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A combined morpho-genetic approach towards identification of *Trichoderma* species

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Twelve of *Trichoderma* species obtained from soil rhizosphere of plantation crops and agricultural fields of Indian Agricultural Research Institute, New Delhi, India, were analyzed based on morphological chatacters viz., colony, conidiophore, phialides, conidia and chlamydospore characters and classified these isolates into two different sections and five groups. Further attempt was made to characterize these 12 isolates of *Trichoderma by* amplifying the ITS1-5.8s-ITS2 and *tef1* regions. Sequence analysis of the amplicons confirmed that the ITS1-5.8s-ITS2 and the *tef1* marker had much capacity to resolve the taxa. It was also seen that there were good correlation between morphological and molecular classification of these *Trichoderma* isolates.

Key words: *Trichoderma*; Internal Transcribed Spacer; Sequencing; Polymorphic Chain Reactions (PCR)

INTRODUCTION

Trichoderma, commonly available in soil and root ecosystems has gained immense importance since last few decades due to its biological control ability against several plant pathogens (Zhang *et.al.*, 2005). As antagonistic microorganisms, it reduces growth, survival or infections of different pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions and enzyme secretion. Despite the importance of *Trichoderma*, the species in this genus are not well identified due to the variability in phenotypic characters. So far the taxonomy in *Trichoderma* is largely based on morphological characters. Species concept in *Trichoderma* is highly complex. Hence development of species concept in *Trichoderma* species requires a combination of phenotypic and genetic information. The advent of molecular tools for investigations in fungal taxonomy prompted research in the mid-nineties to re-assess the morphology based taxonomy in *Trichoderma* (Kubicek and Penttila, 1998) by randomly amplified polymorphic DNA (RAPD) - PCR, restriction fragment length polymorphisms in mitochondrial DNA and ribosomal DNA and sequence analysis of ribosomal DNA (Hjeljord and Tronsmo, 1998; Srivasithamparam and Ghisalberti, 1998; Bissett, 1991a; Rifai, 1969; Gams and Meyer,1998). Authentic identification facilitates the researchers for taxonomy and biotechnological applications. The new taxonomy should be based on a poly-phasic approach using phenotypic characters like micro and macro morphology together with multi-gene DNA sequence (Kubicek *et.al.*, 2001). Fine resolution for identification combining morphological and molecular characters is yet to be done. Therefore, the current research work is proposed to identify twelve different *Trichoderma* isolates by morphological characterization and further confirmed molecularly on the bases of ITS region of 16S rDNA.

MATERIALS AND METHODS

Collection and isolation of fungal cultures

The soil samples were collected from different fields of Indian Agricultural Research Institute, New Delhi, India during 2008-2009. Fungi were isolated by soil dilution plate techniques. *Trichoderma* was isolated on *Trichoderma* selective medium (Samuels ,2002)

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and *Trichoderma* was subsequently grown on Potato dextrose agar medium. Purification of *Trichoderma* was done using single spore isolation method and maintained at 10°C for further use.

Phenotype analysis

Morphological observations were made from cultures grown on Potato dextrose agar at about 25°C under ambient laboratory conditions of diffuse daylight. The major and remarkable macroscopic characters viz. colony growth, colour and texture and microscopic features viz., branching and apex of the conidiophores; disposition and shape of the phialides; size, shape and colour of the conidia and size, shape and production of chlamydospore were studied for species identification (Leuchtmann *et al.*, 1996; Samuels *et al.*, 1994; Cortinas *et al.*,2006) Fungal growth was measured at the reverse side of the colonies with the scale in centimeters at intervals of 24 hrs starting from the second day of incubation.

Molecular analysis

DNA extraction

For the preparation of genomic DNA from *Trichoderma* isolates, colonies from potato dextrose agar medium were transferred to flasks containing potato dextrose broth medium. The flask cultures were incubated in a stationary state at 25ºC for 5-6 days, then mycelia were harvested and freeze-dried. DNA extraction was performed using CTAB (cetiltrimetilamonium bromide) method (Culling, 1992). The DNA pellet was rehydrated in 50 mL TE buffer and allowed to resuspend at 4ºC overnight. The quality and quantity of DNA was estimated using Nanodrop. All DNA samples were diluted to a working concentration of 10 ng/mL.

PCR amplification of ITS and tef1 region

Approximately 650 bp of the *Trichoderma* ITS region in the rDNA fragments were amplified using universal primers ITS1 (5' GGAAGTAAAAGTCGTAACAAGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') that target conserved regions in the 18S and 28S rDNA genes . PCR reaction was carried out in a 100 µl PCR Mix containing 10× assay buffer, 1.5 µM MgCl, 10 µM dNTP mix, 10 pico mole of each primer, 2.5 unit of Taq polymerase (Bangalore Genei, India) and 50 ng DNA. Amplifications were carried out in a PCR thermal cycler (Bioer) using an initial denaturation at 94ºC for 1 min followed by 30 cycles of denaturation for 35 sec at 94ºC, annealing for 1 min at 57ºC and extension for 1 min at 72ºC. This was concluded with a final extension for 10 min at 72ºC. Amplicons were separated in 1.2 % agarose gels in 1X TAE buffer at 50 V for 40 min, stained with ethidium bromide and visualized under UV light.

