

SCREENING OF VARIOUS *TRICHODERMA* ISOLATES COLLECTED FROM PAKISTAN WITH THEIR SUBSEQUENT GENETIC HOMOLOGY BY RAPD ANALYSIS

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ABSTRACT

A total of 23 isolates of *Trichoderma* spp. collected from different areas of Pakistan, were procured from First Fungal Culture Bank of Pakistan (FCBP), Institute of Plant Pathology, University of the Punjab, Lahore. On the basis of morphological and cultural characteristics, *Trichoderma* isolates were identified into four species, namely *T. harzianum*, *T. hamatum*, *T. koningii* and *T. pseudokoningii*. The genomic homology of these isolates of *Trichoderma* was examined using RAPD analysis. Out of 27 random decamer primers, five primers viz., A-11, A-12, A13, B-05 and B-18 were screened out at 25°C optimized T_m (melting temperature). The amplification of bands were observed in the range of 250bp-50000bp by all five primers. In cluster analysis of five primers, isolate of *T. koningii* (wood of *M. indica*) showed least genomic similarity of 47.30% in comparison to all others. The other isolates of *T. harzianum*, *T. hamatum*, *T. koningii* and *T. pseudokoningii* showed maximum genomic homology (~78%). This data will be useful to achieve authentic identification for maximum usage of these microbes in all research fields.

Key words: *T. harzianum*, *T. hamatum*, *T. koningii*, *T. pseudokoningii*, RAPD, random decamers, cluster analysis.

INTRODUCTION

Trichoderma species are common, filamentous fungi and have long been known as non-harmful microorganisms. The genus *Trichoderma* poses a major challenge for systematic classification because the phylogenetic relationships of many of its members are still unclear. In the last few decades an ample array of molecular techniques has been introduced to obtain new disposition for the classification of *Trichoderma* species and each method has its own benefits and constraints. Randomly amplified polymorphic DNA (RAPD) procedure involves simultaneous amplification of several anonymous loci in the genome using primers of arbitrary sequence, used for genetic, taxonomic and ecological studies of several fungi, including *Trichoderma* species (Abbasi *et al.*, 1999). In Pakistan no serious efforts have been made to study this versatile and economically important mold as well as its molecular studies is mostly neglected.

The current research was focused to evaluate the extent that local isolates of *Trichoderma* are diverse genetically. The present study was designed for advanced phylogenetic analysis on molecular basis to differentiate twenty three isolates of *Trichoderma* species (including thirteen isolates of *T. harzianum*, five of *T. koningii*, four of *T. pseudokoningii*, and two of *T. hamatum*.) for maximum usage of these microbes in all research fields.

MATERIALS AND METHODS

Culture procurement and genomic DNA extraction of *Trichoderma* species

Twenty four isolates of *Trichoderma* species from variable substrate sources, including (thirteen isolates of *T. harzianum*, five of *T. koningii*, four of *T. pseudokoningii*, and one of *T. hamatum*.) were acquired from First Fungal Culture Bank of Pakistan (FCBP), Institute of Plant Pathology, University of the Punjab, Lahore. The total genomic DNA of different isolates of *Trichoderma* species were extracted using the CTAB method (Saghai-Marooof *et al.*, 1984) as well as STES method (Park *et al.*, 2005) of DNA extraction. DNA fragments were separated by electrophoresis on 0.8% (w/v) agarose gels by following a method derived from Hoisington *et al.* (1994).

Random Amplification of Polymorphic DNA (RAPD) Analysis

Random Primer Screening

RAPD analysis was carried out following the method described by Ranganath *et al.* (2002). Twenty seven primers of A and B series supplied by Gene Link™ were used against twenty four isolates of *Trichoderma* species in RAPD analysis.

RAPD Reaction Mixture

RAPD reactions were contained PCR Buffer (1.0X), MgCl₂ (1.5-3.0mM), dNTPs (0.2mM), RAPD decamers (100 pMole/μL), Template DNA (0.5-1μg), Taq Polymerase (1U) in a final volume of 50 μL.

RAPD Temperature Cycling Condition

RAPD amplification were carried out in Master cycler gradient PCR (TECHNE TC-412) with initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 25°C for 1 minute, primer extension at 72°C for 1 minute and final extension at 72°C for 5 minute. The reaction was terminated at 4.0°C in 2-3 hours. The five melting temperatures conditions were checked i.e. 20°C, 25°C, 28°C, 30°C, 33°C and 25°C melting temperature was optimized for the decamers used in the study.

Analysis of Amplified DNA fragments

The analysis of RAPD amplified bands were performed on 1% agarose gel with ethidium bromide (10mg/mL), 6X gel loading dye at 100 volts for 45 minutes and examined under UV light and photographed on the Gel Documentation System.

