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Full Length Research Paper

Molecular and morphological characters: An appurtenance for antagonism in *Trichoderma* spp.

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Biocontrol agent *Trichoderma* has attained importance for substitute of chemical pesticides and hence an attempt was intended to corroborate the positive relatedness of molecular and morphological characters with antagonistic ability. Twelve isolates belonging to *Trichoderma harzianum* and *Trichoderma viride* were assessed for their mycoparasitic effect on phytopathogens *Pythium aphanidermatum* and *Sclerotinia sclerotiorum*. Though *T. harzianum* isolates were more aggressive than *T. viride* isolates, the percent inhibitory effect among *T. harzianum* isolates did not vary much (80 to 86%). The inhibitory effect of *T.viride* isolates ranged from 50 to 80%; however, TvChen, Tv4, and TvNir were distinguishable from other *T. viride* isolates in exhibiting higher degree of antagonism. The dataset generated through morphological characters and molecular markers (RAPD and ISSR) showed a comparable output grouping the isolates Tv4, TvChen and TvNir in one cluster and all *T. harzianum* isolates in another cluster. Multiple nucleotide alignment of ITS 1 and ITS 2 region produced 100% homology among *T. harzianum* isolates whilst the nucleotide substitution at 62nd and 150th position of ITS 1 region and 27th and 40th position of ITS 2 region differentiated Tv4, TvChen and TvNir from other *T. viride* isolates. Genetic assessment could not establish substantial disparity among *T. harzianum* isolates which was comparable with its antagonism. The genetic distinctness of Tv4, TvChen and TvNir isolates authenticated their higher degree of antagonism. It is obvious from the present study that genetic diversity analysis had a positive correlation with the antagonistic ability of *Trichoderma* isolates. Thus an integrated approach of morphological and molecular markers can be employed to identify a superior strain of *Trichoderma* for its commercial exploitation.

Key words: *Trichoderma*, antagonism, morphological and molecular characters, RAPD, ISSR, ITS.

INTRODUCTION

Plant disease epidemics have created an ecologically unbalanced system in modern agriculture. Deterrence of such epidemics for the most part achieved through use of chemical fungicides which have greater repercussion on environment and human health. Also progressive confrontation in a midst of pathogen resistance to accessible chemical plant protectants has engrossed the need of alternative methods of disease control. Fungi of the genus *Trichoderma* are important biocontrol agents of several soil borne phytopathogens (Benitez et al., 2004).

The *Trichoderma* species serves as a potential alternative to chemical control measure and growing pathogen resistance crop cultivars.

This may be attributable to their diverse metabolic capability and aggressively competitive nature (Samuels, 1996; Klein and Eveleigh, 1998). *Trichoderma* use different mechanism for control of phytopathogens which includes mycoparasitism, out compete pathogenic fungi for nutrients, secretion for antibiotics and fungal cell wall degrading enzymes (Harman et al., 2004; Renio et al., 2008). *Trichoderma* sp. includes a plethora of strains which differ in their innocuousness and effectiveness as biocontrol agents. Furthermore it is difficult to predict the

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degree of synergism and the behaviour of a biocontrol agent in a natural pathosystem. Thus the present study was to characterize the cryptic species of *Trichoderma* associated with biological control and to establish the relation between bioefficacy and morphological and molecular characters existed if any.

The molecular technique like random amplified polymorphic DNA (RAPD) developed by Williams et al. (1990) has been used for genetic and taxonomic studies for several fungi including *Trichoderma* sp. (Muthumeenakshi and Mills, 1995; Dodd et al., 2004). 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 (Charlesworth et al., 1994). The internal transcribed spacer (ITS) region of the rDNA is perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematic study at species level, and even within species (Ospina-Giraldo et al., 1998; Kubicek et al., 2000; Kulling et al., 2002; Lee and Hseu 2002). Kindermann et al. (1998) attempted a first phylogenetic analysis of the whole genus of *Trichoderma* using sequence analysis of the ITS1 region of rDNA.

MATERIALS AND METHODS

Isolation and identification of *Trichoderma*

Soil samples were collected from various experimental fields of Indian Agricultural Research Institute (IARI), Pusa, New Delhi. A total number of twelve isolates of *Trichoderma* species was isolated and identified in potato dextrose agar (PDA) with low sugar medium (Nirenberg, 1976). The identification of *Trichoderma* isolates were confirmed both by morphological and molecular characters (ITS), and deposited in Indian type culture collection (ITCC), IARI, Pusa, New Delhi.

Confrontation assays *in vitro*

In vitro confrontations were studied by performing dual culture technique described by Dennis and Webster (1971) was used to test the antagonistic ability of *Trichoderma* isolates against the phytopathogenic oomycetes *Pythium aphanidermatum* and fungi *Sclerotinia sclerotiorum*. The host fungus and *Trichoderma* were grown on potato dextrose agar (PDA) for a week at room temperature (28±2°C). Growth parameters in all dual cultures were read after 7 days. The plates containing only the target pathogenic organisms without *Trichoderma* were taken as control to evaluate the percent growth inhibition.

Morphological characterization

The morphological characterization was performed based on the key provided by Bissett (1991 a,b,c) which included the characters classified under colony, mycelia and spore patterns. These characters were used as descriptors and the variation present within each descriptor were taken as descriptor states. Each isolate were scored for every descriptors based on the rank assigned to the respective states. The scored data was finally converted into binary form based on presence (1) or absence (0) for that particular character and subjected to statistical analysis using INDOSTAT package developed by Indostat services, hyderabad, India. Multivariate analysis was carried out based on weighted average linkage clustering and principal component analysis.

Molecular characterization

The total genomic DNA was extracted from each isolate of *Trichoderma* based on cetrimide tetradecyl trimethyl ammonium bromide (CTAB) mini extraction method of Crowhurst et al. (1995) with modification.

Random Amplified Polymorphic DNA (RAPD) analysis

RAPD-PCR conditions for *Trichoderma* isolates in the present investigations were standardized. Forty random primers of 10bp long each (10mer) of OPA and OPX (Operon Technologies, USA) series were used to amplify genomic DNA of the isolates. Out of these, sixteen primers which presented strong band resolution were chosen for the study *viz.,* OPA 1, OPA 2, OPA 4, OPA 5, OPA 9, OPA 11, OPA 12, OPA 16, OPX 2, OPX 5, OPX 8, OPX 12, OPX 13, OPX 17, OPX 18 and OPX 20. Following the experiments for optimization of component concentrations, PCR amplification was carried out with 20 ηg/µl of genomic DNA, 2 mM $MgCl_2$, 1U *Taq* DNA polymerase, 1X PCR buffer with 15 mM $MgCl_2$, 0.6µM decamer primers and 0.2 mM of dNTP mix. The volume was made up to 25 µl. PCR reactions were carried out in a Perkin Elmer Thermocycler under the conditions involving denaturation at 94°C for 2 min; 40 cycles of denaturation at 94°C for 1 min, primer annealing at 35°C for 1 min and primer extension at 72°C for 2 min; final extension step at 72°C for 4 min.

