Int.J.Curr.Microbiol.App.Sci (2023) *12*(03): 256-263

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 12 Number 3 (2023)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2023.1203.031

RAPD Marker Based Determination of Genetic Variability among *Trichoderma* Isolates

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Keywords

Genomic variation - RAPD marker – Trichoderma isolates, soil and root ecosystems

Article Info

Received:
08 December 2022
Accepted:
15 February 2023
Available Online:
10 March 2023

A B S T R A C T

Twenty *Trichoderma* isolates collected from different geographical locations of Tamil Nadu were studied for their genetic relationship. The results showed that the PCR-based Random Amplified Polymorphic DNA (RAPD) marker produced DNA fragments of 0.125 kb to 10 kb size with polymorphism while employing five random primers. The dendrogram constructed using Unweighted Pair Group Method of Arithmetic means (UPGMA) produced two main clusters of *Trichoderma* isolates. Cluster 1 bears 5 isolates with 60 per cent similarity coefficient between them and cluster 2 bears 15 isolates with 57 per cent similarity. Overall, all the isolates have about 49 per cent similarity coefficient indicating that they were genetically varied by their unique banding patterns. Such variability has opened new possibility of using most efficient and more isolates of *Trichoderma* in the preparation of eco-friendly bio-pesticide.

Introduction

Trichoderma spp. are commonly available in soil and root ecosystems have gained immense importance in the biological control of majority of plant pathogens since last few decades. They have many positive effects on plants include increase of rate of seed germination, nutrient uptake, reducing pathogenic microbes in the soil and induced systemic resistance to diseases and finally overall growth and yield. They reduce the growth of deleterious pathogens by different mechanisms include competition for site and nutrient, mycoparasitism, antibiosis and lysis. Closely related genotypes of *Trichoderma* spp. have common mechanisms of actions with different degrees due to variability in their genetic makeup. Molecular markers play a major role in analyzing genetic variability among fungal population. In particular, PCR - based randomly amplified polymorphic DNA (RAPD) marker technique can be used to detect genetic polymorphisms in fungi (Welsh and McClelland, 1990) by simultaneous amplification of several anonymous loci in the genome using primers

of arbitrary sequence. Hence the RAPD analysis being used as a powerful tool for the investigation of genetic relatedness and diversity among closely related strains (Saude *et al.*, 2004). The objective of the present study was to study the genetic relationship among isolates of *Trichoderma* from different host plants and geographic locations of India.

Materials and Methods

Soil sample collection and isolation of *Trichoderma* spp.

Rhizosphere soil samples were collected from different crops such as blackgram, sugarbeet, banana, sunflower, jatropha, brinjal, jasmine, tapioca, tomato, ground nut, forest tree and shrub of lake. Loosely adhering soils were shaken from the roots, just a layer of closely adhering rhizosphere soil was transferred to 10 ml sterilized water and vigorously shaken for 10-15 min. The suspensions from all samples were serially diluted upto 10^4 with three replications for each sample. One ml suspension from each 10^3 and 10^4 dilutions was spread on Rose Bengal agar medium. Three replicative plates were maintained for each sample. *Trichoderma* colonies obtained from each sample were purified using single hyphal tip method (Rangaswami, 1972) and maintained on Potato Dextrose Agar medium.

Growth habits of *Trichoderma* isolates

Twenty isolates of *Trichoderma* sp. collected from rhizosphere of different host plants grown in different regions of Tamil Nadu were studied for their growth habits on Potato Dextrose Agar. Nine mm mycelial disc from the margin of an actively growing colony of each isolate was placed in the centre of the Petri Plate and incubated at normal room temperature (27±2ºC). Observation on mycelial growth rate per day was recorded for each isolate upto four days. After seven days, spores from 6mm dia culture disc were suspended in ten ml of potassium phosphate buffer (0.25M, pH 6.8), filtered using sterile muslin cloth and serially diluted upto 10^{12}. Number of spores was determined microscopically by counting chamber.

