



Molecular characterization through ITS sequencing of the native isolates of *Trichoderma* species

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Abstract

The genus *Trichoderma* are used in biological control programs to control phytopathogens. The secondary metabolites produced by *Trichoderma* have antifungal as well as mycoparasitism actions, defense response induction and resistance in host plants which act synergistically. Different molecular biology techniques used for molecular characterization of the *Trichoderma* genus include UP-PCR (Universal Primer-Polymerase Chain Reaction) and RAPD (Random Amplification of Polymorphic DNA). In the present investigations, molecular characterization of the native isolates of *Trichoderma* species was done using ITS sequencing. Two best strains of *Trichoderma* i.e. UBT-18 and T21 were used and molecular characterization was done using ITS1 and ITS4 Primers. Distance trees of nucleotide sequence of UBT 18 using ITS1 and ITS 4 primers indicated that the test isolates had belong to *Trichoderma harzianum* whereas distance trees of nucleotide sequence of T 21 using ITS 1 and ITS4 primers indicated that the test isolates had belong to *Trichoderma asperellum*.

Keywords: *Trichoderma*, ITS 1 primers, ITS4 primers, molecular characterization

Introduction

Among the widely used biocontrol agents, *Trichoderma* spp. have appeared as the most potential agents for their unique bio control activities and for having a wide array of beneficial plant interactions [1, 2]. Bio control of plant pathogens by the use of *Trichoderma* has become an eco-friendly, effective, farmers' accepted alternative to fungicidal application for reducing the chemical extravagant in farming ecosystem [3]. The new era for fungal systematic to bring about taxonomic changes has been opened from the early 1990's due to introduction of molecular methods and DNA sequence analysis in evolutionary mycology [4]. Strain identification and phylogenetic tree development is possible due to molecular tool development. Different molecular biology techniques are used for molecular characterization of the *Trichoderma* genus which includes UP-PCR (Universal Primer-Polymerase Chain Reaction) and RAPD (Random Amplification of Polymorphic DNA). The analyzing of tef1 (elongation factor 1 alpha) and ITS1 sequence (Internal Transcribed Space region) help in identification of *Trichoderma crissum* which were PCR amplified by using ITS1 and ITS4 primers for ITS and EF1-728F and TEF1 primers for tef1 [5]. Even for other various *Trichoderma* species like *T. atroviride*, *T. harzianum*, *T. koningiopsis*, *T. longibrachiatum* and *T. pseudo-koninngii* molecular characterization was done using rDNA-Its markers and RAPD [6, 7]. In species identification, DNA barcoding are used routinely nowadays and DNA-based methods provides a base for building up of modern taxonomy of the *Trichoderma* genus. Since more strains are isolated from various geographic regions and their novels sequences are made available [8, 9], more *Trichoderma* species and clads are being available and established. