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Randomly Amplified Polymorphic DNA Analysis of Native Trichoderma Isolates

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ABSTRACT

Analysis of all the Trichoderma spp. isolates through Random Amplified Polymorphic DNA (RAPD), a PCR (Polymerase Chain Reaction) based molecular marker system was performed. The procedure was used to examine the genetic similarity among six native isolates of Trichoderma spp. Genomic DNA extracted from the fungal tissues of different isolates of Trichoderma spp. Quality and quantity of DNA checked and subjected to RAPD analysis. Based on the UPGMA dendogram only two clusters were formed, one consisting of the two strains of T. fasciculatum (TFC-1 and TFC-2), T. viride (TVS-1 and TVS-2) and T. harzianum (THCh-1), whereas, T. atroviride (TACh-1) formed the second cluster. The findings of the present experiment undoubtedly indicated all the isolates were moderately (more or less) similar with each other at molecular level by using RAPD analysis. The observed similarity between TFC-1, TFC-2 (Castor) and TVS-1, TVS-2 (Sugarcane) might be due to these species were isolated from the same host.

Keywords: RAPD, Molecular similarity, Native isolates and Trichoderma spp.

1. INTRODUCTION

Despite the growing commercial importance of individuals within the genus *Trichoderma*, the taxonomy of this genus has not kept up with the progress in applied fields. The consequence of this is that the number of species of *Trichoderma* recognized do not adequately account for the diversity of activities attributed to the genus (Papavizas, 1985; Taylor, 1986, Ghisalberti and Sivasithamparam, 1991). Although extensive morphological and cultural studies have been carried out on the taxonomy of *Trichoderma*, there are still not enough variable characters available to differentiate the genus to the species level. It is therefore, proposed that fast and easily repeatable molecular technique may be a useful addition to morphological criteria in the taxonomy of *Trichoderma*. In recent years, number of molecular studies has also been employed to characterize *Trichoderma* species. Molecular techniques used to differentiate isolates by differences in their DNA and RNA. RAPD is a technique where a single oligonucleotide arbitary primer is used in conjuction with a DNA polymerase enzyme, such as *Taq* polymerase, is amplify regions of genomic DNA. A particular DNA fragment which is generated for one individual but not for another represents a DNA polymorphism and can be used as a genetic marker. With this particular technique, individuals are distinguished by differences in their genomic DNA. The technique was termed as random amplified polymorphic DNA (RAPD) by Williams *et al*. (1990). This technique differs from the conventional Polymerase Chain Reaction (PCR) in that it uses only one primer which possesses arbitrary sequences. RAPD techniques have been employed to group isolates of *Trichoderma* species by several workers (Arisan-Atac *et al*., 1995). The RAPD technique has been successfully used to distinguish sub groups within 23 strains of *T. harzianum* and 19 strains of *T. viride* by Zimand *et al*. (1994). However, in the same study, no genetic variation was found between five strains of *T. hamatum*. Further they also found that RAPD based sub groups of *T. harzianum* correlated with isolate geographical origins with strains from the same area producing identical band patterns. The RAPD technique has also been successfully employed to eliminate duplicate strains when screening for metabolite production in *Trichoderma* strains (Fugimori and Okuda, 1994). Choudary *et al.* (2007) isolated thirteen species of *T. harzianum* from the rhizosphere soil samples collected from field grown crops *viz*., castor, chilli, cotton, groundnut, grapes, red gram, tomato and sunflower. To assess the genetic variability in *T. harzianum*, the isolates were analyzed by using random amplified polymorphic DNA markers. Isolates showed 78-80 per cent similarity with standard culture of *T. harzianum* (MTCC 2050). Singh *et al*. (2006) reported that analysis of the *Trichoderma* isolates using molecular markers revealed a high level of genetic diversity among the isolates. *T. virens* isolate, showed only 6 per cent similarity with *T. harzianum* isolate. The diversity was more marked within the *T. harzianum* group. Out of 41 isolates classified as *T. harziamum*, five isolates *viz*., PBAT-34, PBAT-42, PBAT-27, PBAT-9 and PBAT-40 formed separate clusters and showed only 13 to 54 per cent genetic similarity with the remaining *T. harzianum* isolates. Out of the remaining *T. harzianum* isolates, only one-half of isolates showed 90 per cent genetic similarity. By the same way to analyzed genetic similarity of native *Trichoderma* isolates the attempt was done by using the RAPD technique.

