Molecular Characterization of Some Soil Originated *Trichoderma* Species

Toprak Kökenli Bazı *Trichoderma* Türlerinin Moleküler Karakterizasyonu

Research Article

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ABSTRACT

The aim of this study was the molecular characterization of the some *Trichoderma* species which were previously isolated by cultural sampling methods from agricultural soils. A 20 of 39 strains of *Trichoderma* which were identified to species level by morphological data. Their characterization studies were based on the internal transcribed spacer regions 1 and 2 (*ITS 1* and *ITS2*) of ribosomal RNA (rRNA) and a fragment of the translation elongation factor 1-alpha (tef1 gene) sequences. Based on the sequence data and cultural examinations, strains were grouped into four which are included in: *afroharzianum*, *atroviride*, *gamsii* and *harzianum*. Among them,*T. gamsii* was the most encountered species. The species *T. afroharzianum* was reported as the first time for Turkey.

Key Words

Trichoderma, molecular characterization, soil.

ÖZ

Bu çalışmada zirai topraklardan kültürel örnekleme yöntemleri ile izole edilmiş bazı *Trichoderma* türlerinin moleküler karakterizasyonu amaçlanmıştır. Morfolojik olarak tanımlanmış 39 *Trichoderma* strainin 20 si moleküler olarak karakterize edilmiştir. Karakterizasyon çalışmaları ribozomal RNA (rRNA)'nın internal transkript edilen bölgeleri 1 ve 2 (*ITS 1* ve *2*) ve translasyon elongasyon faktör 1-alfa (tef 1 geni) dizilimlerine göre yapılmıştır. Dizilim verisi ve kültürel değerlendirmelere göre strainler dört grup olarak değerlendirilmiştir: *afroharzianum*, *atroviride*, *gamsii* ve *harzianum*. Bunlar arasında *T. gamsii* en çok karşılaşılan türdür. *T. afroharzianum* türü Türkiye için ilk kez bildirilmektedir.

Anahtar Kelimeler

Trichoderma, moleküler karakterizasyon, toprak.

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INTRODUCTION

Fungi of the genus *Trichoderma* are recognized as green spored ascomycetes including more than 200 species, largely based on multi-gene phylogeny, in the different geographical regions and climatic zones of World [1-3]. Most common and natural habitat of these fungi is known to be soil [4].

They are ubiqitous colonizers of cellulosic materials and can thus be found everywhere decaying plant materal is available as well as in the rhizosphere of plants. The members of this genus are characterized by rapid growth, mostly bright green conidia and a repetitively branched conidiophore structure, an ability to assimilate a diverse array of substrates and by their production of a range of antimicrobials [2].

The occurrence of *Trichoderma* spp. in various soils, such as agricultural, forest, prairie, desert or salt marsh, of all climatic zones has been the subject of several investigations. Agricultural soils are rich sources of potential biocontrol agents of genus *Trichoderma*. *T. harzianum* is the most familiar *Trichoderma* species as it is the most frequent *Trichoderma* sp. in the majority of samples worldwide. It was originally defined as a 'species aggregate', but Chaverri et al., reported that it consists of seven genetic lineages which would fulfill the basic criteria of cryptic phylogenetic species wthin a large morphological species [5].

Most of the methodologies to study about the *Trichoderma* biodiversity applied the standard culture-based approaches comprising the collection of samples, isolation of *Trichoderma* strains on the selective media and the identification of species based on conidiophore structure, morphology as well as the size and morphology of conidia can be performed with the aid of taxonomic keys and descriptions available in the literature. However, without professional expertise this may often lead to incorrect diagnoses due to the difficulties of morphology based identification. In order to get around such problems and give precise speies-level diagnoses the use of biochemical and molecular methods is recommended [6,7].

Among the current molecular characterization methods that based on the conserved/coding regions of DNA, the internal transcribed spacer (ITS) region in the ribosomal RNA (rRNA) is the most useful, species specific and accepted as the formal fungal barcode. However, identifications based on ITS sequences must be used with caution because sometimes closely related species cannot be distinguished using this gene. This is especially true of *Trichodema* sect. *Trichoderma*, where more than one species can share the same ITS sequences. Because *tef* is more variable than the ITSrDNA, it is better able to reflect species differences within and among groups of closely related species [8]. A further problem with identifying isolates by BLAST searching GenBank is the number of sequences deposited for misidentified strains. An interactive key to identification of several *Trichoderma* species based on ITS and multiple genes can be found online by the International Submission on *Trichoderma* and *Hypocrea* (ISTH). The ITS based online barcoding program *Trich*OKEY also provides useful tool for the identification of *Hypocrea*/*Trichoderma* strains [9]. The ISTH website (www.isth.info) also provides the primer sequences and protocols for sequencing the genes used for identification [10].