Similar attempt was made to amplify the *tef1* gene using the forward primer EF1 (5´-ATGGGTAAGGAGGACAAGAC-3) and reverse primer *TEF1* rev (GCCATCCTTGGAGATACCAGC) (Samuels *et al.*, 2002) with an initial denaturation at 95ºC for 3 min followed by 30S cycles of denaturation for 1 min at 95ºC, annealing for 1 min at 60ºC, extension for 1 min at 72ºC and final extension for 10 min at 72ºC.

Sequencing of ITS region and tef1 gene

PCR products of *Trichoderma* species were purified from agarose gel using QIAquick gel extraction kit (Qiagen, USA) following manufacturer's instruction. Purified PCR products were sequenced using universal primers, ITS4 for ITS and EF1 for *tef1* in an automated ABI 3100 Genetic Analyser (Applied Biosystems, USA) by Bangalore Genei (Bangalore, India).

Sequence analysis

The sequence data was assembled and analyzed using programme BIOEDIT version 7.0.5. Gene products were predicted using GENERUNNER. Multiple nucleotide sequence alignments were done in BIOEDIT. Comparisons of the ITS and *tef1* sequences were made with ITS1-5.8s-ITS2 region of rDNA and *tef1* region of known *Trichoderma* species obtained from the NCBI (http:/ www.ncbi.nlm.nih.gov) Genbank sequence database. Dendrograms were constructed from the aligned sequences using the Neighbor-Joining method and boot strap option of CLUSTAL X. Phylogenetic trees were made using Tree view 1.6.5.

RESULTS

Morphological studies

On the basis of macroscopic and microscopic characters, all the 12 isolates of *Trichoderma* were divided in to two sections and five groups (3 groups in

section 1 and 2 groups in section 2) (Table 1, Fig.: 1, 2, 3, 4, 5).

SECTION A: Conidiophores thin, long main branches sparingly rebranched with short side branches, branches are mostly at right angles; phialides solitary, alternate, opposite, elongate, directly along the main and side branches; Conidia sub-cylindrical to ellipsoidal, pale or dilute green, smooth, 2.5-6.0x 2.0-4.0 µm; Chlamydospores are frequently absent.

GROUP I: Colony with irregular tufts, compact cushion form lawn at the centre; Reverse colourless to amber; Conidia sub-cylindrical, dilute green, 2.5-6.0 x 2.0-4.0 µm; Chlamydospores mostly absent (Isolates No. R1, R2, R5, R6 and R7).

GROUP II: Colony grey green, compact tufts at the centre, Reverse dark yellow; conidia ellipsoidal, pale green, 3.0-6.0x 3.0-4.0 µm; Chlamydospores present (Isolate No. R8).

GROUP III: Colony olive green, floccose, effuse, loose tufts in circular zone. Reverse yellow; Conidiophore with long main branches, and with rare rebranching; Conidia ellipsoidal, pale green, 3.0-5.0x 2.5-3.0 µm; Chlamydospores few in old culture (Isolate No. R11).

SECTION B: Colony glaucous green to dull blackish green, floccose, effuse, flat marginal conidiation to distributed pustules; Reverse colourless, occasionally drab; Conidiophore main branch is thick, side branches short and thick, covered by short and plump phialides, bigger conidial accumulations to large conidial balls are present; Each branch terminated by cluster of short and stout phialides; Conidia obovoid to broadly ellipsoid, dark green, smooth to slightly rough,3.0-8.0x 3.0-6.0 µm; Chlamydospores abundant, and frequent, globose to sub-globose.

GROUP IV: Colony dull blackish green, floccose with effuse, flat and marginal conidiation, Reverse colourless to amber; Broad conidiophores; Each branch terminated by a cluster of phialides; Phialides swollen and laginiform; Conidia from adjacent phialides coalesce into large conidial ball, smooth, bigger conidia, 3.0-8.0x3.0-6.0 µm; Chlamydospores are bigger in size (Isolates No. R3, R4 and R12).

Table 1. Confirmation of 12 isolates of *Trichoderma* into different species (Based on morphology)

Section	Group	Isolate No.	Species
1. Longibrachiatum	Group I	R1,R2,R5,R6 and R7	*Trichoderma pseudokoningii* Rifai
	Group II	R8	*Trichoderma reesei* Simmons
	Group III	R11	*Trichoderma longibrachiatum* Rifai
2. Pachy basium	Group IV	R3,R4 and R12	*Trichoderma virens* (J.H. Giddens and A.A.Foster)
	Group V	R9 and R10	*Trichoderma hamatum* Rifai

Table 2. Accession No. of NCBI databse of all *Trichoderma* species based on ITS region

Isolate no.	Accession No.
R1	HM439626
R2	HM439627
R3	HM046562
R4	HM046563
R5	HM439628
R6	HM439629
R7	HM439630
R8	HM439631
R9	HM439632
R10	HM439634
R11	HM439633
R12	HM046564

GROUP V: Colony glaucous green, floccose, pustules distributed all over the plate and at the centre there is clear space; Reverse colourless; Conidiophore stout with verticillate branching. Phialides short and plump in the whorls of 3-6 and form bigger conidial accumulations; Conidia are obovoidal, smooth to slightly rough; 4.0-6.0x3.0-5.0µm; Chlamydospores present, smaller in size (Isolates No. R9 and R10).

