

Original Research Article

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Molecular Characterization of *Trichoderma* Mutants with RAPD and ISSR Marker

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ABSTRACT

Trichoderma is an important biocontrol agent of several soil borne plant pathogens. Genetic modification using mutagenesis offers the potential for producing improved bio protection is likely to enhance their biocontrol capabilities against soil borne pathogen. Molecular techniques are important analytical tool to characterized genetic variability and diagnosis of microbial population. Molecular variability of *Trichoderma* mother culture and its mutants was studied by using Random Amplified Polymorphic DNA (RAPD). 20 RAPD primer of OPA series were tested, of which 14 primers produced 72 scorable bands among them 52 bands were polymorphic and level of polymorphism was upto 72%. On the basis of dendrogram, the tested cultures were clearly divided into 2 groups. First group i.e. cluster A included 4 mutants TvM-5, TvM-3, TvM-1 TvM-2 and mother culture TvMC. Second group i.e. cluster B included only one mutant i.e. TvPM-4. In ISSR 6 primers were tested, of which, 6 primer produced 32 scorable bands among which 27 bands were polymorphic and level of polymorphism was upto 84%. Two clusters were observed in Dendrogram i.e. Cluster A includes four mutants TvM-5, TvM-3, TvM-1, TvM-2 and mother culture TvMC and cluster B contained only one mutant i.e. TvPM-4.

Keywords

Trichoderma mutants,
DNA extraction, RAPD
marker, ISSR marker
Scorable bands

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Introduction

Trichoderma is used in different crop like rice, wheat, pulses (black gram, cowpea, and chickpea) vegetable (tomato, brinjal, and chili) against a wide range of plant pathogen. One of the popular species, *Trichoderma viride* is effective against all soil borne fungal pathogen like *Phytophthora*, *Fusarium*, *Pythium*, *Rhizoctonia*, *Sclerotium*. *Trichoderma*, reduce growth, survival or infection caused by pathogen by different mechanism. In addition, some species of the genus are economically important producers of industrial enzymes

(*Trichoderma reesei*, *Hypocreaajecorina*; and antibiotics (Sivasithamparam and Ghisalberti, 1998). *Trichoderma* is easily identified in culture media, which produces large number of characteristics small, green or white conidia, from phialides present on the profusely or meagrely branched conidiophores. However, the identification of isolates to species level is difficult and confusing due to the complexity and closely related characters of the species. *Trichoderma* isolates were differentiated by mycelial growth rate and colony appearance, as well as microscopic morphological features, including

phialides and spores. These can also be distinguished by molecular techniques like DNA sequencing, Random Amplification of Polymorphic DNA (RAPD) analysis, Restriction Fragment Length Polymorphism (RFLP) analysis, internal transcribed sequences (ITS) of the ribosomal DNA (rDNA) analysis (rDNA-ITS1) and universally primed polymerase chain reaction (UP-PCR) have been used to characterize isolates of *Trichoderma* (Cumagun *et al.*, 1999).

Inter simple sequence repeats (ISSR) have been used as another effective method to characterize genetic variability. Since the evolutionary rate within ISSR is considerably higher than other types of DNA, the likelihood of finding polymorphism is greater compared to RAPD. Since the first reports of RAPD markers by (Williams *et al.*, 1990). This method has been widely used for identification of species. This technique has been used in some cases for species identification.

Materials and Methods

Trichoderma viride mother culture and four mutants i.e. (6th generation) irradiated with gamma rays (Cobalt 60) @41.6 gray/minute at BARC, Trombay, Mumbai were collected from Department of Plant Pathology Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola. The applied doses level of gamma rays was 75k-rad with time interval of 15, 30, 45 and 60 minutes. One *T.viride* mutant was collected from G.B. Pant University of Agriculture and Technology, Pantnagar. This mutants can be coded as *Trichoderma viride* mother culture (TvMC), *Trichoderma viride* mutant (*T. Viride*@ 75 k-rad 15 min.TvM-1), *Trichoderma viride* mutant (*T. Viride*@ 75 k-rad 30 min.TvM-2) *Trichoderma viride* mutant (*T. Viride*@ 75 k-rad 45 min. TvM-3) *Trichoderma viride* mutant (Pantnagar TvPM-4), *Trichoderma viride* mutant (*T. Viride*@ 75 k-rad 60 min. TvM-5).