Inter simple sequence repeats (ISSR) analysis

The procedure described by Bornet et al. (2002) with minor modifications was used for carrying out the polymerase chain reaction (PCR) reactions for ISSR analysis. Ten primers were tested for amplification at different annealing temperatures of genomic DNA of the isolates. Out of these, eight primers viz., 5'$(AG)_8$AT3', 5'$(AG)_8$AC3', 5'$(GA)_8$AC3', 5'$(GA)_9$T3', 5'$(GA)_9$AC3', 5'BDB$(GA)_8$ T3', 5'YT$(CA)_7$T3' and 5'YC$(TG)_7$T3' consisting of anchored ISSR gave satisfactory amplification and band resolution. The PCR amplification was carried out with 25 ηg of genomic DNA, 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase, 1 X PCR buffer without $MgCl_2$, 1 µM ISSR primer and 0.2 mM dNTP mix. The volume was made up to 25 µl. PCR reactions were carried out in a Perkin Elmer GeneAmp 9600 thermocycler under the conditions involving denaturation at 94°C for 7 min; 30 cycles of denaturation at 94°C for 30 s, primer annealing at temperature specific to each primer for 45 s and primer extension at 72°C for 2 min; final extension step at 72°C for 7 min.

The PCR products of both random amplified polymorphic DNA (RAPD) and ISSR reactions were resolved on 1.4% agarose gel in 1 X TBE buffer pre-stained with ethidium bromide (1 µg/ml) and electrophoresis was carried out at 90 volts for 1.5 h followed by 70 volts for 2 h and visualized under UV light on a UV- Transilluminator. The gel was photographed using a Gel documentation system.

Data analysis

The amplification products of RAPD and ISSR were scored for the

presence “1” and absence “0” and missing data as “9”. The genetic associations between isolates were evaluated by calculating the Jaccard’s similarity coefficient for pair wise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to cluster analysis by unweighted pair group method for arithmetic mean (UPGMA) and a dendrogram was generated. The computations were performed using the program NTSYS – PC. version 2.02h (Rohlf, 1997). The Jaccard’s similarity matrix was subjected to principal component analysis. This coordination method makes use of multidimensional solution of the observed relationships. PCA resolves complex relationships into a function of fewer and simpler factors. In this technique, the data matrix is derived from the distances (or similarities) between the operational taxonomic units. To simplify the description of these ‘clouds’ of points, the (principal) axes through the hyper-ellipsoid are calculated. The successive principal axes, representing the first major axis, the second axis etc., account for the greatest, the second greatest, etc. amount of variation.

Internal transcribed spacer region

The internal transcribed spacer (ITS) regions of the rDNA repeat from the 3’end of the 18S and the 5’end of the 28S gene were amplified using the two primers, ITS A and D which were synthesized on the basis of conserved regions of the eukaryotic rRNA gene (White et al., 1990). The PCR-amplification reactions were performed in a 50 µl mixture containing 50 mM KCl, 20 mM Tris HCl (pH 8.4), 2.0 mM $MgCl_2$, 200 µM of each of the four deoxynucleotide triphosphates (dNTPs), 0.2 µM of each primer, 40 ηg/µl of template and 2.5 U of Taq polymerase. The cycle parameters included an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min and primer extension at 72°C for 3 min and a final extension for 10 min at 72°C. Amplified products were separated on 1.2% agarose gel in TAE buffer, pre-stained with ethidium bromide (1 µg/ml) and electrophoresis was carried out at 60 volts for 3 h in TAE buffer. One Kb ladder (MBI, Fermentas) was used as a marker. The gel was observed in a trans-illuminator over ultra violet light. The desired bands were cut from the gel with minimum quantity of gel portion using QIAGEN gel extraction kit.

Nucleotide sequencing and *in silico* analysis

The sequencing of the PCR product was carried out in automated sequencer at Genome-Bio, Pune, India. Related sequences were searched using BLAST programme from the Gen-Bank database (http://www.ncbi.nlm.nih.gov/blast/) (Altschul et al., 1997). The multiple sequence alignment and pairwise alignment were made using BioEdit version 5.09 (Hall 1999, Department of Microbiology, North Carolina State University). The neighborhood-joining bootstrap tree was created using CLUSTAL W 1.6 matrix by the CLUSTAL X programme ver. 1.81 (Thompson et al., 1997).

RESULT

Confrontation assay *in vitro*

The isolates of *Trichoderma* spp. *viz*., Th3, Th10, Th30, Th31 and ThAg belonging to *T. harzianum*; Tv2, Tv4, Tv12, Tv15, Tv32, TvChen and TvNir of *T. viride* were evaluated for their antagonistic ability against the phytopathogenic fungi *Pythium aphanidermatum* and *Sclerotinia sclerotiorum* based on percent inhibition mycelial growth and mean mycelial growth (in cm^2). Theisolate Th3 of *T. harzianum* found to have the maximum inhibiting effect on the growth of *P. aphanidermatum* (86.45%) and *S. sclerotiorum* (90%) which directly corresponded to their mycelial growth rate of 23.04 and 25 cm^2 against the target fungus. Among the *T. viride* isolates TvChen was more aggressive with 80% inhibiting effect on *P. aphanidermatum* and TvNir had 85% impact on *S. sclerotiorum*, closely followed by Tv4 in either of the cases.

Morphological characterization

The growth patterns of *Trichoderma* isolates after four days of incubation at 25°C showed significant differences in nature of culture growth and sporulation patterns (Table 1). The conidial wall patterns and shape were rough and subglobose among *T. harzianum,* while they were smooth and globose to obovoid among *T. viride.* The growth characters of culture and sporulation patterns varied noticeably within and between the species.

The morphological characters taken for study were assigned convenient ranks so as to generate a quantitative data for its characterization. The numerical values of the scored codes on nominal/ordinal scale of 15 quantitative and qualitative characters were converted to binary data. The data subjected to simple matching similarity index and weighted average linked clustering resulted in a dendrogram with three distinct clusters (Figure 1). The isolates representing *T. harzianum viz*., Th3, Th31, Th10, Th30 and ThAg clustered into one group with the intra-cluster distance 0.202. The isolates of *T. viride viz*., Tv4, Tv12, Tv2 and Tv15 formed the second group whilst Tv32, TvChen and TvNir grouped in the third cluster. The intra-cluster distance of group two exhibited the maximum diversity (0.332), while the third group showed the least (0.175). The inter-cluster distance between group one and three was highest (0.605). Shaded similarity matrix using pairwise comparison of all isolates confirmed the above results.

The PCA performed on studied traits showed that first two most informative components accounted for about 58% variation and the plot illustrated *T. harzianum* and *T. viride* as two distinct components confirming the cluster analysis.