2. MATERIALS AND METHODS

Analysis of all the *Trichoderma* isolates through RAPD, a PCR (Polymerase Chain Reaction) based molecular marker system (Williams *et. al.,* 1990) was performed as detailed below:

Genomic DNA extraction

Isolates of *Trichoderma* were grown in 100 ml culture tubes overnight at room temperature (27 ± 2^0C) with moderate agitation. After 24 hours of incubation, 1 ml of cell culture from each sample was taken into 2 ml eppendorf tube and centrifuged at 5000 rpm for 10 minutes at room temperature (27 ± 2^0C) using Eltek cooling centrifuge. The supernatant was discarded and fungal tissues were collected at bottom and subjected to genomic DNA extraction. The fungal tissues

collected after centrifugation were suspended in 100 µl of STES buffer {0.2 M Tris HCl pH 7.6, 0.5 M NaCl, 0.1 % (w/v) SDS (Sodium Dodecyl Sulphate), 0.01M EDTA (Ethylene Diamine Tetra Acetic acid)} for 10 minutes. Added 50 µl of TE pH 8.0 (10 mM Tris pH 8.0 and 1 mM EDTA pH 8.0) to the above reaction mixture. After 5-10 minutes, 100 µl of Phenol: chloroform: isoamyl alcohol (25:24:1v/v) was added in each tube and vortex for 1 minute to mix the organic and aqueous phase. Tubes were centrifuged at 8000 rpm for 15 minutes at room temperature. The upper aqueous phase was collected and transferred in sterilized 2 ml eppendorf tube and double volumes of chilled absolute ethanol and 1/10 volume of 3 M sodium acetate pH 5.2 were added and incubated at -20^{o} C for 30 min, followed by centrifugation at 10,000 rpm for 15 minutes at 4° C temperature. The nucleic acid pellet was collected and dissolved in excess of TE pH 8.0 buffer and 2 µl (ug/ ml) of DNase free RNase (supplied from Banglore genei Pvt. Ltd, India) was added and incubated at 37° C for 1 hour to digest RNA. After 1 hour the RNA free DNA was extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) at 8000 rpm for 15 minutes at room temperature. The supernatant was collected and equal volume of Chloroform:isoamyl alcohol(24:1 v/v) was added to the suspension and content were mixed by inversion to form an emulsion. The mixture was centrifuged at 10,000 rpm for 10 min. at room temperature so as to separate the phases. The supernatant was collected in fresh tubes and double volume of chilled absolute ethanol and 1/10 volume of 3 M sodium acetate pH 5.2 was added and incubated at -20^{o} C for 30 min followed by centrifugation at 10,000 rpm for 15 minutes at 4° C. RNA free DNA pellets collected at bottom were washed twice with 70 per cent ethanol and dried at room temperature (27±2^{0}C) to remove the traces of ethanol. The dried DNA pellets were dissolved in 100 µl of TE pH 8.0 and kept overnight at room temperature. The next day, stock suspensions of genomic DNA were preserved at 4°C temperature for further analysis.

Quality check and quantification of DNA

The quantification of DNA was carried as using Nanodrop spectrophotometer (Model ND-1000). From the stock of DNA, 1 µl of sample was loaded to measure the concentration of DNA (ng/µl) and 260:280 ratios for purity confirmation. The quality of DNA was also checked on 0.8 per cent (w/v) agarose gel prepared in 0.5X TBE pH 8.0 (Tris 45 mM, Boric Acid 45 mM and EDTA 1 mM) containing 10 µl of ethidium bromide (EtBr ; 1 mg/ 1ml). Genomic DNA from the stock (5 µl) was mixed with 1 µl of 6X agarose gel loading dye (Supplied by Banglore Genei) and loaded in each well using micropipette. The horizontal gel electrophoresis was used to run the genomic DNA with a potential difference of 5-6 V/cm for an hour. The bands were visualized and captured under UV light using gel documentation system of Gene Genius Syngene, UK. The DNA samples with the ratio of 1.7 – 1.8 at $O.D_{260\ /\ 280}$ were retained for DNA fingerprinting. The stocks were diluted to a final concentration of 50 ng/µl of DNA and used for further analysis.

