soils in Kerman Province was investigated. A total of 131 isolates were obtained and identified at the species level by analyzing their morphological characters. Eighty-seven isolates were identified as *Trichoderma harzianum* and forty-four isolates as *Trichoderma aureoviride.* The sequences of their ribosomal RNA (rRNA) gene internal transcribed spacers (ITS), were determined and used as phylogenetic markers. The complete ITS nucleotide sequences of 17 selected isolates of *T. harzianum* and *T. aureroviride* were determined. Minimal rDNA-ITS sequence variation was observed, and molecular informative differences between *T. harzianum* and *T. aureroviride* were not found in phylogenetic analysis.. RAPD-PCR using the four random primers, OPA4, OPA3, A-5, and Pr3, was evaluated as a technique to separate and differentiate these two species. The four primers produced DNA bands for the 16 *Trichoderma* isolates, of which 81 (95.3%) were polymorphic, and four (4.7%) were monomorphic. The RAPD data was computed into a similarity matrix using the NTSYS computer program. The genetic similarity coefficient between pairwise isolates varied from 0.50 to 0.98 based on an unweighted paired group method of arithmetic average (UPGMA) cluster analysis. The results showed that all isolates were grouped into two main clades. In agreement with observation, data from morphological characteristics resulting in RAPD-PCR banding patterns revealed it was easier to identify and differentiate *T. harzianum* and *T. aureroviride* isolates. The RAPD-PCR technique was practical and efficient for routine high-resolution diversity studies for the two species.

Cite this article: Alaei, H., Mirkhani, F., Mohammadi, A. H., & Haghdel, M. (2024). Genetic diversity of *Trichoderma* spp. isolated from alkaline pistachio soils using morphological and molecular characteristics. *Journal of Advances in Plant Protection, 1*(2), 83–96.

Publisher: Shahid Bahonar University of Kerman

Introduction

Species of the genus *Trichoderma* (Hypocreales, Ascomycota) are well known for their production of a spectacular array of secondary metabolites including polysaccharides, toxins, and antibiotics (Gams & Bissett, 1998. In addition, strains in several species of this genus are widely used in biocontrol of soil-borne plant pathogenic fungi (Samuels, 1996). Given of their economic significance, the taxonomy of this genus and correct identification of the species are essential. However, due to the abundant homoplasy in phenetic characters *Trichoderma* taxonomy and species identification is a difficult issue (Bissett et al., 2003). With the advent of techniques for analyzing DNA sequence polymorphisms and their increased utilization in fungal systematics, the evolutionary relationships among *Trichoderma* species are beginning to unfold. Molecular techniques such as restriction fragment length polymorphism (RFLP) (Lieckfeldt et al., 2001), random amplified polymorphic DNA (RAPD) (Hermosa et al., 2001; Anu et al., 2023), sequence analysis using a single gene (Kindermann et al., 1998) or multiple genes (Kullnig-Gradinger et al., 2002; Chaverri et al., 2003; Samuels, 2006; Samuels et al.,

2006) have been employed to study the identification and genetic diversity of plant pathogenic fungi. Genes used to study the phylogenetic relationships among *Trichoderma* species include the nuclear ribosomal internal transcribed spacers (ITS), the 28S rDNA gene (LSU), and the translation elongation factor 1α (*tef-1α*) (Chaverri et al., 2015; Jambhulkar et al., 2024), encoding endochitinase (Sharma et al., 2024) (*ech42*), the calmodulin (*cal*), the actin (*act*), as well as the RNA polymerase subunit II (*RPB2*) genes. For identification of new species in the genus *Trichoderma*, most authors have utilized a combination of ITS and *tef-1α* (Bissett et al., 2003; Kubicek et al., 2003; Kraus et al., 2004; Lu et al., 2004; Zhang et al., 2005.) However, some closely related species share sequences in their ITS regions (EL-Sobkei et al., 2024). The most commonly used method for inter and intra-specific studies is the analysis of RFLPs. The comparison of different RFLP banding patterns is then used to identify the different species (Lieckfeldt et al., 2002; Garbeva et al., 2004; Bourguignon, 2008). *Trichoderma* species can also be identified using RAPD (Kullnig-Gradinger et al., 2000; Hermosa et al., 2001; Wuczkowski et al., 2003). RAPD is an electrophoretic method used for taxonomy at the species level to discriminate species (Hadrys et al., 1992). It is a DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence (Williams et al., 1990). The RAPD-PCR technique has been developed for various applications including differentiating numerous fungi including *Trichoderma* species (Zimand et al., 1994; Kapteyn & Simion, 2002). RAPD fingerprinting has been used to identify subgroups of *Trichoderma* (Arisan-Atac et al., 1995; Kullnig-Gradinger et al., 2000; Siddiquee et al., 2012) and has shown that biocontrol activity was spread among several distinct groups and subgroups of this genus. Dubey & Suresh (2006) analyzed *Trichoderma* isolates using RAPD-PCR and found it possible to distinguish isolates of *T. harzianum* and *T. viride* into three distinct groups. Narayan et al. (2006) demonstrated that RAPD markers were useful in identifying isolates of *T. aureoviride* within complex collections of *Trichoderma* species. Siameto et al. (2011) also aimed to characterize *T. harzianum* isolates using the RAPD-PCR technique to establish the degree of genetic variation and determine any relationship between molecular variation and antifungal activity. Given the alkaline and saline nature of Iranian pistachio soils and the importance and applications of *Trichoderma* species in biological control of plant pathogens (Fani et al. 2014), in previous study (Mirkhani & Alaei, 2015), we identified *Trichoderma* species using analysis of morphological characteristics and sequence analysis of their ITS rRNA gene cluster. Our data showed minimal variation in the rRNA gene ITS sequence and did not reveal informative molecular differences between *T. harzianum* and *T. aureroviride* in phylogenetic analysis. Due to the prevalence of the species in alkaline pistachio soils, we utilize analysis of morphological characteristics, sequence analysis of their ITS rRNA gene cluster and RAPD-PCR analysis for identification, differentiation and determination of genetic diversity between *T. harzianum* and *T. aureroviride* isolates. To our knowledge there are no reports on the biodiversity of *Trichoderma* species in saline and alkaline soils of pistachio orchards in Iran or worldwide.

