한작지(Korean J. Crop Sci.), 56(1): 35~43(2011)

Rapd Analysis of *Trichoderma* Isolates for Superior Selection for Biopesticide Preparation

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ABSTRACT Thirty five isolates of *Trichoderma* species collected from seven different locations of Bangladesh were studied for morphological characters and molecular variation. Mycelial diameters of the isolates varied from 8.28 cm to 9.00 cm. Based on colony colour, isolates were grouped into five such as dark green, green, light green, yellowish green and whitish green. Maximum isolates were green and light green. On the basis of growth habit and colony consistency, the isolates were categorized into three groups, in which most species had fast growth and were compact in appearance. PCR-based Random Amplified Polymorphic DNA (RAPD) technique employing 3 decamer primers produced 36 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 (13 isolates in cluster 1 and 22 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 bio-pesticide and decomposition of municipality waste.

Keywords : *Trichoderma* isolates, RAPD, biopesticide

Trichoderma spp. are filamentous fungi that are present in nearly all soils and widely distributed in the soil, plant material, decaying vegetation, wood and other diverse habitats. *Trichoderma* is a genus of asexually reproducing fungi that are often the most frequently isolated and most prevalent culturable soil fungi; nearly all temperate and tropical soils contain 101-103 culturable propagules per gram (Samuels and Lodge, 1996). Colonies of *Trichoderma* grow rapidly producing white, yellow or green cushions of sporulating filaments and mature in 5 days.

Some identifying characteristics of *Trichoderma* spp. are: Facultative anaerobic, grows saprophytically or as a parasite on other fungi, grows toward hyphae of other fungi, coils around them and attaches to host mycelium, conidiophores are erect and produce side branches bearing whorls of short phialides. Conidia are one-celled ovoid spores and are produced successively from tips of phialides (Rifai, 1969).

Most *Trichoderma* strains have no sexual stage but instead produce only asexual spores. However, for a few strains the sexual stage is known, but not among strains that have usually been considered for bio-control purposes. Traditional taxonomy was based upon differences in morphology, primarily of the asexual sporulation apparatus, but molecular approaches are now being used. They show a high level of genetic diversity, and can be used to produce a wide range of products of commercial and ecological interest. 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 (Harman, 2000).

Different media for isolation purposes are used to grow *Trichoderma* spp. 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 (Fokkema, 1995).

The Random Amplified Polymorphic DNA (RAPD) procedure developed by Williams *et al.* (1990) and Welsh and McClelland

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(1990) that involves simultaneous amplification of several anonymous loci in the genome using primers of arbitrary sequence has been used for genetic, taxonomic and ecological studies of several fungi. 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 (Random Amplified Polymorphic DNA) is a molecular technique used for such purposes. The advantage of the RAPD's 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. Though RAPD has some limitation 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 (Mailer *et al.*, 1994) and construction of the first linkage maps for certain plant species and pathogens (Yang and Quiros, 1995). RAPD methods have been successfully used for genetic variability analysis.

IPM Lab biopesticide (code- 34/USDA/BG-ARS122) is in commercial use (Meah and Islam, 2005) which was formulated from *Trichoderma*. 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). Morphological and physiological variation of the isolates was studied earlier but not in details. Variation at molecular level has not been studied before. Molecular study may reveal any variation that exists among the isolates in support of the variation in physiological properties. Therefore, the present research work was undertaken to determine morphological and genetic variation among some isolates of *Trichoderma* spp. collected from different regions of Bangladesh using RAPD technique in order to confirm superiority of the isolate CP or select more efficient isolate.

MATERIALS AND METHODS

The *in vitro* experiment was conducted at the IPM Laboratory, Department of Plant Pathology, Central Laboratory, Bangladesh Agricultural University, Mymensingh. Part of molecular experiment was conducted at the Biotechnology Division of Bangladesh Institute of Nuclear Agriculture. Thirty five *Trichoderma* isolates were collected and used. The experiment was designed in a Completely Randomized Design (CRD) with four replications in the laboratory. All activities were conducted during March - December 2009.

Isolation of *Trichoderma* spp.

