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## Molecular variability among the isolates of *Trichoderma viride*

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#### Abstract

The molecular variability was studied among the six isolates of *Trichoderma viride* collected from different region of Maharashtra by using 16 RAPD primers of OPA and OPB series. Out of which 10 primers produced 78 scorable bands. Out of 78 bands, 76 bands were polymorphic and level of polymorphism was 97.32%. The isolate Tv2 (Pune) had higher value of similarity coefficient (0.400) with Tv5 (Sangli) whereas, Tv1 (Akola) had lower value of similarity coefficient (0.087) with Tv2 (Pune) and also with Tv4 (Amravati).

**Keywords:** Random amplification of polymorphic DNA, molecular variability, *Trichoderma viride*

#### Introduction

Plant disease epidemics have created an ecologically unbalanced system in modern agriculture. Pesticides and organic compounds are widely used to manage plant pathogens in many countries. Widely use of chemical fungicides which have greater impact on environment and human health like development of resistance to pesticides in pathogens, fungicides of broad spectrum produce undesirable consequences on non-target organisms and residual effect (Benítez *et al.*, 2004). Recently, the application of biological control agents (BCAs) in agriculture has gained popularity as a way to reduce or eliminate the use of synthetic pesticides (Vinale *et al.*, 2008) [9]. Fungi of the genus *Trichoderma* are important bio-control agents of several soil borne phytopathogens (Benitez *et al.*, 2004) [1]. *Trichoderma* spp. are acclaimed as effective, eco-friendly and cheap, nullifying the ill effect of chemicals. *Trichoderma* spp. are difficult to distinguish morphologically, So needed to give accurate identification of *Trichoderma* spp. However, the identification of isolates to species level is difficult and confusing due to the complexity and closely related characters so that molecular analysis is important.

#### Material and Methods

Soil samples were collected from different districts of Maharashtra i.e Akola (Tv1), Pune (Tv2), Solapur (Tv3), Amravati (Tv4), Sangli (Tv5), Nagpur (Tv6). The *Trichoderma viride* was isolated from the soil by serial dilution technique.

#### Chemicals and reagents required for extraction of DNA

- Extraction buffer [2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, 0.4% β-mercaptoethanol (added at the time of use)]. (The extraction buffer was autoclaved before addition of β-mercaptoethanol)
- Chloroform: Isoamyl alcohol (24:1)
- 100% Isopropanol (Ice-cold)
- Wash buffer: 70% ethanol
- TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) Autoclaved before use.
- 5 M NaCl (solution was sterilized by autoclaving).
- 1 M Tris HCl (pH 8.0).
- 5 M EDTA Na<sub>2</sub> (pH 8.0)

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### Extraction of DNA

The pure culture of fungus grown on potato broth and the mycelial mat was harvested after seven days. It was washed thoroughly and repeatedly and then dried using blotter paper and crushed to powdered form in pestle and mortar with pre-chilled liquid nitrogen. The powdered mass was immediately homogenized by adding pre-warmed (65°C) 1 ml of CTAB extraction buffer and the content was mixed by inversion. The mixture was incubated at 65°C for one hour in hot water bath (inverting the tube after 10 minutes). The tubes were centrifuged at 8000 rpm for 15 minutes. The supernatant was transferred into another 2 ml centrifuge tubes without disturbing the pellet of cell debris. Then equal volume (1ml) of chloroform isoamylalcohol (24:1) was added and mixed gently but thoroughly to emulsify both the components for five minutes. Centrifugation was carried out at 12000 rpm for 15 min. The upper aqueous phase was transferred into another tube. Equal volume of ice-cold isopropanol was added and mixed by inversions. CTAB-DNA complexes formed at the bottom of the tubes. After mixing with isopropanol, the sample were kept at 4°C for 10 minutes and then centrifuged at 10000 rpm for 10 minutes. Pellet was formed at the bottom of the tube. The supernatant was removed and the pellet was washed with 70% ethanol twice and centrifuged at 8000 rpm for 5 minutes. The pellet was air-dried for 30-60 minutes and then dissolved in 0.5 ml of TE buffer. The pellet were allowed to dissolve completely overnight at 4°C without agitation. Quantification and purity of DNA was checked on 0.8% agarose gel at voltage of 60V/cm by using 1X TBE buffer and ethidium bromide (0.5 mg/ml) staining. After completion of 5 cm run, the gel was observed under UV light and the DNA yield and quality was confirmed.