Materials and Methods

Sampling and isolation of *Trichoderma* isolates

During 2010-2012, soil samples were collected from 0 – 40 cm depth and from the rhizosphere of pistachio trees in Kerman Province, Iran. *Trichoderma* isolates were isolated from the soil samples using the dilution plate technique (Johnson, 1960) on a selective *Trichoderma* medium culture (Elad & Chet, 1983). The plates were incubated for a maximum of 10 days at room temperature (25±2 °C). The colonies were counted and transferred to Petri dishes containing PDA (potato dextrose agar, Merck, Germany) for purification of the isolates using the single-spore method (Kullnig-Gradinger et al., 2000). Pure cultures were then transferred to tubes containing PDA and stored at 4 °C for further studies. All *Trichoderma* isolates were deposited in the culture collection of Vali-e-Asr University of Rafsanjan and the Iranian Fungal Culture Collection (IRAN) at the Iranian Research Institute of Plant Protection, Tehran, Iran.

Morphological identification

The preliminary identification of the isolates was conducted based on morphological observation and comparison with taxonomic identification keys from Gams & Bissett (1998) as well as Samuels et al. (2009). The growth rate of colony, formation and shape of tufts or pustules, the occurrence of diffusing pigment in agar plates and sporulation model were observed on PDA and CMD (corn meal agar, Merck, Germany, with %0.5 w/v dextrose). For microscopic criteria, the structure, morphology, size and shape of conidiophores, phialides, conidia and chlamydospores were measured on CMD

colonies at 25 °C under ambient daylight conditions within approximately one week. Morphological studies were carried out based on phialide width at the widest point, phialide length, length/width ratio (L/W), conidium length, width, length/width ratio (L/W), presence of chlamydospores, and chlamydospore width. Fifty units of fungal characters were measured for each isolate.

DNA extraction

For DNA extraction, two mycelial plugs (5 mm in diameter) of *Trichoderma* isolates were transferred from PDA to 250 ml Erlenmeyer flasks containing 100 ml of potato dextrose broth, shaken at 150 rpm and incubated at 25 °C. After three days, mycelia were harvested and genomic DNA was isolated using the 2% CTAB procedure described by Alaei et al. (2009). DNA concentrations were determined using the Nanodrop (BioRad, USA).

PCR amplification and sequencing

PCR amplification of the rDNA-ITS region was conducted using a C-1000 Touch™ thermocycler (Bio Rad, USA). The ITS region was amplified using ITS1F (Gardes & Bruns, 1993) and ITS4 (White et al., 1990) primer set in a final volume of 50 µl by mixing 5 µl of DNA extract (containing 85-300 ng/µl of template genomic DNA), 0.2 µM of each of the primers, 0.2 mM of dNTPs, 2.5 mM of MgCl2, PCR reaction buffer (10 mM Tris-HCl, 50 mMKCl; pH 9.0) and 1.25 units Taq DNA polymerase. The cycle parameters included 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. Subsequently, 5 µl aliquots of PCR products were analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide for visualization under UV light. The PCR products were purified using the Axyprep PCR Cleanup Kit (Axygen Biosciences, USA) following the manufacturer's protocol and sequenced by Bioneer Corporation (Daejeon, South Korea). For species identification, forward and reverse sequences were assembled and edited with ChromasPro ver. 1.7.1 (Technelysium, Australia) and 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 TrichoBLAST interface (http://www.isth.info/tools/blast/index.php) in the ISTH data (Druzhinina et al., 2005). The ITS nucleotide sequences obtained in this study have been submitted to the GenBank databases, and their accession numbers are listed in Table 1.

