Global Journal of Biotechnology & Biochemistry 5 (1): 55-61, 2010
ISSN 2078-466X

Molecular Characterization of *Trichoderma viride* and *Trichoderma harzianum* Isolated from Soils of North Bengal Based on rDNA Markers and Analysis of Their PCR-RAPD Profiles

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Abstract: Nineteen isolates of *Trichoderma viride* and *Trichoderma harzianum* obtained from rhizosphere soil of plantation crops, forest soil and agricultural fields of North Bengal region were studied using RAPD and ITS-PCR. The genetic relatedness among eleven isolates of *T. viride* and eight isolates of *T. harzianum were* analyzed with six random primers. RAPD profiles showed genetic diversity among the isolates with the formation of eight clusters. Analysis of dendrogram revealed that similarity coefficient ranged from 0.67 to 0.95. ITS-PCR of rDNA region with ITS1 and ITS4 primers produced 600bp products in all isolates. This result indicated the identification patterns of *Trichoderma* isolates.

Key words: *Trichoderma viride* · *Trichoderma harzianum* · rDNAmarkers · RAPD

INTRODUCTION

Soil microorganisms influence ecosystems by contributing to plant nutrition [1], plant health [2], soil structure [3] and soil fertility [4]. 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 [5].

Trichoderma, commonly available in soil and root ecosystems has gained immense importance since last few decades due to its biological control ability against several plant pathogens [6]. 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, 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. 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. However, this method does not distinguish between indigenous strains and artificially introduced ones [7]. The *Trichoderma* isolates were differentiated by mycelial growth rate and colony appearance, as well as microscopic morphological features, including phialides and phialospores [8]. These can also be distinguished by randomly amplified polymorphic DNA (RAPD)-PCR, restriction fragment length polymorphisms in mitochondrial DNA and ribosomal DNA and sequence analysis of ribosomal DNA [9-13]. Molecular characterization of the potential biocontrol agents using Random Amplified Ploymorphic DNA (RAPD) and Internal Transcribe Spacer-Polymerase Chain Reaction (ITS-PCR), helps to determine the diversity and identification. In the present study, genetic variability in nineteen isolates of *Trichoderma* spp. from different ecosystems were evaluated with six different RAPD markers.

MATERIALS AND METHODS

Isolation and Identification of *Trichoderma:* Soil samples were collected forests, agricultural field and rhizosphere of plantation crops of three districts of North Bengal. The location of soil samples were recorded through GIS mapping tool (Garmin). *Trichoderma* species were isolated in specific selective medium. TSMC which contained (gm/lit); $MgSO_4.7H_2O$-0.2; K_2HPO_4-0.9;

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Table 1: The nucleotide sequence used for ITS and RAPD PCR

PrinerName	Sequence(5'-3')	Mer	TM	% GC
ITS-Primers pairs				
T/ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57%
T/ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45%
RAPD primers				
AA-04	CAGGCCCTTC	10	38.2	70%
OPA-4	AATCGGGCTG	10	39.3	60%
A-11	AGGGGTCTTG	10	31.8	76%
A-5	AGGGGTCTTG	10	36,8	73%
OPD6	GGGGTCTTGA	10	32.8	83%
OPA1	CAGGCCCTTC	10	38.2	70%

Kcl-0.15; NH_4NO_3-1.0; glucose-3.0, chloramphenicol-0.25, fenaminosulf-0.3, pentachloronitrobenzene-0.2, rose Bengal-0.15, captan-0.02 (post autoclaving), agar-20 as well as in modified TSM (Smith *et al*., 1990): containing (gm/lit): $Ca(NO_3)_2$-1.0, KNO_3.0.26, $MgSO_4,7H_2O$-0.26, KH_2PO_4-0.12, $CaCl_2$, $2H_2O$-1.0, citric acid-0.05, sucrose-2.0, agar-20.0, chlortetracycline-0.05, captan (50% wettable powder)-0.04 [14]. The identification of *Trichoderma* isolates were confirmed by National Center of Fungal Taxonomy (NCFT), New Delhi.

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 hr at 60°C followed by centrifugation at 12,000 rpm for 15 min. The supernatant was then extracted with equal volume of water saturated phenol a nd 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%). 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 (60µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol 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 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: All isolates of *Trichoderma* were taken up for ITS-PCR amplification. 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 sec, annealing at 59 °C for 30 sec 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.

RAPD of *Trichoderma* Isolates: For RAPD, six random primers i.e. OPA-1; OPD-6; OPA-4; A-5; AA-04 and AA-11 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 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 by horizontal electrophoresis.