The morphological characters of all five groups were compared with the reported literature (Rifai, 1969; Bissett, 1984, 1991a, 1991b, 1991c) and were confirmed as five different species. They were: Group I was *T. pseudokoningii*, Group II was *T. reesi*, Group III was *T. longibrachiatum*, Group IV was *T. virens* and Group V was *T. hamatum*, belonging to two different sections viz., *Longibrachiatum* and

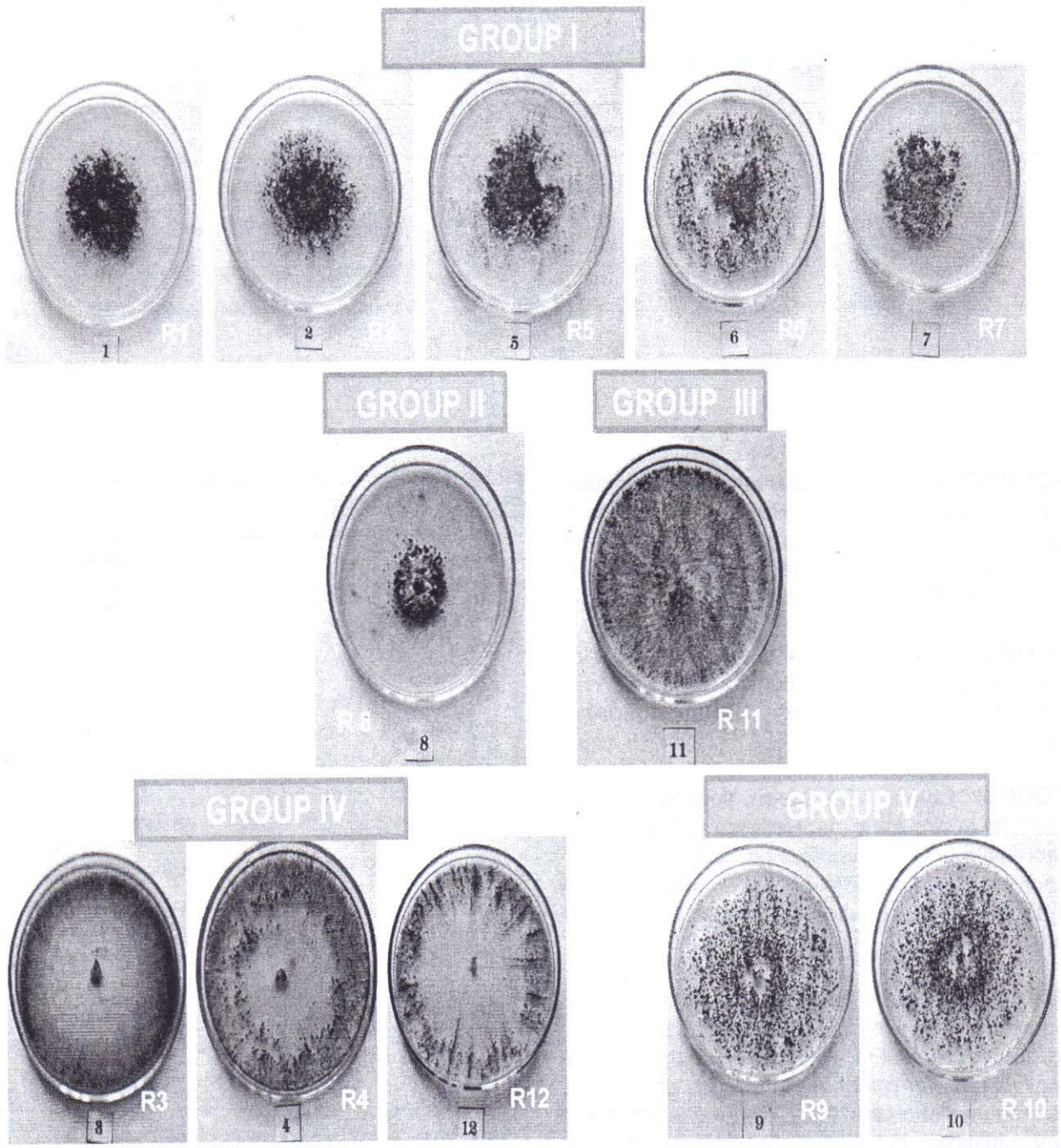

Fig. 1 Grouping of 12 isolates of *Trichoderma* species on the basis of cultural characters

Pachybasium (Table 3). Group I, II and III comes under section 1 ie., *Longibrachiatum* and Group IV and V comes under Section 2 ie *Pachybasium.*

Molecular Analysis

The primers ITS-1 and ITS4 amplified products in the range of 550-600 bp (Fig. 6). These amplified products were then sequenced. The sequence obtained were analyzed though BLAST and identification of the *Trichoderma* species was done which grouped the isolates into three different groups; Group I: *Trichoderma longibrachiatum* consisting of the isolates R1, R2, R5, R6, R7, R8 and R11, Group II: *Trichoderma virens* consisting of the the isolates R3, R4 and R12 and Group III: *Trichoderma asperellum* consisting of the isolates R9 and R10. The sequences of these isolates were submitted to the NCBI and the acession numbers were obtained (Table 3)

Phylogenetic analysis of the ITS and tef1 gene

The amplified fragment size of the ITS1 and ITS2 regions, including the 5.8S rDNA gene, varied from 550 to 600 bp in the isolates studied. The sequence variation in this region was analyzed. The resulting data subjected to phylogenetic analysis. Phylogenetic tree was constructed using Neighbor Joining method to graphically represent the genetic relationship of the isolates (Fig. 7).

