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Determination of genetic variations among different *Trichoderma* isolates using RAPD marker in Bangladesh

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Abstract

Some *Trichoderma* isolates were collected from different locations of Bangladesh for evaluating their bioefficiency by determining their genetic variations. PCR-based Random Amplified Polymorphic DNA (RAPD) Marker employing 3 decamer primers produced 29 scorable bands of which all (100%) were polymorphic. The co-efficient of gene differentiation (Gst) was 1.0000 reflecting the existence of high level of genetic diversity among the isolates. The Unweighted Pair Group Method of Arithmetic Means (UPGMA) dendrogram constructed from Nei's (1972) genetic distance produced 2 main clusters (16 isolates in cluster 1 and 19 isolates in cluster 2). The result indicating their genetic diversity has opened new possibility of using the most efficient and more isolates of *Trichoderma* in the preparation of effective biopesticide.

Keywords: Genetic diversity, *Trichoderma*, RAPD

Introduction

Trichoderma is a soil fungi which reproduces asexually which is frequently isolated and most prevalently culturable. It is widely distributed in plant material, decaying vegetation, wood and other diverse habitats (Ranasingh, 2006). *Trichoderma* spp. are facultative anaerobes, grow saprophytically or as parasites on other *Trichoderma* and are able to grow in soils having a pH range of 2.5 - 9.5, although most of them prefer moderately acidic environment (Grondona, 1997). Most of the *Trichoderma* strains produce only asexual spores. However, a few strains have sexual stage which are not considered for biocontrol purposes. In recent past, morphological features were considered to determine different taxonomical aspects with the help of the asexual sporulation apparatus, but molecular approaches are now being used.

The strains show a high level of genetic diversity which can be used to produce various products of commercial and ecological interests. They are prolific producers of extra-cellular proteins, and also known to produce enzymes that degrade cellulose and chitin. Many other different strains produce more than 100 different metabolites having antibiotic activities. Since the introduction of biocontrol, *Trichoderma* species have been recognized as agents for the control of plant diseases and for their roles in increasing plant growth and development. The most useful strains show a property that is known as 'rhizosphere competence' that is, the ability to colonize and grow in association with plant roots. Much of the known biology and many of the uses of these fungi have been documented recently (Harman, 2000). To isolate *Trichoderma* spp. different media are used. Some selective media are more efficient than others. Depending on the species, *Trichoderma* can show no growth to broadly spreading growth on Potato Dextrose Agar media and Czapek's agar.

The Random Amplified Polymorphic DNA (RAPD) procedure developed by Williams *et al.* (1990) and Welsh & McClelland (1990) involves simultaneous amplification of several anonymous loci in the genome using primers of arbitrary sequence and has been used for genetic, taxonomic and ecological studies of several fungi including *Trichoderma* (Abbasi *et al.*, 1999; Paavanem-Huhtala *et al.*, 2000). The ability to reliably distinguish members of different species, fingerprint of different genotypes and an estimate of the amount of variation within a species is useful for a breeding program. RAPD is a molecular technique used for such purposes. RAPD technique is easy and inexpensive. The advantages of the RAPDs are, the requirement for small amount of DNA (5-20 ng), single short (9 to 10 bp) primers of arbitrary sequence, the rapidity to screen for polymorphisms, the efficiency to generate a large number of markers for genomic mapping and the potential automation of the technique. In addition, no prior knowledge of

sequence is required (Sobral and Honeycutt, 1993). Since primers can be chosen arbitrarily, any organism can be mapped with the same set of primers but there is some loss of information because RAPD markers are dominant rather than co-dominant. If one of the alleles at a RAPD site is unamplifiable, then marker homozygote cannot be distinguished from marker/null heterozygote. Other problems arise if products of different loci have similar molecular weights and so will be indistinguishable on a gel because of comigration. The problem of uncertain homology becomes serious at higher taxonomic levels where it is likely that only a few shared bands are generated. Though RAPD has some limitations, but it is being used as one of the powerful techniques for genetic studies, for example, analysis of genetic variation in plants, fungi and bacteria (Mailier *et al.*, 1994) and construction of the first linkage maps for certain plant species and pathogens (Yang and Quiros, 1995).

IPM Lab biopesticide (code- 34/USDA/BG-ARS122) formulated out of *Trichoderma* is in commercial use (Meah, 2005). The strain *Trichoderma harzianum* CP was used in the formulation. A total of 46 isolates were collected from different regions of Bangladesh. Based on growth rate and sporulation capacity, the strain *T. harzianum* CP was selected and used in the formulation (Meah, 2007). Physiological variations of the isolates were studied earlier but not in details. Variation at molecular level has not been studied. Molecular study may reveal any variation that exists among the isolates in support of the variation in physiological properties. It is well established that previous knowledge about the genetic relationships among breeding materials is crucial for the efficient use of the germplasm in a breeding program (Nienhuis *et al.*, 1994). In Bangladesh, research findings on genetic analysis of different *Trichoderma* isolates are not available. Although no specific markers were found to discriminate different isolates effectively, the RAPD technique revealed some degree of polymorphisms for variation study of different *Trichoderma* isolates and the results obtained from the experiment using RAPD markers have been presented and expressed in tables and figures for ease of understanding. More isolates of *Trichoderma* have been recently collected. The present research work was undertaken to determine genetic variations among some isolates of *Trichoderma* spp. using RAPD technique for evaluating their efficiency as biopesticides.