RAPD-PCR analysis

Four commercial random primers including OPA4, OPA3, A-5 and Pr3 (Table 2) were screened for their effectiveness in producing PCR bands. A total of 16 isolates of *T. harzianum* (10 isolates) and *T. aureroviride* (six isolates) were selected for the genetic diversity study (Table 1). Amplification was carried out in a C-1000 Touch™ thermal cycler (Bio Rad, USA) in a 25 µl volume containing 75 ng of fungal genomic DNA, PCR reaction buffer (10 mM Tris-HCl, 50 mM KCl; pH 9.0), 2.5 mM of MgCl2, 0.2 mM of dNTPs, 0.2 µM of each primer and 1.25 units of Taq DNA polymerase. Only one primer was used in each reaction. The amplification conditions were the same as those described by Chakraborty et al. (2010) with some modifications: 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 2% agarose gel for electrophoresis with 1X TAE buffer, stained with ethidium bromide and visualized under UV light. The image of the gel electrophoresis was documented through gel documentation system and Bio-1D analysis software (Peqlab, UK). All reproducible polymorphic bands were scored and analyzed using the UPGMA cluster analysis protocol and computed in Silico into similarity matrix using Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc, version 2.20, Exeter software, USA). The SIMQUAL program was used to calculate the DICE coefficients. The RAPD patterns of each isolate were assessed, assigning character state 1 to indicate presence in the gel. A data matrix was then used to calculate the DICE similarity coefficient for each pair wise comparison. The DICE coefficient were clustered to generate dendrograms using the SHAN clustering program, selecting the unweighted pair-group method with arithmetic average (UPGMA) algorithm in NTSYSpc.

Phylogenetic analysis

DNA sequences were aligned using the multiple sequence alignment program Clustal-X 1.81 (Thompson et al., 1997). The alignment of sequence data, comprising complete ITS1, 5.8S and ITS2 rDNA sequences of 17 taxa, along with sequences of identified species of section *Pachybasium* B (Kullnig-Gradinger et al., 2002) obtained from Genebank including, *T.*

harzianum, *T. inhamatum*, *T. aureoviride*, and *T. tomentosum* was performed and then visually adjusted. Single gaps were treated as missing data. Phylogenetic analysis was performed in MEGA 4.0 (Tamura et al., 2004). *Trichoderma viride* (accession no. AY665699 belonging to *Trichoderma* sect.) was used as an outgroup. Phylogenetic trees were generated using Neighbor-Joining (NJ) and Maximum Parsimony (MP). The NJ tree was constructed using the Kimura2 parameter model. A MP analysis was performed using a heuristic search, with a starting tree obtained via step-wise addition. The stability of clades was assessed with 1000 bootstrap replications.

Table 1. *Trichoderma* strains and their gene sequence accession numbers obtained in this study

Species name	Isolates number	GenBank accession numbers (ITS1 and ITS2)
T. harzianum	Th 1-1[ab]	KJ000311
T. harzianum	Th4-11[a]	KJ000312
T. harzianum	Th 19-43[ab]	KJ000313
T. harzianum	Th 22-45[ab]	KJ000323
T. harzianum	Th23-53[ab]	KJ000310
T. harzianum	Th 24-61[ab]	KJ000320
T. harzianum	Th26-62[a]	KJ000326
T. harzianum	Th27-65[ab]	KJ000324
T. harzianum	Th33-113[ab]	KJ000314
T. harzianum	Th 38-127[ab]	KJ000321
T. harzianum	Th 52-134[ab]	KJ000325
T. harzianum	Th 55-147[ab]	KJ000322
T. aureoviride	Ta 1-41[ab]	KJ000318
T. aureoviride	Ta 2-43[ab]	KJ000315
T. aureoviride	Ta 3-90[a]	KJ000319
T. aureoviride	Ta 7-116[ab]	KJ000316
T. aureoviride	Ta 9-117[ab]	KJ000317
T. aureoviride	Ta 39-118[b]	-
T. aureoviride	Ta 43-142[b]	-

a. Strains sequenced in this study
b. Strains used in RAPD-PCR analysis

Table 2. Primers used for ITS and RAPD PCR in this study

	Code	DNA sequence (5'-3')	Reference
ITS-Primers	ITS1F	CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns, 1993
	ITS4	TCCTCCGCTTATTGATATGC	White et al., 1990
RAPD primers	OPA 4	AATCGGGCTG	Chakraborty et al., 2010
	OPA 3	AGTCAGCCAC	Latha et al., 2002
	A-5	AGGGGTCTTG	Chakraborty et al., 2010
	Pr3	TCGCCAGCCA	Lopez-Mondejar et al., 2011