Data Analysis

The amplification profiles of different isolates of *Trichoderma* species were compared with each other. In order to assess the overall distribution of genetic diversity, data was analyzed using MINITAB software (MINITAB, 2004).

RESULTS

Initial Primer Screening for RAPD

RAPD results were obtained with only five decamers i.e. A-11(CAATCGCCGT), A-12 (TCGGCGATAG), A-13 (CAGCACCCAC), B-05 (TGCGCCCTTC) and B-18 (CCACAGCAGT) at optimized melting temperature of 25°C.

Amplifications and Dendrogram observed by GL Decamer A-11

The results obtained from decamer A-11 showed band at 250bp, 500bp, 600bp and 750bp. The dendrogram was illustrated that maximum genomic homology ~90% was found among *T. koningii* and *T. harzianum* isolates however least genomic similarity ~82% was observed in *T. pseudokoningii* isolates (Plate and Fig-1A).

Amplifications and Dendrogram observed by GL Decamer A-12

The results demonstrated that 250bp to 500bp bands were observed in GL decamer A-12 and dendrogram indicates that *T. koningii* isolates shared maximum genetic similarities ~85%. While least genetic similarity ~69% was observed in *T. hamatum* isolate with comparison to others. This dendrogram also shows into two major clusters indicating 50.08% genomic homology (Plate and Fig.-1B).

Amplifications and Dendrogram observed by GL Decamer A-13

The results obtained from decamer A-13 showed bands at 250bp, 300bp, 400bp, 500bp, 600bp, 750bp and also at 2000bp and 5000bp. The dendrogram analysis maximum genomic similarity ~92% was quite evident in *T. koningii* isolates. The least genetic homology ~85% was formed in *T. koningii* and *T. pseudokoningii* isolates (Plate 2A and Fig-1C).

Amplifications and Dendrogram observed by GL Decamer B-05

The results of decamer B-05 were subjected in the range of 250bp to 500bp mostly. The *Trichoderma* isolates of *T. hamatum*, *T. koningii*, *T. pseudokoningii* and *T. harzianum* showed maximum genomic similarity ~93% in dendrogram analysis (Plate 2Band Fig-1D).

Amplifications and Dendrogram observed by GL Decamer B-18

The amplifications acquired from decamer B-05 for different isolates of *Trichoderma* species illustrated from 250bp to 1500bp. The dendrogram showed that isolates of *T. harzianum* shared maximum genomic homology ~95% while isolates of *T. hamatum*, *T. koningii* and *T. pseudokoningii* exhibited ~93% similarity with others (Plate 3 and Fig-1E).

Table 1. Genetic homology in different isolates of *Trichoderma* species by RAPD analysis using cluster dendrogram

FCBP ACCE #	SPECIES	SUBSTRATE	GENOMIC SIMILARITY	RAPD GROUP	CLUSTER
908	<i>T. hamatum</i>	Soil mycoflora	~78%	G -a	C -1
213	<i>T. pseudokoningii</i>	Citrus fruit	~78%	G -a	C -1
860	<i>T. harzianum</i>	<i>M. indica</i> , soil	~78%	G -a	C -1
212	<i>T. pseudokoningii</i>	Green Chilli	~78%	G -b	C -1
732	<i>T. harzianum</i>	Air mycoflora	~78%	G -b	C -1
779	<i>T. harzianum</i>	Leaf litter	100%	G -b	C -1
755	<i>T. harzianum</i>	Decaying Wood	100%	G -b	C -1
84	<i>T. harzianum</i>	Rhizospheric soil	100%	G -b	C -1
125	<i>T. harzianum</i>	Decaying wood	100%	G -b	C -1
496	<i>T. harzianum</i>	Basidiocarp of <i>Hydnum</i> sp.	100%	G -b	C -1
210	<i>T. harzianum</i>	Soil mycoflora	100%	G -b	C -1
193	<i>T. harzianum</i>	Air mycoflora	100%	G -b	C -1
140	<i>T. harzianum</i>	Culture of <i>M. phaseolina</i>	100%	G -b	C -1
139	<i>T. harzianum</i>	<i>H. elastica</i> fruiting body	100%	G -b	C -1
585	<i>T. koningii</i>	<i>M. indica</i> stem	~78%	G -c	C -1
54	<i>T. pseudokoningii</i>	Isolate sent by Dr.S.M.Khan	~78%	G -c	C -1
489	<i>T. pseudokoningii</i>	Tannery effluent	~78%	G -c	C -1
692	<i>T. koningii</i>	Oat seeds	~78%	G -c	C -1
769	<i>T. hamatum</i>	<i>S. cumini</i> , stem	~60%	G -d	C -2
191	<i>T. koningii</i>	Citrus fruit	~78%	G -e	C -2
747	<i>T. koningii</i>	Wood	~78%	G -e	C -2
325	<i>T. harzianum</i>	Mushroom contamination	100%	-	C -3
249	<i>T. harzianum</i>	Polluted water	100%	-	C -3
765	<i>T. koningii</i>	<i>M. indica</i> , wood	47.30%	-	C -4

Cluster Dendrogram Analysis

The RAPD amplification data of all five primers (A-11, A-12, A-13, B-05 and B-18) were collectively exhibited that isolate of *T. koningii* showed least genomic similarity 47.30% in comparison with all other isolates. However isolates of *T. hamatum*, *T. koningii*, *T. pseudokoningii* and *T. harzianum* showed maximum genomic homology ~78% (Table-1 and Fig. 2).

