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Molecular characterization of *Trichoderma* sp. isolated from rhizospheric soils of Uttar Pradesh (India) based on microsatellite profiles

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The objectives of this research were to characterize isolates of *Trichoderma* collected from rhizospheres of chickpea, pigeonpea and lentil crop from different places of Uttar Pradesh, India, using microsatellite-primed polymerase chain reaction (MP-PCR) and ribosomal DNA (rDNA) sequence analysis and to combine these results with morphological characteristics for classification. Thirty isolates of *Trichoderma* sp. obtained from rhizosphere soil of plantation crops, and agricultural fields of UP region were studied using inter-simple sequence repeat (ISSR) and Internal transcribed spacer- polymerase chain reaction (ITS-PCR). The genetic relatedness among 15 isolates of *Trichoderma* sp. was analyzed with six micro-satellite primers. ISSR profiles showed 83.7% genetic diversity among the isolates with the formation of four clusters. Analysis of dendrogram revealed that similarity coefficient ranged from 0.27 to 0.95. ITS-PCR of rDNA region with ITS1 and ITS4 primers produced 600 bp products in all isolates. This result presented the identification patterns of *Trichoderma* isolates.

Key words: *Trichoderma sp.*, genetic diversity, polymerase chain reaction (PCR), molecular marker, microsatellite.

INTRODUCTION

Soil microorganisms influence ecosystems by contributing to plant nutrition (Alan et al., 1998), plant health (Bruns et al., 1991), soil structure (Castle et al., 1998) and soil fertility. It has been widely recognized, particularly in the last two decades, that majority of harsh environments are inhabited by surprisingly diverse microbial communities. Bacteria, actinomycetes and fungi are three major groups of soil inhabiting microorganisms.

An estimated 1,500,000 species of fungi exist in the world (Anu et al., 2010).

Trichoderma, commonly available in soil and root eco-systems has gained immense importance since the last few decades due to its biological control ability against several plant pathogens (Elad and Chet, 1983). Antagonistic microorganisms, such as *Trichoderma,* reduce growth, survival or infections caused by pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions and enzyme secretion. In addition,

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the release of biocontrol agents into the environment has created a demand for the development of methods to monitor their presence or absence in soil (Giller et al., 1997). Therefore, monitoring population dynamics in soil is of much importance. Previous methods employed to identify strains of *Trichoderma* spp. in soil samples have included the use of dilution plates on selective media (Lieckfiledt et al., 1999). However, this method does not distinguish between indigenous strains and artificially introduced ones (Knudsen et al., 1996). The *Trichoderma* isolates were differentiated by mycelia growth rate and colony appearance, as well as microscopic morphological features, including phialides and phialospores (Hibbett, 1992). These can also be distinguished by randomly amplified polymorphic DNA (ISSR)-PCR, restriction fragment length polymorphisms in mitochondrial DNA and ribosomal DNA and sequence analysis of ribosomal DNA (Mukherjee et al., 2013; Kubicek and Harman, 1998; Bryan et al., 1995). The use of molecular phylogenetic markers has refined *Trichoderma* taxonomy significantly, and phylogenetic analysis of the large number of *Trichoderma* specie is still a field of active ongoing research. Microsatellites, which are also known as short tandem repeats or simple sequence length polymorphisms, are stretches of tandem mono-, di-, tri-, and tetranucleotide repeats of varying lengths (Sagar et al., 2011). Such sequences are widely dispersed in eukaryotic genomes including those of fungi; they are also present but less frequent in prokaryotic genomes.

