Sequencing of 28SrRNA Gene for Identification of *Trichoderma longibrachiatum* 28CP/7444 Species in Soil Sample

Mohammad Shahid[1,*], Anuradha Singh[1], Mukesh Srivastava[1], Smita Rastogi[2] and Neelam Pathak[2]

[1]*Department of Plant Pathology, C.S.A. University of Agriculture & Technology, Kanpur, U.P., India*

[2]*Department of Biosciences, Integral University, Lucknow, U.P., India*

Abstract: Most of the *Trichoderma* species are morphologically very similar and were considered for many years as a single species. Since new species were discovered, a consolidated taxonomical scheme was needed and proposed and defined nine morphological species aggregates. DNA methods brought additional valuable criteria to the taxonomy of *Trichoderma* which are being used today for studies that include identification and phylogenetic classification. Most isolates of the genus *Trichoderma* that were found to act as mycoparasites of many economically important aerial and soil-borne plant pathogens. *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. A fungal strain of *Trichoderma longibrachiatum* 28CP/7444 was isolated from a soil sample collected from Barabanki district of Uttar Pradesh, India. The universal primers were used for amplification of the 28S rRNA gene fragment and strain characterized by using 28S rRNA gene sequence with the help of ITS marker. It is proposed that the identified strain *Trichoderma longibrachiatum* 28CP be assigned as the type strain of a species of the genus *Trichoderma* based on phylogenetic tree analysis together with the 28S rRNA gene sequence search in Ribosomal Database Project, small subunit rRNA and large subunit rRNA databases. The sequence was deposited in GenBank with the accession number JX978541. Thus an integrated approach of morphological and molecular markers can be employed to identify a superior strain of *Trichoderma* for its commercial exploitation.

Keywords: 28S ribosomal RNA gene, *Trichoderma,* phylogenetic analysis, ITS, DNA sequencing, GenBank.

1. INTRODUCTION

Trichoderma commonly available in soil and root ecosystem has gained immense importance since last few decades due to its biological control ability against several plant pathogens. Some strains of *Trichoderma* like *Trichoderma harzianum, T. longibrachiatum, T. atroviride, T. viride, T. virens and T. koningii* are efficient bio control agent which have the ability to inhibit pathogen growth in the soil, hence improving the overall health of the plant Antagonistic microorganism, such as *Trichoderma* reduce growth, survival of pathogen by different mechanism like competition antibiosis, mycoparasitism, hyphal interactions and enzyme secretion. Such micro organisms are now available commercially and are used in crop management and practices. The use of *Trichoderma* species as biological control agents has been investigated for over 70 years but it is only relatively recently that strains have become available commercially. Biocontrol agents are widely regarded by the general public as “natural” and therefore non-threatening products, although risk assessments must clearly be carried out on their effects on non-target organisms. Moreover, knowledge concerning the behaviour of such antagonists is essential for their effective use. The morphological and microscopic Figures **1** & **2** of *Trichoderma longibrachiatum* 28 CP/ 7444 is given below.

Figure 1: Trichoderma longibrachiatum 28CP/7444 strain in PDA medium.

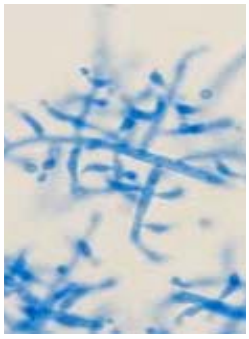

Figure 2: Trichoderma longibrachiatum 28CP/7444 strain under Microscopic observation.

*Address correspondence to this author at the Department of Plant Pathology, C.S.A. University of Agriculture & Technology, Kanpur, U.P., India;
Tel: 0512-2534113; Fax: 0512-2534113;
E-mail: shahid.biotech@rediffmail.com

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Accurate and definitive fungal identification is essential for correct disease diagnosis, treatment of associated with fungal infections. Characterization of fungal species using classical methods is not as specific as the genotyping methods. Genotypic techniques involve the amplification of a phylogenetically informative target, such as the small-subunit (28S) rRNA gene [1]. rRNA is essential for the survival of all cells, and the genes encoding the rRNA are highly conserved in the fungal and other kingdoms. The sequences of the rRNA and proteins comprising the ribosome are highly conserved throughout evolution, because they require complex inter- and intramolecular interactions to maintain the protein-synthesizing machinery [2-4]. *Trichoderma* spp. are common soil inhabitants and are effective in providing bio-control of soil borne pathogens due to antagonistic behaviors. The major aspect of successful biological control strategies includes the production, formulation and delivery system of bio-agents. 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 [5-9] attempted a first phylogenetic analysis of the whole genus of *Trichoderma* using sequence analysis of the ITS region of rDNA.