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 NTSYSpc (Numerical Taxonomy System Biostastiscs, version 2.11W) [15]. The SIMQUAL program was used to calculate the Jaccard's coefficients. The RAPD 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 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.

RESULTS AND DISCUSSION

Nineteen isolates were obtained using the *Trichoderma* selective medium from the rhizosphere soil, forest soil and agricultural field (Table 2). Among them eleven isolates were identified as *Trichoderma viride* and eight isolates as *Trichodema harzianum.*

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 [16]. They also occur in multiple copies with up to 200 copies per haploid genome [17-18] 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 [19]. 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. Amplified products of size in the range of 600bp was produced by the primers (Fig 1). The results are in accordance with Mukherjee [20] who studied the identification and genetic variability of the *Trichoderm*a isolates. These results are also in accordance with several workers who observed the amplified rDNA fragment of approximately 500 to600 bp by ITS-PCR in *Trichoderma* [21-24]. The ITS PCR has helped to detect polymorphism at ITS region of rDNA among the *Trichoderma* isolates.

Table: 2 Isolates of *Trichoderma* spp

				GPS location	
Isolates (Code)	Accession No	Type of soil	Source	Latitude	Longitude
Trichoderma viride (FS/L-20)	NCFT2580	A	Lohagarh forest	N26° 45'13.08''	E88°23'28.72
Trichoderma viride (FS/C-90)	NCFT2581	A	Cinchona forest	N26° 47'49.16''	E88° 21.27.75''
Trichoderma viride (FS/S-455)	NCFT2583	A	Sukna forest	N26° 45'11.75''	E88° 23'28.27''
Trichoderma virid e (FS/S-458)	NCFT2584	A	Sukna forest	N 26° 48'18.68''	E88° 21.14.61''
Trichoderma viride (RHS/T-460)	NCFT2585	B	Tea Rhizosphere	N26° 45.11.75''	E88° 23'28.27''
Trichoderma viride (RHS/T-463)	NCFT2586	B	Tea Rhizosphere	N 26° 48'18.68''	E88° 21.14.61
Trichoderma viride (RHS/T-472)	NCFT2588	B	Tea Rhizosphere	N 26° 45'11.75''	E 88° 23'28.27''
Trichoderma viride (FS/S-473)	NCFT2589	A	Sukna forest	N 26° 48'18.68''	E88° 21'14.61''
Trichoderma viride (FS/S-474)	NCFT2590	A	Sukna forest	N 26° 48'18.68''	E88° 21'14.61''
Trichoderma viride (FS/S-475)	NCFT2591	A	Sukna forest	N 26° 48'18.64''	E88° 21'14.61''
Trichoderma viride (FS/S-478)	NCFT2594	A	Sukna forest	N 26° 48'18.68	E88° 21'14.61
Trichoderma harzianum (Ag/S476)	NCFT2592	C	Potato field	N 25° 01'13.13''	E88° 08'19.98''
Trichoderma harzianum (FS/S-477)	NCFT2593	A	Sukna forest	N 26° 48'18.68	E88° 21'14.61
Trichoderma harzianum (Ag/S471)	NCFT2587	C	Rice field	N 25° 01'13.13''	E88° 08'19.98''
Trichoderma harzianum (Ag/S479)	NCFT2595	C	*Brassica* field	N 25° 01'13.13''	E88° 08'19.98''
Trichoderma harzianum (RHS/AC480)	NCFT2596	B	*Accacia* Rhizosphere	N26° 42'42.56''	E 88.° 21'.15.47''
Trichoderma harzianum (RHS/AC481)	NCFT2597	B	*Accacia* Rhizosphere	N26° 42'42.56''	E 88.° 21'.15.47''
Trichoderma harzianum (RHS/AC482)	NCFT2598	B	*Accacia* Rhizosphere	N26° 42'42.56''	E 88.° 21'.15.47''
Trichoderma harzianum (RHS/AC483)	NCFT2599	B	*Accacia* Rhizosphere	N26° 42'42.56''	E 88.° 21'.15.47''

NCFT-National Center of Fungal Taxonomy

A-Forest soil; B-Rhizosphere soil; C-Agricultural soil

Table 3: Analysis of the polymorphism obtained with RAPD markers in 19 *Trichoderma* isolates