The phylogenetic tree obtained from sequence analysis of ITS clearly grouped the isolates into three

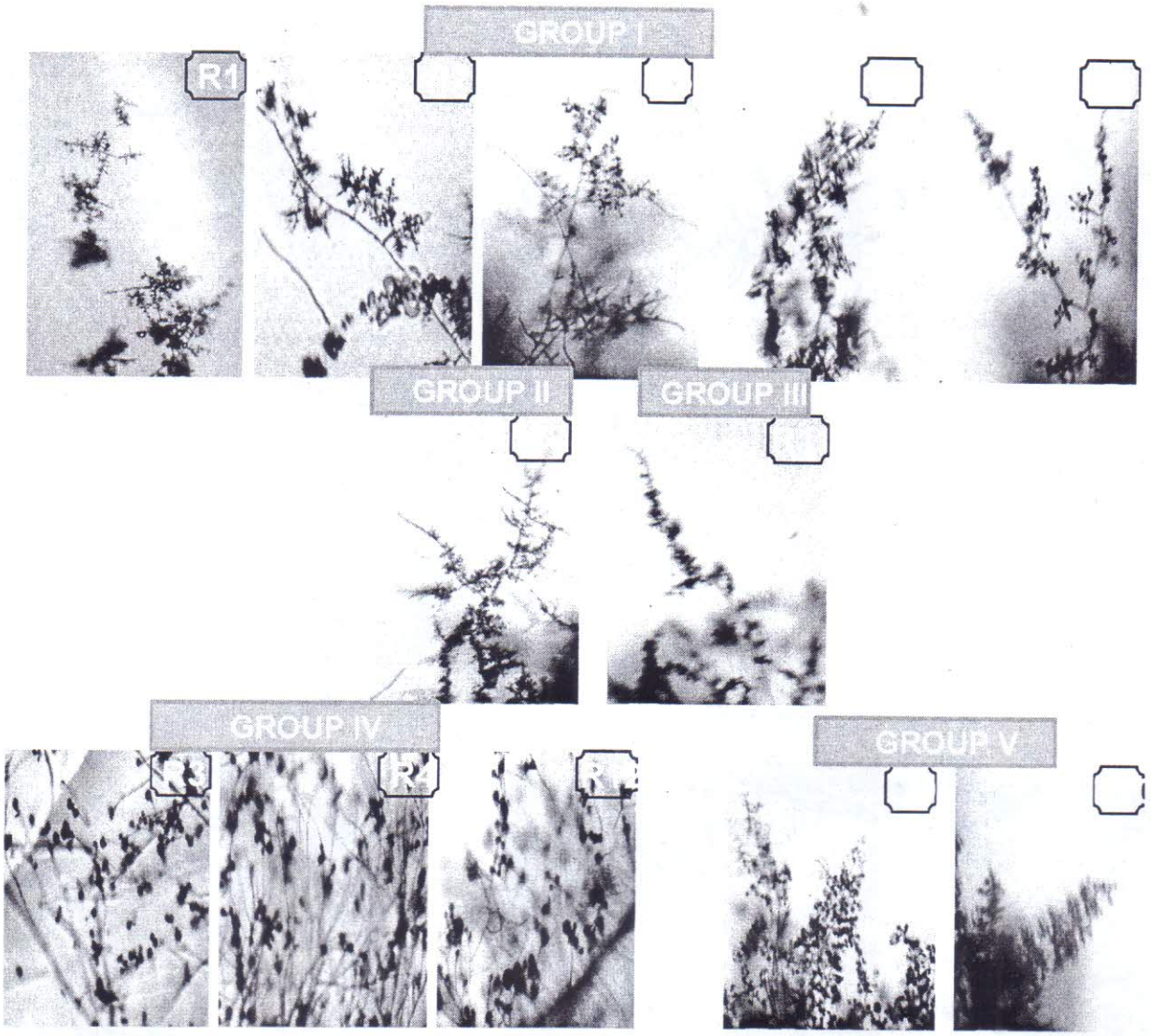

Fig. 2 Grouping of 12 isolates of *Trichoderma* species on the basis of conidiophore characters (100X)

distinct groups. The first group comprises of the *Trichoderma asperellum* consisting of the isolates R9 and R10, the second group comprises *Trichoderma virens* consisting of the the isolates R3, R4 and R12 and the third group comprises of *Trichoderma longibrachiatum* consisting of the isolates R1, R2, R5, R6, R7, R8 and R11.