Genetic analysis of *Trichoderma* isolates

Genomic DNA extraction

Twenty isolates of *Trichoderma* sp. isolated from different regions of Tamil Nadu were studied for their genetic relationship using RAPD analysis. Each isolate was individually inoculated into the Potato Dextrose broth and incubated in arbitrary shaker at 200 rpm for 10 days. Mycelial mat was obtained by filtering through whatman no. 1 filter paper, dried on blotter paper and used for DNA extraction. The CTAB extraction procedure (Zolan and Pukkila, 1996) was followed with some modifications. About 100 mg of mycelium was macerated in liquid nitrogen. The powder was transferred into a test tube containing 5 ml extraction buffer (700 mM of NaCl, 50 mM of Tris HCl with pH 8.0, 10 mM of EDTA, 2% CTAB, and 1% mercapto-ethanol). Samples were incubated for 1 h at 60ºC followed by two consecutive extractions with 5 ml chloroform–isoamylalcohol (24:1). The emulsions were centrifuged at 3500 rpm for 15 min and the aqueous phase was recovered. DNA was precipitated by adding ice cold isopropanol and chilled at -20ºC for at least 30 min. DNA was collected by centrifugation at 10,000 rpm at 4ºC for 15 min. The supernatant was discarded and the pellet was dried at room temperature. The dried pellet was dissolved in 500 µl of TE buffer (10 mM of Tris HCl with pH 8.0, 1mM of EDTA with pH 8.0) and treated with 5 µl of RNAse A (10 mg / ml) at 37ºC for 1 h. The total DNA was tested in the 0.8% agarose gel.

RAPD analysis

The 20 µl PCR reaction mixture contains DNA template 25 ng, 10x Taq buffer, 2.5mM of each of dNTP mixture, 2.5mM of $MgCl_2$, 30 picomole of random primer, and 2 U of Taq DNA polymerase (Bangalore Genei, India). PCR amplification was

performed in a thermocycler (Eppendorf Mastercycler gradient, Westbury, Newyork) using the following conditions: initial denaturation at 94ºC for 5min, 30 cycles of denaturation at 94ºC for 1min, annealing at 36ºC for 1min, extension at 72ºC for 2 min, and a final elongation at 72ºC for 5 min (Gaitan *et al.*, 2002). A set of 5 random primers used in this study; C3 (CGG CTT GGG T), OPA02 (TGC CGA GCT G), OPC20 (ACTT CGC CAC), OPF01 (ACG GAT CCT G) and OPX07 (GAG CGA GGC T). The amplified PCR products were tested on 1.5% agarose gel. The gel was visualized with a UV transilluminator and photographed in the gel documentation system (Alpha Innotech Corporation, California). The experiment was repeated twice and only the RAPD bands which appeared consistently were evaluated.

Cluster analysis

The banding patterns were scored for RAPD primers in each *Trichoderma* isolates starting from the large size fragment to small sized one. Presence and absence of each band in each isolate was coded as 1 and 0 respectively. The scores were used to create a data matrix using the NTSYS-pc program version 2.02 (Exeter Software, New York, USA) described by Rohlf (1993).

The data was subsequently used to construct a dendrogram based on Jaccard's similarity coefficient (Jaccard, 1908) with UPGMA cluster analysis to group the isolates based on their overall similarities. Each RAPD pattern was compared with the other patterns and Euclidean distance matrix was calculated. The relationships among the isolates examined were represented as dendrogram by using UPGMA.

Results and Discussion

Results from the study on growth habits of *Trichoderma* isolates showed that they varied in their mycelial growth rate from 7 to 13 mm per day and spore density from 10^5 to 10^{13} per unit mycelial surface (Table 1). The results from RAPD banding patterns established with five random primers for twenty *Trichoderma* sp. isolates showed the consistent amplicon profiles reproduced in two independent experiments. The largest RAPD product was 10kb by C3 random primer and the smallest was 0.125kb by OPA02 random primer.