Results

Species identification

In the present study, a total of 131 *Trichoderma* isolates were obtained from alkaline pistachio soils in different geographical areas in Kerman Province, Iran (Data not shown). Based on macroscopic and microscopic morphological criteria, 87 and 44 isolates were identified as *T. harzianum* and *T. aureroviride*, respectively. *Trichoderma aureroviride* isolates were differentiated from *T. harzianum* isolates with clavate to ellipsoid 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 typically olive green to brownish green color colonies (Fig. 1). In *T. harzianum*, conidiophores are loosely and regular part of section *Trichoderma*, with spherical and small conidia (1.8-4 × 1.8-3 µm). Phialides are lageniform and short and could be distinguished from other *Trichoderma* species (Fig. 2).

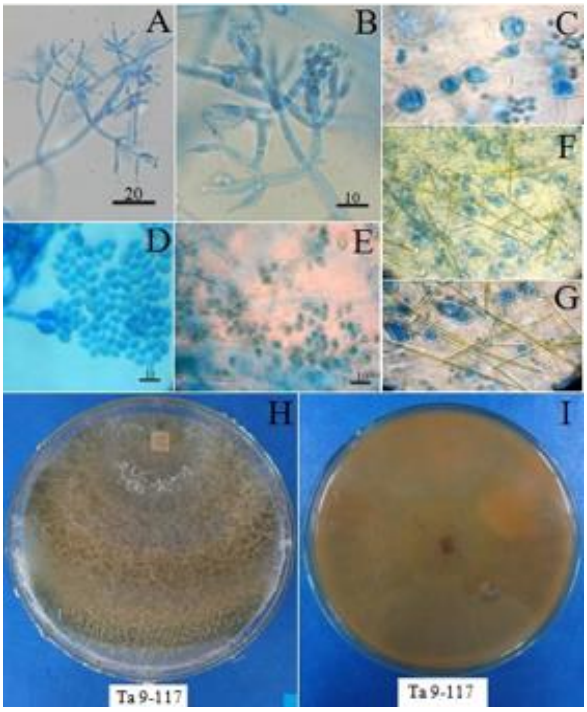

Fig. 1. *Trichoderma aureoviride*: A-B. Conidiophores and phialides. C. Chlamydospores. D–E. Conidia. F–G. Needle-shaped, yellow crystals. H. Colony on PDA after 10 days. I. Reverse colony on PDA after 10 days.

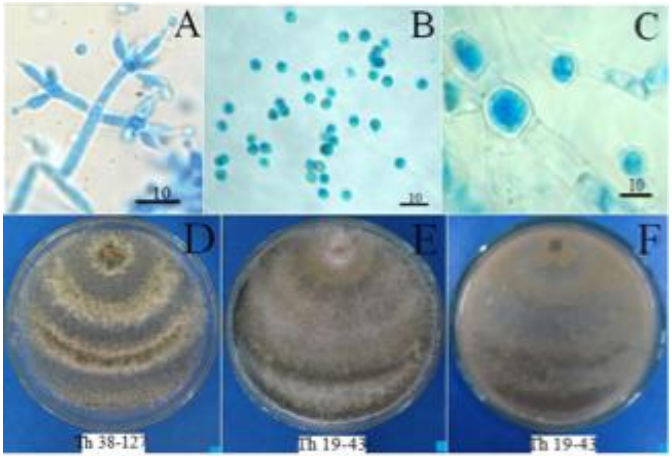

Fig. 2. *Trichoderma harzianum*: A. Conidiophores and phialides. B. Conidia. C. Chlamydospores. D-E. Colonies on PDA after 10 days. F. Reverse colony on PDA after 10 days.

PCR amplification and sequencing

PCR amplification of the rRNA gene ITS sequences of 17 selected *Trichoderma* isolates (listed in Table 1) using the ITS1F/ITS4 primer set was successful (data not shown). Direct sequencing of the gel-purified PCR fragments from *Trichoderma* produced good sequencing reads. All sequences were submitted to GenBank and Their accession codes are listed in Table 1. The rDNA-ITS nucleotide sequences of *T. harzianum* isolates and *T. aureroviride* isolates showed high sequence similarity. Detailed analyses of the rDNA-ITS revealed no intra-isolate variability among *T. harzianum* and *T. aureroviride* obtained from one soil sample. All of the

T. harzianum rDNA-ITS fragments had only 4 bp differences in ITS1 and 7 bp in ITS2. These sequence differences were also found between soil samples (data not shown). The BLAST similarity search using the sequences of the isolates determined in this study for *T. harzianum* revealed E-values of 0.0 (rDNA-ITS) with GenBank entries AY605730 (*Hypocrea lixii*) and for *T. aureroviride* with E-value's of 0.0 (rDNA-ITS) with GenBank entries KF367564 (*Trichoderma* sp.). When the sequences of Th19-43, Th38-127 (*T. harzianum*), and Ts9-117 (*T. aureroviride*) isolates were submitted in Blast search, the most reference sequences obtained were *T. harzianum* and *H. lixii*. Based on the similarity (identity), the reference sequences were AY605730 (E-value 0.0) and AF194011 (E-value 0.0).