DISCUSSION

A polyphasic approach was used that combines data sets from morphological and molecular investigations to study the species identification among the isolates of *Trichoderma* spp. Formal, traditional taxonomy is based solely on morphology or, more recently, and alternatively, on molecular data only. Although this has been a major point of criticism by fungal taxonomists (Seifert *et al.,* 1995), there are only a few examples of the use of combined data sets in studies of fungi (Petrini 1992, Petrini *et. al.,* 1989, Sieber-Canavesi *et. al.,* 1991). Initially, all data were evaluated independently and then combined

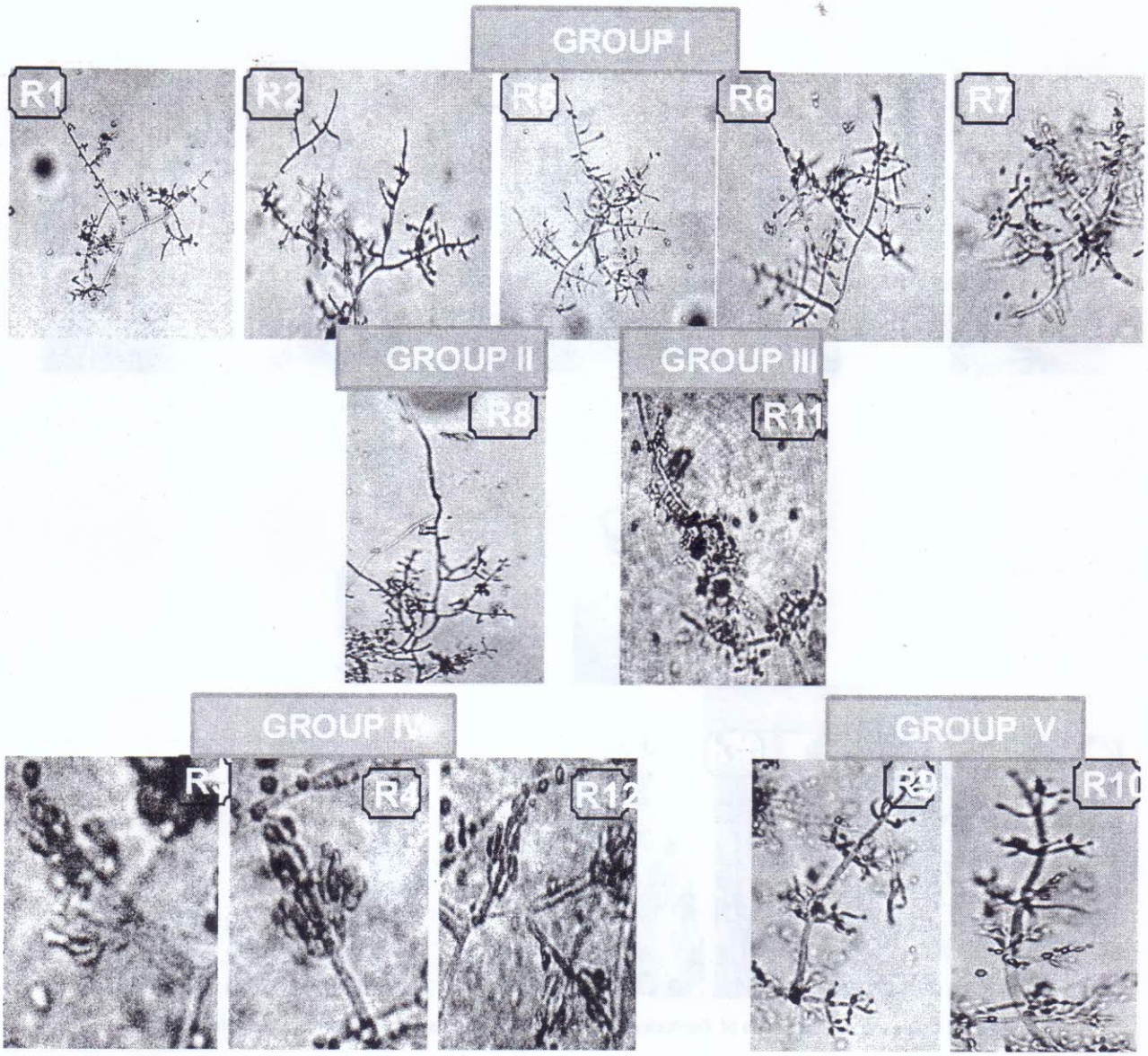

Fig. 3 Grouping of 12 isolates of *Trichoderma* species (on the basis of Conidiophore

Similar results were obtained from the sequence analysis of *tef1* region (Fig. 8). Group I consisting of isolates R1, R2, R5, R6, R7, R8 and R11 which belonged to *Trichoderma longibrachiatum,* Group II consisting of isolates R3, R4 and R12 belonged to *T. virens* and Group III had two isolates R9 and R10 belonged to *T. asperellum.*

in a single analysis to find out whether groupings of strains were still supported and to deduce characteristics that are significant for species or group recognition.

tion 1 and Group IV and V under Section 2). On the basis of the reported literature (Bisset 1984, 1991b, 1991c) they were confirmed as Group I: *T. pseudokoningii*, Group II: *T. reesi*, Group III: *T.*