RAPD analysis

The genomic DNA was subjected to RAPD analysis. Polymerase Chain Reaction was performed in a 200 µl thin walled sterilized PCR tube containing a 25 µl reaction mixture. The PCR reaction mixture consisted of 2.5 µl of 10X Taq Buffer A with1.5 mM $MgCl_2$, 0.5 µl of *Taq*DNA polymerase (3U/ µl), 1 µl of dNTP's (0.2 mM each), 1 µl of primer (10 pmol/ µl) (Supplied by Biogene,USA), 0.5ul of DMSO, 50 ng DNA (1 µl) and 18.5 µl sterilized deionized water. The PCR tubes containing the reaction mixture were tapped gently and spun briefly at 10,000 rpm. Total ninety (90) random decamer primers belonging to OPE and OPJ, OPK, OPI, OPH series obtained from IDT were screened and out of that only five were found to be having complementary sequence present in the set of genome studied from the OPE and OPH series (Table 1). The PCR tube containing 25 µl of reaction mixture was placed in the Biometra thermal cycler along with a control (without genomic DNA). The DNA amplification was carried out in thermal cycler with following PCR program.

Initial Denaturation	:	95° C for 5 minutes
Denaturation	:	95° C for 45 seconds
Primer annealing	:	38° C for 30 seconds
Extension	:	72° C for 45 seconds
Repetition of step 2 to 4 for 35 times		
Final Extension	:	72° C for 10 minutes
Hold	:	at 4° C

The PCR product was collected from the thermal cycler and loaded in 1.8 percent (w/v) agarose gel prepared in 0.5X TBE pH 8.0 with 5 µl of EtBr, required volume of 0.5X TBE pH 8.0 was used as electrode buffer. Whole of the 25 µl PCR product was mixed with 6X agarose gel loading dye and loaded into each well. A known (1kb & 5 kb) molecular weight DNA ladder 3 µl (Supplied by Banglore Genei) was loaded. A potential difference of 5-6 V/cm was provided till the bands separated properly to give better resolution of each and every band of varying molecular weight amplified during PCR. The gel was viewed and photographed under Uv light using the gel documentation system (Gene Genius Syngene, U.K.).

Statistical analysis of RAPD data

Statistical analysis for the RAPD data from all the polymorphic primers were scored by visual observation. The faint bands were not scored as they were not reproducible. The presence of an amplified band (amplicon) in each position was recorded as 1 and the absence as 0. Based on the presence and absence data Jaccard's similarity index was calculated (Sheath and Sokall, 1979). Similarity coefficients were used for cluster analysis, performed using the SAHN (sequencial, agglomerative, heirarchial nested clustering method) sub programme of NTSYS-pc ver 2.2 (Numerical Taxonomy and Multivariate Analysis System; Rohlf, 2005) and dendograms was constructed by UPGMA (unweighted pair group method with arithmetic averages).

3. RESULTS

The genomic DNA extracted from *six* isolates of *Trichoderma spp. viz. T. viride* (Two isolates i.e. TVS-1 and TVS-2), *T. fasciculatum* (Two isolates i.e. TFC-1 and TFC-2), *T. harzianum* (One isolate i.e. THCh-1), and *T. atroviride* (One isolate i.e. TACh-1) *were* subjected to RAPD analysis. Ninety random decamer primers belonging to OPE and OPJ, OPK, OPI, OPH series obtained from Biogene, USA having more than 60 percent GC content were used. Out of the 90 primers screened only five primers *viz.*, OPE-1,