Molecular characterization

Random amplified polymorphic DNA

The isolates of *Trichoderma* were subjected to RAPD analysis where 16 random primers resulted in robust and reproducible RAPD fragment patterns. The selected primers generated 112 RAPD bands and the size of the amplification products ranged from 250 bp to 900 bp. The percent of polymorphism ranged from 60 to 100 with 4

Table 1a. Key morlogical descriptors used for characterization of *T.harzianum* isolates

Isolate/ characters	Th3	Th10	Th30	Th31	ThAg
Colony growth rate (cm/day)	8-9 in 3days	8-9 in 3days	8-9 in 3days	8-9 in 3days	7-8 in 4days
Colony colour	Green to dark green	Green to dark green	Green to dark green	Green to dark green	Green to dark green
Reverse colony colour	Yellow	Colourless	Light yellow	Creamish	Colourless
Colony edge	Smooth	Smooth	Smooth	Smooth	Wavy
Culture smell	Malt	Malt	Malt	Malt	Malt
Mycelial form	Arachnoid	Floccose to Arachnoid	Floccose to Arachnoid	Floccose to Arachnoid	Floccose to Arachnoid
Mycelial colour	Watery white	Watery white	Watery white	Watery white	Watery white
Conidiation	Ring like zones	Ring like zones	Ring like zones	Ring like zones	Ring like zones
Conidiophore branching	Highly branched, regular 2-3µm L	Highly branched, regular 4-6µm L	Branched, regular 1-2µm L	Branched, regular 1-3µm L	Branched, regular 2-3µm L
Phialide disposition	2-3 whorls	Solitary	2/3 whorls	2-3 whorls	2/3 whorls
Phialide shape	Globose 8-15 x 2-3µm	Nine-Pin shape 8-15 x 2-3µm	Nine-Pin shape 8-14 x 2.4-3µm	Globose 8-15 x 2-3µm	Nine-Pin shape 8-15 x 2-3µm
Conidial shape	Subglobose 3.6-4.5µm	Subglobose 3.6-4.5µm	Subglobose 3-4.8x3.5-4µm	Subglobose 3.5-4.5µm	Subglobose 3.5-4.5µm
Conidial wall	Rough	Rough	Rough	Rough	Rough
Conidial colour	Green	Green	Green	Green	Green
Chlamydospores	Not observed	Not observed	Present globose	Not observed	Not observed

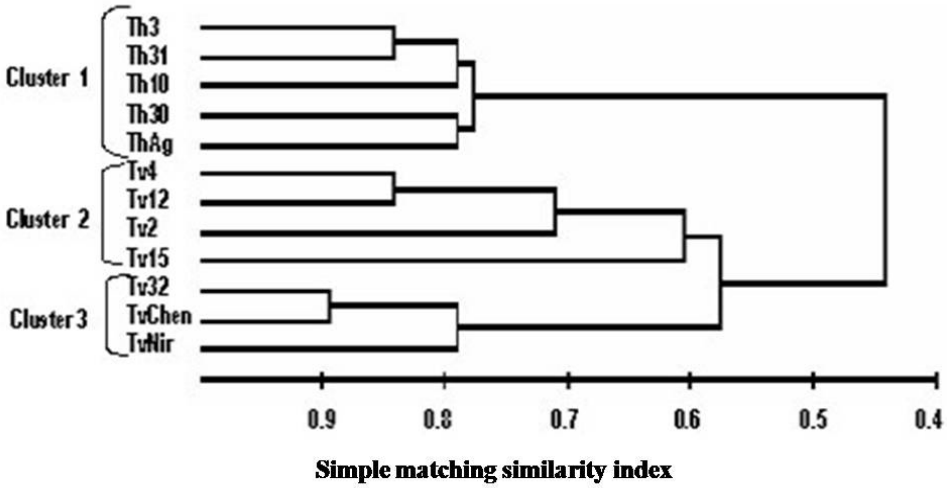

Figure 1. Inter-relationship between the *Trichoderma* isolates based on the morphological characters using simple matching similarity index.

primers OPA4, OPA5, OPX5 and OPX13 displayed 100% polymorphism. The genetic similarity between the isolates of *Trichoderma* was determined on the basis of Jaccard's similarity coefficient. The mean value of the Jaccard's similarity coefficient of the RAPD marker was 0.80. The highest genetic similarity was observed between the

Table 1b. Key morphological descriptors used for characterization of *T. viride* isolates.

Isolate/ characters	Tv2	Tv4	Tv12	Tv15	Tv32	TvChen	TvNir
Colony growth rate (cm)	9-10 cm in 5 days	9-10 cm in 5 days	7-8 cm in 5 days	6-7cm in 5days	9-10cm in 5days	9-10cm in 5days	9-10cm in 5days
Colony colour	Dark green	Dark green	Dark green	Yellow to green	Dark green	Yellow to green	Yellow to green
Reverse colony colour	Deep yellow	Deep yellow	Colourless	Deep yellow	Colourless	Deep yellow	Colourless
Colony edge	Wavy	Wavy	Wavy	Wavy	Wavy	Smooth	Wavy
Culture smell	Coconut	Coconut	Coconut	Coconut	Coconut	Coconut	Coconut
Mycelial form	Floccose to Arachnoid	Arachnoid	Arachnoid	Floccose	Floccose	Floccose	Floccose
Mycelial colour	Watery white	Watery white	White	White	Watery white	Watery white	White
Conidiation	Concentric zones	Concentric zones	Concentric zones	Ring like zones	Ring like zones	Ring like zones	Concentric zones
Conidiophore branching	Moderately branched, irregular 4-5µm L	Moderately branched, irregular 2-2.5µm L	Moderately branched, irregular 4-5µm L	Moderately branched, irregular 4-5µm L	Moderately branched, irregular 1.5-2.5µm L	Highly branched, irregular 1.5-2.5µm L	Highly branched, irregular 1.5-2.5µm L
Phialide disposition	Solitary	2/3 whorls	2/3 whorls	2/3 whorls	Solitary	Solitary	Solitary
Phialide shape	Sigmoid or hooked 8-14 x 2.4-3µm	Sigmoid or hooked 8-14 x 2-3.5µm	Sigmoid or hooked 8-14 x 2-3µm	Sigmoid or hooked 8-14 x 2.4-3µm	Nine-Pin shape15 x 2.5-3.5µm	Nine-Pin shape 4.5-12.5 x 2.5-3.5µm	Nine-Pin shape 5-18 x 2-3.5µm
Conidial shape	Globose to obovoid 3.6-4.5 µm	Globose to obovoid 3-4.5µm	Globose to obovoid 3.6-4.5µm	Globose to subglobose 3.6-4.5µm	Globose to subglobose 3-5µm	Globose to subglobose 2.5-3.5µm	Globose to subglobose 2.5-5µm
Conidial wall	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Conidial colour	Green	Green	Green	Green	Dark Green	Dark Green	Dark Green
Chlamydospores	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed

isolates Th31 and ThAg (80%) and the least was between between the isolates Tv2 and Tv32 (30%). The dendrogram generated using unweighted pair group method arithmetic (UPGMA) based on NTSYS pc version 2.02i software resulted in three major clusters with isolates Tv2 and Tv32 as out-liers (Figure 2). It was evident from the cluster analysis that the isolates representing to *T. harzianum* clustered in one group which had a similarity coefficient of 0.81 while isolates of *T. viride* segregated into two groups. Tv4, TvChen and TvNir formed the second group with 78% similarity and Tv12 and Tv15 formed the third group which shared 82% similarity. The 3D depiction of principle components evidenced the close relatedness of *T. har-zianum* isolates which formed into one subgroup and Tv4, TvChen and TvNir formed the other subgroup. PCA further