Materials and Methods

An *in vitro* experiment was conducted at the IPM Laboratory, Department of Plant Pathology and Central Laboratory, Bangladesh Agricultural University, Mymensingh. Part of molecular experiment was conducted at the Biotechnology Laboratory, Bangladesh Institute of Nuclear Agriculture (BINA). Thirty five *Trichoderma* isolates collected from diverse origin of different hosts and locations in Bangladesh were used in the present experiment. The experiment was designed in a Completely Randomized Design (CRD) with three replications. The activities were performed from November 2009 to June 2010.

Molecular characterization of *Trichoderma* isolates

For DNA extraction, mycelial cultures were raised individually in 150 ml conical flasks containing 100 ml potato dextrose broth. Mycelia were collected on filter paper in a Buchner funnel, washed with distilled water, frozen, and lyophilized. After extraction, mycelia were wrapped with aluminium foil for each isolate and kept in refrigerator before genomic DNA isolation. Isolates of *Trichoderma* spp. were used for the RAPD. The extraction of total genomic DNA of each isolate of *Trichoderma* was made. As a source of genomic DNA, fresh mycelia samples were collected from the 3 days old culture. Genomic DNA samples of each accession were extracted following phenol: chloroform: isoamyl alcohol extraction and ethanol precipitation method from vigorous and fast actively growing mycelial tissue. DNA was extracted using the mini preparation CTAB method.

Genomic DNA isolation

Thirty five isolates of *Trichoderma* spp. were used for the RAPD. Genomic DNA samples of each accession were extracted following phenol: chloroform: isoamyl alcohol extraction and ethanol precipitation method from vigorous and fast actively growing mycelial tissue. Firstly, soft mycelia portion (before spore production in 3 days) of the vigorous and actively growing *Trichoderma* spp. were cut apart with sterile scissors and forceps. Then ethanol was used to wash scissors and forceps to avoid contamination. The DNA from mycelial tissues was then extracted.

Confirmation of DNA preparation

Usually isolated genomic DNA following above protocol contains a large amount of RNA and pigments, which usually cause spuriously high estimation of DNA concentration on a spectrophotometer. For avoiding RNA contamination, RNAase was used in the TE buffer. Therefore DNA samples were evaluated both quantitatively and qualitatively and for the amount of RNA present using spectrophotometer and 1% agarose gel electrophoresis, respectively.

Quantification of DNA

Absorbance reading and DNA concentration varied from (0.005-0.404) μl and (250-20200) μl respectively. It is necessary to optimize the amount DNA used in PCR assay to achieve reproducibility and strong signal. Purity and concentration of genomic DNA was examined by calculating the ratio of optical density using a spectrophotometer.

Using the above absorbance readings, the original concentrations were determined according to the following formula:

$$\text{DNA Conc. (ng/\mu l)} = \text{Absorbance} \times \frac{\text{Volume of distilled water (\mu l)}}{\text{Amount of DNA sample (\mu l)}} \times \text{Conversion factor (0.05)} \times 1000$$

Preparation of working solution of DNA samples

Before PCR, DNA concentrations were adjusted to 25 ng/ μl using the following formula: $V_1 \times S_1 = V_2 \times S_2$. Where, V_1 = Initial volume of DNA solution (μl), S_1 = Initial DNA concentration (ng/ μl), V_2 = Final volume of DNA solution (μl), S_2 = Final DNA concentration (ng/ μl). $V_2 = V_1 \times S_1 / S_2$

PCR conditions

Conditions for RAPD amplification reactions were maintained essentially following (William *et al.*, 1990) with some modifications. PCR reactions were performed on each DNA sample in a 10 μl reaction mix. During the experiment, PCR buffer, dNTPs, and primer solutions were thawed from frozen stocks, mixed by vortexing and placed on ice. DNA samples were also thawed out and mixed gently. The primers were pipetted first into PCR tubes compatible with the thermocycler used (0.2 ml). For each DNA sample being tested, a pre-mix was then prepared including in the following order: buffer, dNTPs, DNA template and sterile distilled water. Taq polymerase enzyme was then added to the pre-mix. The pre-mix was then mixed well and aliquoted into the tubes containing primers. The tubes were then sealed and placed in a thermocycler and the cycling was started immediately.

Banding analyses

Since RAPD markers are dominant, we assumed that each band represented the phenotype at a single allelic locus (Williams *et al.*, 1990). One molecular weight marker, 20 base pair DNA ladder was used to estimate the size of the amplification products by comparing the distance traveled by each fragment with that of the known sized fragments of molecular weight markers. All distinct bands or fragments (RAPD markers) were thereby given identical numbers according to their position on gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. Bands which were not identified considered as non-scorable.