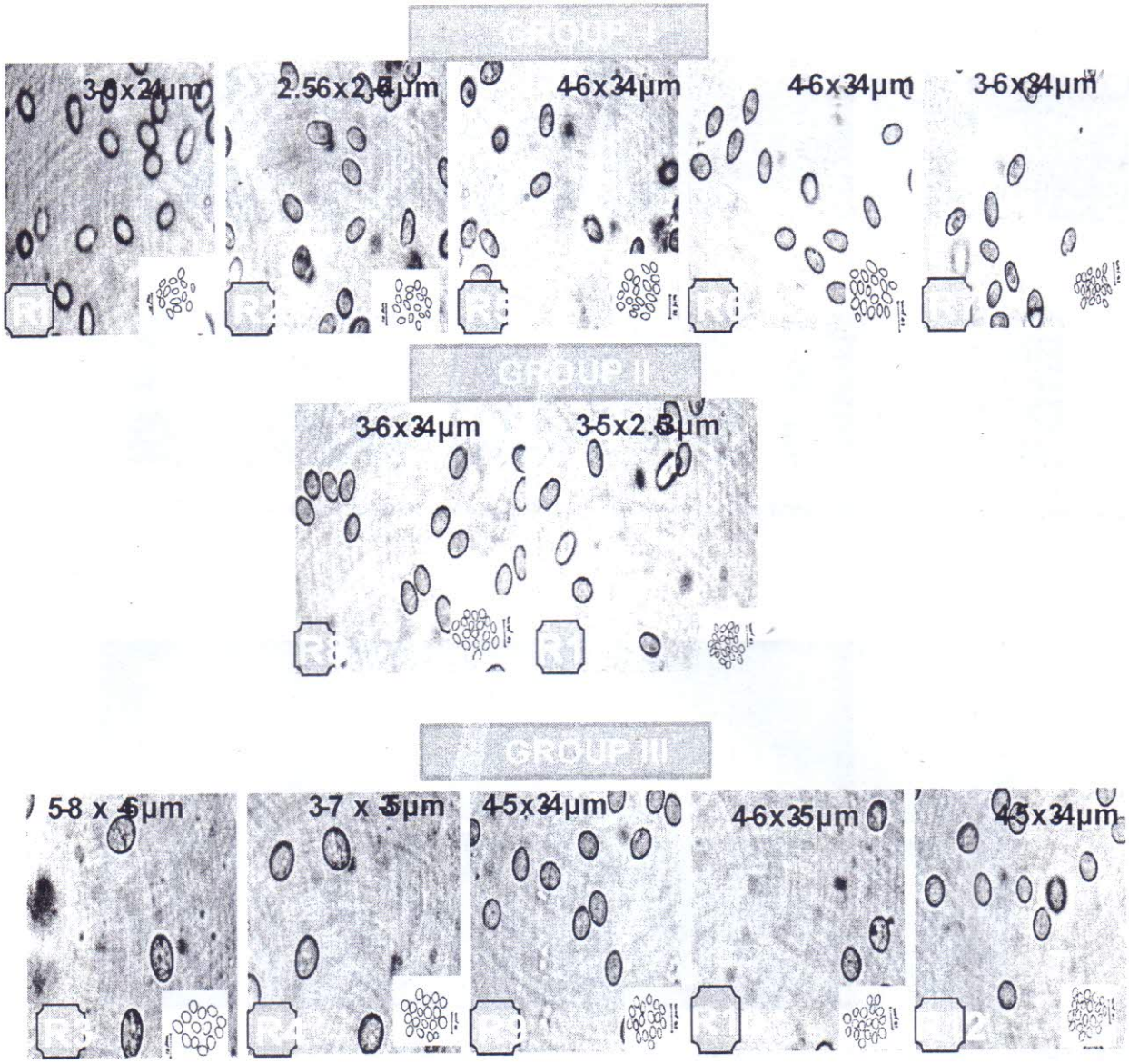

Fig.4 Grouping of 12 isolates of *Trichoderma* species on the basis of spore color, shape and size (400X)

Morphology based taxonomy has divided the genus *Trichoderma* in to five sections: *Trichoderma, Pachybasium, Longibrachiatum, Saturnisporum* and *Hypocreanum* (Kullnig-Gradinger *et al.,* 2002, Doi, 1987). In present study, all 12 isolates of *Trichoderma* were put in to two sections on the basis of morphological data *ie. Longibrachiatum* and *Pachybasium* and in each section, they were divided in to different groups (Group I, II and III under sec-

longibrachiatum, Group IV: *T. virens* and group V: *T. hamatum.*

The result confirms the categorization of the isolates into three groups on the basis of *tef-1* region analysis also as in the case of ITS region. But the *tef-1*a provides much greater resolution of the representatives of the *Trichoderma* isolates than ITS sequences, a result that parallels the findings of

O'Donnell (2000) in *Fusarium*. Sequences of the *tef-1* not only clearly separated the different species of *Trichoderma* but also distinguished them from each other with distinct nucleotide variability. The correlation between the DNA-defined groups

ferences in phenotype.