Sl No.	Seq Name	Total no RAPD bands	Approximate band size (bp). Min	Max.	Monomorphic band	Polymorphic bands	Polymorphic (%)
1.	A-11	09	100	2000	0	9	100
2	OPA-4	12	100	1000	0	12	100
3.	A-5	18	100	2000	0	18	100
4.	OPD-6	14	100	1000	0	14	100
5.	AA-04	11	100	1000	0	11	100
6.	OPA1	09	100	1000	0	09	100
	Total	73			0	73	100

Table 4: RAPD-based genetic similarity within groups

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	1.00																		
2	1.00	1.00																	
3	40.90	0.90	1.00																
4	05.81	0.81	0.90	1.00															
5	0.58	0.58	0.66	0.75	1.00														
6	0.50	0.50	0.58	0.66	0.90	1.00													
7	0.58	0.58	0.66	0.75	1.00	0.90	1.00												
8	0.58	0.58	0.66	0.75	1.00	0.90	1.00	1.00											
9	0.58	0.58	0.66	0.75	1.00	0.90	1.00	1.00	1.00										
10	0.63	0.63	0.72	0.66	0.90	0.80	0.90	0.90	0.90	1.00									
11	0.54	0.54	0.63	0.58	0.80	0.88	0.80	0.80	0.80	0.88	1.00								
12	0.28	0.28	0.26	0.25	0.26	0.28	0.26	0.26	0.26	0.285	0.30	1.00							
13	0.28	0.28	0.26	0.25	0.26	0.28	0.26	0.26	0.26	0.28	0.30	1.00	1.00						
14	0.28	0.28	0.26	0.25	0.26	0.28	0.26	0.26	0.26	0.28	0.30	1.00	1.00	1.00					
15	0.28	0.28	0.26	0.25	0.26	0.28	0.26	0.26	0.26	0.28	0.30	1.00	1.00	1.00	1.00				
16	0.26	0.26	0.25	0.23	0.25	0.26	0.25	0.25	0.25	0.26	0.28	0.72	0.72	0.72	0.72	1.00			
17	0.26	0.26	0.25	0.23	0.25	0.26	0.25	0.25	0.25	0.26	0.28	0.72	0.72	0.72	0.72	1.00	1.00		
18	0.26	0.26	0.25	0.23	0.25	0.26	0.25	0.25	0.25	0.26	0.28	0.72	0.72	0.72	0.72	1.00	1.00	1.00	
19	0.26	0.26	0.25	0.23	0.25	0.26	0.25	0.25	0.25	0.26	0.28	0.72	0.72	0.72	0.72	1.00	1.00	1.00	1.00

T.viride (1-11) and *T.harzianum* (12-19) 1-NCFT2580, 2-NCFT2581, 3-NCFT2583, 4-NCFT2584, 5-NCFT2585, 6-NCFT2586, 7-NCFT2588, 8-NCFT-2589, 9-NCFT2590, 10-NCFT2591, 11-NCFT2594, 12-NCFT2592, 13-NCFT2593, 14-NCFT2587, 15-NCFT2595, 16-NCFT2596, 17-NCFT2597, 18-NCFT2598, 19-NCFT2599)

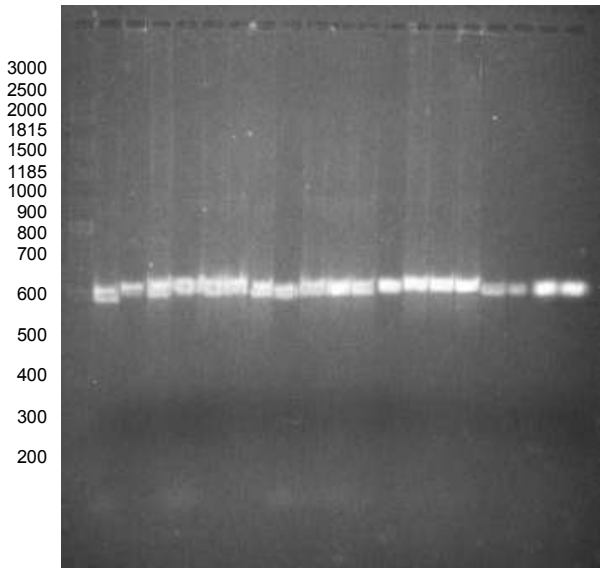

Fig. 1: PCR amplification of ITS region of *Trichoderma* isolates. *T. viride* (Lane 1-11) and *T. harzianum* (Lane12-19) Lane M: Low range DNA Marker, Lane 1-NCFT2580, Lane2-NCFT2581, Lane3-NCFT2583, Lane4-NCFT2584, Lane5-NCFT2585, Lane6-NCFT2586, Lane7-NCFT2588, Lane8-NCFT-2589, Lane9-NCFT2590, Lane10-NCFT2591, Lane11-NCFT2594, Lane12-NCFT2592, Lane13-NCFT2593, Lane14-NCFT2587, Lane15-NCFT2595, Lane16-NCFT2596, Lane17-NCFT2597, Lane18-NCFT2598, Lan19-NCFT2599