In this study, the method of isolation and identification of an unknown fungal from the Indian Barabanki district using 28S rRNA gene sequence as reported in bacterial rRNA gene [10] to characterize the strain 28CP as a member of the *Trichoderma* spp.. The Soil sample has received great attention from the public, due to its potential for biodiversity and biological conservation. 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 [11]. Kindermann *et al.* attempted a first phylogenetic analysis of the whole genus of *Trichoderma* using sequence analysis of the ITS1 region of rDNA.

2. MATERIALS AND METHODS

2.1. Isolation and Identification of *Trichoderma*

Soil samples were collected from various experimental fields of Indian district Barabanki. Isolate of *Trichoderma* species was isolated and identified in potato dextrose agar (PDA) with low sugar medium [12]. The identification of *Trichoderma* isolates were confirmed both by morphological and molecular characters (ITS), and deposited in India type culture collection (ITCC), IARI, Pusa, New Delhi accession number allotted 7444/09.

DNA Isolation of Trichoderma

Pure culture of the target fungal was grown overnight in liquid PD Broth medium for the isolation of genomic DNA using a method described by Hiney [13].

2.2. Molecular Characterization

The total genomic DNA was extracted from isolate of *Trichoderma longibrachiatum* 28CP/7444 based on cetrimide tetradecyl trimethyl ammonium bromide (CTAB) mini extraction method of Crowhurst *et al.* [14] with minor modification.

2.3. Agarose Gel Electrophoresis

Ten microlitre of the reaction mixture was then analyzed by submarine gel electrophoresis using 1.0% agarose with ethidium bromide at 8 V/cm and the reaction product was visualized under Gel' doc/UV trans-illuminator.

2.4. Internal Transcribed Spacer Region

The internal transcribed spacer (ITS) regions of the rDNA repeat from the 3'end of the 28S and the 5'end of the 28S gene were amplified using the two primers, ITS 1 and 4 which were synthesized on the basis of conserved regions of the eukaryotic rRNA gene [15]. 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 mM 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 (1µg/ml) and electrophoresis was carried out at 60 volts for 3 hours 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.

2.5. Purification of PCR Product

The PCR product was purified by Qiagen gel extraction kit using the following protocol described

below. The DNA fragment was excised from the agarose gel with a clean sharp scalpel. Then the gel slice was weighed in an eppendorf. We then added 3 volumes of buffer QG to 1 volume of gel (100 mg ~ 100 µl). The mixture was then incubated at 50°C for 10 min. The gel was dissolved by vortexing the tube every 2 to 3 min during the incubation until the mixture color is uniformly yellow. We then added 1 gel volume of Isopropanol to the sample and mixed. A QIAquick spin column is then placed in a 2 ml collection tube provided. The sample is applied to the QIA quick column followed by centrifugation for one minute so that DNA binds to the column. The flow-through is discarded and the QIAquick column is placed back in the collection tube. We then added 0.75 ml of buffer PE to QIAquick column and centrifuged for 1 min to wash. The flow through is again discarded and the QIAquick column centrifuged for an additional 1 min at 10,000 x g. The QIAquick column is now placed into a clean 1.5 ml eppendorf. We then added 50µ l of buffer EB (10 mM Tris-Cl, pH 8.5) to the center of the QIAquick membrane and centrifuged the column for 1 min to elute DNA.

2.6. DNA Sequencing of the 28S rDNA Fragment

The 28S rDNA amplified PCR product (100 ng concentration) was used for the sequencing with the single 28S rDNA 20F Forward, ITS 1 primer: 5'-TCCTCCGCTTATTGATATGC3' and 22R Reverse ITS 4 primer: 5'GGAAGTAAAAGTCGTAACAAGG3'-synthesized by DNA Sequencer by (Merck laboratory, Bangalore).