Dendrogram construction

The scores obtained using all primers in the RAPD analysis were pooled to create a single data matrix. This was used to estimate polymorphic loci, (Nei's, 1972) gene diversity, population differentiation (G_{st}), gene flow (Nm), genetic distance (D) and to construct a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among populations using a computer program, POPGENE (Version 1.31). The same program was also used to perform test of homogeneity in different loci between population pairs. Gene frequency estimation for RAPD loci was based on the assumption of a two-allele system. Only one of the two alleles is capable to amplify a RAPD marker by primer annealing, at an unknown genomic position (locus). The other is the "null" allele incapable of amplification, mainly because of loss of primer-annealing site by mutation. The two-allele assumption is in most cases acceptable because, co-dominant

loci showing band shifts are few. In this system only a null homozygote is detectable as negative for the RAPD - band of interest. Under the assumption of Hardy-Weinberg equilibrium, the null allele frequency (q) may be $(N/n)^{1/2}$, where N and n are the number of band negative individuals observed and the sample size, respectively. The frequency of the other allele (P) is $1 - q$. The assumption of the two-allele system enables us to calculate the Nei's, genetic distance (Nei, 1972) from the RAPD pattern. Nei's genetic distance and identity values were computed from frequencies of polymorphic markers to estimate genetic relationship between the studied thirty five *Trichoderma* isolates using the unweighted pair-group method of arithmetic means (UPGMA). The dendrogram was then constructed using the POPGENE (Version 1.31) computer program.

Results and Discussion

The stated experiment was undertaken to determine genetic variation among some isolates of *Trichoderma* spp. using RAPD analysis. The results are presented here.

RAPD Analyses

Primer selection and RAPD pattern: Six primers were initially screened for their ability to produce polymorphic patterns among 35 isolates of *Trichoderma* spp. and three primers viz., OPB-09, OPB-10, and OPC-01 which showed comparatively maximum number of high resolution bands and distinct polymorphic amplified products were selected. Selected three primers generated 29 bands all were polymorphic, i.e. there were no monomorphic bands. The three different primers generated various banding patterns, ranging from 12 (OPB-9), 8 (OPB-10) to 9 (OPC-01). The primer OPB-9 and OPC-01 produced the highest numbers of polymorphic bands (12 and 9). Thus it showed a higher level of polymorphism. On the other hand, the primer OPB10 generated least number of polymorphic bands (8). (Table 1).

Table 1. RAPD primers with corresponding bands score and their size range together with polymorphic bands observed in 35 isolates

Primer code	Sequences (5'-3')	Total number of bands scored	GC content	Number of polymorphic bands	Proportion of polymorphic loci (%)
OPB-09	TGGGGGACC	12	70	12	100
OPB-10	CTGCTGGGC	8	70	8	100
OPC-01	TTCGAGCCG	9	60	9	100
Total		29		29	300
Average		9.67		9.67	100

In the present study the average percentage of polymorphic loci across primers was 100%. The banding patterns of different *Trichoderma* isolates using primers OPB-9, OPB-10, and OPC-1 are shown in Plate-1

Grouping of different *Trichoderma* isolates based on similar bands produced by three primers to determine polymorphism

In case of primer OPB-09 total number of bands were 12 and some isolates produced similar bands against it, such as T(22, 23), T(25, 33) and T(31, 42). The isolates were rearranged by keeping only one lane from two similar lanes and another lane was discarded. In this way the highest polymorphic loci were found for the primer OPB-09 and the isolates number were reduced from 35 to 32 (Plate 2). Similarly for the primers OPB-10 and OPC-01 the polymorphic loci of the isolates were detected by reducing one lane from two similar lane and the isolates were reduced from 35 to 31 (Plate 3, Plate 4). It can be concluded that, among the three primers OPB-09 produced maximum polymorphism (32 polymorphic bands out of 35) as compared to OPB-10 and OPC-01 (31 from 35). So OPB-09 was the better primer for its capacity to produce the highest polymorphic bands. The DNA polymorphisms were detected according to presence or absence of bands. Absence of bands may be caused by failure of primers to anneal a site in some individuals due to nucleotide sequence differences or by insertions or deletions between primer sites (Clark and Lanigan, 1993) as shown in the Table 2.