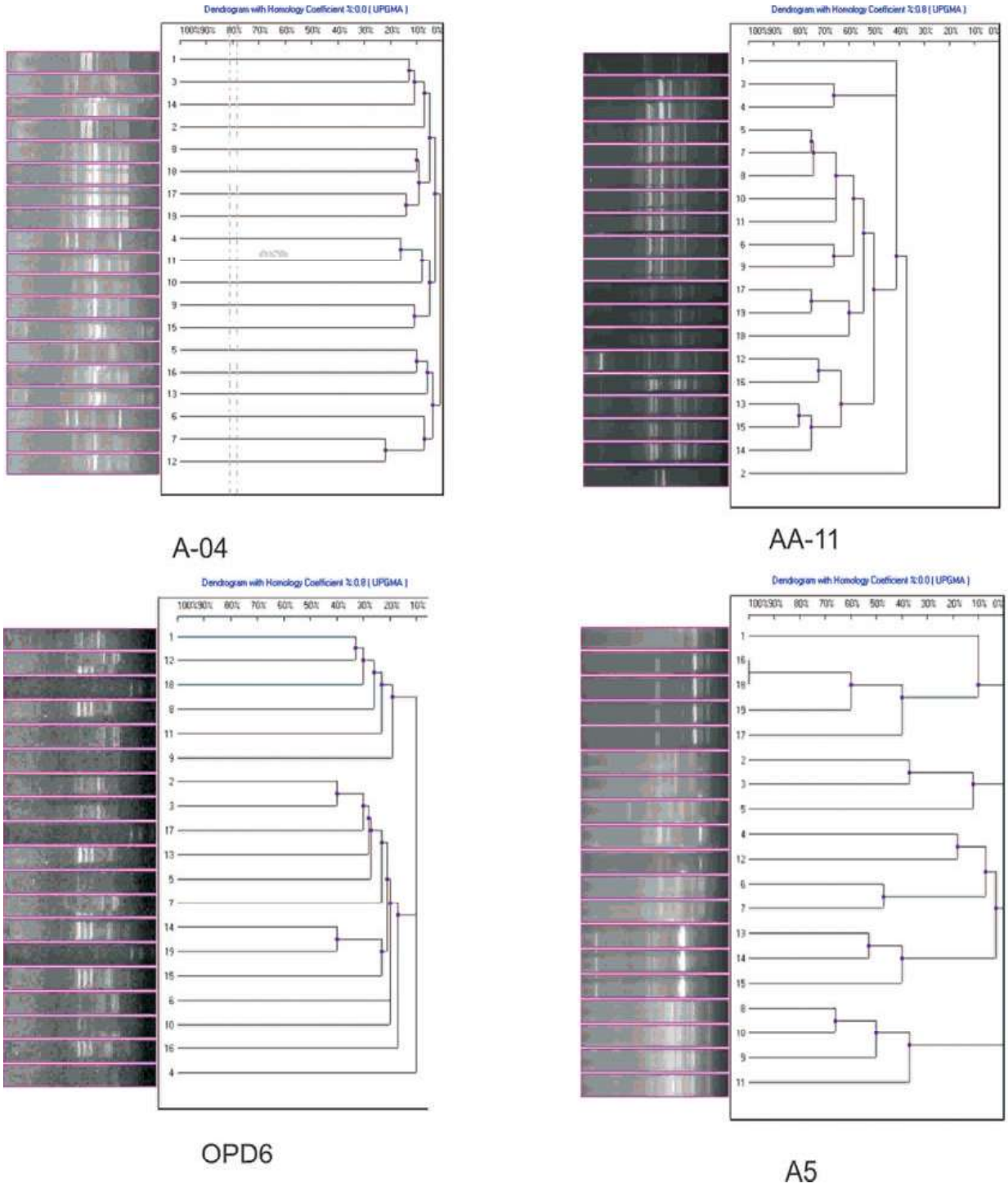

Fig. 2: RAPD analysis of isolates of *T.viride* (1-11) and *T.harzianum* (12-19) using BioProfil 1D software with primers (A-04, AA-11, OPD6 and A-5) 1-NCFT2580, 2-NCFT2581, 3-NCFT2583, 4-NCFT2584, 5-NCFT2585, 6-NCFT2586, 7-NCFT2588, 8-NCFT-2589, 9-NCFT2590, 10-NCFT2591, 11-NCFT2594, 12-NCFT2592, 13-NCFT2593, 14-NCFT2587, 15-NCFT2595, 16-NCFT2596, 17-NCFT2597, 18-NCFT2598, 19-NCFT2599).