Extraction of DNA

Genomic DNA was isolated from the twenty four selected isolates by the cetyl dimethyl ethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980) with some modifications. For extraction of DNA seven days old mycelial mat was transferred on sterilized blotter paper, air dried to remove moisture. Dried mycelium mat was used for DNA isolation. Approximately, one gram of air dried fungal mat was quickly frozen in liquid nitrogen (-196⁰C) and crushed into powder form with the help of sterilized mortar and pestle. The powder was immediately homogenized by adding pre-warmed (65⁰C) extraction buffer and transferred to two ml Eppendorf tubes. These tubes were incubated in a water bath at 65⁰C for one hour with gentle shaking at every 15 minutes then add an equal volume of chloroform : isoamyl alcohol (24:1) and centrifuged at 12000 rpm at room temperature for 20 minutes. Then aqueous phase transferred to another tube and equal volume of ice chilled Isopropanol was added. Centrifuged for 10 minutes at 12000 rpm and decanted the supernatant. The pellet was washed with 70 per cent ethanol twice and suspended in T₅₀E₁₀ buffer (50 mMTris-HCl, pH 8.0, 10 mM EDTA). The DNA solution was treated with RNase at 37⁰C for 1 hr and stored at -20⁰C until needed.

PCR Reaction for RAPD and ISSR marker

The PCR cycle was carried out in small reaction tubes, containing a reaction volume typically of 12.5 µl in which 10x *Taq* buffer 1.25 µl, MgCl₂ (25 mM 1.25 µl), dNTPs (10 mM 0.3 µl), *Taq* polymerase (5 U/µl 0.3 µl), Sterile distilled water (5.4 µl), Primer(1.0 µl), Template DNA (37.5 ng 2.0 µl) that was inserted into a thermal cycler (Eppendorf) that heated and cooled the reaction tubes within it to the precise temperature required for each step of the reaction. The PCR cycle was as

follows initial denaturation at 94°C for 5min, Denaturation at 94°C for 1min, Annealing 35°C for 1min (for RAPD and its specific to each primer for ISSR) Extension 72°C for 3min and Final Extension 72°C for 10min. With 35 cycles. 20 primers were screened, consisting of RAPD primer of OPA series 1 to 20 from Genaxys Scientific Pvt. Ltd. to evaluate the variability among *Trichoderma viride* mother culture and mutants. The PCR amplified product of each primer was resolved on 1.2% agarose gel electrophoresis and the amplified product was compared with 1kb DNA ladder for RAPD and 100bp for ISSR.

Results and Discussion

Internal Transcript Spacer (ITS) analysis

For the confirmation of *Trichoderma viride* mother culture and mutants ITS marker was done with using ITS-1 and ITS-4 primer which gives band size in the range of 500bp to 600bp. The results are in accordance with Chakraborty (2010) who studied the identification and genetic variability of

Trichoderma isolates which observed amplified DNA fragment approximately 600bp.

RAPD analysis

The genomic DNA of five *Trichoderma* mutants and one mother culture of *Trichoderma viride* was amplified. Molecular variability of *Trichoderma viride* mother culture and mutants was studied by using 14 RAPD primers of OPA series of which 14 primers produced 72 scorable bands. Among the 72 scorable bands the 52 bands were polymorphic and level of polymorphism was 72% (Table 1).

ISSR analysis

The genomic DNA of five *Trichoderma* mutants and one mother culture of *Trichoderma viride* was amplified. Six ISSR primers screened for *Trichoderma viride* mother culture and mutants produced reproducible and scorable bands with high degree of polymorphism (Table 2).