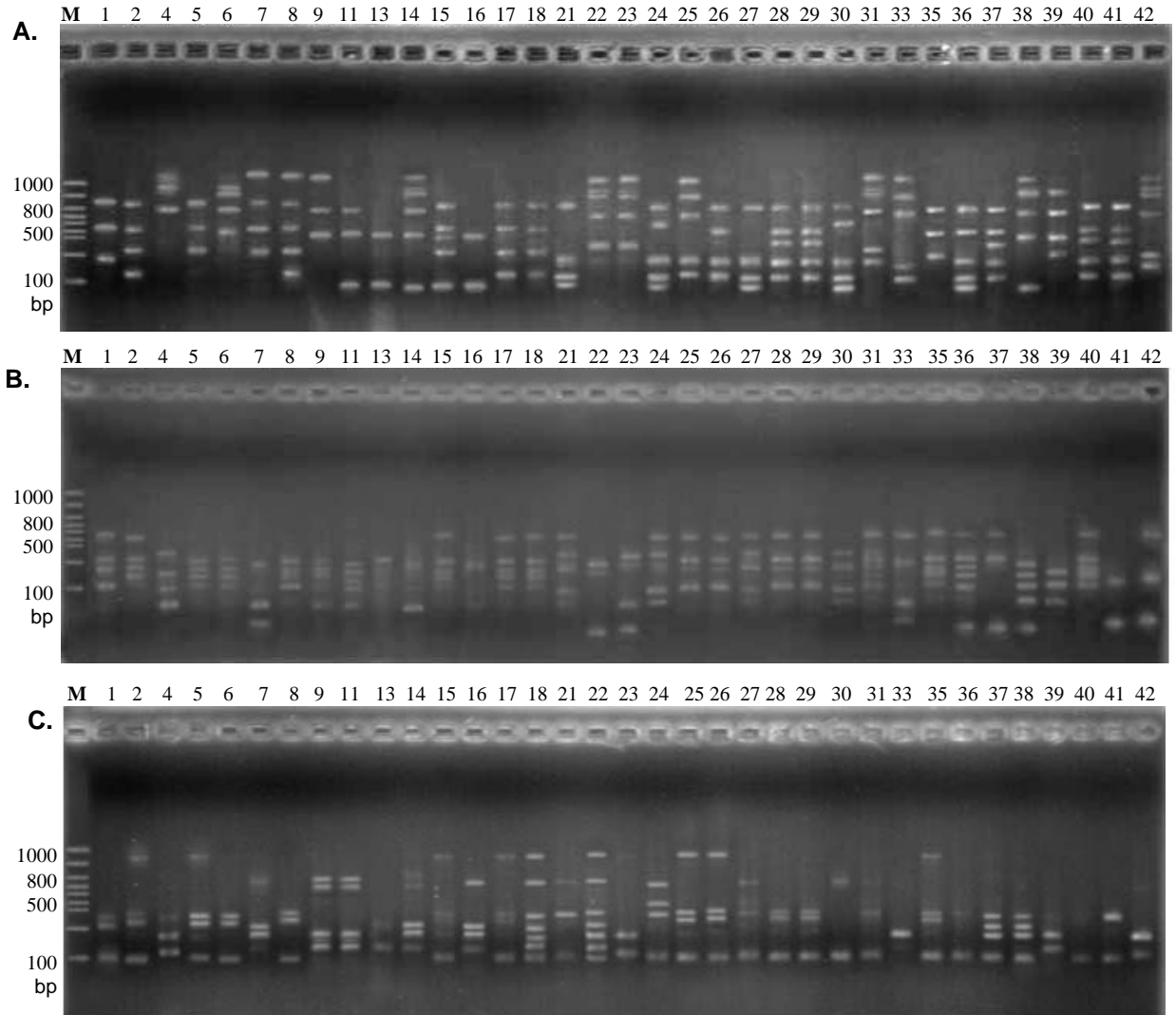


Plate 1. RAPD profiles of different isolates of *Trichoderma* spp. using primers OPB-9, OPB-10, OPC-01 for bands A, B, C, respectively. (Lane M: 20 bp ladder, Lane 1-2: T1 to T2, Lane 4-9: T4 to T9, Lane 11: T11, Lane 13-18 :T13 to T18, Lane 21-31: T21 to T31, Lane 33: T33, Lane 35-42: T35 to T42 on agarose gel)

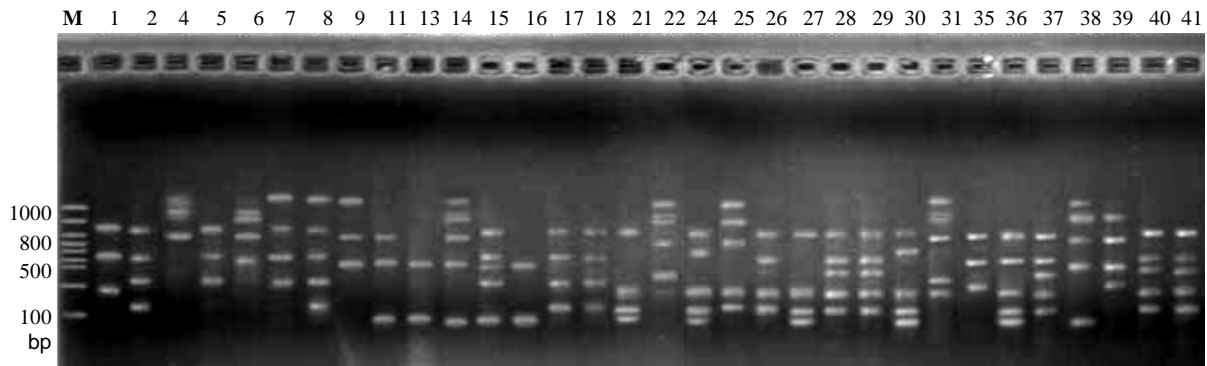


Plate 2. RAPD profiles of different isolates of *Trichoderma* spp. using primer OPB-9 (Lane M: 20 bp ladder, Lane 1-2: T1 to T2, Lane 4-9: T4 to T9, Lane 11: T11, Lane 13-18: T13 to T18, Lane 21-22: T21-T22, Lane 24-31: T24-T31, Lane 35-41: T35-T41 on agarose gel)