Chaverri et al. [10] studied a wide morphological and geographic diversity of isolates using four genes. They concluded that, this common, cosmopolitan species complex with distinct, partly geographically defined phylogenetic lineages that lacked diagnostic morphological characters.

Altough the genus *Trichoderma* is represented in soils and other organic matter collected at all latitudes, some species are widely distributed while other species are geographically limited in their distribution. Some species of *Trichoderma* such as *T. harzianum* and *T. asperellum* are truly cosmopolitan. In this study the characterization of some *Trichoderma* strains previously isolated from agricultural soils using molecular techniques was aimed.

MATERIALS and METHODS

Trichoderma strains

A total of 20 strains of *Trichoderma* which were previously isolated from the soil that planted vi-

neyard, wheat and corn were investigated in this study. They are maintained in the stock cultures in our laboratory culture collection.

Identification of *Trichoderma*

In this study, obtained isolates were identified at the species level by a combination of morphological and molecular examinations.

Morphological observations of colonies were based on isolates grown on SNA8 (Synthetischer Nahrstoffarmer Agar, Nirebnerg, 1976), PDA (Merck potato dextrose agar) and CMD (corn meal dextrose agar) for up to three weeks in 25ºC and 35ºC with alternating 12h/12h fluorescent light/darkness. Characters of the conidium bearing structures and conidia were assessed from the edges of the cultures grown on SNA. Morphological traits (e.g. phialide lenghts and width, conidium lenghts and widths) were measured and recorded [11].

Growth rate examinations were done in 9 cm petri dishes with the PDA, SNA and CMD at 25, 30, 35ºC. Cultures were grown in darkness up to 1 week until they covered the whole agar surface. Measurements of colony radius were taken daily.

For DNA extraction strains were grown in 9 cm petri dishes containing PDA (Merck) for 3-4 days at 25ºC. DNA was extracted for the mycelium from the surface of the medium. The DNA was isolated by the EurX geneMatrix DNA Isolation Kit according to the manufacturer's instructions. Both the purity and quantity of DNA were checked by agarose gel electrophoresis, determined by the NanoDrop spectrophotometer (Figure 1).

DNA sequences of nuclear internal transcribed spacers of rRNA regions (*ITS* 1&2) and *tef* 1 gene was used to amplify by PCR in thermal cycler (Veriti, Applied Biosystems). The conditions and primers were applied as indicated in Hojos-Carvajal et al. [11]. Primers used are given in below (Table 1).

Amplicons were purified and sequenced by MacroGene. For species identification, sequences were subjected to analysis by BlastN (NCBI).

Bioinformatic analysis and *Trichoderma* species identification

The phylogenetic analyses were based on the selected *ITS* 1&2 and *tef* 1 gene sequences obtained in this work as well as on the sequences retrieved from NCBI GenBank as the closest matches. The raw sequence reads of *ITS*1&*ITS*2, *tef*1 were manually edited. Then, DNA sequences were initially aligned with multiple sequence alignment program CLUSTAL W. Phylogenetic relationships were reconstructed with MEGA 7 software version using the maximum parsimony approach with complete deletion all positions containing gaps and missing data. All reconstructions were tested by bootstrapping with 1000 replicates.

Sequences of the selected strains are available at the National Center for Biotechnology Information (NCBI) GenBank (accession numbers available are provided in Table 2).

RESULTS and DISCUSSION

39 *Trichoderma* isolates obtained from the cultivated soils were identified at the species level on morphological as well as molecular analyses. Combined analyses of the strains based on sequences of *ITS*1, *ITS*2 and the fragment of *tef*1 gene confirmed that all the strains were belong to 4 species group which are; *gamsii*, *atroviride*, *afroharzianum* and *harzianum*. Recognized species are in given in the Table 2.

The results of the phylogenetic relationship of the strain groups are given in the Figures 2-4.

The different soil types have been studied for the diversity of *Trichoderma* spp., worlwide. Alkaline pistachio soils at different geographic areas in Kerman province, Iran was investigated. Isola-

Table 1. Primers used for the amplification of ITS region and tef1.

Locus	Primer name	Sequence (5'- 3')	Average Amplicon lenght (kb)
*ITS*1 and 2	NS5-fw	CTTCCGTCAATTCCTTTAAG	0.6
	NS4-rev	TCCTCCGCTTATTGATATGC	
tef 1 (eEF1a1)	Tef71f	C AAA ATG GGT AAG GAG GAS AAG AC) CA GTA CCG GCR GCR ATR ATS AG	1.2
	Tef 997R		

Table 2. Selected 20 *Trichoderma* strains used for the molecular characterization in this study and their accession numbers in NCBI GenBank.