Table.1 Per cent polymorphism observed in RAPD primer

Sr.	Primer	Total bands	Polymorphic bands	% polymorphism
1	OPA-1	6	4	66.66%
2	OPA-4	7	5	71.42%
3	OPA-5	3	2	66.6%
4	OPA-9	6	5	83.33%
5	OPA-10	7	6	85.7%
6	OPA-11	5	4	80%
7	OPA-12	6	3	50%
8	OPA-13	5	3	60%
9	OPA-14	2	2	100%
10	OPA-15	4	3	75%
11	OPA-16	9	6	66.6%
12	OPA-17	5	4	80%
13	OPA-18	4	3	75%
14	OPA-19	3	2	66%
	TOTAL	72	52	72%

Table.2 Percent polymorphism observed in ISSR primer

Sr. No.	Primer	Total bands	Polymorphic band	% polymorphism
1	AC8YT	5	4	80%
2	GA8C	5	5	100%
3	CT8RG	6	4	66.66%
4	GA8YT	5	4	80%
5	AG8T	4	4	100%
6	GA9RY	7	5	71%
	TOTAL	32	27	84%

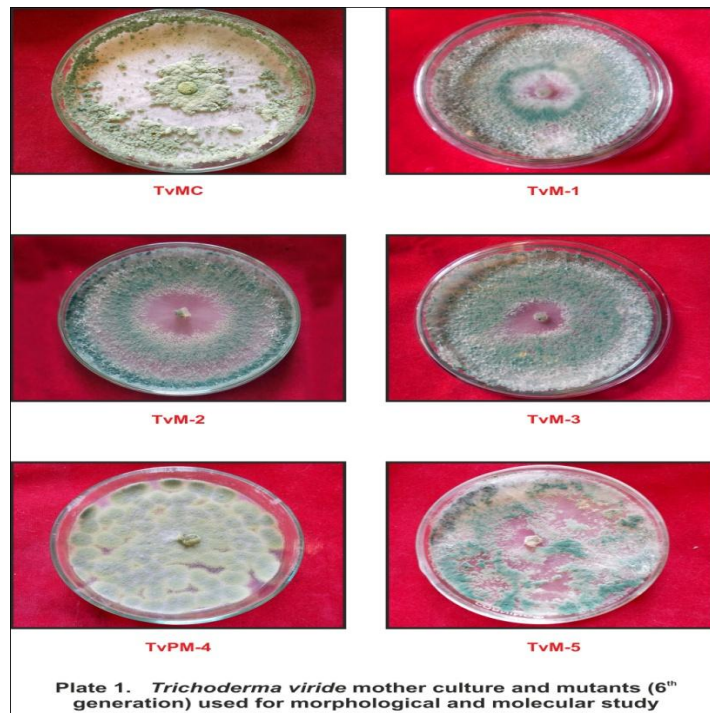


Plate.2 Conformation and identification of *Trichoderma* mutants by using ITS primer

