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Phylogenetic Relationships of *Trichoderma* isolates of North Bengal Based on Sequence Analysis of ITS Region of rDNA

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Abstract: The aim of this study was to determine whether sequence analysis of internal transcribed spacer-1 (ITS1) region of the ribosomal DNA can be used to detect phylogenetic relationships of *Trichoderma* isolates obtained from North Bengal region. ITS 1 of the rDNA of thirteen *Trichoderma* isolates was amplified by polymerase chain reaction (PCR) using universal primers (ITS1 and ITS4). PCR products were purified and these purified products were used to amplify the ITS 4 region of all *Trichoderma* isolates. The amplified DNA was sequenced and aligned against ex-type strain sequences from TrichoBLAST and established *Trichoderma* taxonomy. Nine isolates were positively identified as *Trichoderma harzianum* and four as *Trichoderma erinaceum* which were used as an outgroup in these analyses. The ITS-1 region sequences have been used as the reference's sequence and may be used for future study involving the identification and taxonomy of *Trichoderma harzianum.* Amplification of ITS1 region of the rDNA has showed potential as a rapid technique for identifying *Trichoderma harzianum* successfully in all cases.

Key words: *Trichoderma harzianum, T. erinaceum,* rDNA, ITS

INTRODUCTION

The fungal genus *Trichoderma* (Ascomycetes, Hyprocreales) contains species that are of vast economic importance owing to their production of industrial enzymes (cellulose and hemi-cellulose) [1,2], antibiotics, and their ability to act as biological control agents against plant pathogens since 1920s[3]. Taxonomy of *Trichoderma* is currently based largely on morphological character such as conidial form, size, color and ornamentation, branching pattern with short side branches, short inflated phialides and the formation of sterile or fertile hyphal elongations from conidiophores[4,5]. *T. harzianum* is an aggregate species, divided into three, four or five subspecific groups, depending on the strains. However, most species descriptions are based on examination of a limited number of strains where the morphological differences are clear but these differences become less clear as more strains are studied. This suggests that there are not enough morphological and cultural characters for reliable species level definition. Identification of *Trichoderma* isolates at the species level has proved difficult, due to the degree of morphological similarities. Rifai[4] adopted the concept of "species aggregate" and distinguished nine aggregates, some of which comprised two or more morphologically indistinguishable species. Gams and Bissett[6], in an attempt to differentiate phenotypically similar species, proposed 'sections' based on morphology, to accommodate similar forms within the species concept of Rifai[4]. Current studies find that morphological analysis was highly prone to error and roughly 50% of the *Trichoderma* spp. obtained by morphological analysis alone was wrongly identified[7].

Molecular methods have recently been introduced into *Trichoderma* taxonomy with revision of *Longibrachiatum* and *Trichoderma*, respectively and related teleomorphs[8]. These techniques have been proven to be valuable tools in fungal taxonomy and their application has led to the reconsideration of several genera[9]. In *Trichoderma,* isoenzyme analysis[10,11], RAMS[12,13] and rDNA sequencing[14,15] have been used to distinguish species within specific groups of strains.

Kindermann *et al.*[16] attempted a first phylogenetic analysis of the genus *Trichoderma*, using sequence analysis of the ITS 1 region of the rDNA. Nevertheless, the use of phylogenies based on single gene sequences is now normally discredited, especially as regards the use of ITS1 and/ or ITS2, as some fungi and plants have been shown to contain paralogous copies[17]. Taylor, *et al*.[18] proposed basing phylogenetic species concepts between five or more gene trees.

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These results demonstrated that molecular techniques indicating interrelations among species combined with phenotypic characters, can lead to a reliable taxonomy that is reflective of phylogenetic relationships.

Druzhinina *et al*.,[19] was able to identify 70 out of a total of 77 strains investigated within the genus of *Trichoderma*. Kubicek *et al*.,[20] demonstrated out of seventy-eight isolates of *Trichoderma,* 37 strains could be positively identified as *T. harzianum*, 16 as *T. virens*, 8 as *T. spirale* 3as *T. koningii*, 3 as *T. aureoviride*, 4 as *T. asperellum*, 2 as *Hypocrea jecorina*, 2 as *T. viride*, and each of *T. hamatum* and *T. ghanense*. Ospina-Giraldo *et al*.[21] showed that phylogentic analyses were closely related to isolates of *T. harzianum* compared others *Trichoderma* isolates.

The purpose of the present study was to establish a species of *Trichoderma harzianum* gene sequence in North Bengal isolates based on the sequence analysis of ITS-4 region of the rDNA gene.