DISCUSSION

In the present study the attempt has been made to elucidate the variations at molecular level of a group of *Trichoderma* isolates which have already been characterized on the basis of phenotypic features. The results of *T. harzianum* in this analysis are largely correlated with the morphological characterization recently been carried out by Bajwa *et al.* (2008), however the isolates of *T. koningii*, *T. pseudokoningii* and *T. hamatum* have shown 21%, 22% and 31% deviations respectively from the expected trends of affinities. RAPD is an initial step for screening the genome of any organism. This is the reason that genomic diversity among twenty four different local isolates of *Trichoderma* was compared by means of RAPD technique. Nelson *et al.*, in 1997 grouped out *Fusarium oxysporum* into two subpopulations based on RAPD analysis, and no polymorphism in RAPD pattern was observed among isolates. The conditions for RAPD analysis were optimized by investigating each factor individually. This included genomic DNA quality and concentration, primer annealing and extension temperature as well as denaturation time and melting temperature.

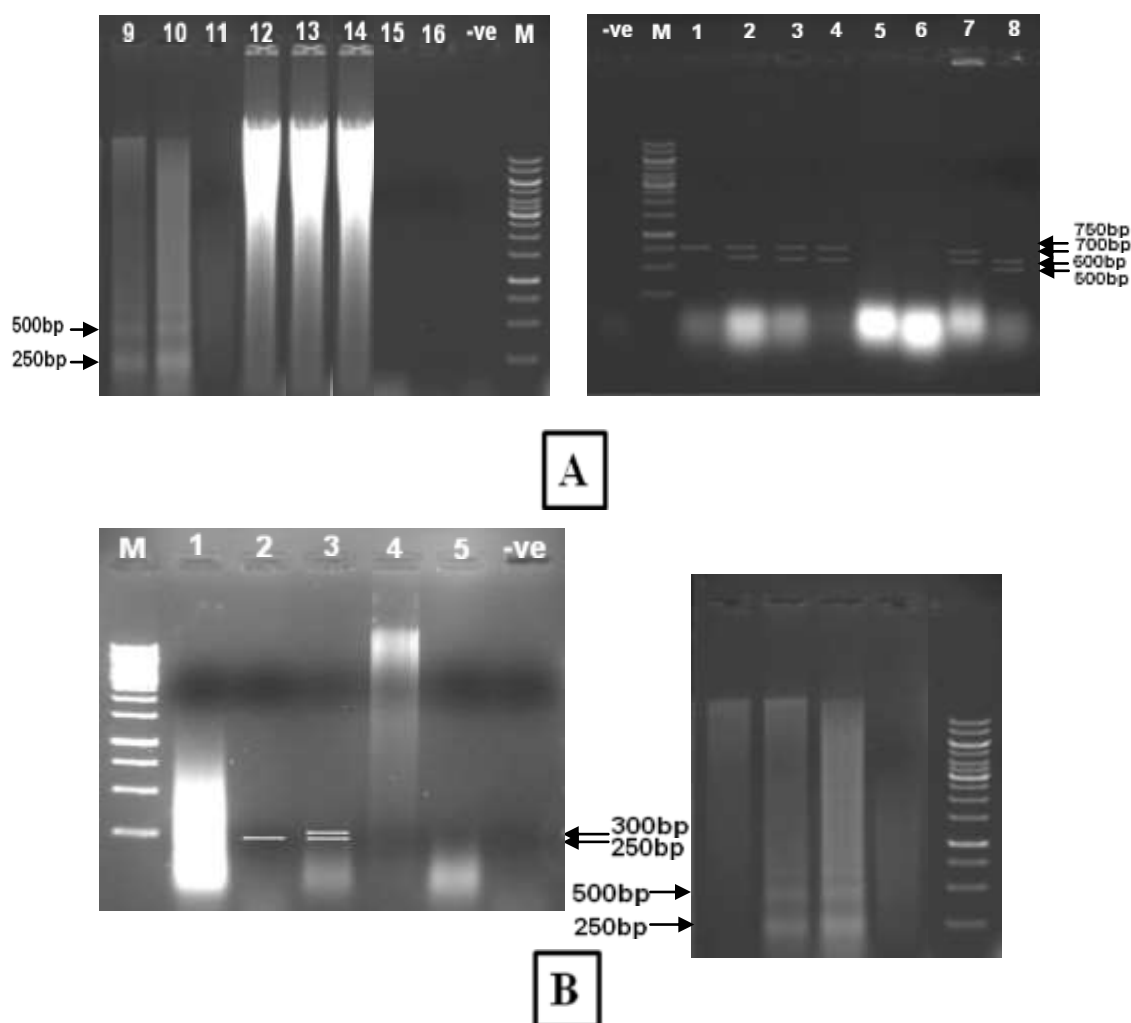


Plate 1. RAPD analysis of different isolates of *Trichoderma* species by using (A) Primer A-11 and (B) Primer A-12. -ve: negative control, M: DNA marker (1Kb ladder), 1: *T. hamatum* (*Syzygium cumini*, stem bark), 2: *T. hamatum* (soil mycoflora), 3: *T. koningii* (citrus fruit), 4: *T. koningii* (*M. indica* stem), 5: *T. koningii* (Oat seeds), 6: *T. koningii* (wood), 7: *T. koningii* (*M. indica*, wood), 8: *T. pseudokoningii* (Green Chilli), 9: *T. pseudokoningii* (Citrus fruit), 10: *T. pseudokoningii* (Tannery effluent), 11: *T. pseudokoningii* (isolate sent by Dr. S.M. Khan), 12: *T. harzianum* (*Mangifera indica*, rhizospheric soil), 13: *T. harzianum* (Leaf litter), 14: *T. harzianum* (Decaying Wood), 15: *T. harzianum* (Air mycoflora) and 16: *T. harzianum* (Basidiocarp of *Hydnum* sp.).