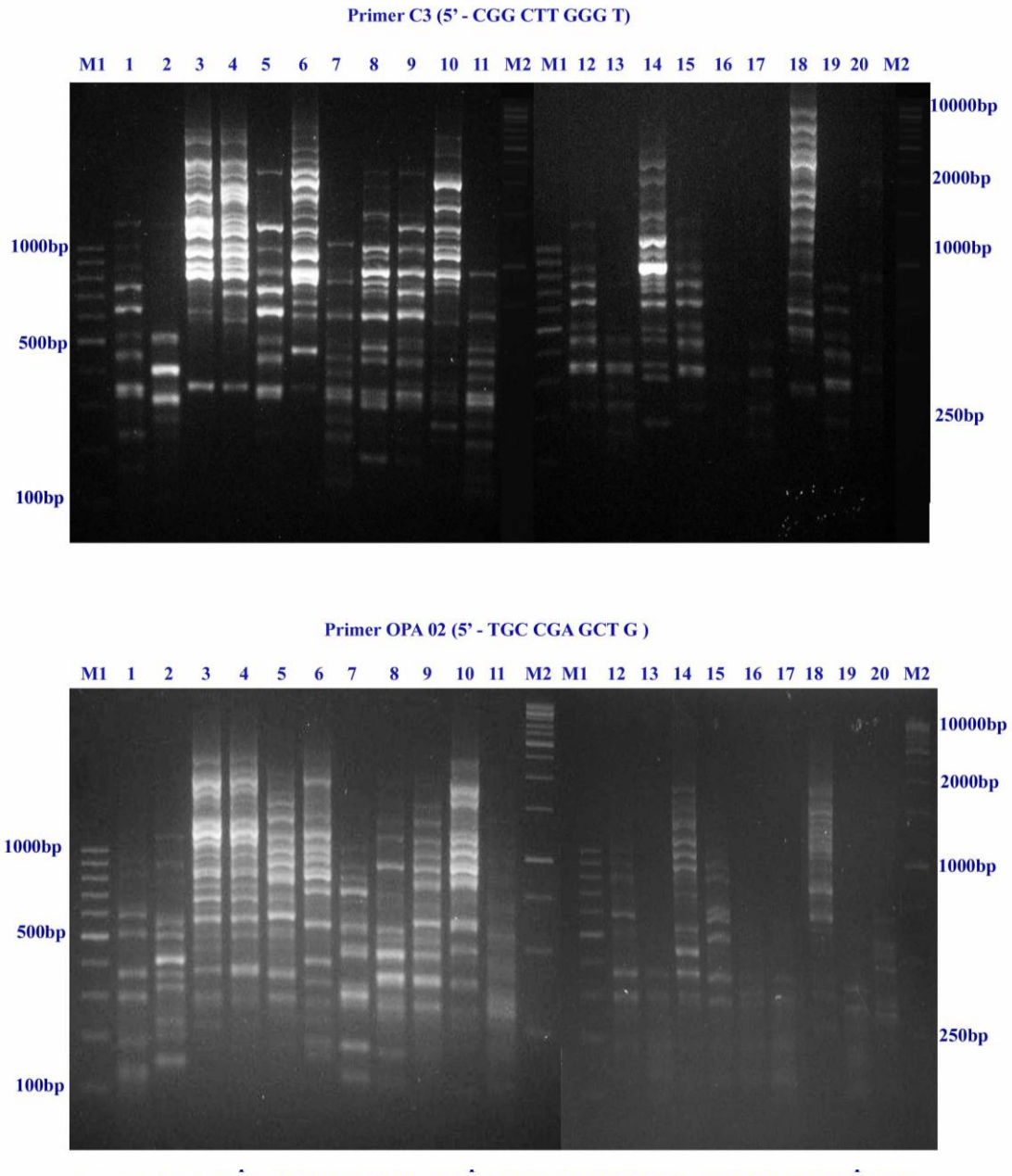
All the five random primers produced >2 kb products in the isolates TV1, TDHA1, TSAT1 and TKAN 1. The random primers C3, OPF01 and OPC20 were not produced visible bands in the isolate TDHE1. Similarly, the random primer OPC20 was not amplified visible bands in the isolate TSBO1. The number of scorable bands for corresponding primers ranged from 3 to 15. An average of 215 bands were scored against 20 isolates for each random primers (Fig. 1, 2 &3). Based on the dendrogram generated using UPGMA, the *Trichoderma* isolates were divided into two main clusters, 1 and 2 which were further subdivided into subclusters. The isolates TV1, TED1, TSBO3, TDHA1 and TKAN1 fall in cluster 1, which showed 60 per cent similarity coefficient between them. The isolates TTH1, TSAT1, TED3, TED2, TSAL1, TSBO2, TSFCE1, TVR1, TDHE1, TCHI1, TBHA1, TCOO1, TPOL1, TTNJ1 and TSBO1 fall in cluster 2, which showed 57 per cent similarity. Isolates TCHI1 and TBHA1 showed 88 per cent similarity and TPOL1 and TTNJ1 isolates showed 86 per cent between them which fall in cluster 2. Overall, all the isolates have about 49 per cent similarity coefficient indicating that they were genetically varied by their unique banding patterns (Fig. 4). Similarly, several researchers used RAPD markers to distinguish isolates of *Trichoderma* (Sagar *et al.*, 2011; Chakraborty *et al.*, 2010**;** Shanmugam *et al.*, 2008; Shalini *et al.*, 2006). Hence this study clearly indicated that the genetic variability existed among *Trichoderma* isolates. Such variability has opened new possibility of using the most efficient and more isolates of *Trichoderma* in the preparation of ecofriendly bio-pesticide.