2.7. Sequence Analysis

A comparison of the 28S rRNA gene sequence of the test strain against nucleotide collection (nr/nt) as a database was done using BLAST [16]. A number of sequence of *Trichoderma* were selected on the basis of similarity score 90 % of the determined sequence with a reference sequence. Multiple sequence alignment of these selected homologous sequences and 28S rRNA gene sequence of test strain was performed using ClustalW [17]. Subsequently, an evolutionary distance matrix was then generated from these nucleotide sequences in the dataset. A phylogenetic tree was then drawn using the Neighbour joining method [18]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA (Molecular Evolutionary Genetics analysis) version 4.0 [19]. We again compared the 28S rRNA gene sequence of test strain with different set of sequence database such as small subunit ribosomal RNA (SSU rRNA) and large subunit ribosomal RNA (LSU rRNA) using Ribosomal RNA BLAST [20]. 28S rRNA gene sequence of test strain is also compared against those sequences, in Ribosomal Database Project [21] by using the RDP Classifier check Program. The annotated information for the sequence in the database to which 28S rRNA aligns is used for the fungal identification.

3. RESULTS

Rapid identification of bioagent is very necessary and important in the pathological laboratory to take decision for installment of commercial based bioformulation. The rRNA based analysis is a central method in pathology used not only to explore microbial diversity but also to identify new strains. The genomic DNA was extracted from isolated fungal strain *Trichoderma longibrachiatum* 28CP-7444/09 and universal ITS -1 primers 20F and ITS -4 primers 22R were used for the amplification and sequencing of the 28S rRNA gene fragment. A total of 666 bp of the 28S rRNA gene was sequenced and used for the identification of isolated fungal strain. Subsequently, a 28S rRNA gene sequence based phylogenetic tree showing the relationships between the test strain 28CP and selected representatives of the genus *Trichoderma* is given in Figure **3**. It is evident from phylogenetic analysis of 28S rRNA gene that the isolate 28CP represents a genomic species in the genus *Trichoderma*. Comparison of test strain against known sequences of SSU rRNA and LSU rRNA databases showed that the gene sequence of isolate 28CP has 90% sequence similarity (Score=666 bits, Expect=0.0) with 28S rRNA gene sequence of *Trichoderma* (Genbank Acc. No.: JX978541). Thus, data shows that the isolate 28CP is a member of the genus *Trichoderma*. Similarity rank program classifier available at the Ribosomal Database Project [21] classified the isolate 28CP as a novel genomic species of the genus *Trichoderma* with a confidence threshold of 90% (Figure **1**). The 28S rRNA gene sequence of isolate 28CP was deposited in GenBank and alloted the accession number JX978541.

4. DISCUSSION

4.1. Molecular Analysis Using Internal Transcribed Spacer Region (ITS)

Ribosomal RNA (rRNA) sequence analysis has been well-documented as a means of determining phylogenetic relationships in all of the major organismal