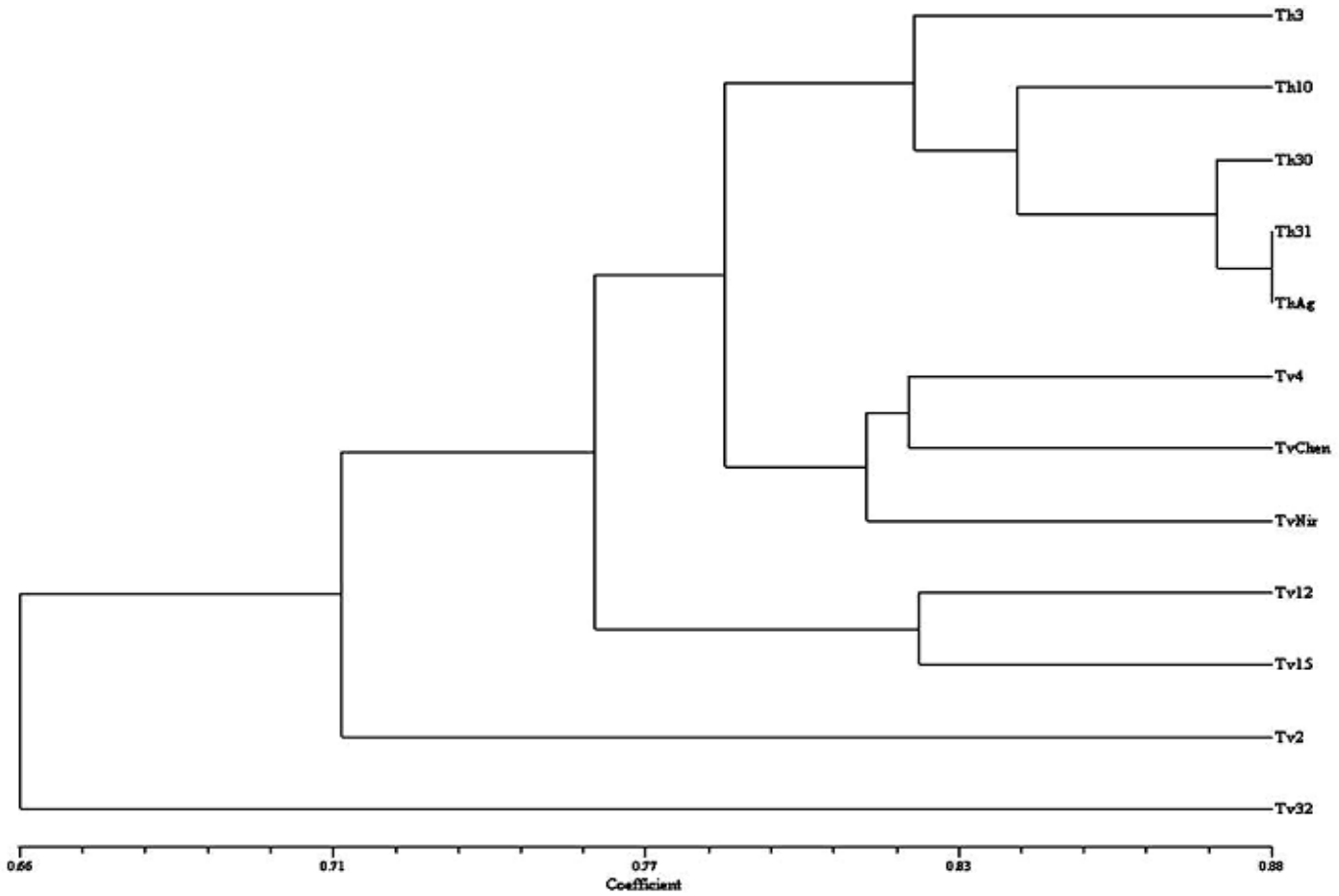

Figure 2. Jaccard's similarity based dendrogram of *Trichoderma* isolates for RAPD markers.

confirmed the genetic distinctness of Tv32 and Tv2 which stood as outliers in the dendrogram as shown in Figure 3

Inter simple sequence repeats

Fifteen ISSR primers were screened and eight of them giving satisfactory amplification and band resolution were taken for the study. The selected primers generated 70 ISSR bands and the size of the amplification products ranged from 100 to 950 bp. The primer 5' $(GA)_8AC$ 3' produced the maximum number of polymorphic loci (6) out of the total loci (7) followed by 5' $(GA)_9AC$ 3' (6). The percent of polymorphism ranged from 40 to 86. The mean value of the Jaccard's similarity coefficient of the ISSR marker was 0.76. The dendrogram based on the above data separated the isolates into two major clusters with similarities ranging from 76 to 94%. All the five *T. harzianum* isolates clustered into a single group sharing similarity around 80%. The second major cluster consisted of isolates *T. viride,* however these isolates formed two subgroups within the cluster. The isolates Tv2, T15 and Tv32 formed the first subgroup while Tv4, TvChen and TvNir formed the other. However, Tv12 did not cluster with any of these two subgroups but stood separately with only 65% similarity with main branch as evident from Figure 4. The 3D depiction of principle components reconfirmed the results obtained through dendrogram analysis (Figure 5).

Internal transcribed spacer

The universal primers ITS A and ITS D were used to amplify the internal transcribed spacer regions of rDNA yielding products of approximately 600 bp as estimated by agarose gel electrophoresis. The amplified PCR products was eluded and sequenced by automated sequencer which encompass ITS 1, ITS 2 and 5.8S rDNA gene and also 18S rDNA gene having base pairs ranging 35-49 bp at the 3' end and 20-25 bp of the 5' end of 28S rDNA. The sequences so obtained were subjected to BLAST search for its identity and confirmation, and subsequently submitted to National Centre for Biotechnology Information (NCBI) GenBank. The accession number for