In this regard, the power of using independently derived data—in this case DNA sequence data—as a grouping factor cannot be overstated. Several

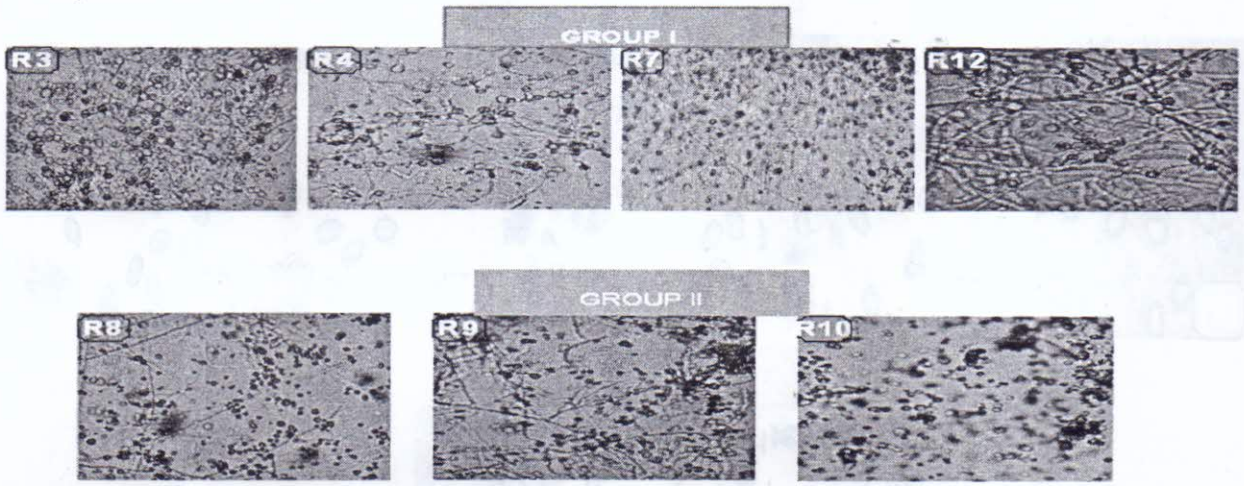

Fig. 5 : Grouping of isolates of *Trichoderma* species on the basis of Chlamydospore shape and size (400X)

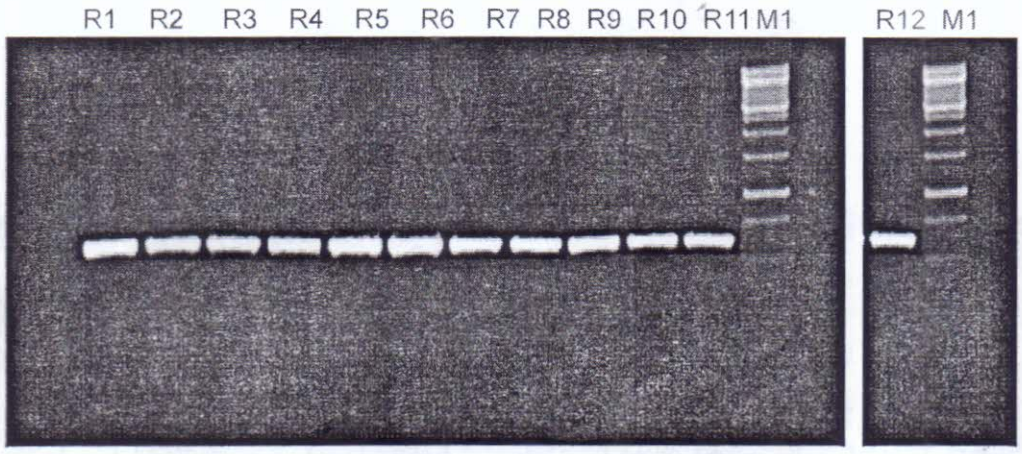

Fig. 6 : Agrose gel electrophoresis of PCR product of ITS region. Lane M1= 1 Kb ladder, Lanes R1-R12=Amplification with DNA extraction from R1-R-12 isolates of *Trichoderma* species

based on ITS and *tef-1* region analyses and their respective phenotypes was almost perfect. Despite its diverse phenotype, all *Trichoderma* isolates were clearly separated from the other groups. The analysis depends upon study of a large number of geographically separated specimens and cultures in order to accurately account for intra-taxon variation. Multivariate analysis of quantifiable phenotype characters was an effort to document both the phylogenetic proximity and to highlight diagnostic differences in phenotype.

recent publications indicate that even the restricted morphological concept of *Trichoderma* species (Bissett 1991a, Gams and Meyer 1998)is genetically and phenotypically diverse. But in the present study, it was confirmed that the ITS and *tef-1* marker had much capacity to resolve taxa in contrast to the reports of Hermosa *et al.* (2004) and a good correlation between morphological and molecular characteristics among isolates of *Trichoderma* spp. was observed in most of the isolates, up to the sec-