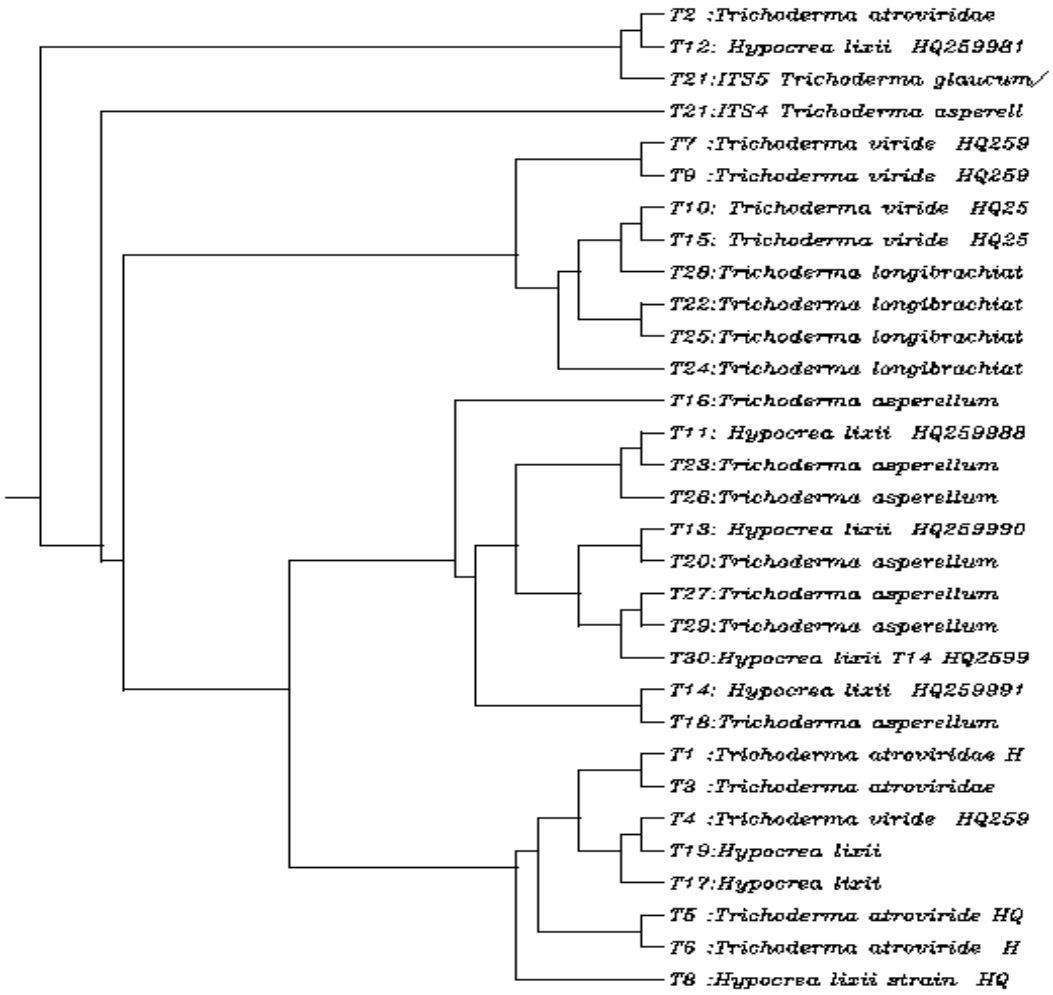

Figure 3: Phylogenetic and molecular evolutionary analysis of *Trichoderma* Species

domains. Variable sequences within the mature small subunit (SSU) and large-subunit (LSU) rRNA genes have been found to be appropriate for assessing subgeneric relationships in many eukaryotes. One of these variable regions, the D2 region of the LSU, has been used to determine phylogenetic has been used to determine phylogenetic relationships in a number of pathogenic fungal genera [22]. The ITS region of the rDNA operon, which is more variable than the D2 region, has proven useful in distinguishing relationships at the species level [23].

The genetic variability within 69 bio-control isolates of *Trichoderma* collected from different geographic locations and culture collections and their phylogenetic analysis were done with the help of the sequence data obtained from the inter Transcribed spacer 1(ITS1) region of Ribosomal DNA and a fragment of the translation elongation factor 1(tef1) and reported that more than 50% of the potential bio-control strains were grouped within *Trichoderma* section Pachybasium [24].

Trichoderma isolates with different biocontrol capabilities and identification by molecular methods and further characterized into three main clades by internal transcribed spacer (ITS) Sequence analyses. Consequently, a reliable phylogenetic tree was constructed containing isolates belonging to the *T. herziunum* clade [25].

In eukaryotes, the genes encoding ribosomal RNAs are organized in arrays which contain repetitive transcriptional units involving 16 – 18S, 5.8S and 23 – 28S rRNAs, two transcribed intergenic spacers ITS1 and ITS2 and two external spacer sequences (5´and 3´ ETS). These units are transcribed by RNA polymerase I and separated by non-transcribed intergenic spacers (IGS) as represented on Figure **4**. The product of RNA polymerase I is processed in the nucleolus, where ITS1 and ITS2 are excised and three types of rRNAs produced. In eukaryotic genomes the ITS regions vary greatly in size and sequence. In *S. cerevisiae* the ITS1 spans 361 bp and ITS2 is 232 bp long, the polymerase