Phylogenetic analyses

The phylogenetic analyses were aimed to determine the taxonomic position and relationship of *T. harzianum* and *T. aureroviride* among the identified species of *Trichoderma*. The phylogenetic tree obtained by NJ (Fig. 3) was identical to the MP tree (Fig. 4). These trees showed that the sequences of the two species are very similar and exhibit species-overlapping isolate variations. Due to identical ITS sequences of *T. harzianum* and *T. aureoviride* isolates, the final identification of them were performed by morphological observations and to further support their unique nature, five representative isolates (of 131 isolates) were also characterized by the sequence of a fragment of *tef-1α*., but they were identified as *the T. harzianum* on the GenBank (Mirkhani & Alaei, 2015). However, 34 nucleotide differences were observed between the two groups of the isolates (isolates related to *T. harzianum* and *T. aureoviride*). Thus, the final identification of these isolates was determined by morphological observations and confirmed by RAPD-PCR using four random primers OPA4, OPA3, A-5, Pr3.

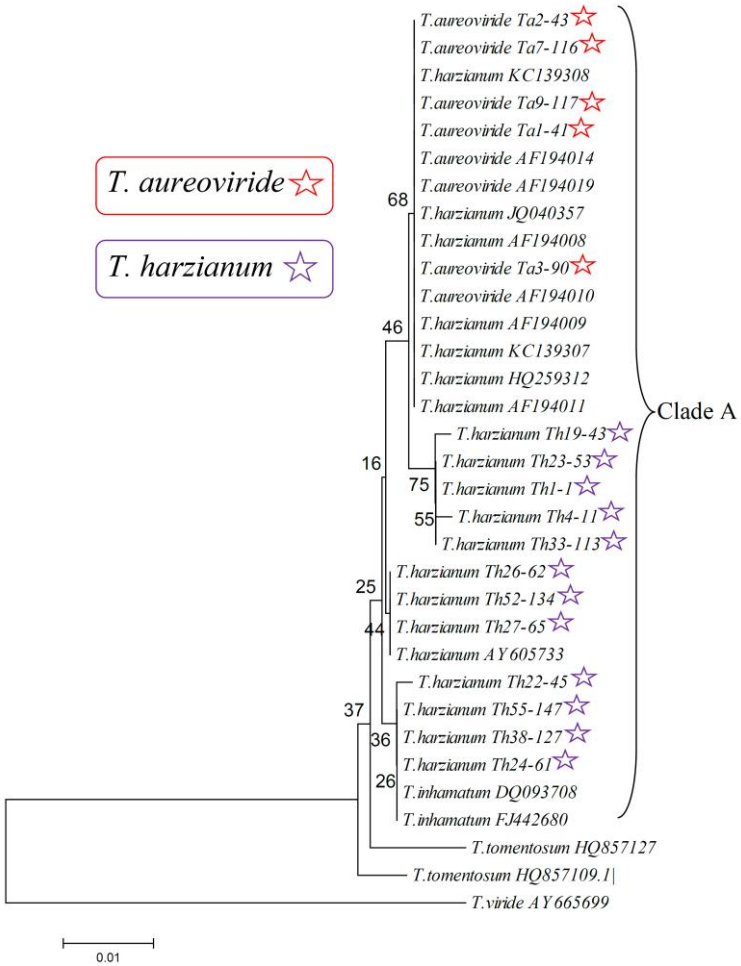

Fig. 3. Phylogenetic tree of representative isolates of *Trichoderma* species belonging to section *Pachybasium*, inferred by Neighbour Joining analysis of ITS1, 5.8s, and ITS2 sequences. The rDNA ITS sequence of *Trichoderma viride* (Accession no. AY665699) was used as an outgroup.

