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Full Length Research Paper

Molecular characterization of *Trichoderma longibrachiatum 21PP* isolated from rhizospheric soil based on universal ITS primers

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***Trichoderma* species are morphologically very similar and were considered for many years as a single species. A new strain *Trichoderma longibrachiatum 21PP* was isolated from a rhizospheric soil sample collected from Kaushambi district of an Indian State (Uttar Pradesh). The universal primers (ITS-1& ITS-4) were used for the amplification of 28S rRNA gene fragment that produced a sharp band of about 700 bp on the gel. The amplified gene fragment was then sequenced (664 bp) and then deposited in GenBank with the Accession No. JX978542. Thus, the molecular identification of the specified strain enabled us for further characterization as this strain of *Trichoderma* species can be used as a biocontrol agent against pathogenic fungi such as *Fusarium, Alternaria, Sclerotinia*, etc. It is proposed that the identified strain *T. longibrachiatum 21PP* be assigned as the type strain of a species of the genus together with the 28S rRNA gene sequence search in Ribosomal Database Project, small subunit rRNA and large subunit rRNA databases.**

Key words: 28S ribosomal RNA gene, *Trichoderma,* polymerase chain reaction, phylogenetic analysis, DNA isolation, DNA sequencing.

INTRODUCTION

Accurate and definitive fungal identification is essential for correct disease diagnosis and treatment of associated 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 (Woese and Fox, 1977). 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 (Sacchi et al., 2002; Hillis et al., 1991; Woese, 1987). *Trichoderma* spp. is common soil inhabitants and is effective in providing biocontrol of soil borne pathogens due to its antagonistic behaviour. The major aspect of successful biological control strategies includes the production, formulation and delivery system of bioagents. The Internal Transcribed Spacer (ITS) regions of the rDNA are perhaps the most widely sequenced DNA regions 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.,

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Table 1. Gene sequence with universal primers.

Primer Name	Sequence (5'-3')	Length	T_m (˚C)	GC content (%)
ITS-1	TCTGTAGGTGAACCTGCGG	19	53	58
ITS-4	TCCTCCGCTTATTGATATGC	20	50	45

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 ITS-1 region of rDNA.

The study aims at identifying a potential strain of Trichoderma species as it can help us in the commercialization and eventually developing a better solution to the eradication of soil borne pathogens. Here, the method of isolation and identification of an unknown fungus from the Indian Kaushambi (25° 31′ 50.68″ N, 81° 22′ 38.25″ E) district using 28S rRNA gene sequence is described (Srivastava et al., 2008; Yadav et al., 2009) to characterize the strain *Trichoderma longibrachiatum 21PP* as a member of the *Trichoderma* spp. We initiated a systematic screening program to catalogue the microbial composition of the Indian district Kaushambi.

MATERIALS AND METHODS

Isolation of *Trichoderma* from soil sample

Rhizospheric soil samples were collected from various experimental fields of an Indian district Kaushambi (25° 31′ 50.68″ N, 81° 22′ 38.25″ E). The strain was isolated and identified in Potato Dextrose Agar (PDA) with low sugar medium by serial dilution method (Johnson and Crul, 1972; Nirenberg, 1976).

DNA isolation of *Trichoderma*

Pure culture of the target fungal was grown overnight in liquid PDB for the isolation of genomic DNA using a method described by Hiney et al. (1992).

Identification of *Trichoderma*

The isolated strain was then identified at molecular level using PCR amplification of the specific gene sequence with universal ITS-1 (forward) and ITS-4 (reverse) primers. It is then deposited to the Indian Type Culture Collection (ITCC), IARI (New Delhi, India).

Molecular characterization

The total genomic DNA was extracted from isolate of *Trichoderma* based on Cetrimide Tetradecyl Trimethyl Ammonium Bromide (CTAB) mini extraction method as was described by Crowhurst et al. (1995) in their study.

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 a UV transilluminator.