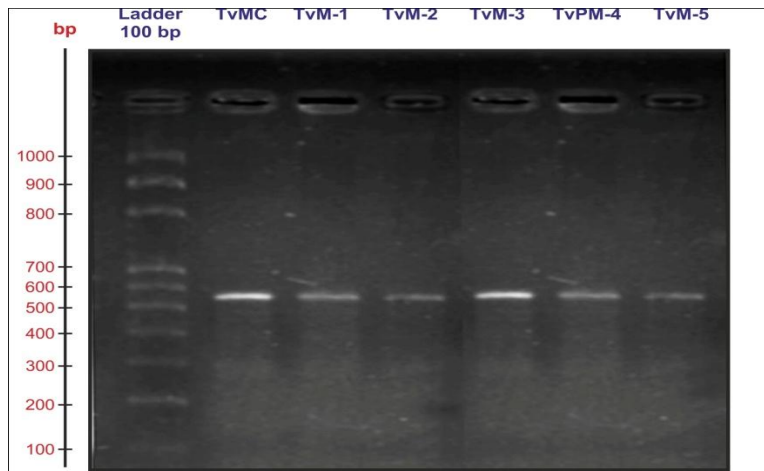


Plate.3 RAPD analysis of *Trichoderma* mutants using OPA-12 and OPA-4 primers

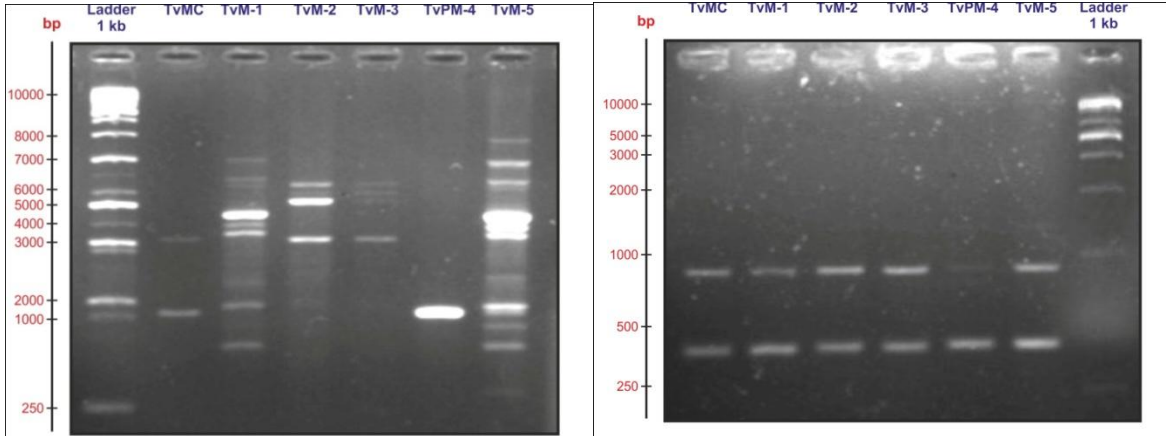


Plate.4 RAPD analysis of *Trichoderma* mutants using OPA-12 and OPA-13 primers

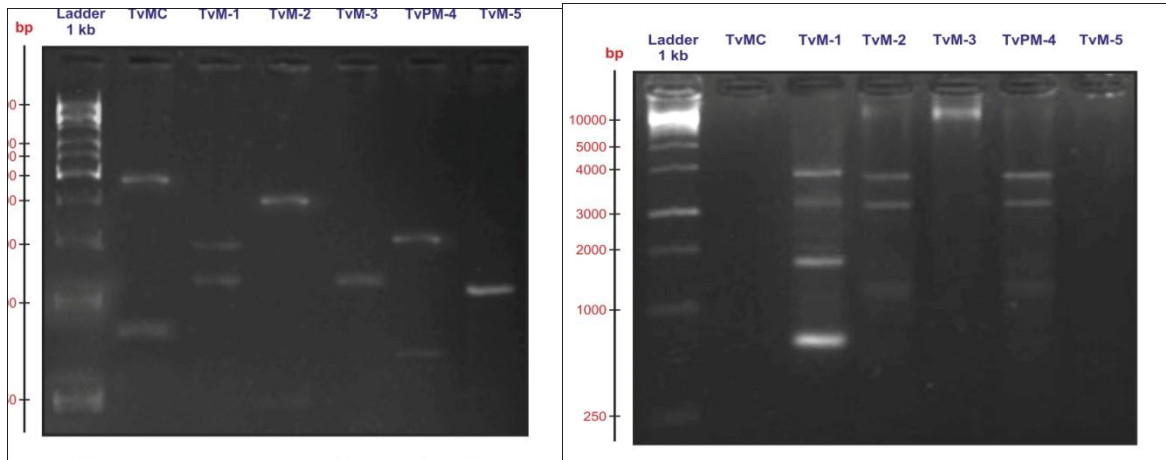


Plate.5 RAPD analysis of *Trichoderma* mutants using OPA-10 and OPA-11 primers

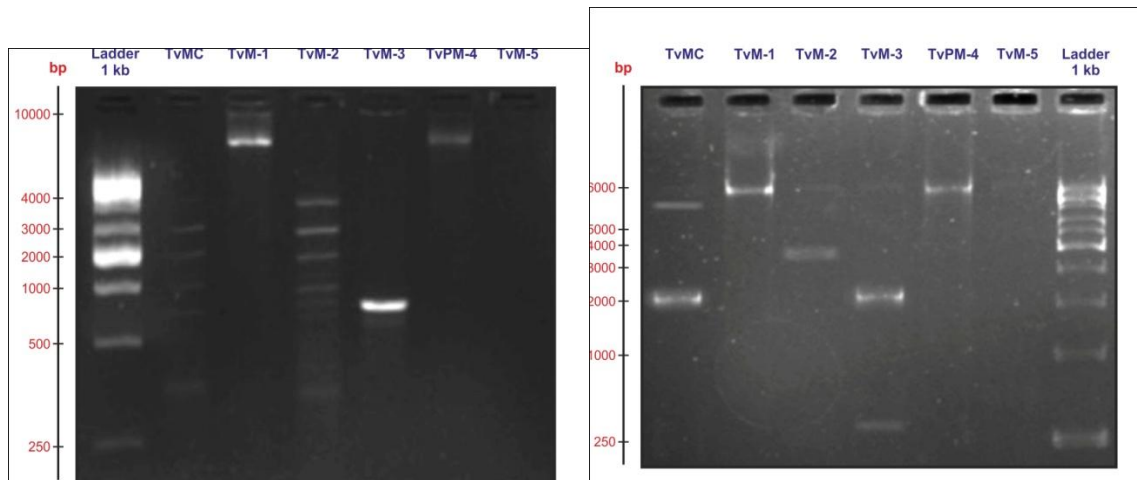


Plate.6 ISSR analysis of *Trichoderma* mutants using AC8YT and GA8C primers.