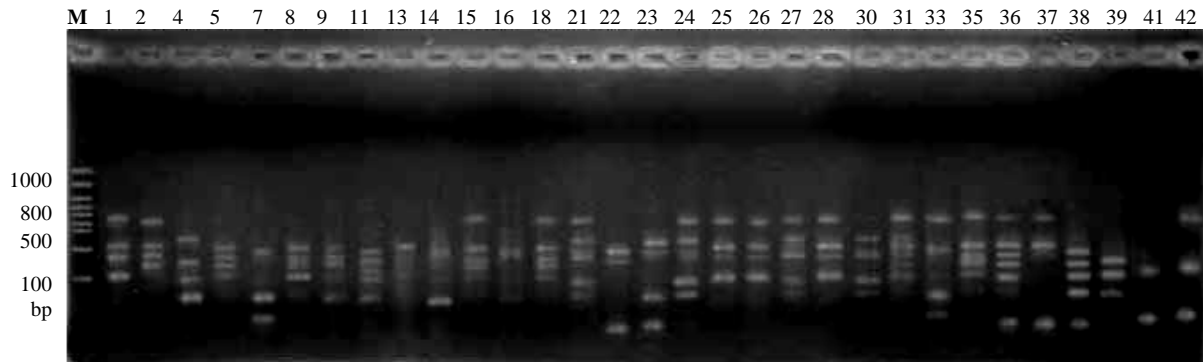


Plate 3. RAPD profiles of different isolates of *Trichoderma* spp. using primer OPB-10 (Lane M: 20bp ladder, Lane 1-2: T1-T2, Lane 4-5: T4-T5, Lane 7-9: T7-T9, Lane 11: T11, Lane 13-16: T13-16, Lane 18: T18, Lane 21-28: T21-T28, Lane 30-31: T30 -T₃₁ , Lane 33: T33, Lane 35-39: T35 to T39 , Lane 41-42: T₄₁ - T₄₂ on agarose gel)

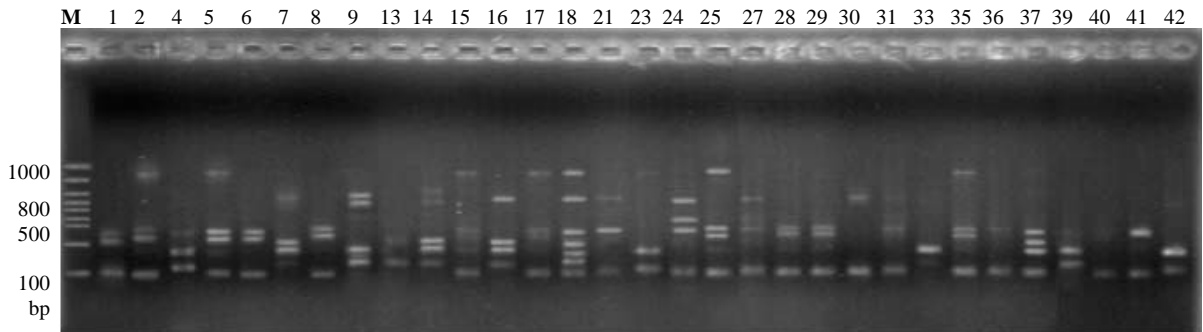


Plate 4. RAPD profiles of different isolates of *Trichoderma* spp. using primer OPC-1 (Lane M:20 bp ladder, Lane 1-2: T1-T2, Lane 3-4: T4-T5, Lane 5-7: T7-T9, Lane 8-12:T13-T17, Lane 13-17: T21-T25, Lane 18-22: T27-T31, Lane 23: T33, Lane 24-31: T35 to T42 on agarose gel)

Table 2. Frequencies of polymorphic RAPD markers in 35 Isolates of *Trichoderma* spp

RAPD Markers	Gene frequency	RAPD Markers	Gene frequency
OPB09-1	0.3429	OPB10-4	0.5143
OPB09-2	0.2286	OPB10-5	0.4571
OPB09-3	0.2857	OPB10-6	0.6286
OPB09-4	0.5714	OPB10-7	0.4000
OPB09-5	0.3714	OPB10-8	0.2286
OPB09-6	0.0857	OPC01-1	0.2857
OPB09-7	0.4857	OPC01-2	0.1143
OPB09-8	0.3429	OPC01-3	0.3143
OPB09-9	0.3429	OPC01-4	0.0571
OPB09-10	0.4571	OPC01-5	0.6571
OPB09-11	0.4286	OPC01-6	0.4571
OPB09-12	0.3143	OPC01-7	0.4000
OPB10-1	0.5143	OPC01-8	0.3143
OPB10-2	0.1714	OPC01-9	0.7429
OPB10-3	0.7429		

Gene flow and population differentiation

The average estimated gene flow (N_m) was 0.0000 and co-efficient of gene differentiation (G_{ST}) was 1.0000 across all loci (Table 3). Hardy-Weinberg expectation of average heterozygosity in subpopulation (H_t) was 0.4136 whereas obtained heterozygosity (H_s) was 0.0000 across all primers. RAPD marker revealed high level of differentiation ($G_{ST}=1.0000$) that supports the presence of sufficient polymorphisms in 35 *Trichoderma* isolates.