MATERIALS AND METHODS

Isolation and identification of *Trichoderma:* Soil samples were collected from forests, rhizophere soil of plantation crops and agricultural fields of North Bengal. The location of soil samples were recorded through GIS mapping tool (Garmin). *Trichoderma* species were isolated in specific selective medium (TSMC) which contained (gm/lit); $MgSO_4.7H_2O$ -0.2; K_2HPO_4- 0.9; KCl - 0.15; NH_4NO_3-1.0; glucose-3.0, chloramphenicol-0.25, fenaminosulf-0.3, pentachloronitrobenzene-0.2, rose Bengal-0.15, captan-0.02 (post autoclaving), agar-20 as well as in modified TSM (Smith et al., 1990): containing (gm/lit): $Ca(NO_3)_2$-1.0, KNO_3 0.26, $MgSO_4 \cdot 7H_2O$-0.26, KH_2PO_4-0.12,$CaCl_2 \cdot 2H_2O$-1.0, citric acid-0.05, sucrose-2.0, agar-20.0, chlortetracycline-0.05,captan (50% wettable powder) - 0.04 [22].

Genomic DNA Extraction from isolates of *Trichoderma:* Isolation of fungal genomic DNA was done following standard technique [23]. Fungal mycelia from 3-4 days old culture was crushed with liquid nitrogen and incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 100 mM NaCl and 2% SDS, for 1 hr at 65^{0}C followed by centrifugation at 12,000 rpm for 15 min. The supernatant was extracted with equal volume of water saturated phenol, centrifuged at 12,000 rpm for 15 min, and further extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) by centrifugation at 12000 rpm for 15 min; the aqueous phase was transferred in a fresh tube and chloroform (in the ratio of 1:4 v/v) was added followed by 0.5M Na-acetate (in the ratio of 1:10 v/v). Next Isopropanol was added to the above mixture (0.7 times the final volume) and centrifuged. DNA was precipitated from the aqueous phase with chilled ethanol (100%) and pelleted by centrifuging at 12000 rpm for 15 min followed by washing in 70% ethanol and centrifugation. The pellets were air dried and suspended in TE buffer pH 8.

Qualitative and quantitative estimation of DNA: The extraction of total genomic DNA as per the above procedure was followed by RNAase treatment. Genomic DNA was re suspended in 100 µl 1 X TE buffer and incubated at 37ºC for 30 min with RNAse (60µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel.

PCR amplification of ITS region of *Trichoderma* isolates: All isolates of *Trichoderma* were taken up for ITS-PCR amplification. Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. For amplification of the ITS1–5.8S–ITS2 region of *Trichoderma* isolates, the primer pair T/ITS1 TCTGTAGGTGAACCTGCGG and T/ITS4 TCCTCCGCTTATTGATATGC was used.

PCR was programmed with an initial denaturing at 94ºC for 5 min. followed by 30 cycles of denaturation at 94 ºC for 30 sec, annealing at 59 ºC for 30 sec and extension at 70 ºC for 2 min and the final extension at 72 ºC for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 m1) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examining by with horizontal electrophoresis. PCR products were sent for sequencing to Genie, Bangalore.

Data analysis: Sequences were aligned following the Clustal Walgorithm included in the Megalign module (DNASTAR Inc.).Multiple alignment parameters used were gap penalty = 10 andgap length penalty = 10. Both of these values are aimed to prevent lengthy or excessive numbers of gaps. The default parameters (Ktuple= 2, gap penalty = 5, window = 4, and diagonals saved = 4)were used for the pairwise alignment. The use of Clustal W determines that, once

a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were made manually in order to remove artificial gaps. Phylogenetic analyses were completed using the MEGA package (version 4.01; Institute of Molecular Evolutionary Genetics, University Park, PA). Neither gaps (due to insertion-deletion events) nor equivocal sites were considered phylogenetically informative. Hence, complete deletion prevented the use of any of these sites in further analyses. Phylogenetic inference was performed by the UPGMA method. Bootstrap tests with 1,000 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained. An additional standard error test was performed with the data set using the same characters in order to evaluate the statistical confidence of the inferred phylogeny.

RESULTS AND DISCUSSION

Thirteen isolates were obtained using the *Trichoderma* selective medium from the rhizosphere soil, forest soil and agricultural field (Table 1). Among them nine isolates were identified as *Trichoderma harzianum.* The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome [24]. They also occur in multiple copies with up to 200 copies per haploid genome [25-26] arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S, and the 28S large subunit (LSU) genes. Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species [27]. In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies, In the present study, we focused on the ITS regions of ribosomal genes for the construction of primers that can be used to identify *Trichoderma* spp. ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 primers. Amplified products of size in the range of 600bp was produced by the primers (Fig 1). The results are in accordance with Mukherjee [28] who studied the identification and genetic variability of the *Trichoderm*a isolates. These results are also in accordance with several workers who observed the amplified rDNA fragment of approximately 500 to600 bp by ITS-PCR in *Trichoderma* [29-32]. After direct sequencing of the PCR products, a total of 13 isolates of *Trichoderma* PCR products produced sequences that could be aligned and showed satisfactory homology with ex-type strain (THVA) of *T. harzianum* sequences from the NCBI Genbank data base. The priming site of the ITS1 and ITS4 primers were determined in order to confirm that the sequences obtained corresponded to the actual ITS 4 region. A multiple sequence alignment was carried out that included the ITS 1 region, including gaps and the complete sequences align. There were quite a number of gaps that were introduced in the multiple sequence alignment within the ITS-4 region that were closely related and similar sequence indicated. These 13 *Trichoderma* isolates were used in the pair wise and multiple sequence alignment. From the sequence alignment, variations were observed between *T.harzianum* isolates and *Trichoderma erinaceum* isolates. The ITS PCR has helped to detect polymorphism at ITS region of rDNA among the *Trichoderma harzianum* and *T. erinaceum* isolates.