### Internal Transcribed Spacer (ITS) amplification

The PCR was carried out in small reaction tubes, containing a reaction volume 12.5 µl (Table 1 and 2) that was inserted into a thermal cycler (Table 3)

**Table 1:** PCR reaction mix for 1x of 12.5 µl reaction

Sr. No.	Master Mix	1x
1	10x <i>Taq</i> buffer	1.25 µl
2	MgCl <sub>2</sub> (25 mM)	1.25 µl
3	dNTPs (10 mM)	0.3 µl
4	<i>Taq</i> polymerase (5 U/µl)	0.3 µl
5	Sterile distilled water	5.4 µl
	Total Volume	8.5 µl

**Table 2:** Constituents of PCR reaction

Sr. No.	PCR Reaction	Quantity
1	Master Mix vol.	8.5 µl
2	Primer (Forward)	1.0 µl
3	Primer (Reverse)	1.0 µl
4	Template DNA (37.5 ng)	2.0 µl
	Total Reaction Volume	12.5 µl

**Table 3:** Steps used for PCR-ITS reaction

Name of step		Temperature	Time
Initial Denaturation		94 °C	5 min.
30 cycles	Denaturation	94 °C	1 min.
	Annealing	55 °C	1 min.
	Extension	72 °C	30 sec.
Final Extension		72 °C	10 min.

### Random Amplification of Polymorphic DNA analysis

A total of 16 OPA and OPB series were screened for RAPD analysis. The RAPD primers were used for the analysis of random amplification of polymorphic DNA to study the polymorphism present in the isolates of *T. viride*.

**Table 4:** PCR reaction mix for 1x of 20 µl reaction

Sr. No.	Master Mix	1x
1	10x <i>Taq</i> buffer	2.0 µl
2	MgCl <sub>2</sub> (25 mM)	2.0 µl
3	dNTPs (10 mM)	0.5 µl
4	<i>Taq</i> polymerase (5 U/µl)	0.2 µl
5	Sterile distilled water	12.3 µl
	Total Volume	17 µl

**Table 5:** Constituents of PCR reaction for RAPD

Sr. No.	PCR Reaction	Quantity
1	Master Mix vol.	17 µl
2	Primer	2.0 µl
3	Template DNA (37.5 ng)	1.0 µl
	Total volume	20 µl

The PCR tubes containing reaction mixture were placed in the thermal cycler for 40 cycles with the following profiles-

**Table 6:** Steps used for PCR-RAPD reaction

Name of step		Temperature	Time
Initial Denaturation		94 °C	5 min.
30 cycles	- Denaturation	94 °C	1 min.
	- Annealing	34 °C	1 min.
	- Extension	72 °C	30 sec.
Final Extension		72 °C	10 min.

PCR products were separated by electrophoresis in 1.2 per cent agarose gels run in 1x TBE, stained with ethidium bromide and visualized with a UV transilluminator.

### Data analysis and dendrogram

The gel images were captured and visualized in gel documentation system. The data was scored as the presence (1) or absence (0) of individual band for each isolates of *T. viride* in RAPD-PCR analysis. The data was used to generate similarity coefficient using simple matching coefficient based on RAPD bands scoring. The Dice coefficient between each pair of accessions were used to construct a dendrogram using the Unweighted Pair Group Method with Arithmetic Average (UPGMA).

### Results and Discussion

The *Trichoderma* specific ITS primers pair ITS-1 (TCCGTAGGTGAACCTGCGG) and ITS-4 (TCCTCCGCTTATTGATATGC) were used for molecular confirmation of the isolates. All the isolates of *Trichoderma viride* yielded the 550 bp band with the ITS marker.

Total 16 RAPD primers were evaluated for the molecular variability in six isolates of *Trichoderma viride* and the size of the amplified product was compared with 100bp+500bp DNA ladder. Out of these 16 primers screened for RAPD, 10 primers produced 78 scorable band and OPA-1, OPA-4, OPA-12, OPA-15, OPB-11 and OPF-6 didn't showed any banding pattern. Out of 78 bands, 76 were found to be polymorphic and the level of polymorphism was 97.32 per cents (Table 7).

The primer OPA-2 amplified maximum 9 bands within the size of 6410 to 200 bp and OPA-16 primer amplified minimum 6 bands within the size of 5810 to 210 bp.