Trichoderma spp. were isolated from soil following dilution plate technique (Askew and Laing, 1993). One gram of composite soil sample collected from the field was taken in a test tube containing 9 ml of sterilized water to make dilution as 1:10. Similarly a series of dilution process was continued until the sample was diluted to 1:10000. All primary samples were diluted in the same process.

One ml of each sample (10^{-4}) was placed in each PDA plate, mixed thoroughly with the medium using a glass spreader.

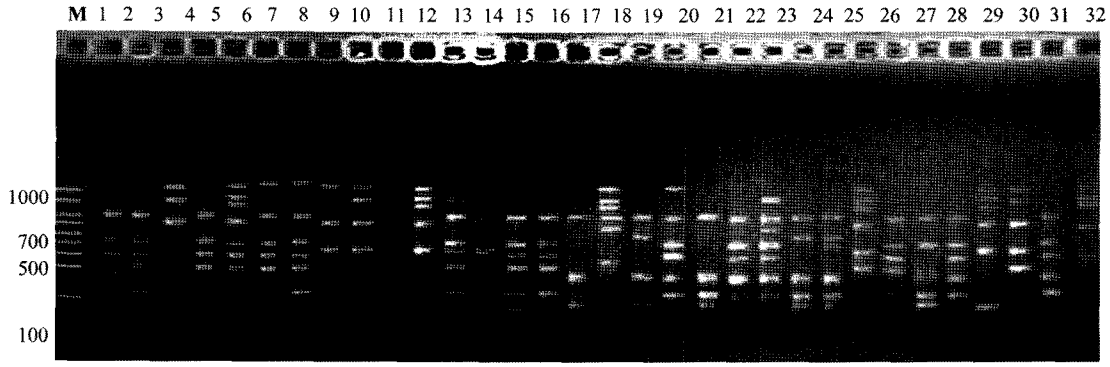

Plate 1. RAPD profiles of different isolates of *Trichoderma* spp. using primer OPB-9 (Lane M: 20 bp ladder, Lane 1-2: T_1 to T_2, Lane 3-8: T_4 to T_9, Lane 9: T_{11}, Lane 10-15: T_{13} to T_{18}, Lane 16-17: T_{21}-T_{22}, Lane 18-19:T_{24}-T_{25}, Lane 20-24: T_{27}-T_{31}, Lane 25: T_{33}, Lane 26-31: T_{35}-T40, Lane 32: T_{42} on agarose gel).

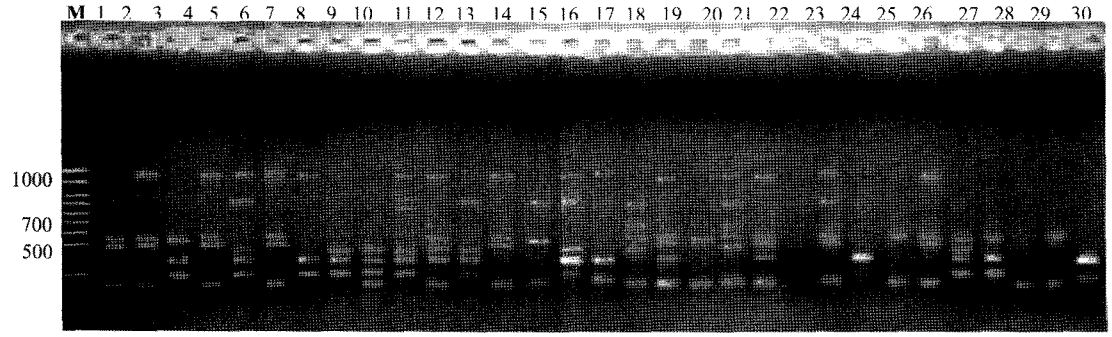

Plate 2. RAPD profiles of different isolates of *Trichoderma* spp.using primerOPB-10(Lane M: 20bp ladder, Lane1-2: T_1-T_2, Lane3-4: T_4-T_5, Lane5-7: T_7-T_9, Lane8: T_{11}, Lane 9-13: T_{13}-T_{17}, Lane14-21: T_{21}-T_{28}, Lane22-23: T_{30}-T_{31}, Lane24: T_{33}, Lane25-26: T_{35}-T_{36}, Lane27-31:T_{38} toT_{42} on agarose gel).