The evolutionary history was inferred using the UPGMA method [33]. The optimal tree with the sum of branch length = 1.84709756 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [34]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method [35] and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 189 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 [36].

In conclusion, above results strongly indicated a "*Trichoderma* aggregate species" with similar "DNA-based sequence", which could be accommodated with similar forms as referred in the species identifying concept. Nine *Trichoderma* isolates comprise the largest group and similar gene sequence with respect to phylogenetic analyses and have been assigned to *T. harzianum*, while the other four isolates which comprise a smaller group have been assigned to *T.erinaceum* in the phylogram.

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Table 1: Isolates of *Trichoderma* spp

Type of soil	GPS location		Isolates
	Latitude	Longitude	
Forest Soil	N26 º 45'13.08''	E88º23'28.72	*Trichoderma erinaceum* (FS/L-20)
	N26 º 47'49.16''	E88 º 21.27.75''	*Trichoderma harzinum* (FS/C-90)
	N26 º 45'11.75''	E88 º 23'28.27''	*Trichoderma harzianum* (FS/S-455)
	N 26 º 48'18.68''	E88 º 21.14.61''	*Trichoderma harzianum*(FS/S-458)
	N 26 º 48'18.68''	E88 º 21'14.61''	*Trichoderma erinaceum* (FS/S-474)
	N 26 º 48'18.64''	E88 º 21'14.61''	*Trichoderma erinaceum* (FS/S-475)
	N 26 º 48'18.68	E88 º 21'14.61	*Trichoderma erinaceum* FS/S-478)
Rhizosphere soil	N26 º 45.11.75''	E88 º 23'28.27''	*Trichoderma harzianum* (RHS/T- 460)
	N 26 º 48'18.68''	E88 º 21'14.61''	*Trichoderma harzianum* (RHS/T- 477)
	N26 º 42'42.56''	E 88. º 21'.15.47''	*Trichoderma harzianum*(RHS/M511)
Agricultural soil	N 25 º 01'13.13''	E88 º 08'98''	*Trichoderma harzianum* (Ag/S476)
	N 25 º 01'13.13''	E88 º 08'98''	*Trichoderma harzianum* (Ag/S471)
	N 25 º 01'11.13''	E88 º 68'88''	*Trichoderma harzianum* (Ag/S479)

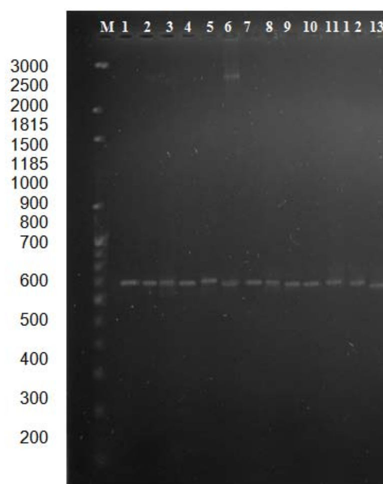

Fig. 1: PCR amplification of ITS region of *Trichoderma* isolates (Lane 1-13) Lane M: Low range DNA Marker, 1 *T.erinaceum* (FS/L-20), 2 *T. harzainum* (FS/C-90), 3 *T. harzianum* (FS/S-455), 4 *T.harzianum* (FS/S-458) 5. *T. erinaceum* (FS/S-474) 6. *T. erinaceum* (FS/S-475),7. *T. erinaceum* (FS/S-478), 8. *T. harzianum* (RHS/T- 460), 9. *T. harzianum* (RHS/T- 477), 10. *T. harzianum* (RHS/M511), 11. *T. harzianum* (Ag/S476), 12. *T. harzianum* (Ag/S471), 13. *T. harzianum* (Ag/S479).

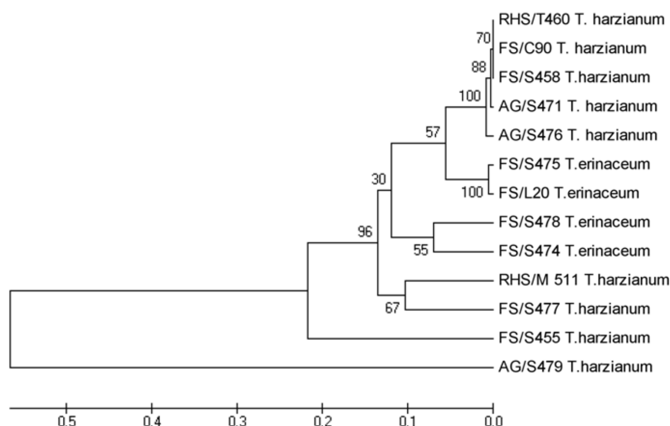

Fig. 2: The phylogenetic analyses of *Trichoderma* isolates was inferred using the UPGMA method conducted in MEGA 4.

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