MATERIALS AND METHODS

Isolation and Identification of *Trichoderma*

Trichoderma isolates were originally isolated from soil collected from rhizospheres of chickpea, pigeonpea and lentil crop from different places of Uttar Pradesh, India, and *Trichoderma* isolates were isolated on Potato Dextrose Agar medium by following serial dilution plate technique. They were cultured on PDA 25°C for seven days. After an incubation period, colonies were purified and determined to be *Trichoderma* species and confirmed using *Trichoderma* morphological key. The identity of the purified bioagents was then confirmed by ITCC, Division of Plant Pathology IARI, New Delhi-12. Single-spore isolates of 30 *Trichoderma* isolates were cultured in Erlenmeyer flasks (250 ml) containing 100 ml potato dextrose broth at 25°C for seven days. Mycelia were harvested by filtration through whatman filter paper. Samples were frozen in liquid nitrogen and ground to fine powder using a mortar and pestle (Shahid et al., 2014).

Genomic DNA extraction from *Trichoderma* Isolates

Isolation of fungal genomic DNA was done by growing the fungi for 3-4 days. The mycelia were incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 100 mM NaCl and 2% SDS, for 1 h at 60°C followed by centrifugation at 12,000 rpm for 15 min (Shahid et al., 2014). The supernatant was then extracted with equal volume of water saturated phenol and further centrifuged at 12,000 rpm for 10 min; the aqueous phase was further extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuge at 12,000 rpm for 15 min; the aqueous phase was then transferred in a fresh tube and the DNA was precipitated with chilled ehanol (100%) (Yao et al., 1992). DNA was pelleted by centrifuging at 12000 rpm for 15 min and washed in 70% ethanol by centrifugation. The pellets were air dried and suspended in TE buffer (pH 8.0).

Qualitative and quantitative estimation of DNA

The extraction of total genomic DNA from the *Trichodrma* isolates as per the above procedure was followed by RNAase treatment. Genomic DNA was re suspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse (60 µg). After incubation the sample was reextracted with PCI (phenol: chloroform: isoamyl-alcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both and isolates of *Trichoderma* were taken up for ITS-PCR spectrophotometrically and in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

PCR Amplification of its region of *Trichoderma* Isolates

Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase chain reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s and extension at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µ1) containing 0.25% bromophenol blue, 40% w/v sucrose in water and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination with horizontal electrophoresis.

ISSR of *Trichoderma* Isolates

For ISSR, six microsatellite primers that is, A-1; A-2; A-3; A-4; A-5 and A-6 were selected (Table 1). PCR was programmed with an initial denaturing at 94°C for 4 min followed by 35cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 70°C for 90 s and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25% bromophenol blue, 40% w/v sucrose in water and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination by horizontal electrophoresis (Venkateswarlu et al., 2008).

Scoring and data analysis

The image of the gel electrophoresis was documented through Bio-Profil Bio-1D gel documentation system and analysis software. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed *In silico* into similarity matrix using Numerical Taxonomy System Biostastiscs, (NTSYSpc version 2.11W) (Muthumeenakshi et al, 1994). The SIMQUAL program was used to calculate the Jaccard's coefficients. The ISSR patterns of each isolate was evaluated, assigning character state "1" to indicate the presence of band in the gel and "0" for its absence in the gel.

Thus a data matrix was created which was used to calculate the

Table 1. The nucleotide sequence used for ITS and *Trichoderma* PCR.

Priner Name	Sequence(5'-3')	Mer	TM	GC (%)
ITS-Primers pairs				
T/ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57
T/ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45
ISSR primers				
A-1	5'YC $(TG)_7$T3'	17	49.77	47
A -2	5'$(GA)_9$AC3'	20	53.70	55
A-3	5'$(GA)_9$T3'	20	58.01	47
A-4	5'$(GA)_8$AC3'	18	56.35	40
A-5	5'$(AG)_8$AC3'	18	60.17	50
A-6	5'$(AG)_8$AT3'	18	60.26	47

Table. 2 Isolates of *Trichoderma* spp.