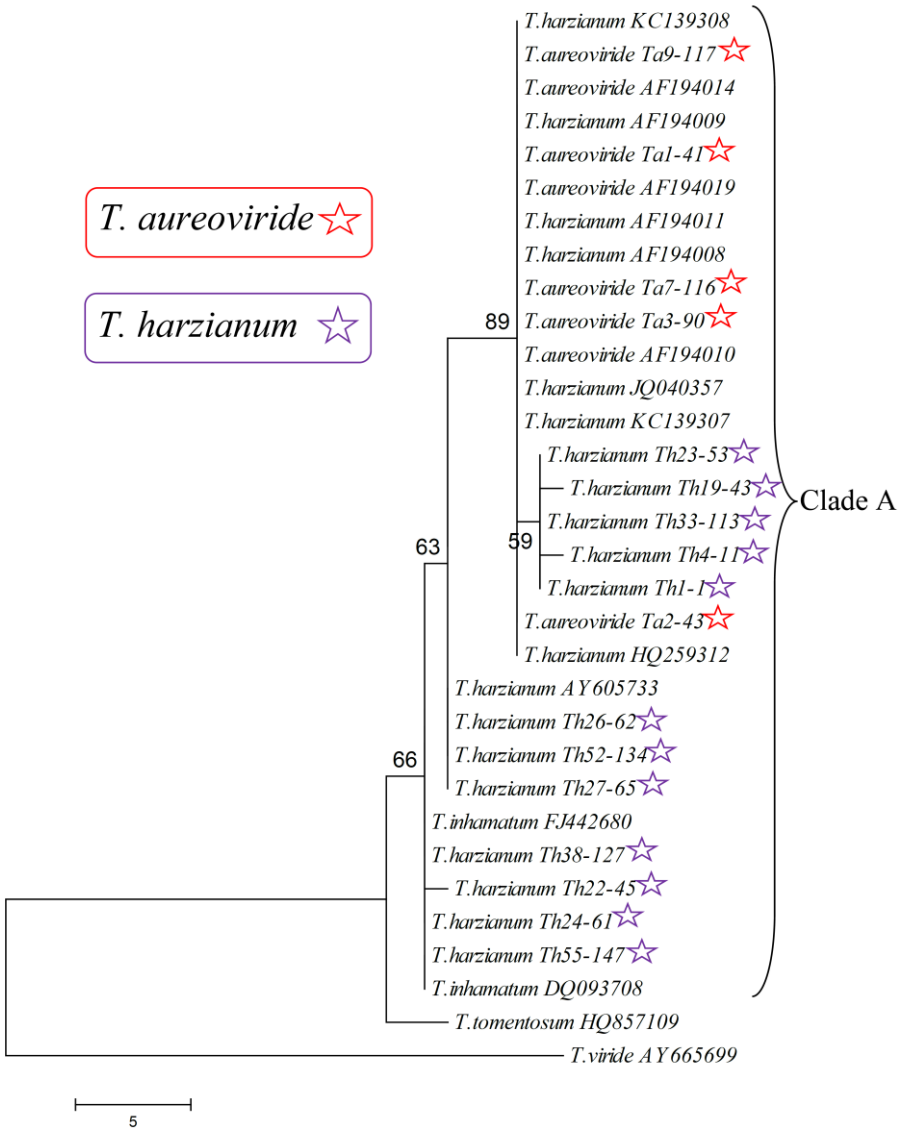

Fig. 4. Phylogenetic tree of representative isolates of *Trichoderma* species belonging to section *Pachybasium*, inferred by Maximum Parsimony analysis of ITS1, 5.8s, and ITS2 sequences. The rDNA ITS sequence of *Trichoderma viride* (Accession no. AY665699) was used as an outgroup.

RAPD-PCR analysis

The genetic relatedness among 10 isolates of *T. harzianum* and six isolates of *T. aureroviride* were analyzed by four random primers: OPA4, OPA3, A-5, and Pr3, to generate reproducible polymorphisms. Amplification with primers showed polymorphism at intra- and inter-specific levels within *T. harzianum* and *T. aureroviride* isolates. The size of the amplified nucleotides obtained with the four selected RAPD primers ranged from 200 bp to 3000 bp. The four primers produced a total of 85 bands for the 16 *Trichoderma* isolates, of which 81 (95.3%) were polymorphic and four (4.7%) were monomorphic (Table 3). Four common bands (monomorphic) were produced by all of the isolates using OPA4 and Pr3 primers. These consisted of a single band at 500 bp produced by primer OPA 4, while three bands at 350, 700, and 1200 bp produced by primer Pr3. Using an OPA4 primer, two bands (0.5 and 0.6 kb patterns) are found in all *T. aureroviride* isolates, but only the first band (0.5 kb pattern) exists at the *T. harzianum* isolates (Figure 5A). Amplification with OPA3 primer generated the five bands of 0.6 to 1 kb in all the *T. aureroviride* isolates (Fig. 5B). These bands are diagnostic for *T. harzianum* and *T. aureroviride*. A-5 and OPA3 primers did not produce any monomorphic band (Fig. 5B). The RAPD data was computed into a similarity matrix using the NTSYS computer program. The genetic similarity coefficient between pairwise isolates varied from 0.47

to 0.96 based on an unweighted paired group method of arithmetic average (UPGMA) cluster analysis. Based on the results, 16 isolates can be grouped into two major clusters, which were labeled as I and II and shared 0.47% similarity (Fig. 6). Major cluster I consisted of 10 isolates identified as *T. harzianum* based on morphological and molecular analyses. Major cluster II consisted of six isolates, which were morphologically identified as *T. aureroviride* cluster I is subdivided into two sub-clusters where the first sub-cluster has eight isolates and the second one has two isolates of two subclusters. *Trichoderma aureroviride* cluster is divided into two different clusters, containing two and four isolates. The RAPD primers OPA4, OPA3, A-5, and Pr3 could discriminate *T. aureroviride* from the other isolates.