The collective dendrogram based on the amplification results of all the five primers indicated that isolates of *Trichoderma* were divided into four clusters (1, 2, 3 and 4). Cluster 1 were supplementary separated into three groups (a, b and c) and these exhibited proximately ~78% genomic similarity. Likewise, two groups (d and e) observed in cluster 2, in which the single isolate of *T. hamatum* (*Syzygium cumini*, stem bark) exhibited ~60% genomic similarity, *T. pseudokoningii* (green chilli) and *T. harzianum* (air mycoflora) displayed ~78% genomic similarities. Conversely, these isolates are distinguished from one another through conidial ornamentation, conidium morphology and enzyme production potential. The differences observed in percentage genetic homology is due to unique nature of the genomes of isolates under study. On the other hand, morphologically similar isolates of *T. harzianum* (mushroom contamination and polluted water) showed 100% genomic similarity to each other in cluster 3. However, only one isolate of *T. koningii* (*M. indica* wood) belonged to cluster 4 and showed that it was genetically distinct from all other isolates. The combined homology of *Trichoderma* isolates suggested that some phenotypically similar isolates were distinguished genetically. Whereas, isolates of *T. harzianum* gave 100% same results as compared to morphological data except two isolates. Although interspecific variations revealed that *T. pseudokoningii* was exactly similar to *T. harzianum* in cluster 1. Similarly, *T. hamatum* that have morphologically distinct characters of oblong or ellipsoidal phialospores was genetically very close to *T. koningii* and *T. harzianum*.

The abundant homoplasy in phenetic characters may be the likely reason, given that the number of morphologically distinct species is significantly lower than the number of genetically distinct species recognized using methods of genomic analysis (Druzhinina *et al.*, 2006).