S/N	ITCC number IARI, New Delhi	Culture number	Reference number	Source	Fungus identified
T1	ITCC-7437/09	21PP	01	Kausambi	*Trichoderma longibrachiatum*
T2	ITCC-7438/09	31PP	02	Allahabad	*Trichoderma longibrachiatum*
T3	ITCC-7439/09	81PP	03	Mirzapur	*Trichoderma longibrachiatum*
T4	ITCC-7440/09	100PP	04	Sonbhadra	*Trichoderma longibrachiatum*
T5	ITCC-7441/09	120PP	05	Bhadohi	*Trichoderma longibrachiatum*
T6	ITCC-7442/09	06 CP	06	Sultanpur	*Trichoderma atroviride*
T7	ITCC-7443/09	24CP	07	Sitapur	*Trichoderma atroviride*
T8	ITCC-7444/09	28CP	08	Barabanki	*Trichoderma longibrachiatum*
T9	ITCC-7445/09	71L	09	Hardoi	*Trichoderma atroviride*
T10	ITCC-7446/09	115L	10	Bahraich	*Trichoderma atroviride*
T11	ITCC-7447/09	52L	11	Unnao	*Trichoderma atroviride*
T12	ITCC-7448/09	75PP	12	Auriya	*Trichoderma atroviride*
T13	ITCC-7449/09	126PP	13	Kanpur Dehat	*Trichoderma atroviride*
T14	ITCC-7450/09	5 CP	14	Kanpur Nagar	*Trichoderma longibrachiatum*
T15	ITCC-7451/09	105PP	15	Etawah	*Trichoderma atroviride*

Jaccard similarity coefficient for each pair wise comparison. Jaccard coefficients were clustered to generate dendograms using the SHAN clustering programme, selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc (Knudsen et al., 1996).

RESULTS AND DISCUSSION

Thirty isolates were obtained using the *Trichoderma* selective medium from the rhizosphere soil (Table 2). The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Muthumeenakshi et al., 1994). They also occur in multiple copies with up to 200 copies per haploid genome (Ospina-Giraldo et al., 1999; Ospina-Giraldo et al., 1998) arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S and the 28S large subunit (LSU) genes. Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Rohlf, 1993). In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies. In the present study, we focused on the ITS regions of ribosomal genes for the construction of primers that can be used to identify *Trichoderma* spp. ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 primers. These results are in accordance with several workers who observed the amplified rDNA fragment of approximately 500 to 600 bp by ITS-PCR in *Trichoderma* (Sagar et al., 2011; Seaby, 1996). The ITS PCR has helped to detect polymorphism at ITS region of rDNA among the *Trichoderma* isolates (Figure 1). Products of size in the range of 600 bp were

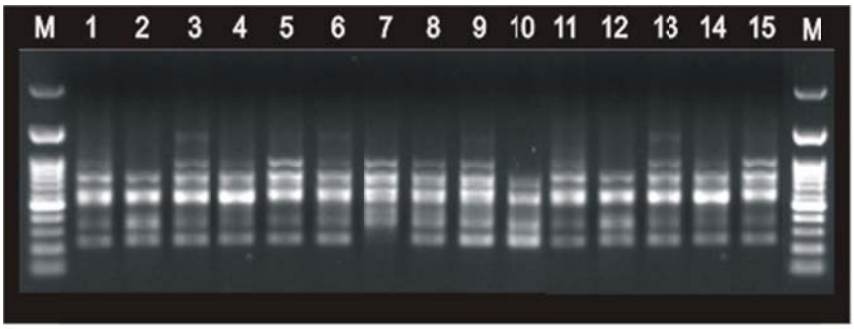

Figure 1. PCR amplification of ISSR (A1 marker) of *Trichoderma* species (Lane 1-15). Lane M, Low range DNA Marker (1 kb); Lane 1, ITCC,7437/21PP; lane 2, ITCC,7438/31PP; Lane 3, ITCC,7439/81PP; Lane 4, ITCC,7440/100PP; Lane 5, ITCC,7441/120PP; lane 6, ITCC,7442/06 CP; lane 7, ITCC,7443/24CP; lane 8, ITCC,7444/28CP; Lane9, ITCC,7445/71L; lane 10, ITCC,7446/115L; Lane 11, ITCC,7447/52L; lane 12, ITCC,7448/75PP; lane 13, ITCC,7449/126PP; lane 14, ITCC,7450/5 CP; lane 15, ITCC,7451/105PP.