Amplification of isolated DNA using ITS

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 ITS-4 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 µmM of each primer, 40 ng/µl of template and 2.5 U of Taq polymerase (Fermentas). The cycle parameters included an initial denaturation for 5 min at 94°C, followed by 40 cycles of denaturation for 1 min at 94°C, primer annealing for 2 min at 55°C, primer extension for 3 min at 72°C, 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 V for 3 h in TAE buffer. One Kb ladder (MBI, Fementas) was used as a marker. The gel was observed in a transilluminator over ultraviolet light. The desired bands were cut from the gel with minimum quantity of gel portion using QIAGEN gel extraction kit.

Purification of PCR product

The PCR product was purified by QIAGEN gel extraction kit using the protocol described in the manufacturer's manual.

DNA sequencing of the 28S rDNA fragment

A pair of universal ITS primers ITS-1 (forward) and ITS-4 (reverse) was used for sequencing of the amplified product and this step was carried out at the Merck Laboratory (Bangalore, India). The details of the primers are mentioned in Table 1.

Sequence analysis and phylogeny

A comparison of 28S rRNA gene sequence of the test strain against nucleotide collection (nr/nt) database was done using BLAST program (Zhang et al., 2000). Sequences that shared about 90% similarity with the test sequence were selected for a multiple sequence alignment that was carried out using ClustalW (Thompson et al., 1994). Subsequently, an evolutionary distance matrix was generated from these nucleotide sequences in the dataset. A phylogenetic tree was then constructed using the Neighbor Joining method (Saitou and Nei, 1987). Phylogenetic and molecular evolutionary analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 (Tamura et al., 2007). The 16S rRNA gene sequence of test strain was compared with a different set of sequence database such as small subunit ribosomal RNA (SSU rRNA) and large subunit ribosomal RNA (LSU rRNA)

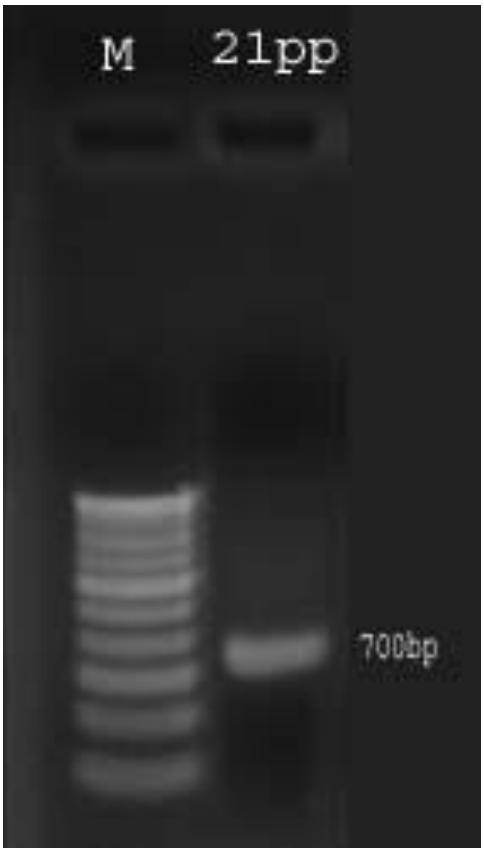

Figure 1. Shows the amplified PCR product of the *Trichoderma longibrachiatum 21PP*.

using Ribosomal RNA BLAST (Altschul et al., 1997). 28S rRNA gene sequence of the test strain was also compared against those sequences, in Ribosomal Database Project (Cole et al., 2009) by using the RDP Classifier check program (Wang et al., 2007). The annotated information for the sequence in the database to which 28S rRNA aligns is used for fungal identification.

RESULTS

Rapid identification of microorganisms is necessary in the pathological laboratory in an attempt to proceed for the preparation of a bioformulation that can be useful for the farmers. 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 *T. longibrachiatum* 21PP and universal ITS-1 (19F) and ITS-4 (20R) primers were used for the amplification and sequencing of the 28S rRNA gene fragment. A total of 664 bp of the 28S rRNA gene was sequenced (Figure 1).