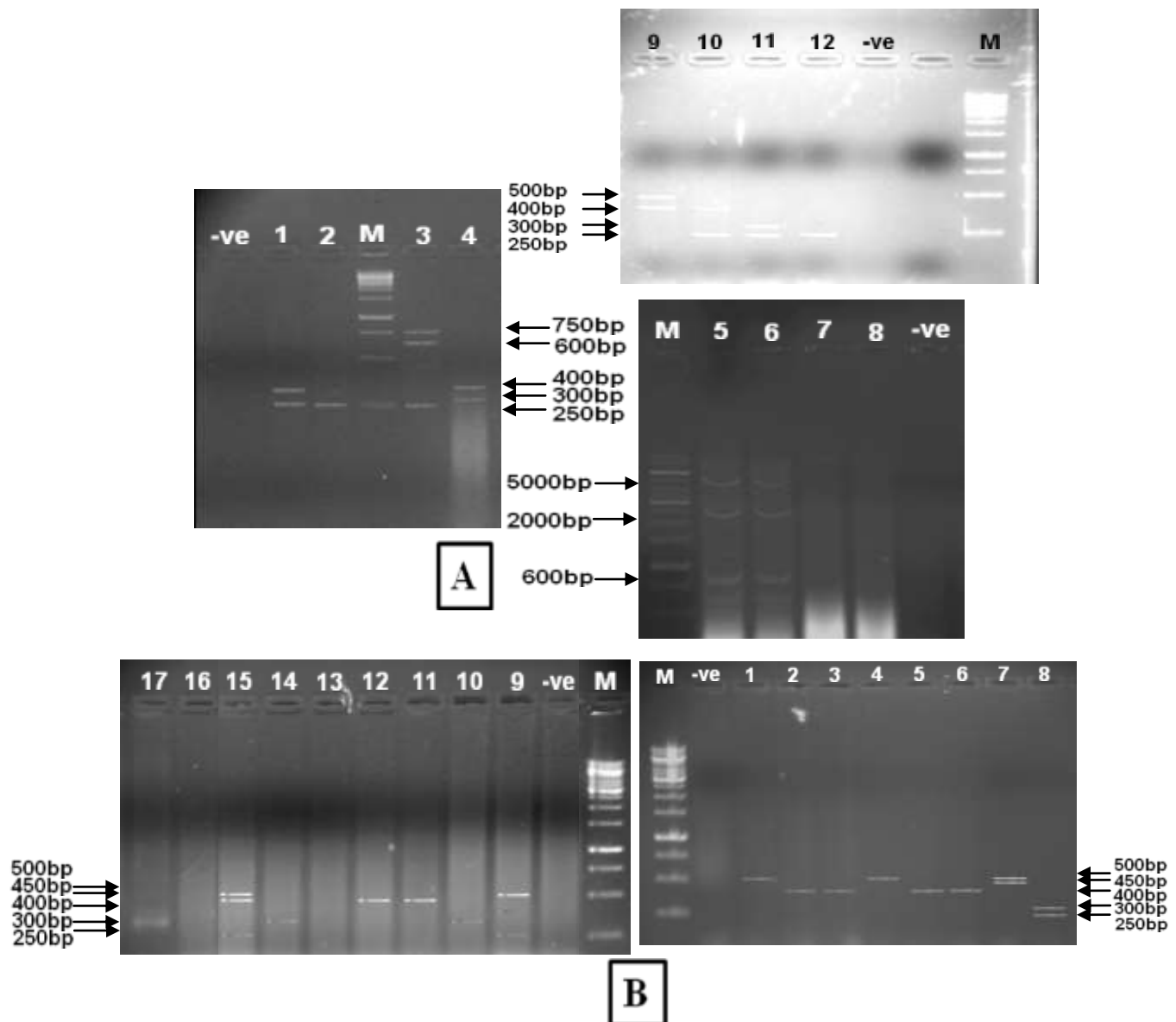


Plate 2. RAPD analysis of different isolates of *Trichoderma* species by using (A) Primer A-13 and (B) Primer B-05 –ve: negative control, M: DNA marker (1Kb ladder), 1: *T. hamatum* (*Syzygium cumini*, stem bark), 2: *T. hamatum* (soil mycoflora), 3: *T. koningii* (citrus fruit), 4: *T. koningii* (*M. indica* stem), 5: *T. koningii* (Oat seeds), 6: *T. koningii* (wood), 7: *T. koningii* (*M. indica*, wood), 8: *T. pseudokoningii* (Green Chilli), 9: *T. pseudokoningii* (Citrus fruit), 10: *T. pseudokoningii* (Tannery effluent), 11: *T. pseudokoningii* (Isolate sent by Dr. S.M. Khan), 12: *T. harzianum* (*Mangifera indica*, rhizospheric soil), 13: *T. harzianum* (Leaf litter), 14: *T. harzianum* (Decaying Wood), 15: *T. harzianum* (Air mycoflora), 16: *T. harzianum* (Basidiocarp of *Hydnum* sp.) and 17: *T. harzianum* (Mushroom contamination).