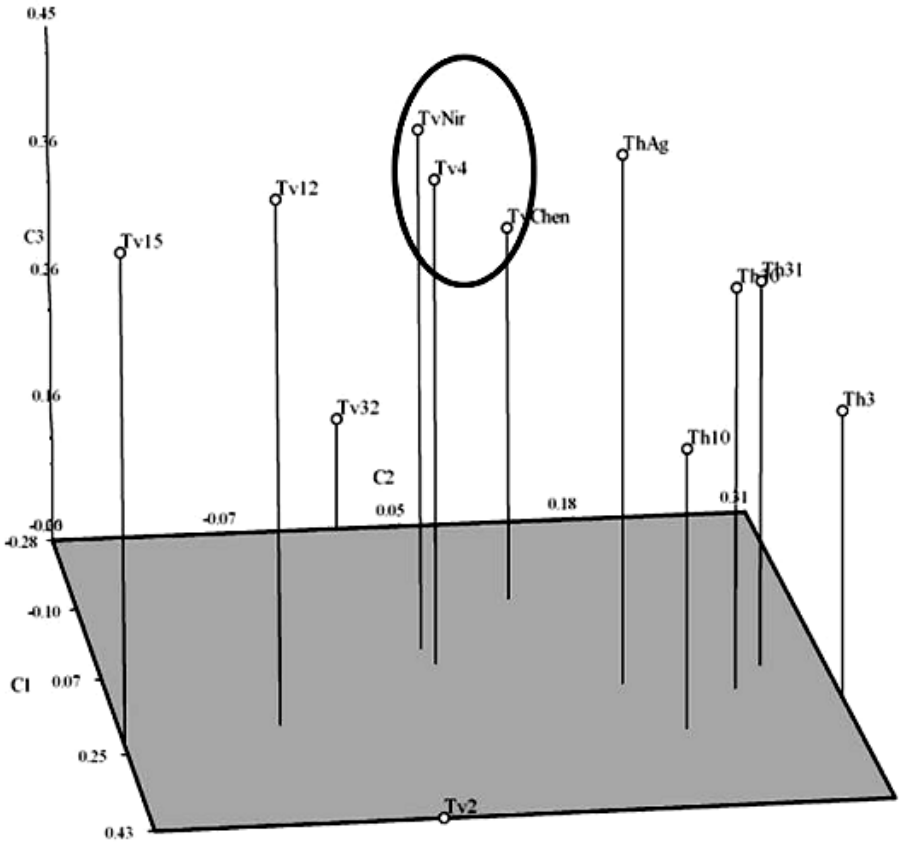

Figure 3. 3-D Plot depicting the principle components of *Trichoderma* isolates based on RAPD primers.

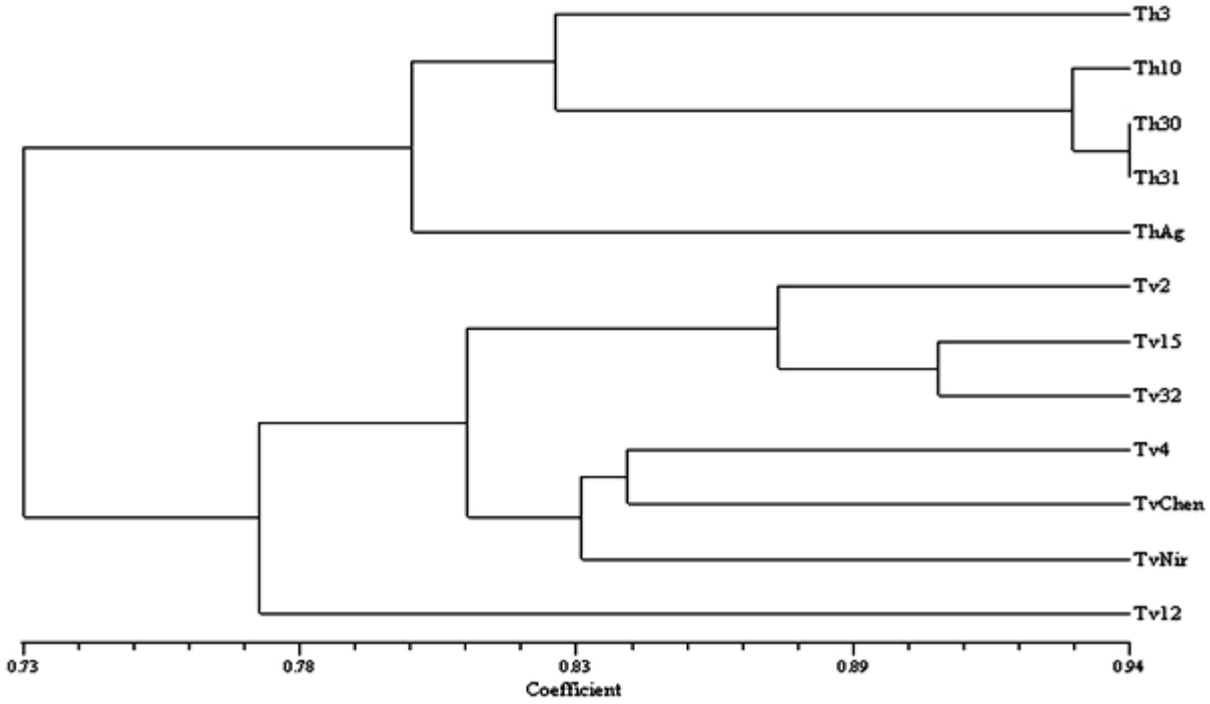

Figure 4. Jaccard's similarity based dendrogram of *Trichoderma* isolates for ISSR markers.

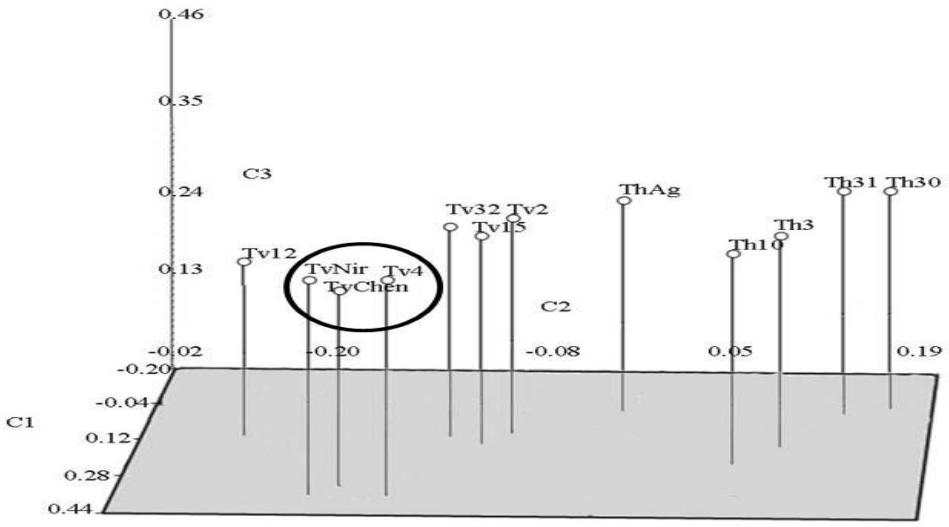

Figure 5. 3-D Plot depicting the principle components of *Trichoderma* isolates based on ISSR markers.

Table 2. Accession numbers for twelve isolates of Trichoderma provided by NCBI.