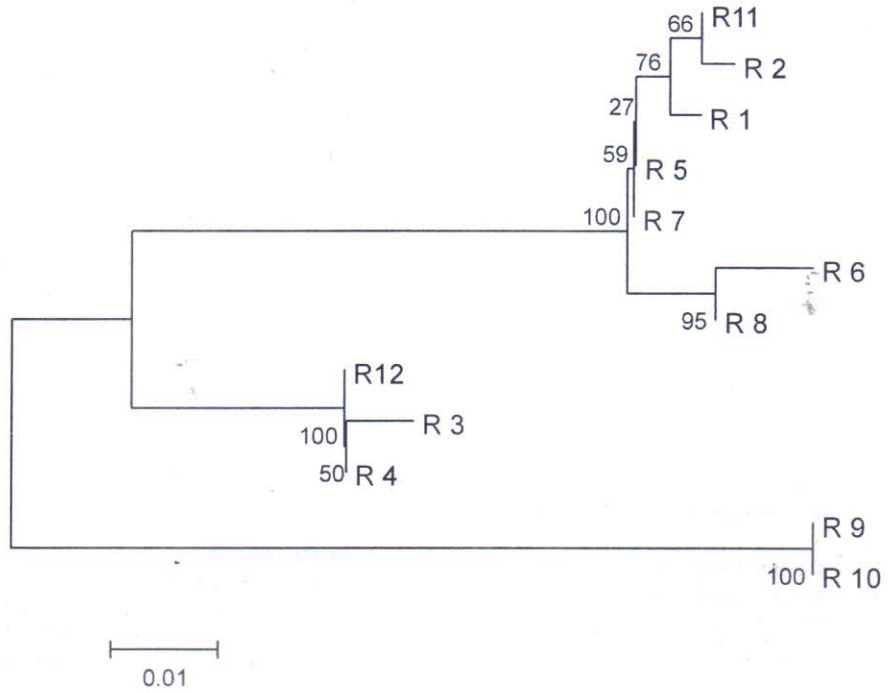

Fig.7 : Phylogenetic relationship among the isolates of *Trichoderma* species based on ITS1-5.8s-ITS2 region

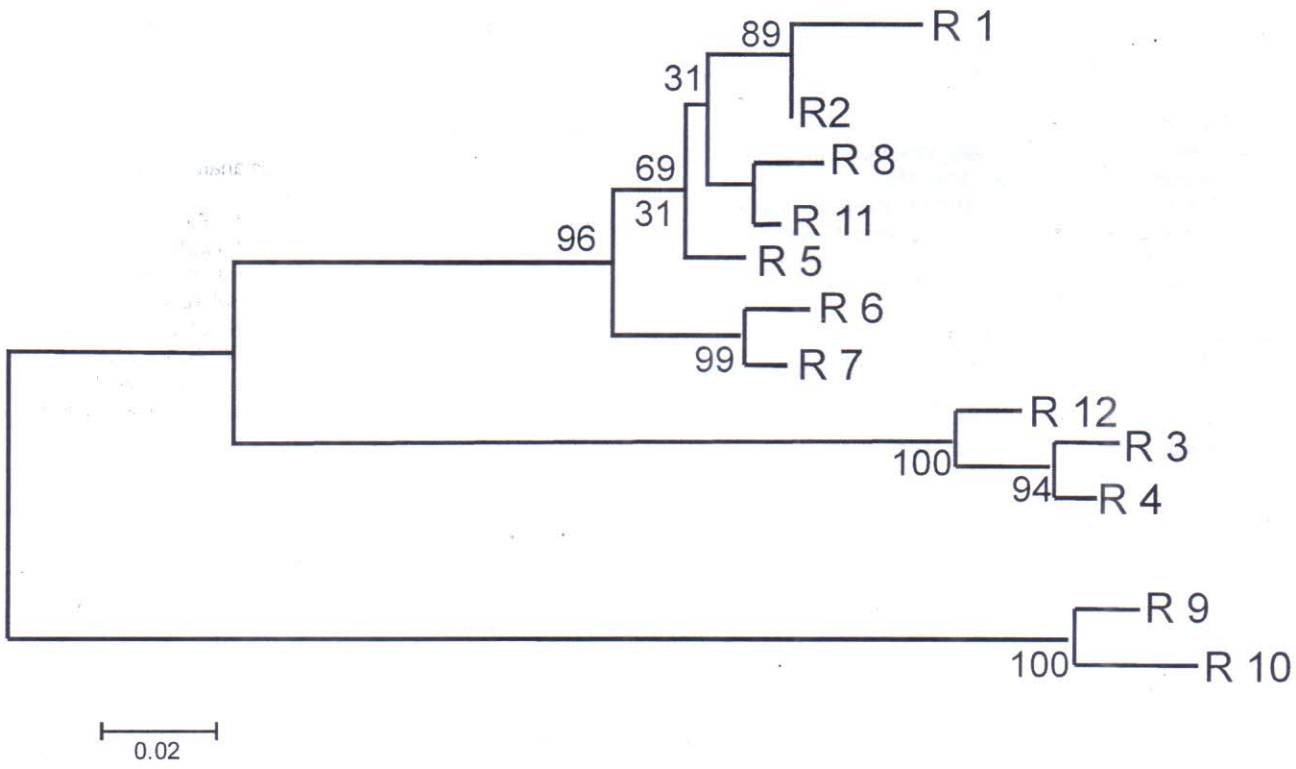

Fig 8. Phylogenetic relationship among the isolates of *Trichoderma* species based on *tef1* region

tional level classification. Only isolates R9 and R10 showed the characters of different section ie. section *Trichoderma* at molecular level. At least some of the genetic and phenotypic diversity could be accounted for by sexual or parasexual outcrossing, as it has been suggested for *Aspergillus flavus* (Geiser *et al.*, 2004).

Thus we can conclude that the molecular approaches are useful in the characterization of the *Trichoderma* isolates along with the morphology. They are robust, simple and convenient to use.

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