Table.1 Growth habits of *Trichoderma* isolates under *in vitro*

Isolates of *Trichoderma* sp.	Origin	Host	Mycelial growth rate (mm day^{-1})	No. of spores culture disc $^{-1}$
Tv1	TNAU, Coimbatore	Blackgram	13.3^{b}	**59 x 10^{11}**
TTH 1	Thondamuthur	Sugarbeet	18.3^{a}	**61.7 x 10^{12}**
TED 1	Erode-1	Banana	11.0^{c}	**94x 10 ^{9}**
TSBO 1	Ooty	Sugarbeet	9.3^{e}	**78.3 x 10^{7}**
TSBO 2	Ooty	Forest tree	8.0^{f}	**53.7 x 10^{6}**
TSBO 3	Ooty	Forest tree	9.0^{e}	**90 x 10^{9}**
TSFCE 1	Coimabatore	Sunflower	10.3^{d}	**64.3x 10^{8}**
TED 2	Erode	Jatropha	8.0^{f}	**28x 10^{6}**
TED 3	Erode	Jatropha	7.0^{g}	**42x 10^{5}**
TDHA 1	Dharmapuri	Brinjal	9.0^{e}	**41.7x 10^{7}**
TCOO 1	Conoor	Jasmine	10.3^{d}	**75x 10^{6}**
TTNJ 1	Thanjavur	Tapioca	10.3^{d}	**59.3x 10^{8}**
TBHA 1	Bhavanisagar	Blackgram	8.0^{f}	**36.7x 10^{6}**
TSAT 1	Sathyamangalam	Banana	8.3^{f}	**66.3x 10^{7}**
TPOL	Oddenchutrum	Jasmine	7.0^{g}	**45x 10^{5}**
TDHE 1	Dhevarayapuram	Tomato	8.3^{f}	**39x 10^{6}**
TCHI 1	Chidambaram	Shrub at Pitchavaram Lake	9.0^{e}	**36.7x 10^{9}**
TKAN 1	Kanchipuram	Ground nut	9.0^{e}	**93.3x 10^{8}**
TVRI 1	Virudhachalam	Ground nut	7.3^{g}	**88.7x 10 ^{7}**
TSAL 1	**Salem**	**Tapioca**	**9.0^{e}**	**58.3x 10 ^{9}**