Trichoderma isolates	Accession number of ITS 1 region	Accession number of ITS 2 region
Th3	*EU365835*	*HM358882*
Th10	*EU382224*	*HM358883*
Th30	*EU443841*	*HM358884*
Th31	*EU443842*	*HM358885*
ThAg	*EU443843*	*HM358886*
Tv2	*EU443844*	*HM358887*
Tv4	*EU443845*	*HM358888*
Tv12	*EU443846*	*HM358889*
Tv15	*EU443847*	*HM358890*
Tv32	*EU443848*	*HM358891*
TvChen	*EU443849*	*HM358892*
TvNir	*EU443850*	*HM358893*

sequences of ITS 1 and ITS 2 region for the twelve isolates of *Trichoderma* is represented in Table 2. The multiple nucleotide alignment of ITS regions was analyzed using BioEdit programme. There was substantial disparity in length of ITS sequences between *T. harzianum* (199 bp) and *T. viride* (181 bp) isolates. The isolates of *T. harzianum* showed 100% homology in nucleotide sequence. Conversely *T. viride* isolates showed nucleotide divergence of 2.2% in ITS 1 and 2.26% in ITS 2 region. The isolates Tv4, TvChen and TvNir displayed 100% homology in nucleotide sequence, however they differed from other isolates at 62nd (A instead of C) and 150th (A instead of T) nucleotide position in ITS 1 region and 27th (T instead of G) and 40th (C instead of T) in ITS 2 region (Figure 6a and 6b). To elucidate the genetic closeness of the *Trichoderma* isolates a phylogenetic tree was constructed based on sequence analysis of ITS 1 and ITS 2 regions using the neighbour-joining method in treecon for windows version 1.3b on sequences aligned using ClustalW 1.7 version. A random sequence was used as an out-group to demonstrate the situation of the root. Bootstrap analysis of ITS 1 region with 1000 bootstrap replication demonstrated two main branches. All the isolates of *T. harzianum* formed one group which supported with a bootstrap value of 99.9%. The other cluster consisted of *T. viride* isolates which separated into two subgroups supported by bootstrap value higher than 75%. The isolates Tv4, TvChen and TvNir formed one subgroup, while Tv15, Tv2 and Tv32 formed the other subgroup. Tv12 did not group with any of the two subgroups but stood separately in this cluster. The bootstrap analysis of ITS 2 region also showed similar results, however Tv12 which stood separately in ITS 1 clustered into one of the subgroups consisting of

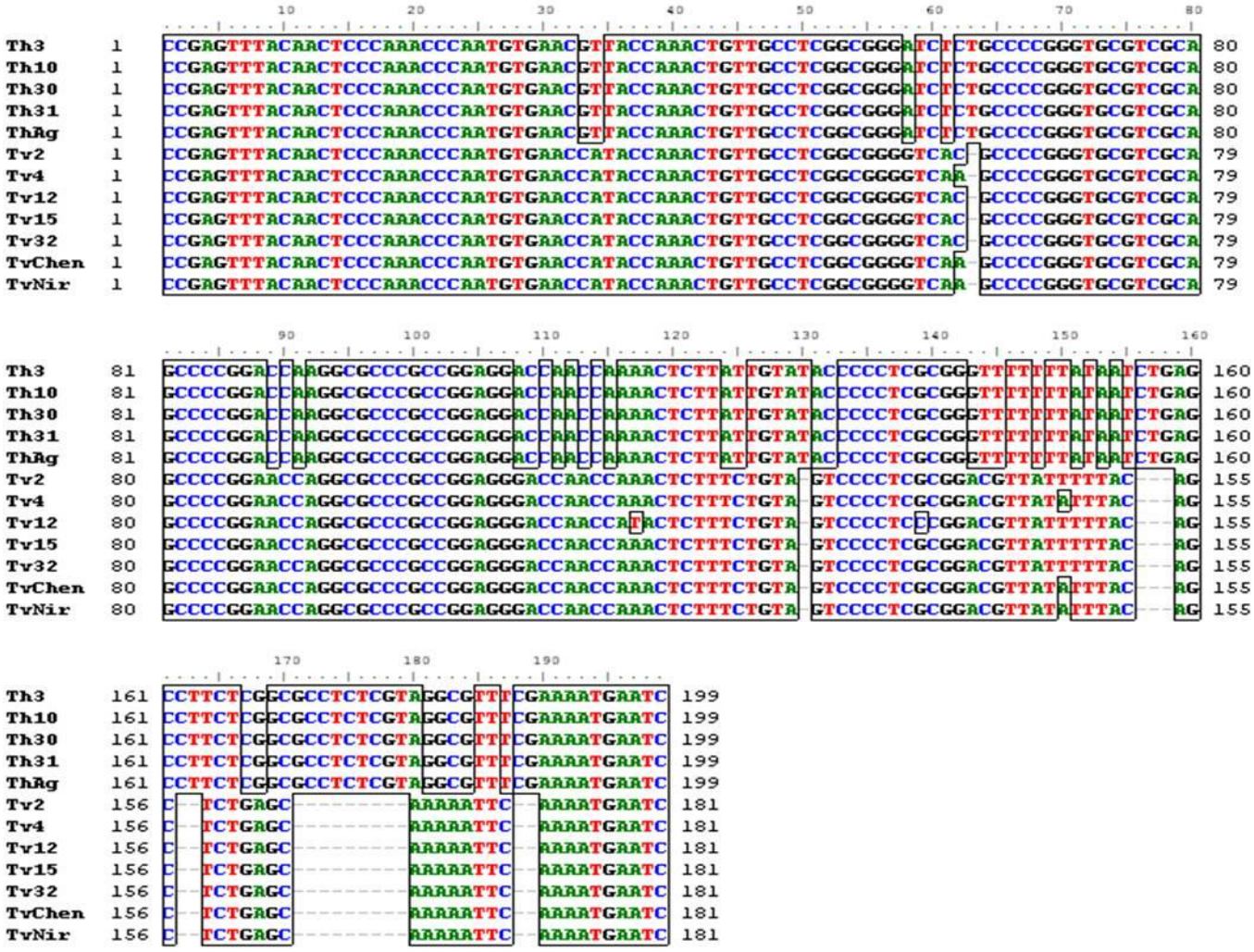

Figure 6a. Multiple nucleotide alignment of ITS 1 region of *Trichoderma* isolates (conserved regions are showed in boxes).

Tv15, Tv2 and Tv32 (Figure 7 and 8). This grouping was supported by more than 70 percent bootstrap values.

DISCUSSION

In pursuit for a probability of corroborating the mycoparasitic antagonism ability of *Trichoderma* with respect to their morphological and molecular characters the current study was undertaken. Such corroboration will certainly be a needful aspect in elucidating the molecular mechanism involved in mycoparasitic antagonism. The source isolates were subjected for morphotaxon observation which is the pedestal for any taxonomic work and we identified the isolates as two groups, *T. harzianum* and *T. viride.*

Dual petri-plate marriage of *Trichoderma* with pathogen *P. aphanidermatum*, and similarly with *S. sclerotiorum* endorsed the mycoparasitic antagonism of *T. harzianum* and *T. viride. T. harzianum* isolates were more aggressive than *T. viride* in their antagonistic effect which ranged between 80 to 86% against *P. aphanidermatum* and 83 to 90% against *S. sclerotiorum,* on the other hand *T. viride* isolates ranged between 56 to 81% against *P. aphanidermatum* and 50 to 80% against *S. sclerotiorum*. However, the percent inhibition of mycelial growth of Tv4, TvChen and TvNir was around 80% which was comparable with antagonistic ability of *T. harzianum*.