Strain no	Species name	NCBI GenBank accession numbers	
		ITS1 and 2	***tef*** 1
TR1	***T. gamsii***	KP211554	*
TR3	***T. atroviride***	KP211541	KY769053
TR5	***T.atroviride***	KP211542	*
TR8	***T. atroviride***	KP211543	*
TR9	***T. atroviride***	KP211544	*
TR12	***T. atroviride***	KP211545	KY769054
TR17	***T. gamsii***	KP211546	*
TR18	***T. gamsii***	KP211547	KY769055
TR22	***T. gamsii***	KP211548	KY784145
TR25	***T. gamsii***	KP211549	*
TR27	***T. gamsii***	KP211550	KY784146
TR28	***T. atroviride***	KP211539	*
TR29	***T. gamsii***	KP211551	*
TR31	***T. gamsii***	KP211552	*
TR32	***T. gamsii***	KP211553	*
TR33	***T. atroviride***	KP211540	*
TR37	***T. afroharzianum***	*	KY784147
TR41	***T. harzianum***	*	KY769056
TR43	***T. afroharzianum***	*	KY76905
TR47	***T. afroharzianum***	*	KY784148

*: not submitted

Figure 1. (a) Genomic DNAs extracted from different isolates of *Trichoderma* species, (b) DNA fragments amplified in PCR using specific primers for *Trichoderma*.

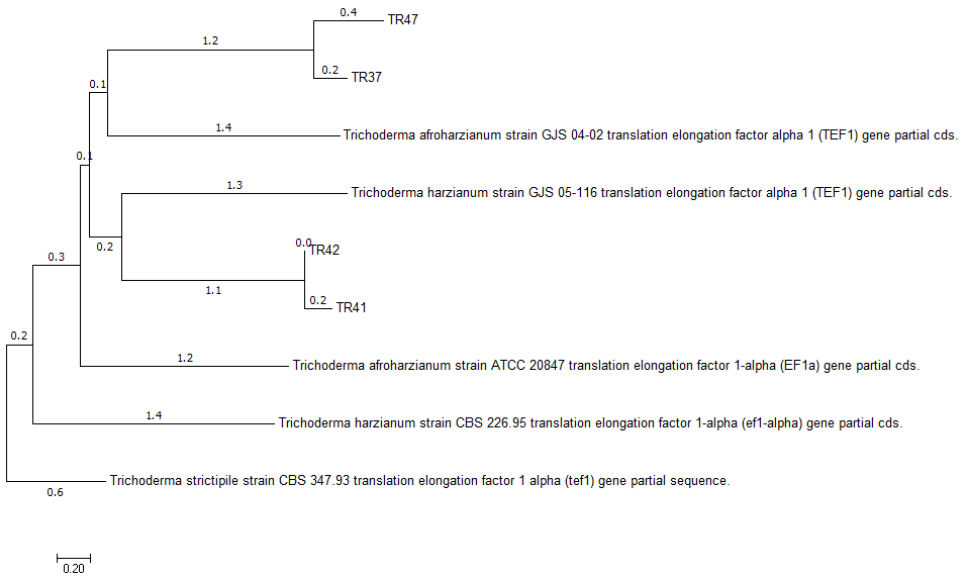

Figure 2. Tree of *T. atroviride* isolates obtained by phylogenetic analyses of *ITS* 1&2 sequences according to Maximum Composite Likelihood Model, Neighbour-Joining Method. Bootstrap; 1000. Out group: *Trichoderma gamsii* GJS 07-111.

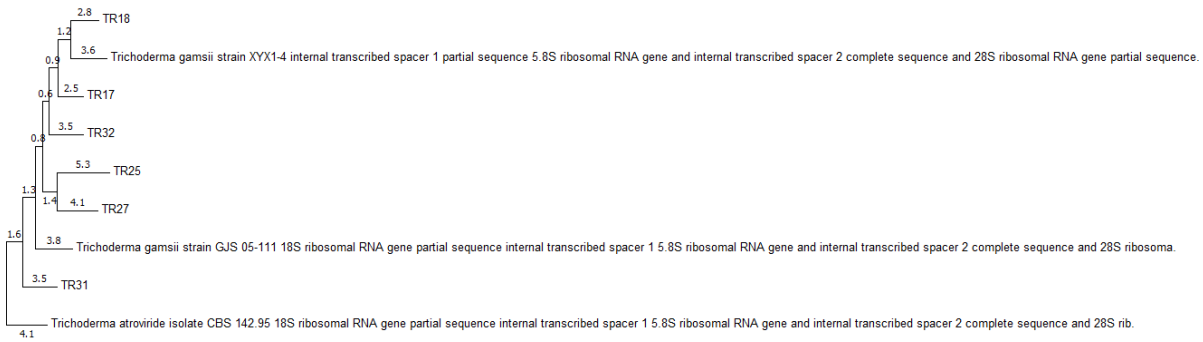

Figure 3. Tree of *T.gamsii* isolates obtained by phylogenetic analyses of *ITS 1&2* sequences according to Maximum Composite Likelihood Model, Neighbour-Joining Method. Bootstrap; 1000. Out group: *Trichoderma* atroviride isolate CBS142.95.