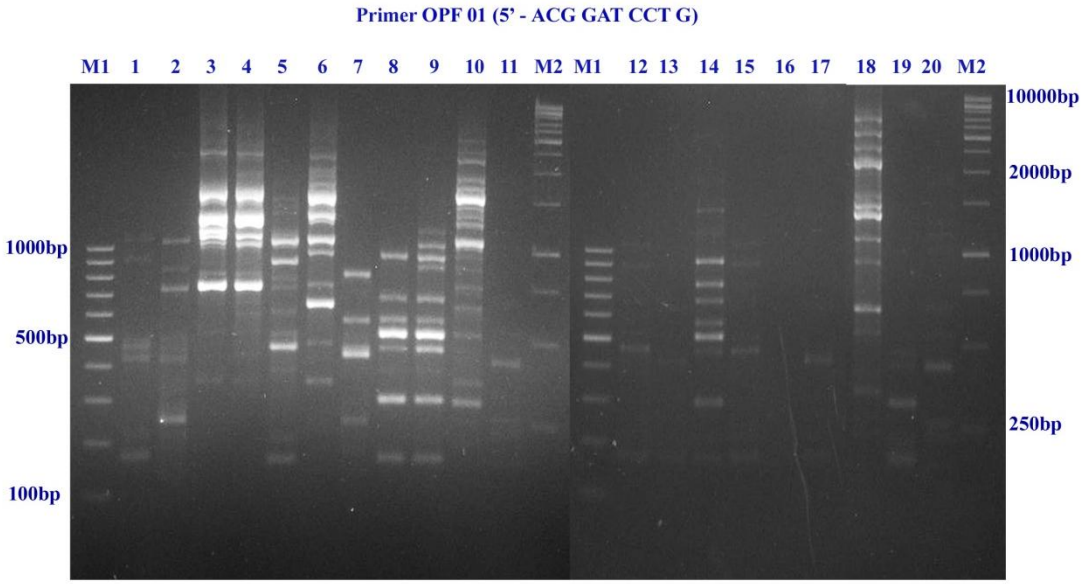
Values are mean of three replications. Means followed by a common letter are not significantly different at 5% level by DMRT

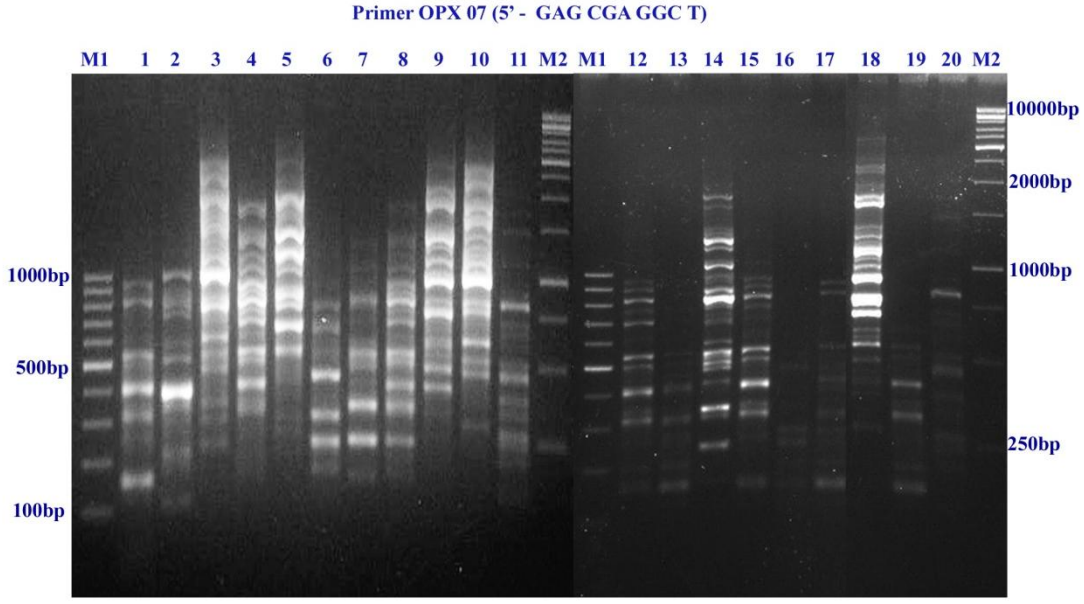
Fig.1 RAPD profiles of *Trichoderma* isolates obtained with C3 and OPA02 primers