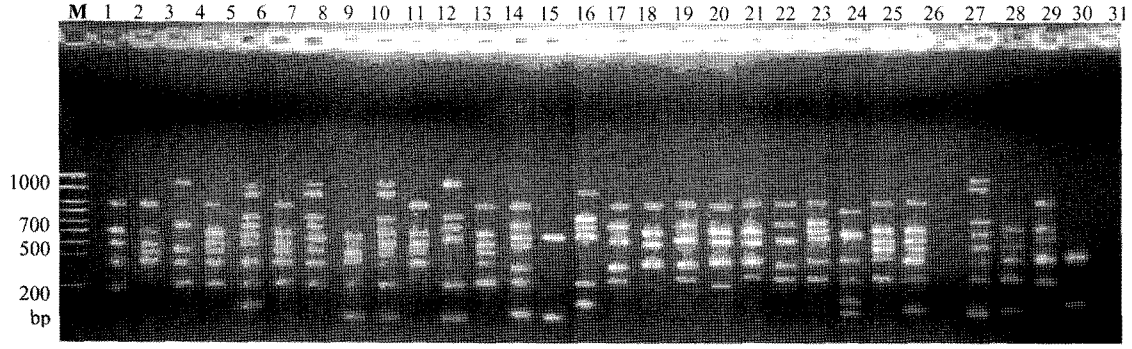

Plate 3. RAPD profiles of different isolates of *Trichoderma* spp. using primer OPC-1 (Lane M:20 bp ladder, Lane 1-2: T_1-T_2, Lane 3-4: T_4-T_5, Lane 5-7: T_7-T_9, Lane 8-12: T_{13}-T_{17}, Lane 13-17: T_{21}-T_{25}, Lane 18-22: T_{27}-T_{31}, Lane 23:T_{33}, Lane 24-31: T_{35} to T_{42} on agarose gel).

The Petri-plates were incubated for 5 days for growth at room temperature (25±2℃). After 3 days of incubation, plates were observed for *Trichoderma* colony. The growing margin of *Trichoderma* colony was cut into 5 mm blocks with the help of a cork borer. The blocks were carefully placed in a new PDA plates for pure culture of *Trichoderma*. The new plates were incubated as before. The pure culture of *Trichoderma* was found to grow sufficiently within 3 days of incubation. The well developed pure cultures were sub-cultured to PDA plate and transferred to PDA slants for preservation. The fully grown *Trichoderma* in slants were preserved in the refrigerator at 4℃ for further use. The isolates were identified following the key of Kubicek and Harman (1998).

Morphologcal characters of different *Trichoderma* isolates

Radial mycelial growths of the isolates were studied following method of Sultana *et al.* (2001). The fungus started growth at 24 hours of inoculation. Radial mycelial growth (cm) was measured at 24, 48 and 72 hours of inoculation. Mean of three replications were taken as growth of each isolate. Colony characters such as shape, colour, growth habit, compactness and spore density /ml were measured.

The number of spores per 1 ml was determined putting the average number of spore per unit cell counted in Haemacytometer and using the following formula (Ashrafuzzaman, 1976):

Number of spores per cubic mm suspension=

$$\frac{\text{Number of spores counted X dilution}}{\text{Number of smallest square counted}} X\,4000$$

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 aluminum foil for each isolate and kept in refrigerator (-20℃) before genomic DNA isolation.

Thirty five isolates of *Trichoderma* spp. were used for the RAPD. The extraction of total genomic DNA of each isolate of *Trichoderma* was made as described by Raeder and Broda (1985). As a source of genomic DNA, fresh mycelia samples were collected from the 3 days old culture.

Methods of DNA isolation are variable. 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 (Raeder and Broda, 1985).

Confirmation of DNA preparation

Usually isolated genomic DNA contains a large amount of RNA and pigments, which usually cause spuriously high estimation of DNA concentration on a spectrophotometer. Therefore DNA samples were evaluated both quantitatively and qualitatively and for the amount of RNA present using spectrophotometer and 1% agarose gel electrophoresis, respectively.

Documentation of the DNA samples

After electrophoresis, the gel was viewed under UV transilluminator (Fig. 1).

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 of 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 and stored in the freezer.