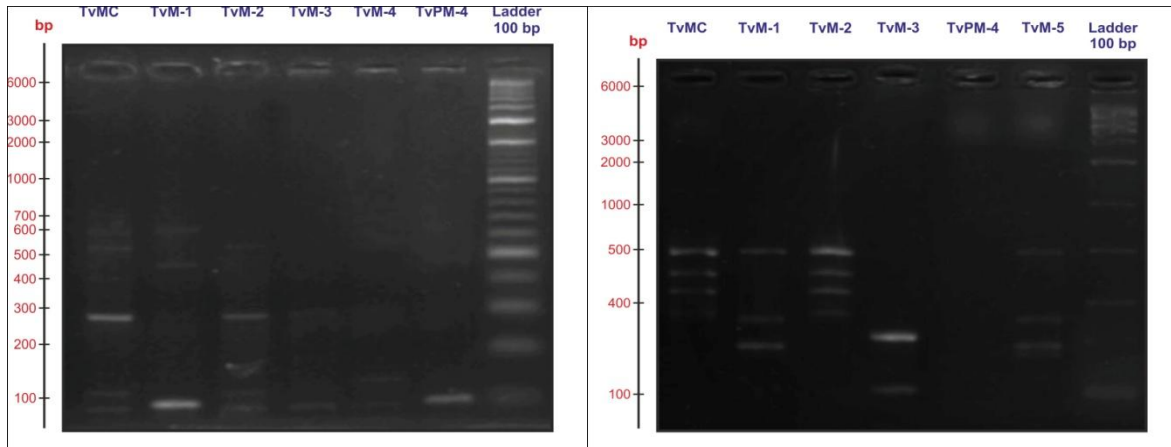


Plate.7 ISSR analysis of *Trichoderma* mutants using AG8T and GA9RY