Lanes 1-20: Isolates TSBO1,TSBO2 , TV1, TED1, TTH1, TSBO3, TSFCE1, TED2, TED3, TDHA1, TCOO1, TTNJ1, TBHA1, TSAT1, TPOL, TDHE1, TCHI1, TKAN1, TVRI1,TSAL1
M1 - 100bp marker; M2 - 1kb marker.

Fig.2 RAPD profiles of *Trichoderma* isolates obtained with OPF 01 and OPX 07 primers





Lanes 1-20: Isolates TSBO1,TSBO2 , TV1, TED1, TTH1, TSBO3, TSFCE1, TED2, TED3, TDHA1, TCOO1, TTNJ1, TBHA1, TSAT1, TPOL, TDHE1, TCHI1, TKAN1, TVRI1,TSAL1
M1 - 100bp marker; M2 - 1kb marker.

Fig.3 RAPD profiles of *Trichoderma* isolates obtained with OPC 20 primer

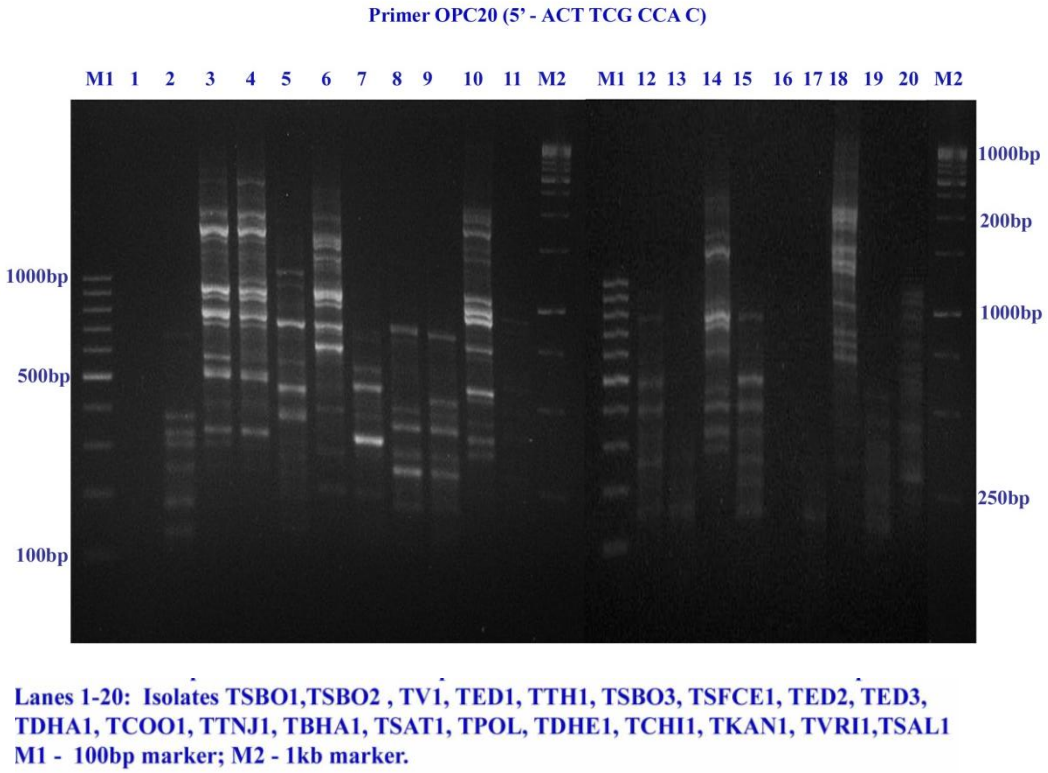
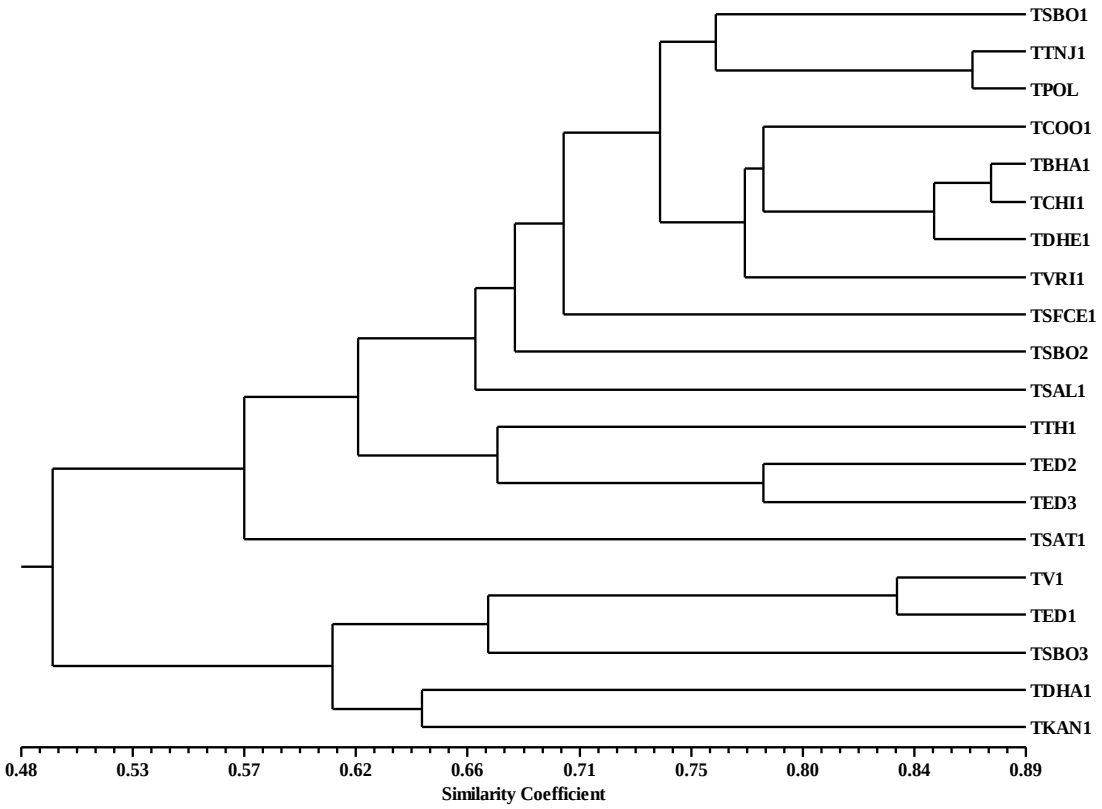


Fig.4 Dendrogram generated for the isolates of *Trichoderma* spp. from different host plants and locations of Tamil Nadu using RAPD primers



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How to cite this article:

Thilagavathi, R. 2023. RAPD Marker Based Determination of Genetic Variability among *Trichoderma* Isolates. *Int.J.Curr.Microbiol.App.Sci.* 12(03): 256-263. **doi:** https://doi.org/10.20546/ijcmas.2023.1203.031