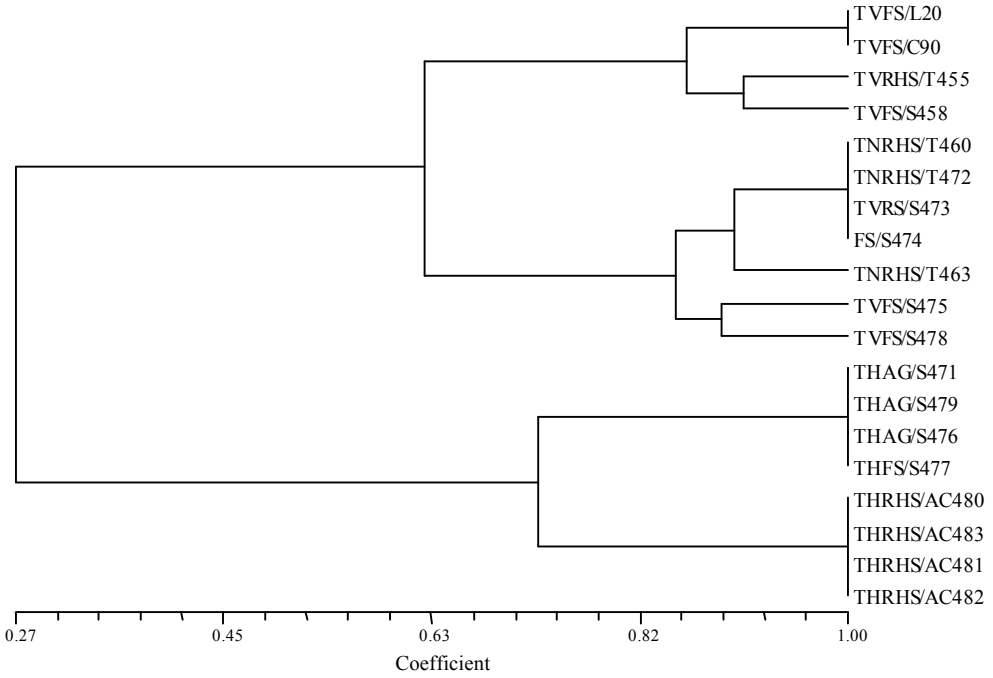

Fig. 3: Dendrogram showing the genetic relationships among 19 *Trichoderma* fungal isolates based on RAPD analysis

The genetic relatedness among eleven isolates of *Trichoderma viride* and eight isolates of *Trichodema hurzianum* were analyzed by six random primers OPA-1, OPD-6, OPA-4, A-5, AA-04 and AA-11 to generate reproducible polymorphisms. All amplified products with the primers had shown polymorphic and distinguishable banding patterns which indicate the genctic diversity of *Trichoderma* isolates. A total of 73 reproducible and scorable polymorphic bands ranging from approximately 100bp to 2000bp were generated with six primers among the nineteen *Trichoderma* isolates (Table 3). RAPD profiles showed that primer A-5 scored highest bands which ranged between 100bp to 2000bp. Relationships among the isolates was evaluated by cluster analysis of the data based on the similarity matrix (Table 4). The Dendogram was generated by unweighted pair-group methods with arithmetic mean (UPGMA) using BIO Profil 1D image software for each primer (Fig 2) and NTSYSpc software (Fig 3). Based on the results obtained all the nineteen isolates can be grouped into two main clusters. One cluster represents *T.viride* and other *T. harzianum*. Again the *T. viride* cluster is also sub grouped into two. First subgroup with four isolates and second one is with seven isolates of two sub clusters. The cluster of *T. harzianum* divided into two different cluster contains four different isolates.

ACKNOWLEDGEMENT

Financial support from Indian Council of Agricultural Research (ICAR), Govt. of India, New Delhi under network project of National Bureau of Agriculturally Important Microorganisms (NBAIM), is gratefully acknowledged.

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