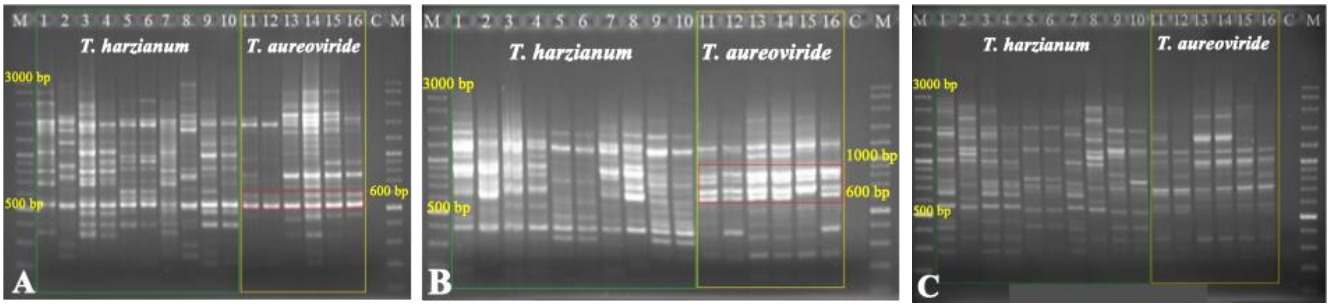

Fig. 5. RAPD PCR fingerprinting patterns from genomic DNA of *Trichoderma harzianum* (1.Th 1-1, 2.Th 27-65,3.Th 19-43,4.Th 23-53,5.Th 22-45,6.Th 24-61,7.Th 33-113,8.Th 52-134,9.Th 55-147 and10.Th 38-127) and *Trichoderma aureoviride*(11.Ta 1-41,12.Ta 2-43,13.Ta 9-117,14.Ta 39-118,15.Ta 7-116 and 16.Ta 43-142) generated with primers OPA 4 (A), OPA 3 (B), and Pr3 (C). M: 3 kb ladder (VC 100 bp Plus DNA Ladder, Mass ruler Mix, 0.1–3kb).

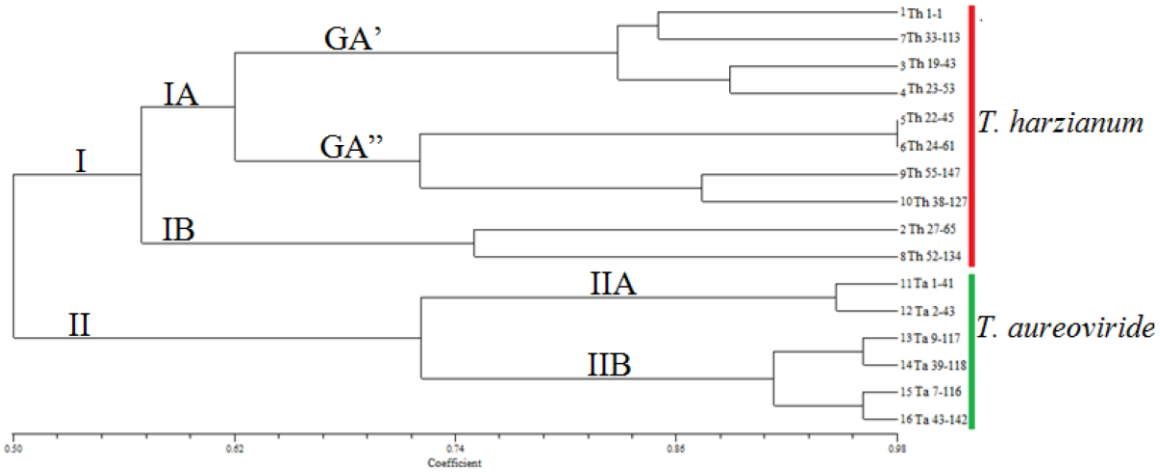

Fig. 6. Dendrogram derived from the RAPD analysis of *Trichoderma harzianum* (10 isolates) and *Trichoderma aureoviride* (six isolates) using primers A-5, OPA 4, OPA 3, Pr3. The downscale is the percentage of similarity by the Dice similarity coefficient.