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

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

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), $V_2 = V_1 \times S_1 / S_2$

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 constructed using the POPGENE (Version 1.31) computer program.

Fig. 1. Electrophoregram of genomic DNA samples of different *Trichoderma* spp.

RESULTS

Morphological characterization of *Trichoderma* isolates

Isolates of *Trichoderma* spp. distinctly differed on their cultural and morphological properties like mycelial growth, colony color, and colony consistency and sporulation rate.

Mycelial growth

Mycelial growth rate of different isolates varied considerably up to 72 hours (3 days). Based on the growth *Trichoderma* spp. were grouped, in which 18 isolates had the higher mycelial growth, 6 had medium and 1 showed slow growth rate. Mycelial growth ranged between 2.76 and 3.00 cm/24h. Isolate 33 with dark green colour had the highest mycelial growth rate (3.00cm/24h) while isolate 7 had the lowest with light green colony colour. A similar study was conducted in Hebron University, Palestine by Radwan and Mohammad (2006) who measured radial mycelial growth of forty seven isolates of *Trichoderma* and found different level of growth.

Colony shape

All 35 isolates of *Trichoderma* spp. produced regular shaped colony.

Colony colour

Five isolates produced dark green colony, 10 isolates showed green colony, 10 showed light green, four isolates produced yellowish green and six produced whitish green colony (Fig. 2).

Growth habit

On the basis of growth habit isolates were divided into three groups such as fast, medium and slow.

Colony consistency

Colonies were either very compact, compact or loose (Table 1).

Geographical distribution

26 isolates of *Trichoderma* collected from Thakurgaon and Dinajpur were mostly green and light green. Five isolates collected from Kishoregonj were well distributed to four colour groups. Dark green and yellowish green isolates were less frequent (Table2, Fig. 3).

Spore density of *Trichoderma* isolates

Higher spore density was recorded among isolates of dark green and yellowish green colour. Whereas lower was found

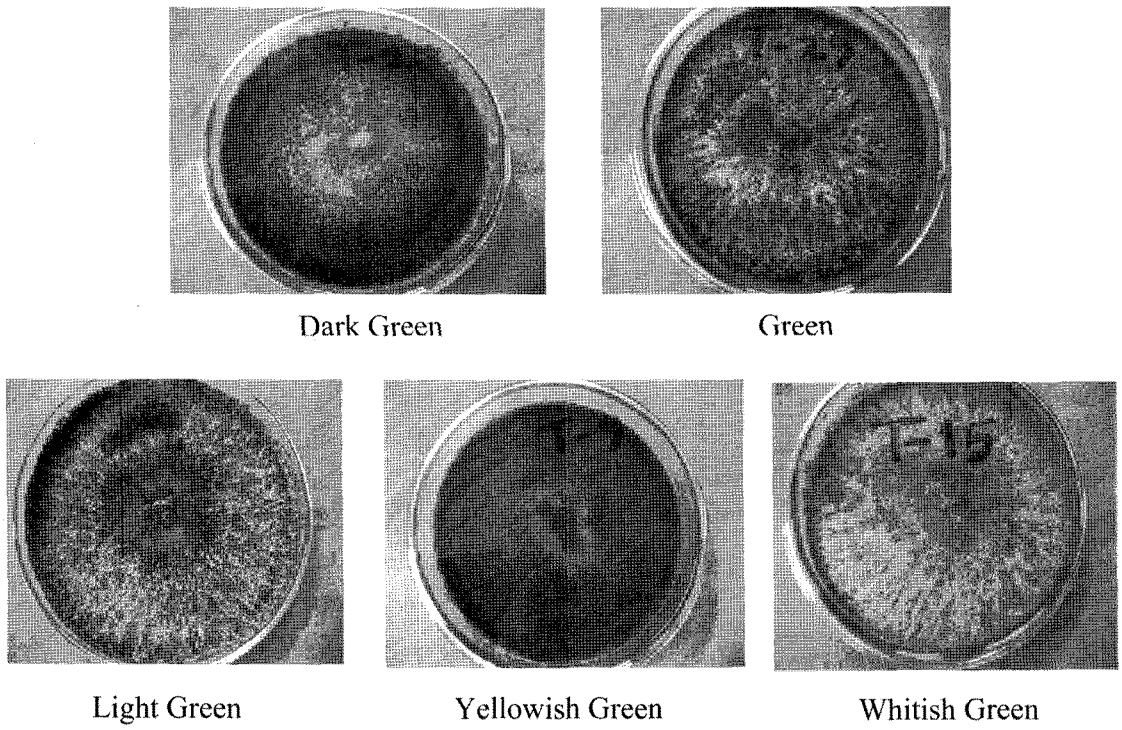

Fig. 2. Five colour groups of *Trichoderma* isolates.