The sequenced gene fragment of the specific strain was then searched for other closely related and similar templates using BLAST program. Sequences that shared 90% similarity to the test sequence were selected for a multiple sequence alignment. Figure 2 shows the phylogenetic relationship of the test strain (*T. longibrachiatum 21PP*) with others strains of *Trichoderma*. Comparison of test strain against known sequences of SSU and LSU rRNA databases showed that the gene sequence of the isolate *21PP* has 90% sequence similarity (Score=664 bits, Expect=0.0) with 28S rRNA gene sequence of *Trichoderma* (GenBank Acc. No.: JX978542). Thus, it can be concluded from the above data that the isolate *21PP* is a member of the genus *T. longibrachiatum*.

Conclusion

The study is focused on the identification of bioagent *T. longibrachiatum 21PP* at species level. Biocontrol activity of fungal strains in laboratory is generally determined by biological assays. To check the presence or absence of these biocontrol agents is a need to develop some efficient method. In past, serial dilution method had some disadvantages as it cannot distinguish between indigenous and artificially introduced strains that is why the first step of utilizing the full potential of fungal strains is identified at molecular level.

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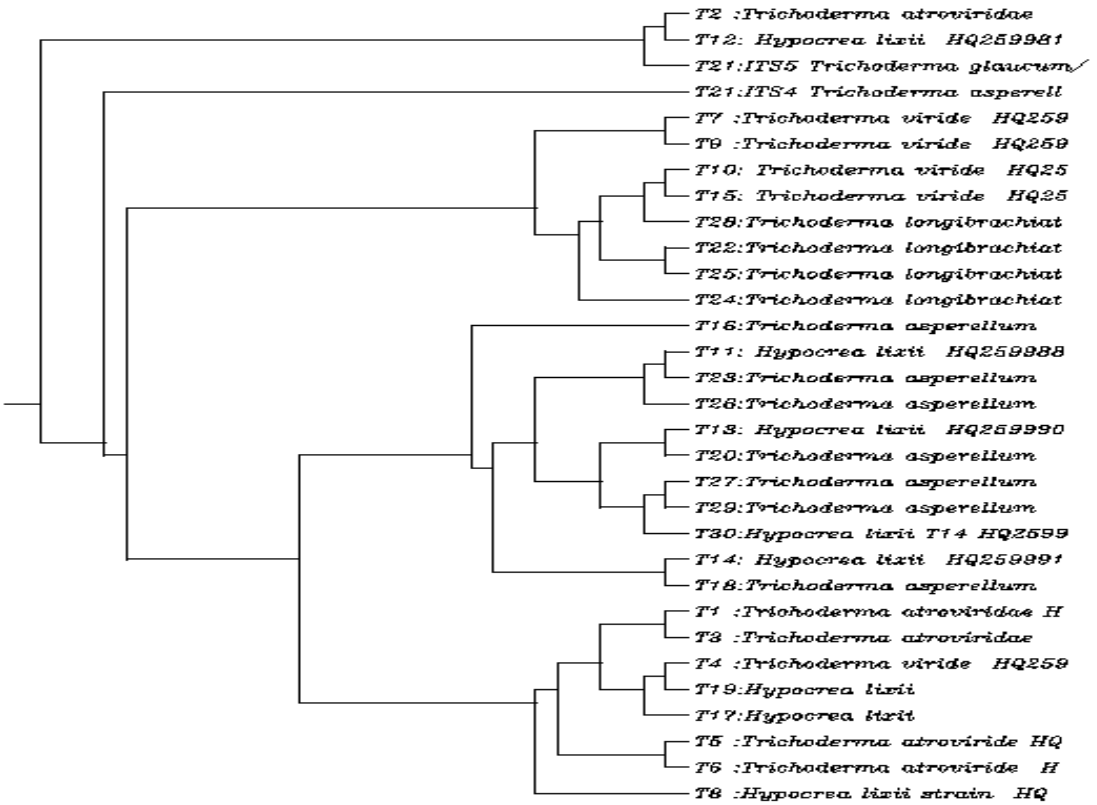

Figure 2. Shows the phylogenetic relationship of the test strain with other strains of *Trichoderma*.

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