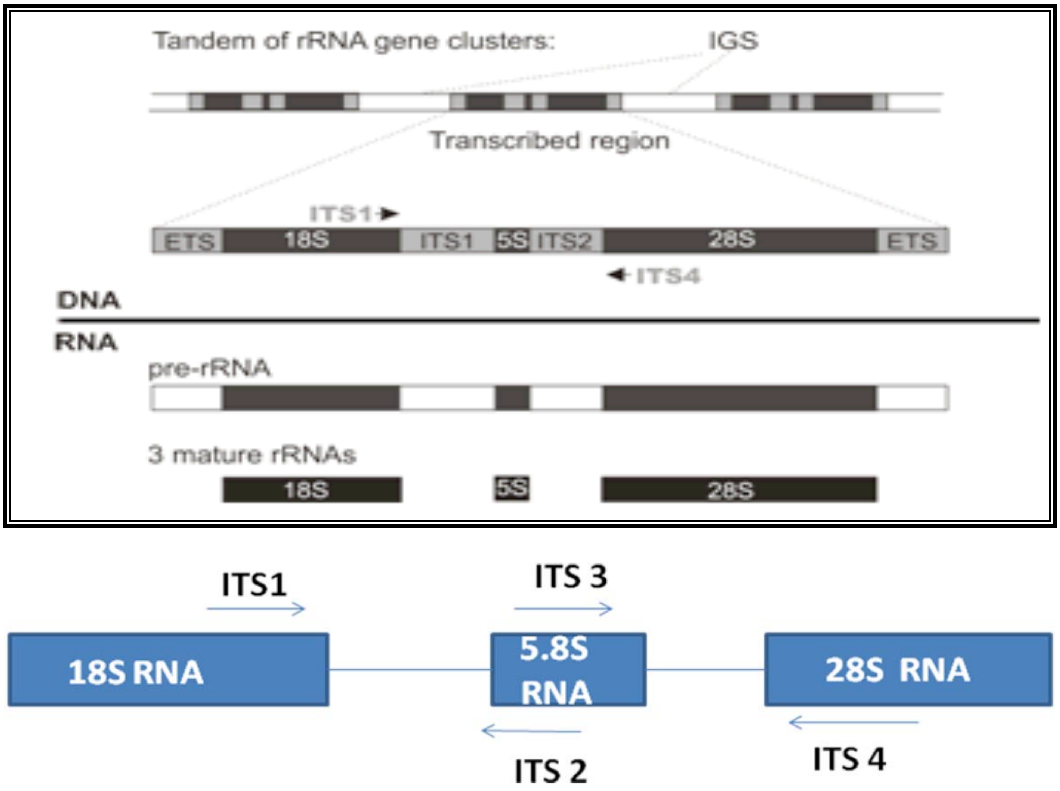

Figure 4: Location of Internal Transcribed Spacer sequence 1 & 2 (**a**) and the position of primers for PCR based amplification of the region (**b**).

chain reaction (PCR) and subsequent analysis of amplified rDNA using restriction endonucleases were employed in different studies to achieve efficient interspecies discrimination in medical and food mycology. The ITS regions have important biological meaning in rRNA processing. The structures of analyzed ITS1 and ITS2 contain four or three helical arms. The changes in size and sequence of these regions are then biologically permissible as long as they do not disturb the formation of secondary structures which facilitate the rRNA processing. It is thus presents a simple method for determination of inter- and intraspecies variability in fungal isolates.

Molecular phylogenetic analyses of biological control strains of *Trichoderma* (*Ascomycetes*, *Hypocreales*) strains that have warted conidia are traditionally identified as *T. viride*, the type species of *Trichoderma*. However, two morphologically distinct types of conidial warts (I and II) have been found. Because each type corresponds to a unique mitochondrial DNA pattern, it has been questioned whether *T. viride* comprises more than one species. Combined molecular data (sequences of the internal transcribed spacer 1 [ITS-1] and ITS-2 regions and of part of the 28S rRNA gene along with results of restriction fragment length polymorphism analysis of the endochitinase gene and PCR fingerprinting), morphology, physiology, and colony characteristics distinguish type I and type II as different species. Type I corresponds to “true” *T. viride*, the anamorphic of *Hypocrea rufa*. Type II represents a new species, *T. asperellum*, which is, in terms of molecular characteristics, close to the neotype of *T. hamatum*.

Analysis of ITS1-5.8S-ITS2 region of the cDNA showed that approximate 600 bp and size variation was observed. Restriction analysis of this region showed that inter and intra -specific polymorphism [26].

6. CONCLUSION

Biocontrol, or Biological Control, can be defined as the use of natural organisms, or genetically modified, genes or gene products, to reduce the effects of undesirable organisms to favour organisms useful to human, such as crops, trees, animals and beneficial microorganisms. This strategy of control is ecologicaly clean and compatible with different models of agriculture: organic, biological and integrated pest/pathogen management (IPM) programmes.

Trichoderma strains used as biocontrol agents can act: a) colonizing the soil and/or parts of the plant,

occupying a physical space and avoiding the multiplication of the pathogens; b) producing cell wall degrading enzymes against the pathogens; c) producing antibiotics that can kill the pathogens; d) promoting the plant development and e) inducing the defensive mechanisms of the plant. Antifungal formulations based on Trichoderma strains and proteins require, as in the case of chemical fungicides, a costly and sound registration process previous to their commercialization.

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