The molecular and morphological markers could not establish any variation within *T. harzianum* isolates instead grouped all the isolates into one cluster. The sequence identity matrix of ITS 1 and ITS 2 region showed 100% homology among these isolates of *T. harzianum*. None of the markers were able to identify distinct variation among *T. 1harzianum* isolates and also it is prominent that these isolates exhibited less variation in their antagonism. Therefore it can be interpreted that these isolates taken for study had no much dissimilarity

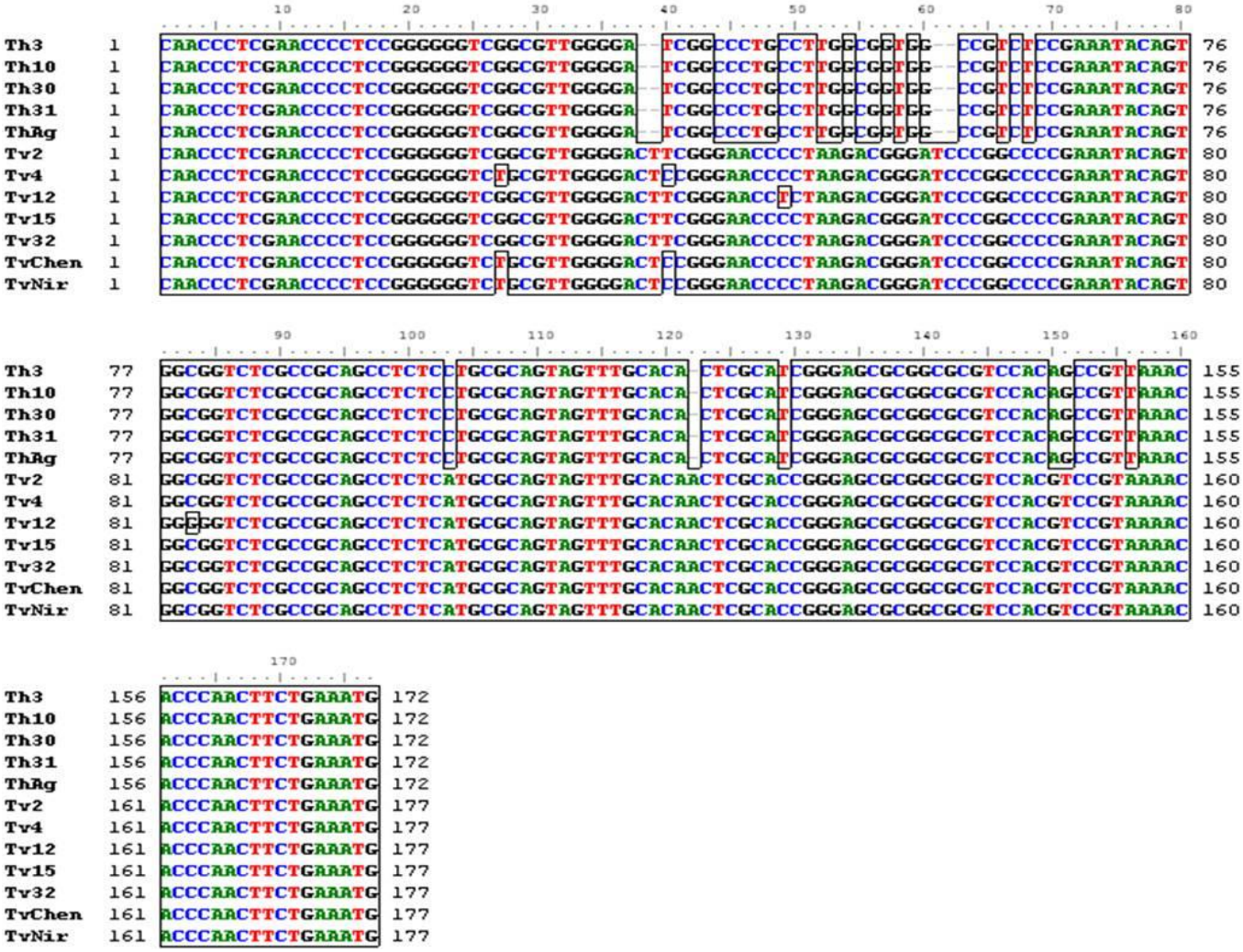

Figure 6b. Multiple nucleotide alignment of ITS 2 region of *Trichoderma* isolates (conserved regions are showed in boxes).

among them genetically.

The quantitative and qualitative characters taken for morphological characterization were able to differentiate *T. viride* isolates into two clusters. The cluster two (Tv4, Tv12, Tv2 and Tv15) and three (Tv32, TvChen and TvNir) of the dendrogram in Figure 2 consisted of *T. viride* isolates, they shared only 60% in common. Though the morphological characterization had resulted in classifying the *Trichoderma* isolates, it can give only a broader picture. Waalwijk et al. (1996) indicated the difficulties to distinguish species based on morphology alone. Seaby (1996) was also of the same opinion where he reported that the morphological traits are subjected to environmental influence and can vary substantially from culture to culture. The taxonomic consideration based solely on phenotype may be subjected to ambiguities induced by environmental conditions. Thus to improve the reliability of morphological characters and to resolve the ambiguities the characterization should be complemented with molecular data.

The RAPD marker like morphological characters divided the isolates of *T. viride* into two subgroups, but here Tv4, TvChen and TvNir formed a single group. Though RAPD marker formed 80% polymorphism it failed to differentiate Tv2 and Tv32. Singh et al. (2006) reported that RAPD primers exhibited both inter and intra specific variation in the isolates and were able to distinguish the pathogenic isolates *T. harzianum* and *T. virens* causing green mould diseases in cultivated mushroom which was in conformity with our results. Though RAPD analysis provided good discrimination within and between species it is generally not considered a robust enough technique because of its poor reproducibility.