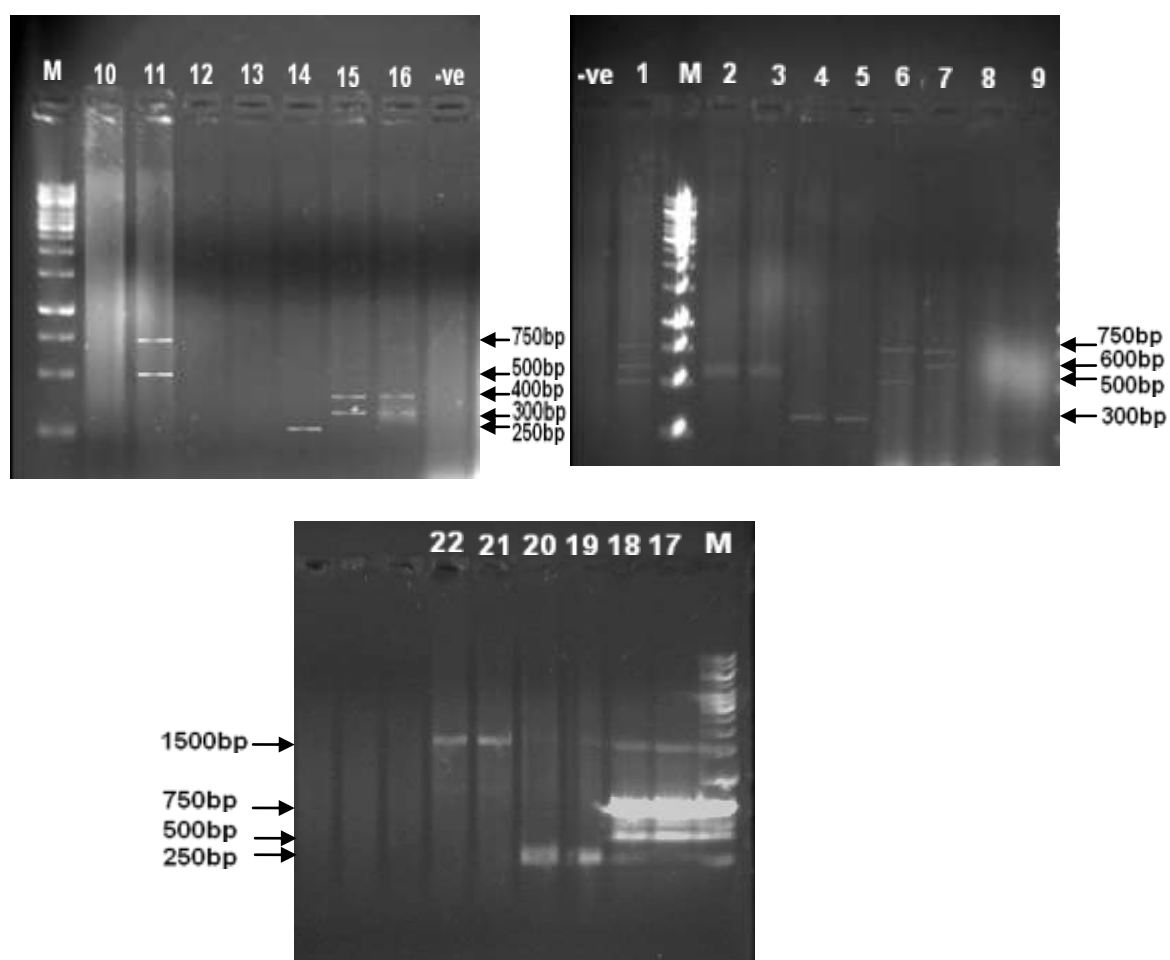


Plate 3. RAPD analysis of different isolates of *Trichoderma* species by using Primer B-18. **-ve**: negative control, **M**: DNA marker (1Kb ladder), **1**: *T. hamatum* (*Syzygium cumini*, stem bark), **2**: *T. hamatum* (soil mycoflora), **3**: *T. koningii* (citrus fruit), **4**: *T. koningii* (*M. indica* stem), **5**: *T. koningii* (Oat seeds), **6**: *T. koningii* (wood), **7**: *T. koningii* (*M. indica*, wood), **8**: *T. pseudokoningii* (Green Chilli), **9**: *T. pseudokoningii* (Citrus fruit), **10**: *T. pseudokoningii* (Tannery effluent), **11**: *T. pseudokoningii* (Isolate sent by Dr. S.M. Khan), **12**: *T. harzianum* (*Mangifera indica*, rhizospheric soil), **13**: *T. harzianum* (Leaf litter), **14**: *T. harzianum* (Decaying Wood), **15**: *T. harzianum* (Air mycoflora), **16**: *T. harzianum* (Basidiocarp of *Hydnum* sp.), **17**: *T. harzianum* (Mushroom contamination) **18**: *T. harzianum* (Polluted water), **19**: *T. harzianum* (Soil mycoflora), **20**: *T. harzianum* (Air mycoflora), **21**: *T. harzianum* (contamination of *M. phaseolina*) and **22**: *T. harzianum* (*Helvela elastica* fruiting body).

It confirms that the RAPD technique is an effective tool in the search for inter/intra specific variations within *Trichoderma* species and serve as the preliminary genome level analysis. DNA polymorphisms recorded with RAPD markers revealed that morphologically distinguishable isolates also exhibit a high level of genetic variation. During the past few years, numerous publications have demonstrated the utility of RAPD markers for the analysis of the genetic diversity among species and within fungal populations and plant populations (Bagheri *et al.*, 1995; Debener *et al.*, 1996; Saker *et al.*, 2000; Sharma *et al.*, 2002; Swelim, 2005 and Aiat, 2006).

It is concluded that integration of molecular attributes, together with morphological criteria, has served to establish new interspecific variations within the species and also intraspecific differences among twenty three isolates of *Trichoderma*.