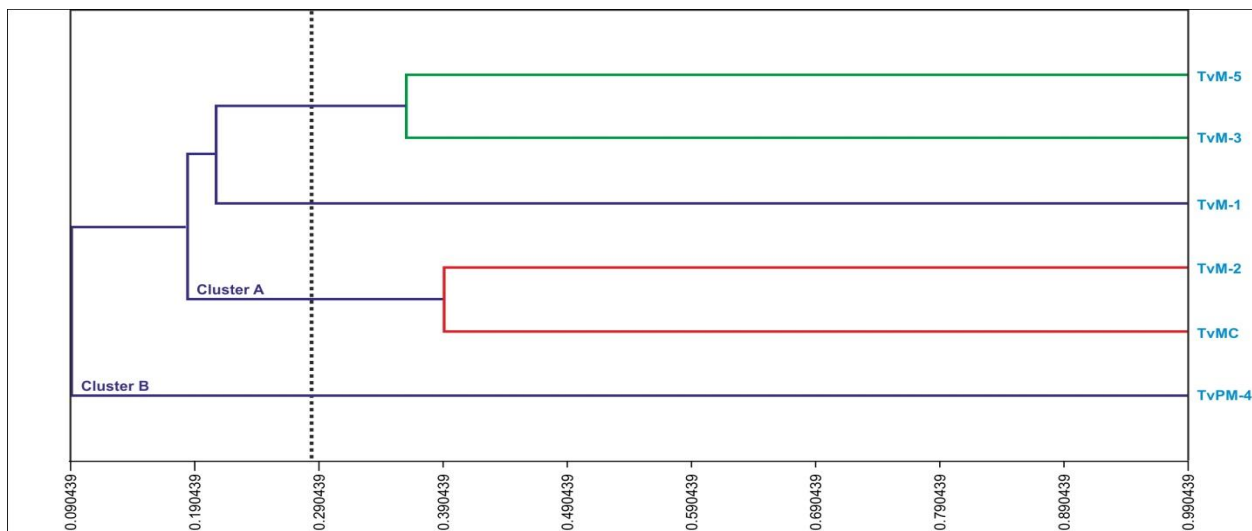
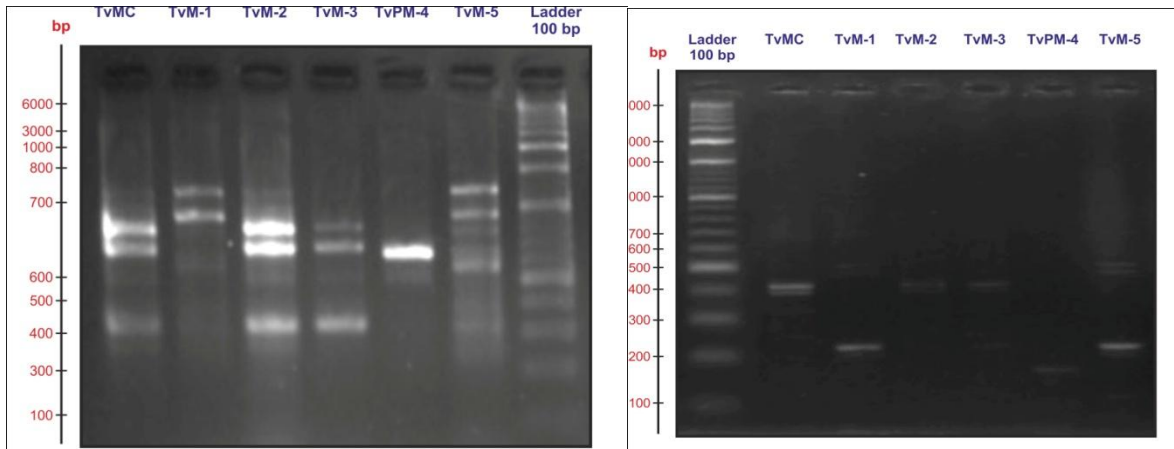
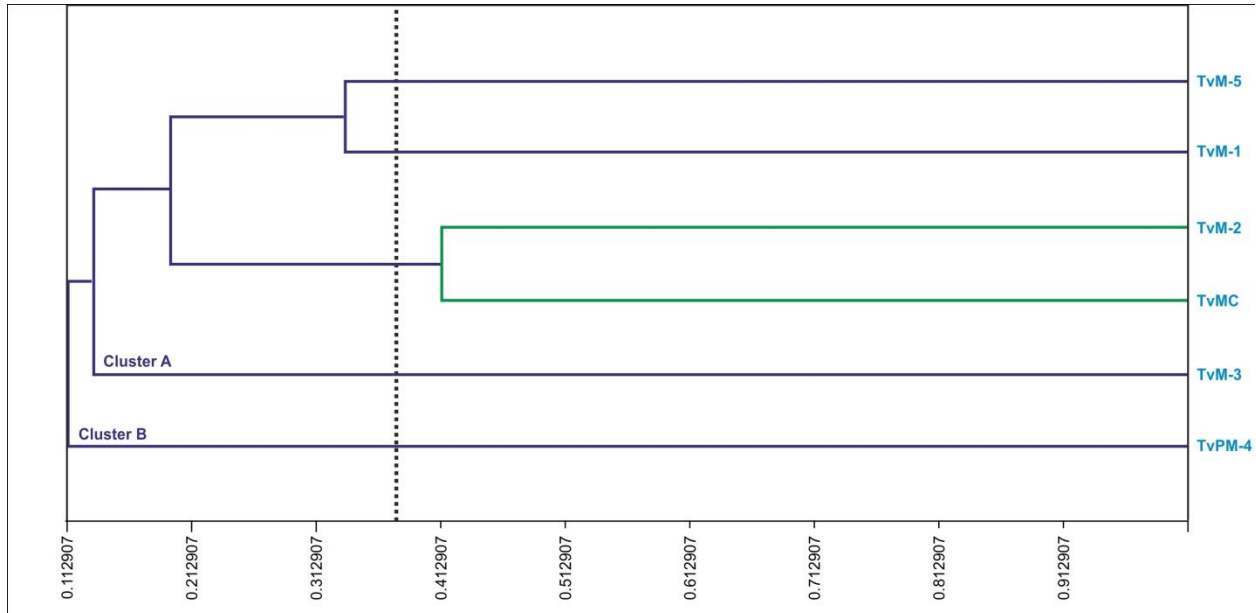