Table 3. Gene flow (N_m) and the proportion of co-efficient of gene differentiation (G_{ST}) across different RAPD markers of studied *Trichoderma* isolates

Locus	Sample Size	Ht	Hs	Gst	Nm*
B09-1	35	0.4506	0.0000	1.0000	0.0000
B09-2	35	0.3527	0.0000	1.0000	0.0000
B09-3	35	0.4082	0.0000	1.0000	0.0000
B09-4	35	0.4898	0.0000	1.0000	0.0000
B09-5	35	0.4669	0.0000	1.0000	0.0000
B09-6	35	0.1567	0.0000	1.0000	0.0000
B09-7	35	0.4996	0.0000	1.0000	0.0000
B09-8	35	0.4506	0.0000	1.0000	0.0000
B09-9	35	0.4506	0.0000	1.0000	0.0000
B09-10	35	0.4963	0.0000	1.0000	0.0000
B09-11	35	0.4898	0.0000	1.0000	0.0000
B09-12	35	0.4310	0.0000	1.0000	0.0000
B10-1	35	0.4996	0.0000	1.0000	0.0000
B10-2	35	0.2841	0.0000	1.0000	0.0000
B10-3	35	0.3820	0.0000	1.0000	0.0000
B10-4	35	0.4996	0.0000	1.0000	0.0000
B10-5	35	0.4963	0.0000	1.0000	0.0000
B10-6	35	0.4669	0.0000	1.0000	0.0000
B10-7	35	0.4800	0.0000	1.0000	0.0000
B10-8	35	0.3527	0.0000	1.0000	0.0000
C01-1	35	0.4082	0.0000	1.0000	0.0000
C01-2	35	0.2024	0.0000	1.0000	0.0000
C01-3	35	0.4310	0.0000	1.0000	0.0000
C01-4	35	0.1078	0.0000	1.0000	0.0000
C01-5	35	0.4506	0.0000	1.0000	0.0000
C01-6	35	0.4963	0.0000	1.0000	0.0000
C01-7	35	0.4800	0.0000	1.0000	0.0000
C01-8	35	0.4310	0.0000	1.0000	0.0000
C01-9	35	0.3820	0.0000	1.0000	0.0000
Mean	35	0.4136	0.0000		
St. Dev		0.0109	0.0000		

N_m = estimate of gene flow from G_{ST} , $N_m = 0.5(1 - G_{ST})/G_{ST}$

G_{ST} = Co-efficient of gene differentiation

H_t = Hardy – Weinberg expectation of average heterozygosity in subpopulations

H_s = Hardy – Weinberg expectation of average heterozygosity obtained

The number of polymorphic loci is : 29

The percentage of polymorphic loci is : 100.00

Gene diversity for the RAPD Primer

Genetic diversity values across all *Trichoderma* isolates for 3 primers are given in Table 4. Average gene diversity (h) and Shannon's Information index (I) for three primers across different isolates for all loci were found 0.4136 and 0.5984, respectively. High level of gene diversity value and Shannon's Information index was found in locus OPB09-7, OPB10-1, OPC01-6, (0.4996, 0.4996, and 0.4963, respectively). Lowest level of gene diversity value and Shannon's Information index (0.1567, 0.2841 and 0.1078) was found in locus OPB09-6, OPB10-2, OPC01-4.

Table 4. Genetic diversity for three primers in thirty five *Trichoderma* species

Locus	Sample Size	na*	ne*	h*	I*
B09-1	35	2.0000	1.8202	0.4506	0.6429
B09-2	35	2.0000	1.5448	0.3527	0.5375
B09-3	35	2.0000	1.6897	0.4082	0.5983
B09-4	35	2.0000	1.9600	0.4898	0.6829
B09-5	35	2.0000	1.8760	0.4669	0.6597
B09-6	35	2.0000	1.1859	0.1567	0.2925
B09-7	35	2.0000	1.9984	0.4996	0.6927
B09-8	35	2.0000	1.8202	0.4506	0.6429
B09-9	35	2.0000	1.8202	0.4506	0.6429
B09-10	35	2.0000	1.9854	0.4963	0.6895
B09-11	35	2.0000	1.9600	0.4898	0.6829
B09-12	35	2.0000	1.7575	0.4310	0.6225
B10-1	35	2.0000	1.9984	0.4996	0.6927
B10-2	35	2.0000	1.3968	0.2841	0.4581
B10-3	35	2.0000	1.6182	0.3820	0.5700
B10-4	35	2.0000	1.9984	0.4996	0.6927
B10-5	35	2.0000	1.9854	0.4963	0.6895
B10-6	35	2.0000	1.8760	0.4669	0.6597
B10-7	35	2.0000	1.9231	0.4800	0.6730
B10-8	35	2.0000	1.5448	0.3527	0.5375
C01-1	35	2.0000	1.6897	0.4082	0.5983
C01-2	35	2.0000	1.2538	0.2024	0.3554
C01-3	35	2.0000	1.7575	0.4310	0.6225
C01-4	35	2.0000	1.1208	0.1078	0.2190
C01-5	35	2.0000	1.8202	0.4506	0.6429
C01-6	35	2.0000	1.9854	0.4963	0.6895
C01-7	35	2.0000	1.9231	0.4800	0.6730
C01-8	35	2.0000	1.7575	0.4310	0.6225
C01-9	35	2.0000	1.6182	0.3820	0.5700
Mean	35	2.0000	1.7478	0.4136	0.5984
St. Dev		0.0000	0.2509	0.1045	0.1224

* na = Observed number of alleles

* ne = Effective number of alleles [Kimura and Crow (1964)]

* h = Nei's (1973) gene diversity

* I = Shannon's Information index [Lewontin (1972)].