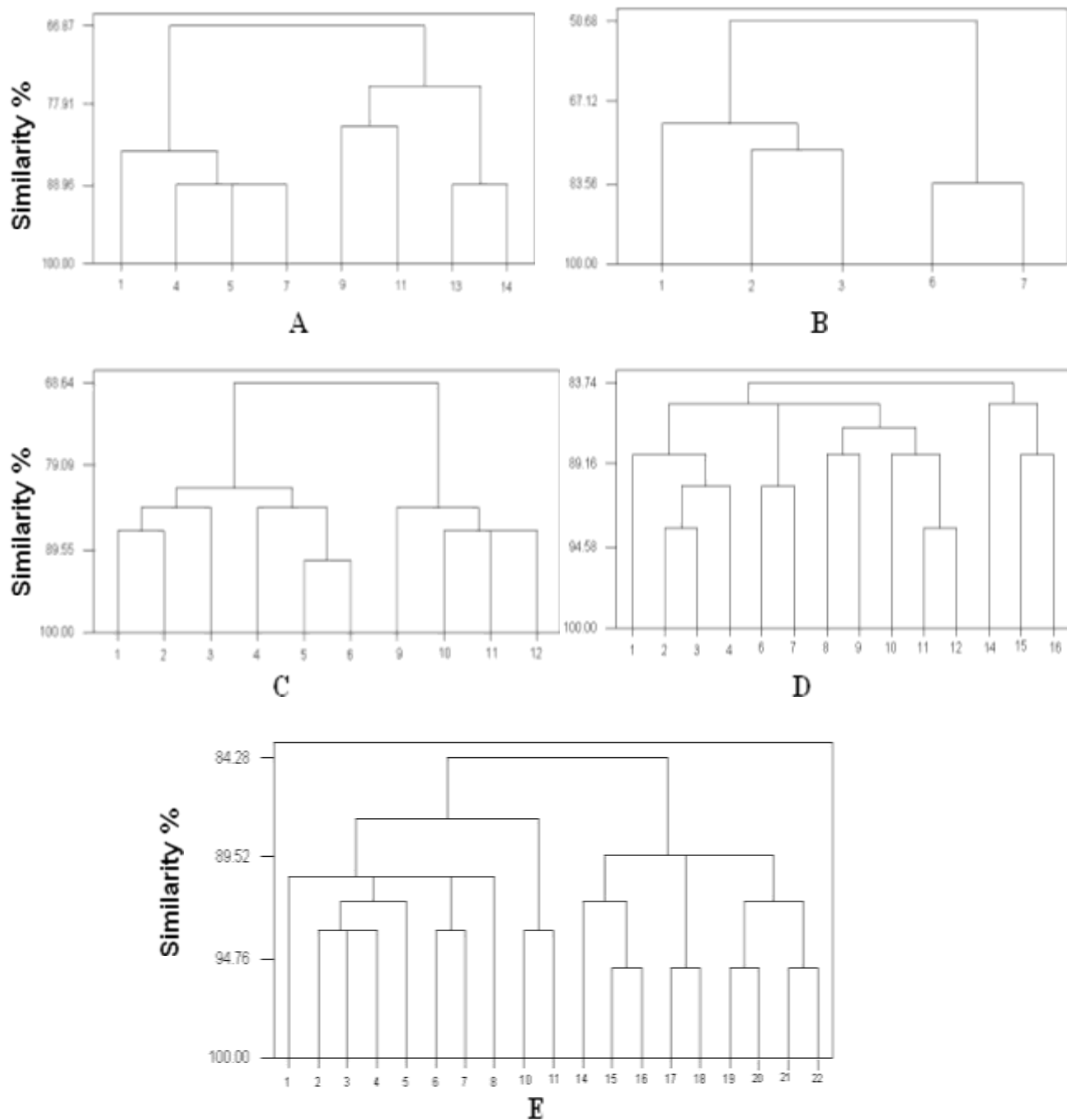


Fig. 1. Dendrogram obtained from amplification by (A) Primer A-11, (B) Primer A-12, (C) Primer A-13, (D) Primer B-05 and (E) Primer B-18. **1:** *T. hamatum* (*Syzygium cumini*, stem bark), **2:** *T. hamatum* (soil mycoflora), **3:** *T. koningii* (citrus fruit), **4:** *T. koningii* (*M. indica* stem), **5:** *T. koningii* (Oat seeds), **6:** *T. koningii* (wood) **7:** *T. koningii* (*M. indica*, wood), **8:** *T. pseudokoningii* (Green Chilli), **9:** *T. pseudokoningii* (Citrus fruit), **10:** *T. pseudokoningii* (Tannery effluent), **11:** *T. pseudokoningii* (isolate sent by Dr. S.M. Khan), **12:** *T. harzianum* (*Mangifera indica*), **13:** *T. harzianum* (Leaf litter), **14:** *T. harzianum* (Decaying Wood), **15:** *T. harzianum* (Air mycoflora), **16:** *T. harzianum* (Basidiocarp of *Hydnum* sp.), **17:** *T. harzianum* (Mushroom contamination) **18:** *T. harzianum* (Polluted water), **19:** *T. harzianum* (Soil mycoflora), **20:** *T. harzianum* (Air mycoflora), **21:** *T. harzianum* (contamination of *M. phaseolina*) and **22:** *T. harzianum* (*Helveta elastica* fruiting body).