OPE-3, OPE-5, OPH-5 and OPH-8 were found to be having complementary sequence present in the set of genome studied. From these five primers a total of 34 scorable bands were generated, out of which 26 were found to be polymorphic. The highest numbers of bands (8) were obtained with primer OPE-1 and OPE-5, while the lowest numbers of bands (5) were obtained with primer OPH-5. Different primers showed a variation in their ability to detect polymorphism. The percentage of polymorphism ranged from 66.66% to 87.50%. Primer OPE-5 revealed the highest polymorphism (87.50%) while primer OPH-8 exhibited the lowest polymorphism (66.66%) (Table 2). The RAPD profiles generated through PCR are presented in Fig 1. For each primer, RAPD fragments were scored on the basis of presence and absence of amplified product. The Jaccard's similarity index was estimated among the *six* isolates *of Trichoderma spp. viz. T. viride* (Two isolate), *T. fasciculatum* (two isolate), *T. harzianum*, and *T. atroviride* (Table 3). The similarity index among the fungus studied ranged from 0.430 to 0.920 indicating presence of moderate genetic similarity between set of genomes. The two strains of *T. fasciculatum* had a similarity coefficient of 0.920 which were found more similar with each other. Whereas, strains of the *T. viride* (TVS-1) showed moderately similar with *T. viride* (TVS-2) (0.767). The strain of the *T. harzianum* showing similarity with the two strains of *T. viride* (0.640), while *T. atroviride* showed most divergent with other strains (0.430). The Jaccard's similarity coefficient matrix was further used to obtain a dendrogram based on UPGMA using the NTSYSpc version 2.2 software (Fig 2). Only two clusters were formed, one consisting of the two strains of *T. fasciculatum*, *T. viride* and *T. harzianum*, whereas, *T. atroviride* formed the second cluster.

4. DISCUSSION

All the six native isolates of *Trichoderma* species were found moderately (more or less) similar at molecular level by using RAPD analysis. The observed similarity between TFC-1, TFC-2 (Castor) and TVS-1, TVS-2 (Sugarcane) might be due to these species were isolated from the same host. Results obtained are in agreement with Zimand *et al*. (1994); Choudary *et al.* (2007) and Singh *et al*. (2006). They reported the genetic similarity between different *Trichoderma* spp. isolates.

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Table 1: List of primers with sequence and melting temperature details used for RAPD amplification reaction

Primer Name	Sequence Details	GC content	Melting Temperature (^{o}C)
OPE-01	5^{l}- ACG GAT CCT G-3^{l}	60%	33.3
OPE-03	5^{l}- CCT GAT CAC C-3^{l}	60%	30.6
OPE-05	5^{l}- CCG AAT TCC C-3^{l}	60%	32.0
OPH-05	5'-AGT CGT CCC C-3'	70%	34.0
OPH-08	5'-GAA ACA CCC C-3'	60%	32.0

Table 2: Details of amplification obtained with different RAPD primers in different accessions of *Trichoderma* spp.

Primer	Number of monomorphic bands	Number of polymorphic bands	Total number of bands	Percentage of polymorphism
OPE-01	2	6	8	75.00
OPE-03	2	5	7	71.42
OPE-05	1	7	8	87.50
OPH-05	1	4	5	80.00
OPH-08	2	4	6	66.66
Total	**8**	**26**	**34**	**82.35**

Table 3: Jaccard's similarity coefficient among different accessions based on the RAPD data

Accessions	TFC-1	TFC-2	TVS-1	TVS-2	THCh-1	TACh-1
TFC-1	1.000					
TFC-2	0.920	1.000				
TVS-1	0.345	0.367	1.000			
TVS-2	0.633	0.645	0.400	1.000		
THCh-1	0.613	0.677	0.483	0.606	1.000	
TACh-1	0.529	0.588	0.552	0.774	0.697	1.000

TFC-1 & TFC-2: *T. fasciculatum;* TVS-1 & TVS-2: *T. viride*; THCh-1: *T. harzianum*; TACh-1: *T. atroviride*