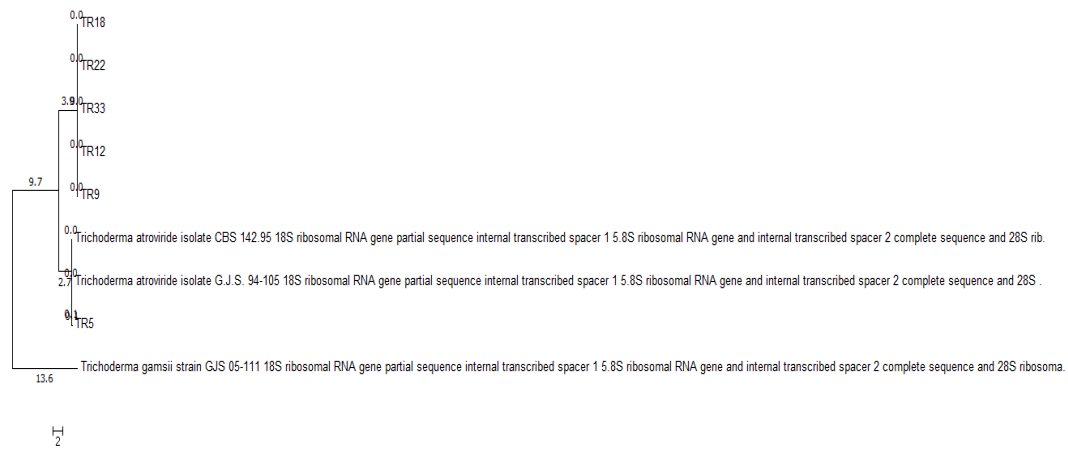

Figure 4. Tree obtained by phylogenetic analyses of *tef*1 sequences of *T. afroharzianum* and *T. harzianum* strains according to Maximum Composite Likelihood Model, Neighbour-Joining Method. Bootstrap; 1000. Out group: *Trichoderma strictipile* (AY865644).

tes were examined by analysis of their morphological caharacters and sequence analysis of their *ITS*1-2 and *tef1* gene. *T. harzianum* as reported as most encountered species among the five species [12]. In order to determine the presence of *Trichoderma* and *Gliocladium*, different soil samples from nine regions of Turkey were collected. *T. harzianum* has been reported as the most common species [13].

Chakraborty et al. [14] have isolated *Trichoderma* stains from the soil of agricultural, forest and the rhizosphere of plantation crops. They have isolated 19 species which are identified as *T. viride* (11 strains) and *T. harzianum* (8 strains), based on molecular characterization with ITS PCR. They have also studied the polymorphism of ITS region of rDNA among the isolates.

18 *Trichoderma* spp. isolates were obtained from the soil of different provinces located in central and northern regions of Turkey and their identification was done on the basis of microscopic examination. The most common identified species was *T. harzianum*. They have tested the strains for the controlling studies by targeting the protozoal vector responsible for sugar beet disease as the first time [15].

Küçük and Kıvanç [16] have investigated the different features of *Trichoderma* spp. that were isolated from different soil samples in Eskişehir. *T. harzianum* was isolated as common species.

Migheli et al. [17] have studied the distribution of *Trichoderma* spp. from 15 soils comprising undisturbed and disturbed environments (forest, shrublands and undisturbed or extensively grazed gras steppes) in Island of Sardinia (Italy). Majority of isolates were identified as pan-European and/or pan-global *Hypocrea/Trichoderma* species. *H. lixii/T .harzianum* was the most abundant species (57%) among the identified 14 and 1 unidentified species and present in 10 of 15 sites investigated. They have found the total diversity index very low as 0.03.

With the increasing importance of sequence data in species definition and recognition, morphological species defnition and delimitation becomes less applicable or even impossible. While *tef*1 the main and best resolving marker used for species definition in *Trichoderma* may overemphasize seperation at the species level, it demonstrates the presence of a high biodiversity of genetically clearly seperated cryptic species [18]. By the intensively surveyed studies biogeographical and ecological disitribution of *Trichoderma* has expanded, recently.

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