**Table 7:** Per cent polymorphism observed in RAPDprimers

Sr. No.	Primers	Total amplicons	Polymorphic amplicons	Monomorphic amplicons	% Polymorphism
1	OPA2	9	9	0	100
2	OPA3	8	7	1	87.5
3	OPA 5	9	9	0	100
4	OPA6	8	8	0	100
5	OPA9	8	8	0	100
6	OPA10	7	7	0	100
7	OPA11	9	9	0	100
8	OPA14	7	7	0	100
9	OPA16	6	6	0	100
10	OPA18	7	6	1	85.71
Total		78	76	2	97.32%

**Table 8:** Binary similarity matrix for RAPD analysis

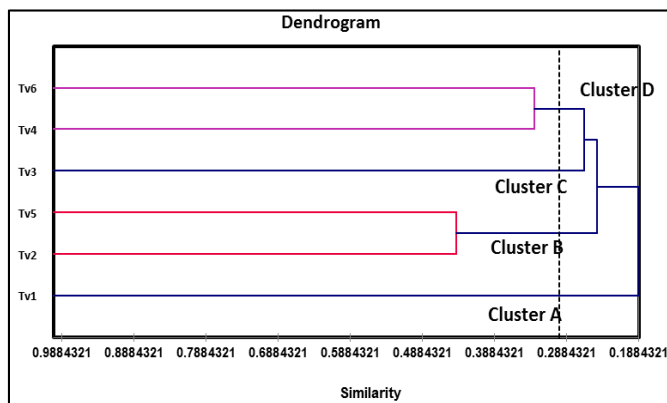
Isolates	Tv1	Tv2	Tv3	Tv4	Tv5	Tv6
Tv1	1					
Tv2	0.087	1				
Tv3	0.143	0.216	1			
Tv4	0.087	0.306	0.240	1		
Tv5	0.105	0.400	0.125	0.143	1	
Tv6	0.231	0.240	0.200	0.292	0.174	1

In present study, the similarity coefficient value ranged from 0.087 to 0.400 across six isolates indicating high degree of genetic variation. This ultimately means high range of genetic diversity among the isolates studied. The highest genetic similarity to an extent of 0.400 was recorded between Tv2 and Tv5 isolates followed by 0.306 similarity between Tv2 and Tv4 isolates. Least genetic similarity was observed that is 0.087 between Tv1 and Tv2 also inTv1 and Tv4 (Table 8).

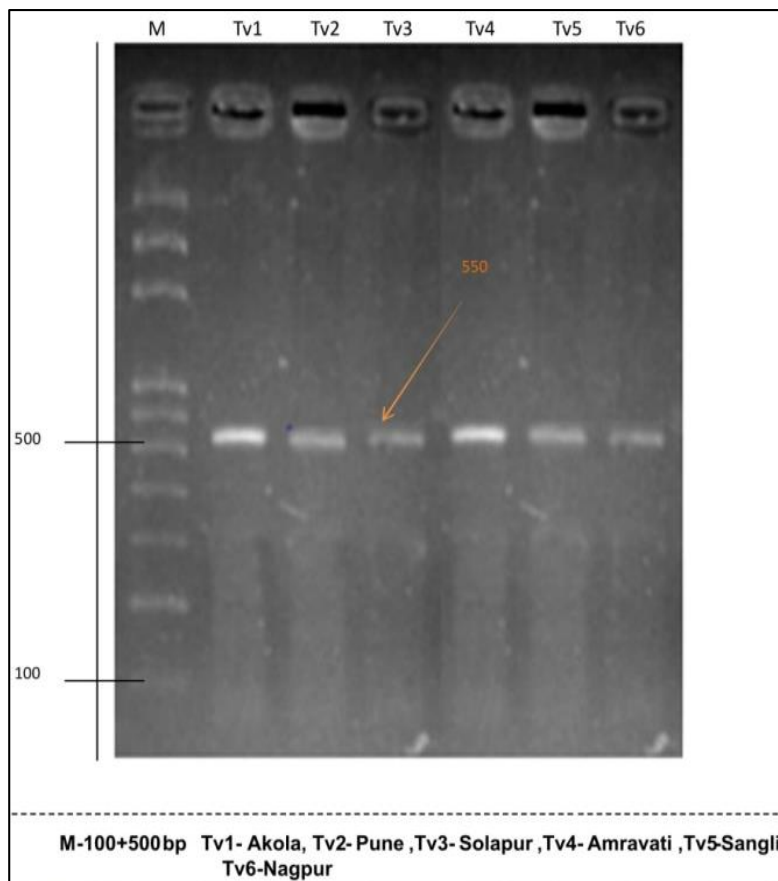
Chakraborty *et al.* (2010) [2] studied nineteen isolates of *Trichoderma viride* and *T. harzianum* obtained from North Bengal region using RAPD and ITS-PCR. RAPD profiles showed genetic diversity among the isolates with the formation of eight clusters. Analysis of dendrogram revealed that similarity coefficient ranged from 0.67 to 0.95. ITS-PCR of rDNA region with ITS1 and ITS4 primers produced 600bp products in all isolates.