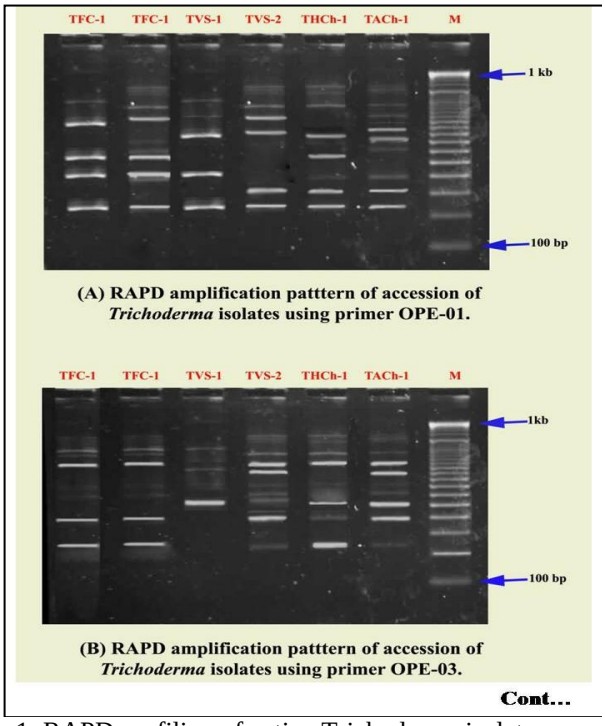
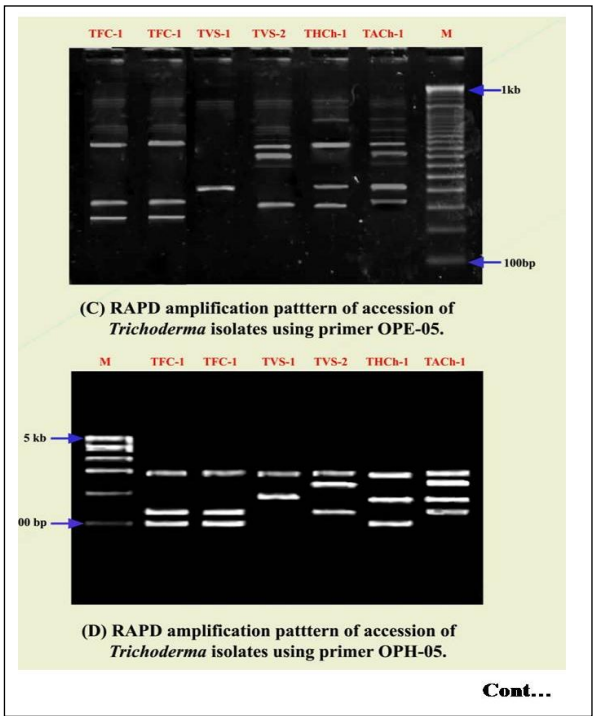
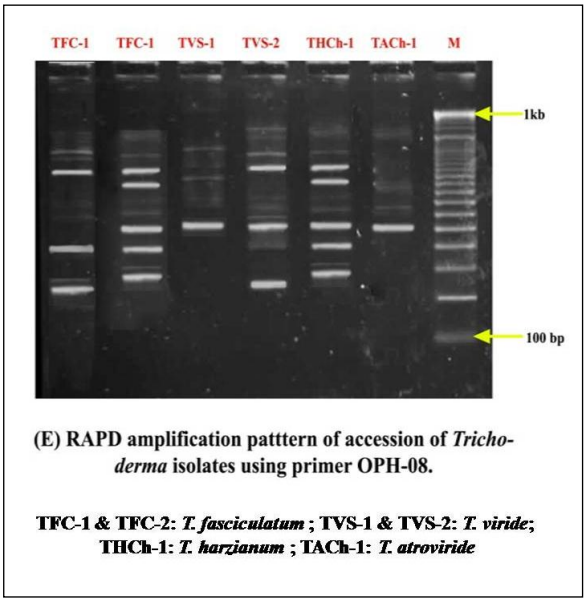


Fig. 1: RAPD profiling of native Trichoderma isolates





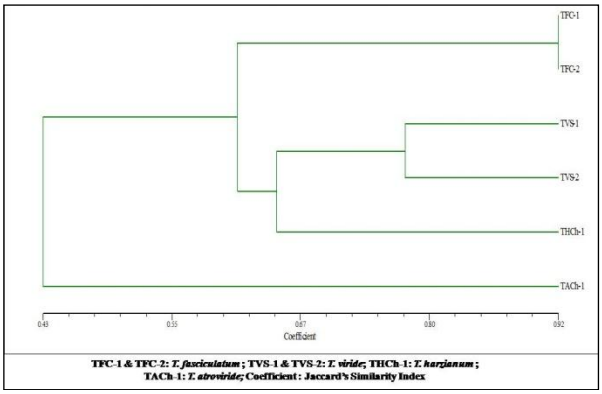


Fig. 2: UPGMA based dendogram of Jaccard's similarity index for RAPD data generated for Trichoderma isolates