Table 1. Morphological characters of *Trichoderma* isolates

Group	Colour	Isolates	Growth habit	Colony consistency
Group-1	Dark green	22, 23, 33, 39, 42	Fast	Very compact, compact
Group-2	Green	4, 6, 25, 27, 29, 30, 31, 35, 37, 41	Fast, slow (37, 41)	Compact
Group-3	Light green	1, 7, 13, 16, 21, 24, 26, 28, 36, 40	Fast, medium, slow (7)	Very compact (24), compact, loose (7, 26)
Group-4	Yellowish green	9, 11, 14, 38	Fast	Very compact (14), compact
Group-5	Whitish green	2, 5, 8, 15, 17, 18	Fast (2, 5), medium	Compact, loose (5)

Table 2. Distribution of morphological groups of Trichoderma isolates in different locations of Bangladesh.

Location	Group No					Total
	Group-1 (Dark green)	Group-2 (Green)	Group-3 (Light green)	Group-4 (Yellowish green)	Group-5 (Whitish green)	
Thakurgaon	22, 23	27, 29, 31, 35	1, 7, 13, 24, 26, 28	11	2, 5, 18	16
Dinajpur	42	4, 6, 25, 30	21	9, 14, 38	15	10
Birgonj, Dinajpur	-	-	40	-	-	1
Boyra, Mymensingh	-	-	16	-	-	1
BAU, Mymensingh	-	37	-	-	-	1
Kishoregonj	39	41	36	-	8, 17	5
Natore	33	-	-	-		1
Total	5	10	10	4	6	35

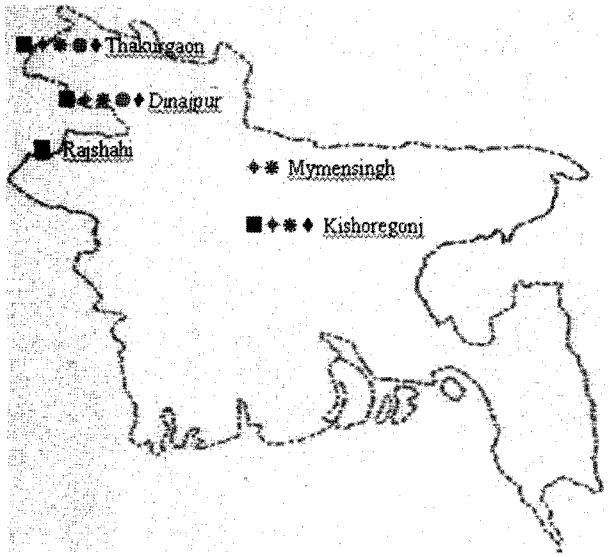

Fig. 3. Distribution of morphological groups of *Trichoderma* isolates in different locations of Bangladesh.

among the isolates of light green, green and whitish green colour (Fig. 4). From this result, it is evident that *Trichoderma* isolates showed differences in spore production and no relationship could be drawn between sporulation capacity and colony colour or colony consistency which agrees with Singh and Sharma (2006) who had similar observation while working with some *Trichoderma* isolates.

Primer selection and RAPD pattern

Three primers viz., OPB-09, 0PB-10, and OPC-01 which showed comparatively maximum number of high resolution bands and distinct polymorphic amplified products were selected.