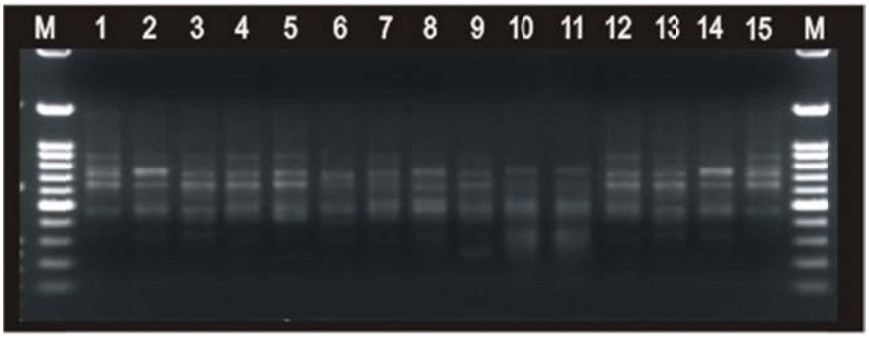

Figure 2. PCR amplification of ISSR (A2 marker) of *Trichoderma* species (Lane 1-15). Lane M, Low range DNA Marker (1 kb); Lane 1, ITCC,7437/21PP; lane 2, ITCC,7438/31PP; Lane 3, ITCC,7439/81PP; Lane 4, ITCC,7440/100PP; Lane 5, ITCC,7441/120PP; lane 6, ITCC,7442/06 CP; lane 7, ITCC,7443/24CP; lane 8, ITCC,7444/28CP; Lane9, ITCC,7445/71L; lane 10, ITCC,7446/115L; Lane 11, ITCC,7447/52L; lane 12, ITCC,7448/75PP; lane 13, ITCC,7449/126PP; lane 14, ITCC,7450/5 CP; lane 15, ITCC,7451/105PP.

produced by Mukherjee et al. (2002) who studied the identification and genetic diversity analysis.

The genetic relatedness among 30 isolates of *Trichoderma* sp. and were analyzed by six microsattelite primers A-1 (Figure 1) give four polymorphic loci; A-2 (Figure 2) give six polymorphic loci; A-3 (Figure 3); give 9 polymorphic loci A-4 (Figure 4); give three polymorphic loci A-5 (Figure 5) give four polymorphic loci (Smith and Goodman, 1999). All amplified products with the primers had shown polymorphic and distinguishable banding patterns which indicate the 83.7% genetic diversity of Trichoderma isolates. A total of 31 reproducible and 26 scorable polymorphic bands ranging from approximately 100 to 2000 bp were generated with six primers among the 15 *Trichoderma* isolates (Table 3). ISSR profiles showed that primer A-2 and A3 scored highest bands which ranged between 100 to 2000 bp. Relationships among the isolates was evaluated by cluster analysis of the data based on the similarity matrix (Figure 6). The dendogram was generated UPGMA using NTSYSpc software. Analysis of dendrogram revealed that similarity coefficient ranged from 0.27 to 0.95. Based on the results obtained all the 15 isolates can be grouped into four main clusters. First cluster represents five *Trichoderma*.isolates, second contains three, third cluster contain three and finally fourth cluster contain four isolates, respectively. Shahid et al. (2014) also revealed that *Trichoderma* sp. also showed the robust polymorphism which were collected from different geographical locations of Uttar Pradesh, India (Yao et al., 2010; Wright and Upadhyaya, 1998).