**Dendrogram and cluster analysis**

Cluster analysis using UPGMA clearly separated the isolates in 4 clusters viz. Cluster A, B, C and D. The cluster A consists of only one isolate of Tv1 (Akola).The isolate Tv2 (Pune) and Tv5 (Sangli) showed 40.00% similarity in cluster B. Cluster C consists of one isolate *i.e.* Tv3 from Solapur district. And the cluster D consists of two sub cluster Tv4 (Amravati) and Tv6 (Nagpur) showed 29.00% similarity (Fig.1).



**Fig 1:** RAPD UPGMA dendrogram of six isolates of *Trichoderma viride* on Jaccard's similarity coefficient



**Fig 2:** Internal Transcribed Spacer (ITS) of *Trichoderma viride* isolates by primers ITS-1 and ITS-4

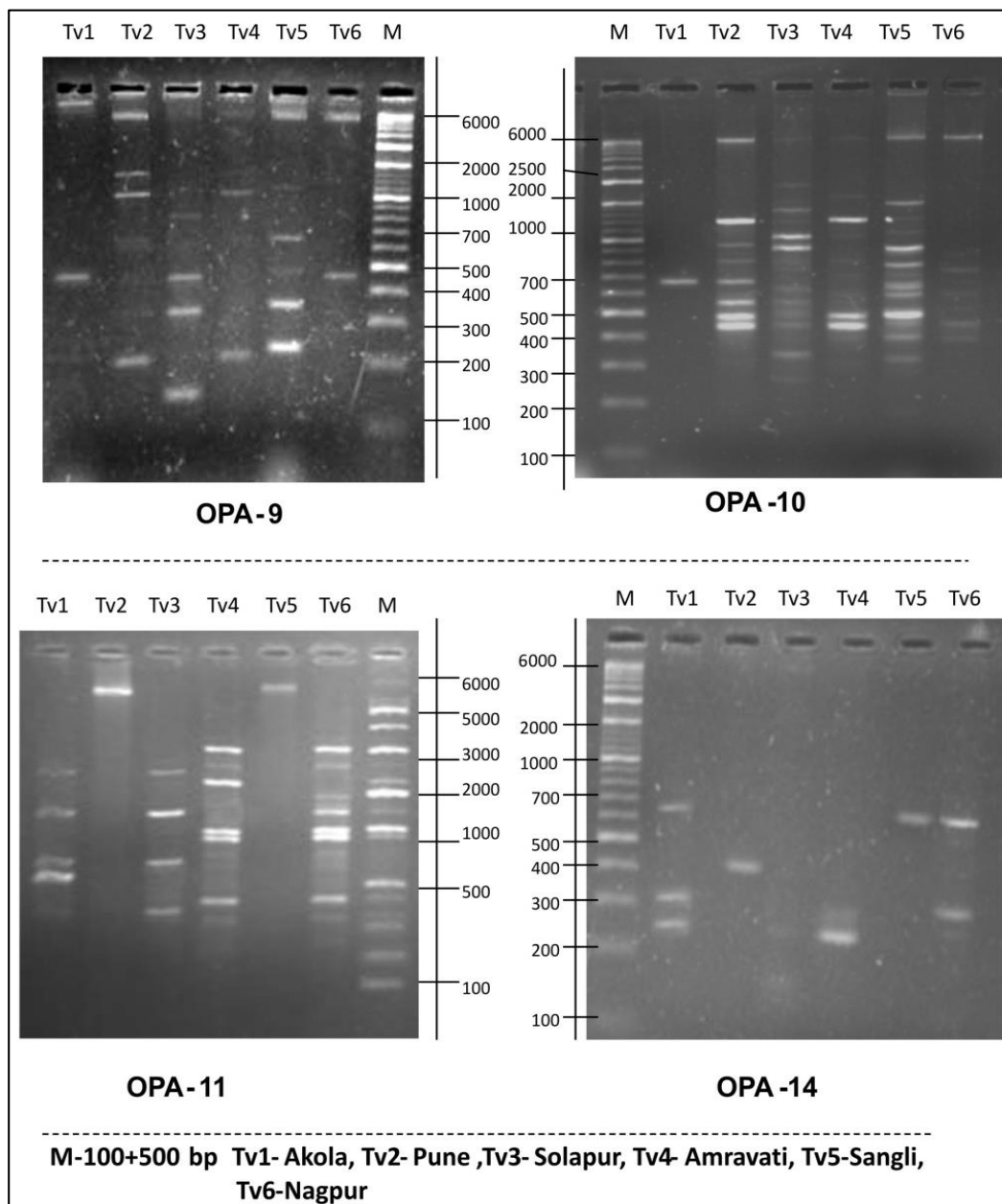


Fig 3: RAPD banding pattern of primers OPA-9, OPA-10, OPA-11 and OPA-14

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