Table 3 Analysis of the polymorphism obtained with RAPD markers in 16 *Trichoderma* isolates

Primers	Total no. of bands amplified	Range of size of the amplification products	No. of monomorphic bands	No. of polymorphic bands
OPA 4	30	200-3000	1	29
OPA 3	14	250-1800	0	14
A-5	16	400-2900	0	16
Pr3	25	280-2100	3	22
Total	85	200-3000		

Discussion

Trichoderma species, as biological fungi exist in a wide range of ecological environments such as soil, air, plant surface, and other environments. These fungi are widely used for the control of plant pest as well as a variety of plant diseases (Zheng et al., 2021; Wang et al., 2022). However, the choice of active *Trichoderma* isolates is important in designing effective and safe biocontrol strategies. In fact, acidity and alkaline conditions are factors that could be affected by *Trichoderma* species such as its presence, density, longevity, and production of enzymes (Michel-Aceves et al., 2001; Kredics et al., 2003; Samaniego, 2008). To this, we aimed to isolate and identify indigenous *Trichoderma* isolates from alkaline soils and use as a biocontrol agent. The results of the present study showed that *T. harzianum* and *T. aureroviride* were predominant species in alkaline soils. These results are in general agreement with the earlier investigation (Gherbawy et al., 2004). Based on morphological criteria, the producing of brownish yellow colony with the development of needle shape, golden yellow crystals are observed as the characteristics of the *T. aureroviride* 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 related to *T. harzianum* and *T. aureroviride* are grown fast at 25, 30 and 35°C on PDA. Jaklitsch (2009) reported that the anamorph isolates related to *T. lixii* that were obtained from Europe, was grown 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 have a clear shape. In our study, *T. harzianum* isolates did not produce crystals, whereas all *T. aureroviride* isolates produced a needle shape crystals bodies with golden yellow color and were stable in medium. Inter-and intra-specific variation was assessed by two different approaches including the rRNA gene ITS sequencing and RAPD PCR analyses. Detailed analyses of the rRNA gene ITS sequences from the *T. harzianum* and *T. aureroviride* isolates revealed high similarity and existence very little of intra or inter-isolates variability. In this study, isolates of both species *T. harzianum* and *T. aureroviride* with having identical ITS sequence regions were not differentiated, but morphological characteristics could be identified as the both *Trichoderma* species. The dendrogram based on the ITS rRNA gene analysis was shown that 17 isolates located within *Trichoderma* sect. *Pachybasium* were grouped in a complex including *T. harzianum* and *T. aureroviride* (Fig. 3 and 4). Consistent with the results obtained in this study, several authors have also reported a genetic variability and diversity among *T. harzianum* isolates (Bisby, 1939; Bowen et al., 1996) as well as *T. aureoviride*, (Samaniego, 2008; Siddiquee et al., 2012). The results of RAPD PCR analyses using four random primers were distinguished *T. harzianum* from *T. aureoviride.* The RAPD technique produced three common bands (500 bp by OPA4, 350, 700bp and 1200 bp by Pr3) among all the isolates. These common bands are informative and have the potential to be developed as a diagnostic tool for the detection of *Trichoderma* species. Sequence characterized amplified region markers have been developed for identification and detection of *Trichoderma* species such as *T. asperellum* (Liu et al., 2010) and *T. viride* (Song et al., 2010) as well as other fungal taxa (*Aspergillus niger*) (Awan et al., 2011). Narayan et al. (2006) showed that some of the RAPD markers were useful for identifying isolates of *T. aureoviride* within the complex collections. Siddiquee et al. (2012) showed different degrees of the genetic similarity among 42 isolates of *T. aureoviride* by using five primers for the Random Amplified Microsatellites (RAMS) analysis. The dendrogram based on the UPGMA analysis from RAPD data analyses consisted of two major clusters. Cluster I consisted of 10 isolates which were morphologically identified as *T. harzianum*. Cluster II consisted of six isolates which were morphologically identified as *Trichoderma* sp. In conclusion, the results of the present study revealed that the PCR based fingerprinting technique, RAPD, is informative to assess the amount of genetic variability as well as to elucidate the genetic relationships among isolates of different *Trichoderma* species. Ample polymorphism levels were found using the tested primer sets. They enabled the establishment of informative fingerprints for the analyzed isolates. In our study the RAPD primers used amplified DNA fragments that are diagnostic for *T. harzianum* and *T. aureoviride* species. The RAPD technique seems to be a useful diagnostic tool for the distinguishing of *Trichoderma* species.

Acknowledgments

We express our appreciation to Vali-e-Asr University Research Council of Rafsanjan for financial support. We greatly appreciate M. Gorgi (Jihad-e-Agriculture center of Anar) and H. Masoumi (Iranian Pistachio Research Institute) for providing the pistachio soil samples.

Conflict of interest

The authors declare that there are no conflicts of interest present.

CRediT author statement

H. Alaei: Supervision, methodology and data analysis**. F. Mirkhani:** Field and laboratory works. **A. H. Mohammadi:** Supervision and data analysis**. M. Haghdel:** reviewing & editing.

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