Genetic distance

The values of pair-wise comparisons of Nei's (1972) genetic distance between 35 *Trichoderma* isolates were computed from combined data sets for the three primers ranging from 0.0034 to 1.4214. Comparatively higher genetic distance (1.4214) was found between T14 and T (2, 17). T14 was dark green, fast growing and very compact isolate, whereas T2 and T17 were whitish green, medium growing and compact isolate. As the isolates T14 and T (2, 17) were genetically apart, can be used in breeding programme for improving some desired traits. The lowest genetic distance (0.0034) was revealed between T24 and T27, also in T28 and T29 where all the isolates were fast growing, compact and green and light green in colour. Considering this, it may be concluded that T24, T27, T28 and T29 were originated from same parent or closely related parent.

Dendrogram

Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated segregation of the 35 isolates of *Trichoderma* spp. into two main clusters: T4, T6, T7, T9, T11, T13, T14, T16, T22, T23, T25, T31, T33, T38, T39, T42 were grouped into cluster 1, most of which were dark green and yellowish green based on colour and other characters were also similar. On the other hand T1, T2, T5, T8, T15, T17, T18, T21, T24, T26, T27, T28, T29, T30, T35, T36, T37, T40, T41 were grouped in cluster 2, most of which were green, light green in colour and all were probably identical based on growth habit, colony consistency etc. (Figure 1). Cluster1 was again divided into two sub-clusters. Sub-cluster 1 has isolate-T (4, 7, 9, 11, 13, 14, 16, 39) which are dark green, green, light green, yellow green and isolates T (6, 22, 23, 25, 31, 33, 38, 42) belonged to sub-cluster 2. Sub-cluster 2 was again divided into two sub sub-cluster. Sub sub-cluster 2 were dark green colour. Sub sub-cluster 1 was then divided into two groups. Group1 has isolates green, yellowish green colour and rest was in group 2. Group 2 was divided into two sub groups which were dark green and green. Further, the isolates of cluster2 were divided into two sub-clusters, sub-cluster 2 contained T21, T24, T27, T30, in which T21, T24, were light green where as T27, T30, were green colour. Rest of the isolates belonged to sub-cluster 1. After that isolates of sub-cluster 1 were divided into two sub sub-cluster. Sub sub-cluster 2, all of which were whitish green except T35 which were green colour. Sub sub-cluster 1 was then divided into two groups. Group 2 contained green colour isolates and Group 1 was divided into two sub-groups, in which sub-group 1 contained light green and dark green whereas sub-group 2 had light green colour isolates. The highest genetic distance (1.4214) was found between T14 and T (2, 17) and they remained in different cluster. The genetic distance between T₂₄, T₂₇ and T₂₈, T₂₉ was lowest (0.0034) and found in a group of the same cluster.

The primers OPB-09 and OPC-01 produced maximum number of bands (12 and 9), whereas OPB10 generated the least number (8). The three primers generated 29 total number of bands and average 10 scorable bands where all were polymorphic bands per primer. Strong and weak bands were produced in the RAPD reactions which is a well known and widely used marker throughout the world. Weak bands resulted from low homology between the primer and the pairing site on the DNA strand (Thormann *et al.*, 1994). In the present study the average percentage of polymorphic loci across primers was 100%. DNA polymorphism of the *Trichoderma* isolates were studied in Brazil (Brazilian archives of Biology and technology) by Goes and Costa (2002). In their study a total of 197 bands were produced with the 7 primers OPX12, OPA13, OPX1, OPX2, OPX3, OPX7, OPA-5 and found no monomorphic bands while polymorphic bands were evident. This report strongly supports the current study. Fujimori and Okuda (1993) examined 74 strains of *Trichoderma* by RAPD profiles and the results were consistent with the morphological, physiological and ecological data of these strains which agree the present study. Schlick *et al.* (1994) and Zimand *et al.* (1994) used RAPD markers obtained from arbitrary primers to distinguish strains of *Trichoderma*. This report agrees the findings of the present investigation.

Molecular characterization of different *Trichoderma* isolates are not available and there were no specific markers to differentiate the isolates effectively. The RAPD technique revealed some degree of polymorphisms for variation study of thirty five *Trichoderma* isolates in the present study and the results obtained from the experiment showed significant molecular variation among the isolates in relation to morphological characters. Therefore, findings of the present investigation agree with the report of Kullnig *et al.* (2000) who recognized about 35 *Trichoderma* species on the basis of morphological and molecular characters and revealed genetic variability among the isolates through RAPD analysis. Hadrys *et al.* (1992) have provided relatively simple and inexpensive method for examining variation in the total genome through RAPD-PCR technique. On the other hand Peteira *et al.* (2001) presented the genetic diversity among *Trichoderma* isolates by RAPD technique which also supports the results of the present investigation.