Fig. 1. RAPD UPGMA dendrogram of RAPD analysis of *Trichoderma viride* mother culture and mutants based on Jaccard's similarity coefficient

Fig.2 ISSR UPGMA Dendrogram of ISSR analysis of *Trichoderma* mother culture and mutants



Molecular variability of *Trichoderma viride* mother culture and mutants was studied by using 6 primers of which 6 primers produced 32 scorable bands. Among the 32 scorable bands the 27 bands were polymorphic and level of polymorphism was 84%.

A binary similarity matrix of combined data from 14 RAPD primers and 6 ISSR primers of *Trichoderma viride* mother culture and mutants were prepared by scoring presence or absence of band (Fig. 1 and 2).

The same molecular weight was assumed to be identical. On the basis of calculated similarity matrix the similarity between genotypes can be predicted. The genotypes showing similarity index “1” are presumed to be 100% similar while that of “0” are 100% genetically dissimilar.

In present study of RAPD Primer the similarity coefficient value ranged from 0.370 to 0.000 across *Trichoderma viride* mother culture and mutants while it in ISSR is ranged from 0.409 to 0.050 indicating high degree of polymorphism in respect to genetic similarity. Genetic similarity estimate (Jaccard’s

coefficient) based on RAPD and ISSR banding pattern was used for cluster analysis to present genetic relationship in the form of dendrogram.

Two major clusters were obtained on the basis of analysis in both RAPD and ISSR marke, First group is named as cluster –A, includes TvM-5, TvM-3, TvM-1, TvM-2, TvMC. Second group is named as cluster –B which include TvPM-4.

In RAPD the TvMC was found to have a higher similarity index with TvM-2. The TvPM-4 with TvM-3 and TvPM-4 with TvMC was found to have a lower similarity index and in ISSR the TvM-2 was found to have a higher similarity index with TvMC. The TvM-5 with TvPM-4 was found to have a lower similarity index.

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