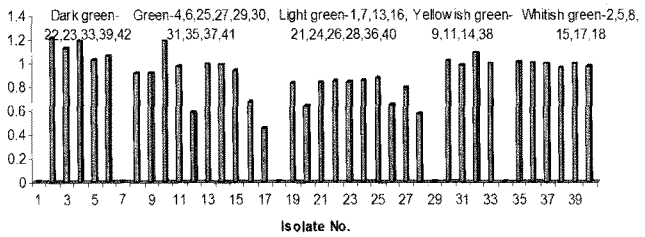

Fig. 4. Spore production by different isolates of *Trichoderma harzianum*.

Selected three primers generated 36 bands, all were polymorphic. The three different primers generated various banding patterns, ranging from 13 (OPB-9), 9 (OPB-10) to 14(OPC-01). The primer OPB-9 and OPC-01 produced the highest numbers of polymorphic bands (13 and 14). Thus it showed a higher level of polymorphism. On the other hand, the primer OPB10 generated least number of polymorphic bands (9). The present results have support of Schlick *et al.* (1994) and Zimand *et al.* (1994) who used RAPD markers obtained from arbitrary primers to distinguish strains of Trichoderma.

Grouping of *Trichoderma* isolates based on similar bands

The isolates were rearranged by keeping only one lane from two similar lanes. In this way the highest polymorphic loci was found for the primer OPB-09 and the isolates number were reduced from 35 to 32. Similarly for the primers OPB-10 and OPC-01 the polymorphic loci were detected by reducing the isolates from 35 to 31.Grouping of the isolates is shown below:

Primers	Isolates	Grouping
OPB-09	(22,23), (25,26), (40,41)	22, 25, 40
OPB-10	(5,6), (17,18), (28,29), (35,37)	5, 17, 28, 35
OPC-01	(5,6), (9,11), (17,18), (25,26)	5,9,17,25

Gene flow and population differentiation

The average estimated gene flow (Nm) was 0.0000 and co-efficient of gene differentiation (GST) was 1.0000 across all loci. Hardy-Weinberg expectation of average heterozygosity in subpopulation (H_t) was 0.3946 whereas obtained heterozygosity (H_S) was 0.0000 across all primers. RAPD marker revealed high level of differentiation (GST=1.0000) that supports the presence of sufficient polymorphisms in 35 *Trichoderma* isolates.

Gene diversity for the RAPD Primer

Average gene diversity (h) and Shannon's Information index (I) for three primers across different isolates for all loci were found 0.3946 and 0.5749 respectively. High level of gene diversity value and Shannon's Information index was found in locus OPB09-1, OPB09-7, OPB09-9, OPB09-12, OPB10-1, OPC01-3, OPC01-4, OPB10-6, and OPB10-7 (0.4898, 0.4963, and 0.4996, respectively). Lowest level of gene diversity value and Shannon's Information index (0.0555 and 0.1567) was found in locus OPB09-6, OPB10-3, OPB10-4.

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 0.0282 to 1.3863. Comparatively higher genetic distance (1.3863) was found between T14 and T(37,41). Genetic relationships among 14 *Trichoderma* isolates were described by Costa and Góes (2002) that partially agree with the findings of present study.

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: 13 isolates 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 22 isolates were grouped in cluster 2, most of which