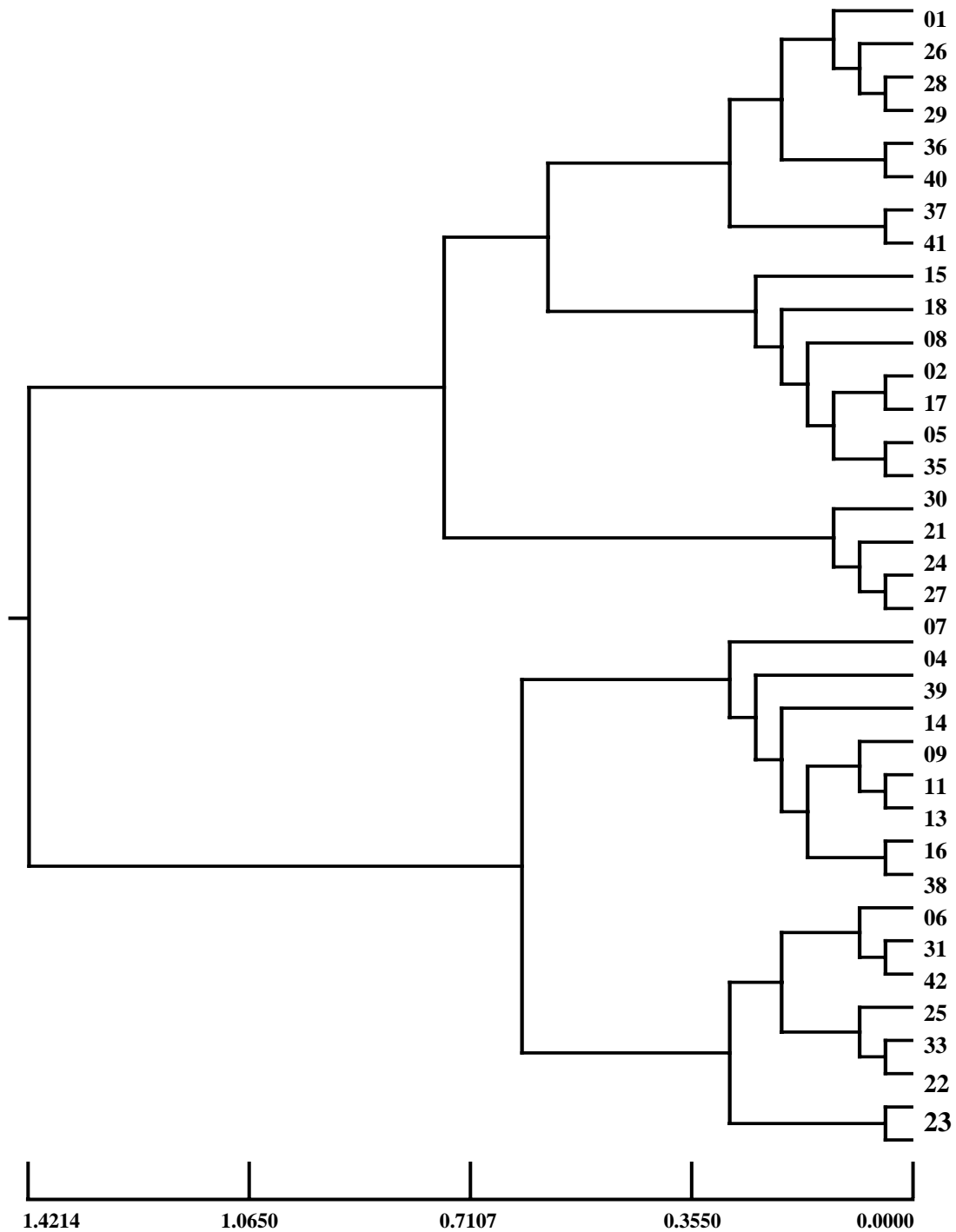


Fig. 1. Unweighted pair group method of arithmetic mean (UPGMA) dendrogram based on Nei's (1972) genetic distance, summarizing data on differentiation in 35 isolates according to RAPD analysis

Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated segregation of the 35 isolates of *Trichoderma* spp. into two main clusters: T4, T6, T7, T9, T11, T13, T14, T16, T22, T23, T25, T31, T33, T38, T39, T42 were grouped into cluster -1, most of which were dark green and yellowish green in colour. On the other hand T1, T2, T5, T8, T15, T17, T18, T21, T24, T26, T27, T28, T29, T30, T35, T36, T37, T40, T41 were grouped in cluster-2, most of which were green, light green and whitish green in colour and probably identical in growth habit, colony consistency which are in agreement with the opinion of Meah (2007) on the study of other research. The highest genetic distance (1.4214) was found between T14 and T (2, 17) and they remained in different cluster. The genetic distance between T24, T₂₇ and T₂₈, T₂₉ was lowest (0.0034) and found in a group of the same cluster. This result indicates that highest genetic diversity remains between the isolates T14 and T (2, 17). On the other hand, lowest genetic diversity remains between the isolates T24 and T27, also in T28 and T29.

This study indicates that T14 Vs. T (2, 17) and T24 Vs. T27 also T28 Vs. T29 can be used in genetic diversity and more developed analysis for the improvement of beneficial fungi *Trichoderma* spp. UPGMA dendrogram based on the DICE similarity index (SD) illustrated the genetic relationships among 14 *Trichoderma* isolates was described by Goes and Costa (2002) and that partially agree to the present study.

Conclusion

Highest genetic distance (1.4214) was found between T14 and T (2, 17) and they remained in different clusters. The genetic distance between T24 and T27, also in T28 and T29 was the lowest (0.0034) and found in a group of the same cluster. The lowest genetic distance (0.0034) was revealed between T24 and T27, also in T28 and T29. Considering this, it may be concluded that T24, T27, T28 and T29 were originated from same parent or closely related parents.

From the study, it can be concluded that RAPD markers can be sensitive, simple, efficient and powerful tool for genetic diversity analysis among and within *Trichoderma* isolates and effectively trace their genetic relationships and which could be useful for distinguishing the most efficient isolate for its use in different beneficial purposes.

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