Conclusion

Preliminary studies indicates that the *Trichoderma* isolates

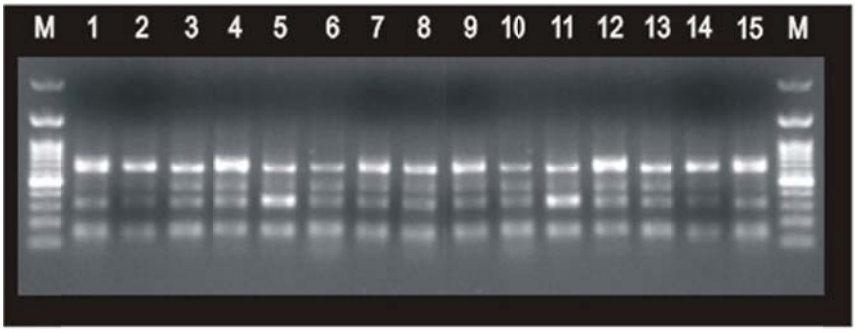

Figure 3. PCR amplification of ISSR (A3 marker) of *Trichoderma* species (Lane 1-15). Lane M, Low range DNA Marker (1 kb); Lane 1, ITCC,7437/21PP; lane 2, ITCC,7438/31PP; Lane 3, ITCC,7439/81PP; Lane 4, ITCC,7440/100PP; Lane 5, ITCC,7441/120PP; lane 6, ITCC,7442/06 CP; lane 7, ITCC,7443/24CP; lane 8, ITCC,7444/28CP; Lane9, ITCC,7445/71L; lane 10, ITCC,7446/115L; Lane 11, ITCC,7447/52L; lane 12, ITCC,7448/75PP; lane 13, ITCC,7449/126PP; lane 14, ITCC,7450/5 CP; lane 15, ITCC,7451/105PP.

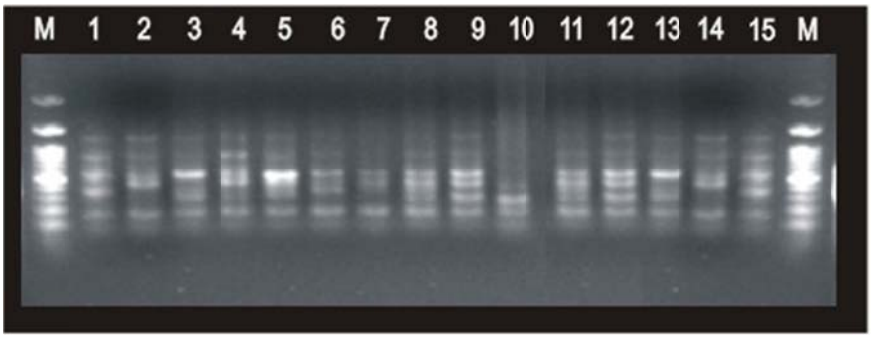

Figure 4. PCR amplification of ISSR (A4 marker) of *Trichoderma* species (Lane 1-15). Lane M, Low range DNA Marker (1 kb); Lane 1, ITCC,7437/21PP; lane 2, ITCC,7438/31PP; Lane 3, ITCC,7439/81PP; Lane 4, ITCC,7440/100PP; Lane 5, ITCC,7441/120PP; lane 6, ITCC,7442/06 CP; lane 7, ITCC,7443/24CP; lane 8, ITCC,7444/28CP; Lane9, ITCC,7445/71L; lane 10, ITCC,7446/115L; Lane 11, ITCC,7447/52L; lane 12, ITCC,7448/75PP; lane 13, ITCC,7449/126PP; lane 14, ITCC,7450/5 CP; lane 15, ITCC,7451/105PP.