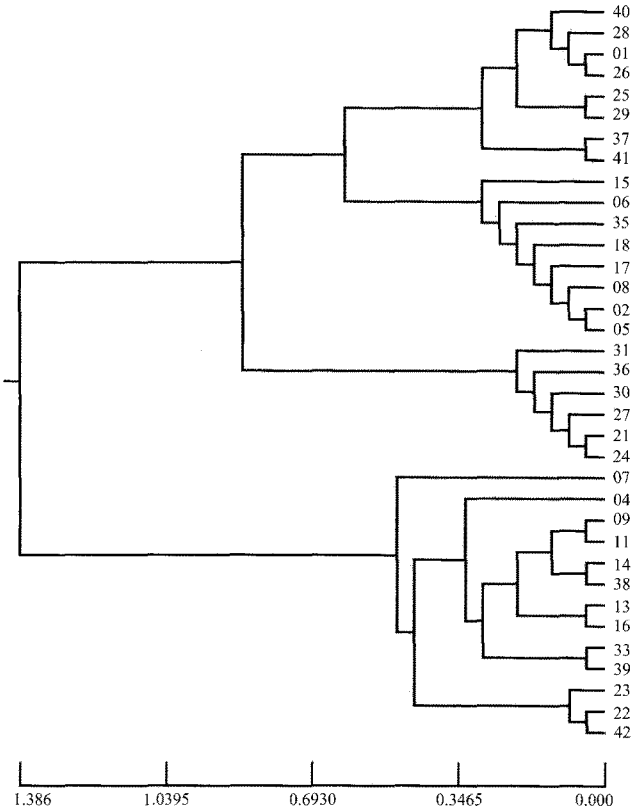

Fig. 5. Unweighted pair group method of arithmetic mean (UPGMA) dendrogram based on Nei's (1972) genetic distance, summarizing data on differentiation in 35 isolates according to RAPD analysis.

were green, light green and whitish green in colour and all were probably identical based on growth habit and colony consistency(Fig. 5).

Cluster 2 was again divided into two sub-clusters. Sub-cluster 1 has isolate T7 which is light green and other isolates T(4,9,11,14,38,13,16,33,39,22,23,42) belonged to sub-cluster 2. Sub-cluster 2 was again divided into two sub sub-cluster. Sub sub-cluster 2 contained T22, T23 and T42, all of which were dark green colour. Sub sub-cluster 1 was then divided into two groups. Group1 has one isolate T4 having green colour and rest was in group 2. Group 2 was divided into two sub groups. T33 and T39 which were dark green belonged to sub group 2, whereas sub group 1 was again divided into two sub sub-groups. Sub sub-group 2 contained T13 and T16 which were light green colour and sub sub-group1 contained T9, T11, T14, and T38 all of which were yellowish green.

Further, the isolates of cluster 1 were divided into two sub-clusters, sub-cluster 2 contained T21, T24, T27, T30,

T31, T36 in which T21, T24, T36 were light green whereas T27, T30, T31 were green colour. Rest of the isolates belonged to sub-cluster 1.

DISCUSSIONS

Trichoderma species have been investigated as biological control agents (BCAs) for over 70 years. They are free-living fungi and highly interactive in root, soil and foliar environments. Thirty five isolates of *Trichoderma* spp. were collected from different strategic areas of Bangladesh. Collected isolates were categorized into groups depending on their cultural, morphological and molecular characterizations like colony colour, mycelial growth and pattern, DNA concentration, DNA banding pattern. Among the groups, DNA concentration ranged from 250 to 20,200 ng/μl.

Mycelial growth was measured on Potato Dextrose Agar media. The mycelial growth varied from 8.28 to 9.00cm.

The isolates were classified into 5 categories depending on colour, which were dark green, green, light green, yellowish green and whitish green. Among thirty five isolates, five were dark green, ten were green, ten light green, four whitish green and six were yellowish green in colour.

Sporulation capacity of the isolates was significantly different in different isolates. The maximum number of spores was produced by the isolates of dark green and yellowish green colour, whereas lowest spore density was found in the isolates of light green and green colour. The genetic variations in thirty five *Trichoderma* isolates were analyzed by Polymerase Chain Reaction (PCR)-based RAPD markers by using three arbitrary decamer primers (OPB-09, OPB-10, and OPC-01). The 3 different primers generated various banding patterns, ranging from 9 to14. Selected 3 primers produced 36 bands from which all bands (100%) were polymorphic. The 3 primers generated average 12 scorable bands per primer and 12 polymorphic RAPD markers per primer.

Relationships between colony colour, mycelial growth rate and sporulation capacity of the isolates were evident. Molecular groupings are partly in line with the cultural characters particularly with sporulation capacity. Dark green isolates having faster mycelial growth and higher sporulation were mostly grouped in the same cluster.

Isolate 33 representing the strain *Trichoderma harzianum* CP (already established at IPM Lab, BAU) a dark green colony produced spores (1.19×10^{10} per ml) is in the group of other four isolates (22, 23, 39 and 42). The range of sporulation in this group is $1.06\text{-}1.22 \times 10^{10}$ per ml. The strain CP is second to isolate 22 which having sporulation 1.22×10^{10}. So the present research has created scope for options for choosing isolate 22 for the replacement of the strain CP.

ACKNOWLEDGEMENTS

The USDA Grants (USDA-ARS-122) for conducting the research is gratefully acknowledged.

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