The ISSR technique which is more reliable reproduced similar dendrogram pattern as that of RAPD marker grouping Tv4, TvChen and TvNir separately. The isolates Tv2 and Tv32 which stood as outliers in RAPD clubbed to form a subgroup, but Tv12 stood separately within the

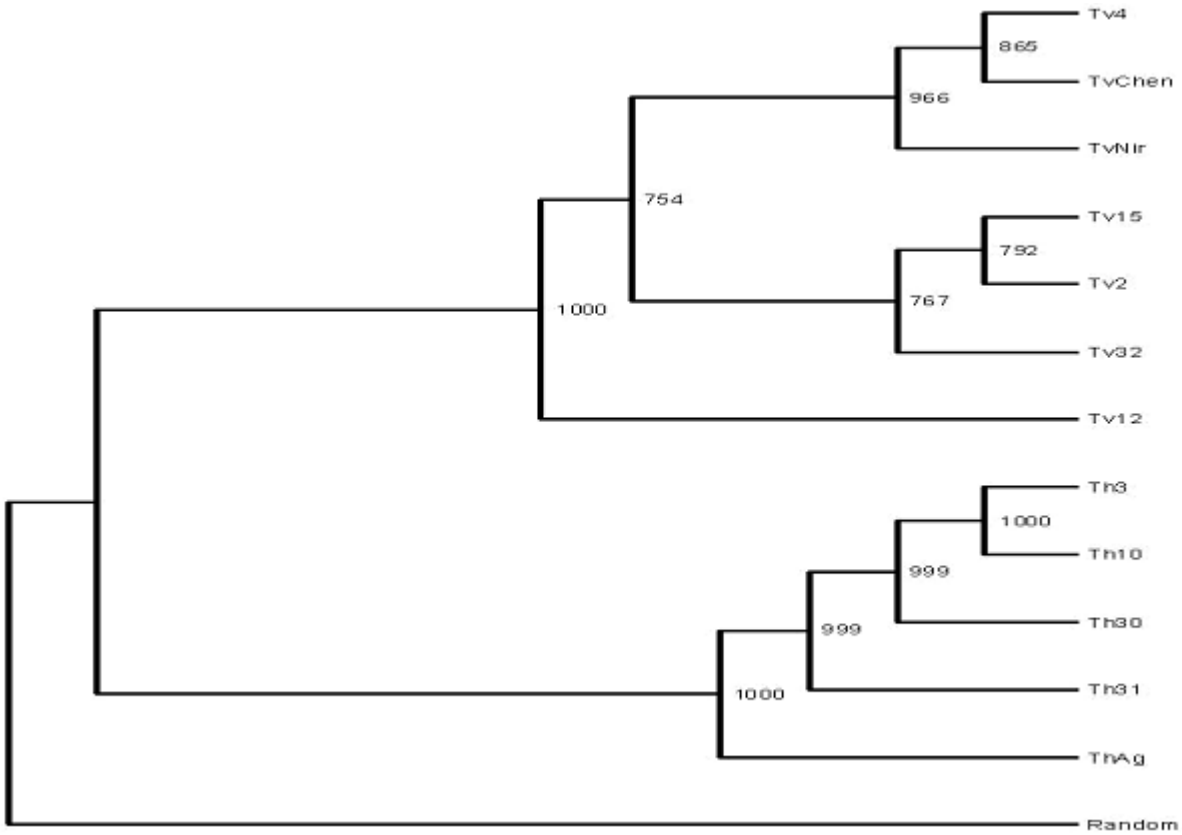

Figure 7. Phylogenetic relationships of *Trichoderma* isolates based on analysis of ITS 1 sequences.

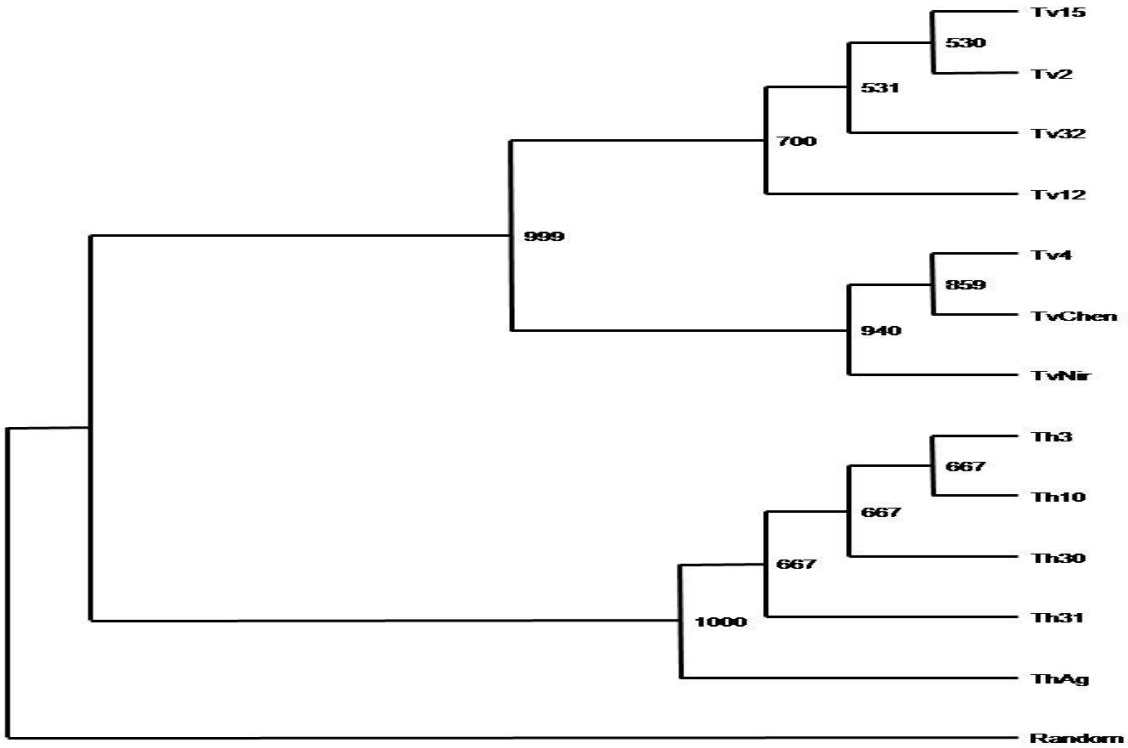

Figure 8. Phylogenetic relationships of *Trichoderma* isolates based on analysis of ITS 2 sequences.

main branch. The combined ISSR and RAPD dendrogram also authenticated the above results. The mantel test using Euclidean distances and Neis genetic distances validated the comparison of morphological characters with molecular markers RAPD and ISSR.

The nucleotide sequence alignment of ITS 1 and ITS 2 region revealed the presence of hotspots with nucleotide substitution at four positions supported the distribution of Tv4, TvChen and TvNir into one subgroup. The dendrogram pattern obtained through ISSR was almost indistinguishable from the phylogenetic tree obtained from ITS 1 and ITS 2 sequences. This authenticates the soundness of our perception that the bioefficacy of these three isolates were manifested at the molecular level which made them to distinguish from other *T. viride* isolates. Conversely Goes et al. (2002) reported that there was no relationship between the polymorphism showed by the *Trichoderma* isolates and their hardness against *R. solani* based on RAPD marker. Similar kind of result was reported by Shalini et al. (2006) and Shanmugam and Sharma (2008). However the results of molecular markers employed in this present study was complementary and confirmatory in nature which substantiates our corroboration between bioefficacy of *Trichoderma* isolates and molecular characters. Thus we proceed to conclude that combination of more than one molecular marker especially ISSR and ITS can be used as a diagnostic kit to identify a superior *Trichoderma* strain for its commercial application.

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