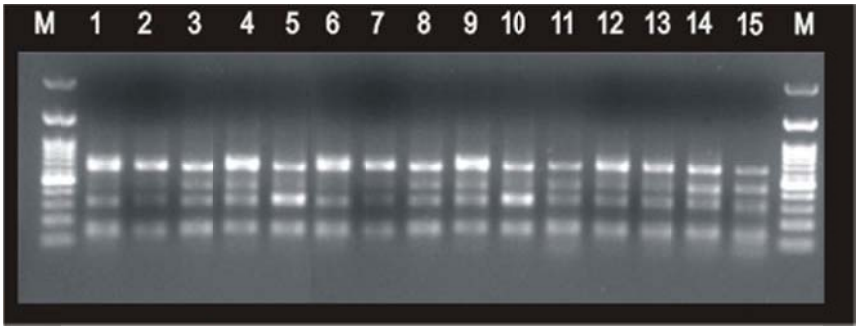

Figure 5. PCR amplification of ISSR (A5 marker) of *Trichoderma* species (Lane 1-15). Lane M, Low range DNA Marker (1 kb); Lane 1, ITCC,7437/21PP; lane 2, ITCC,7438/31PP; Lane 3, ITCC,7439/81PP; Lane 4, ITCC,7440/100PP; Lane 5, ITCC,7441/120PP; lane 6, ITCC,7442/06 CP; lane 7, ITCC,7443/24CP; lane 8, ITCC,7444/28CP; Lane9, ITCC,7445/71L; lane 10, ITCC,7446/115L; Lane 11, ITCC,7447/52L; lane 12, ITCC,7448/75PP; lane 13, ITCC,7449/126PP; lane 14, ITCC,7450/5 CP; lane 15, ITCC,7451/105PP.

Table 3. Analysis of the polymorphism obtained with ISSR markers in 15 *Trichoderma* sp.

S/N	Primers	Total loci	Polymorphic loci	Polymorphism (%)
A-1	5'$(GA)_9$AC3'	6	4	67
A -2	5'$(GA)_9$T3'	6	6	100
A-3	5'$(GA)_8$AC3'	9	9	100
A-4	5'$(AG)_8$AC3'	5	3	60
A-5	5'$(AG)_8$AT3'	5	4	80
Total		31	26	83.87

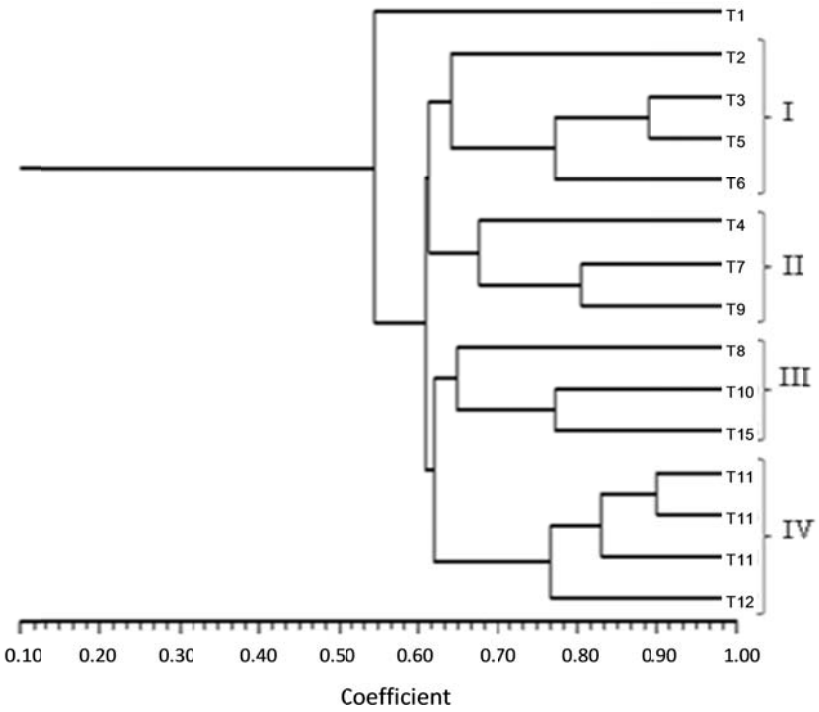

Figure 6. ISSR based phylogenetic trees.

had very good diversity and there are strong possibility to get the isolate specific primers that will be utilized for identifying the particular *Trichoderma* isolates with good biological potential from the field isolates without carrying out the cumbersome bioassay again

Conflict of Interests

The author(s) have not declared any conflict of interests.

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