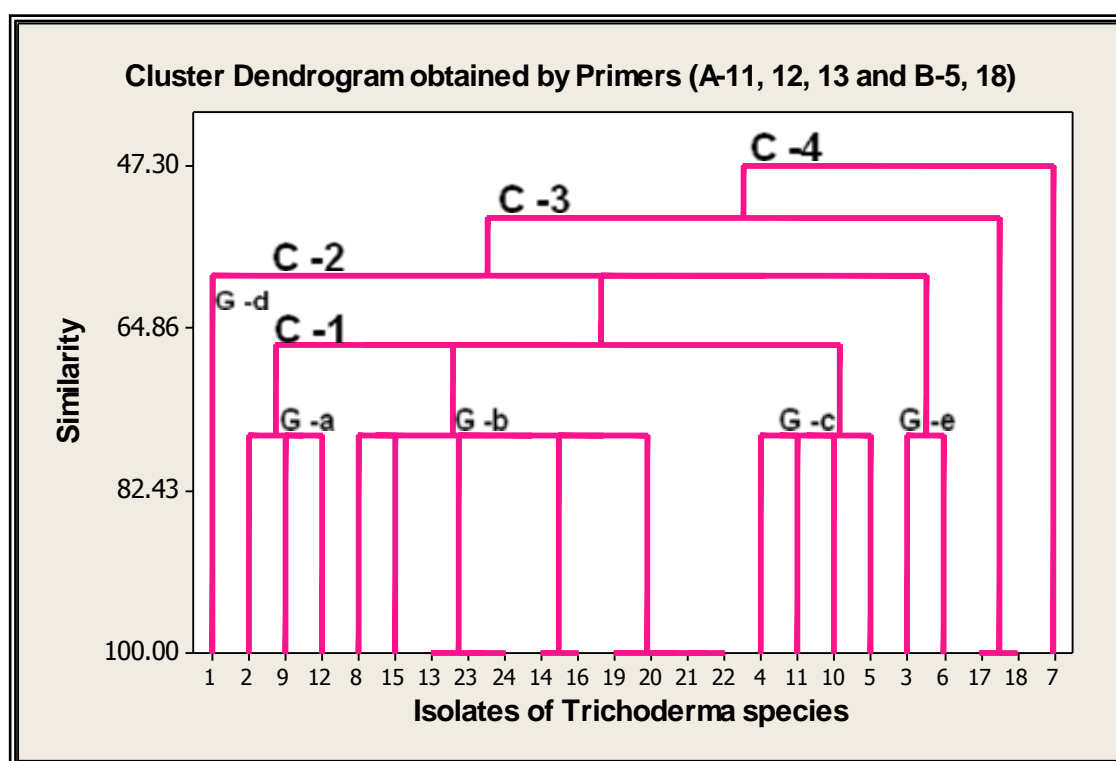


Fig. 2. Dendrogram obtained from amplification by Cluster of Primers. C: cluster, G: group. **C -1:** [G -a = 2: *T. hamatum* (soil mycoflora), 9: *T. pseudokoningii* (Citrus fruit), 12: *T. harzianum* (*M. indica*, soil) G -b = 8: *T. pseudokoningii* (Green Chilli) 15: *T. harzianum* (Air mycoflora), 13: *T. harzianum* (Leaf litter), 23: *T. harzianum* (Decaying wood), 24: *T. harzianum* (Rhizospheric soil). 14: *T. harzianum* (Decaying Wood), 16: *T. harzianum* (Basidiocarp of *Hydnum* sp.), 19: *T. harzianum* (Soil mycoflora), 20: *T. harzianum* (Air mycoflora), 21: *T. harzianum* (contamination of *M. phaseolina*), 22: *T. harzianum* (*Helvela elastica* fruiting body) G -c = 4: *T. koningii* (*M. indica* stem), 5: *T. koningii* (Oat seeds), 10: *T. pseudokoningii* (Tannery effluent). 11: *T. pseudokoningii* (Isolate sent by Dr. S.M. Khan)] **C -2:** [G -d = *T. hamatum* (*Syzygium cumini*, stem bark) G -e = 3: *T. koningii* (citrus fruit), 6: *T. koningii* (wood)] **C -3:** 17: *T. harzianum* (Mushroom contamination) 18: *T. harzianum* (Polluted water) and **C -4:** 7: *T. koningii* (*M. indica*, wood).

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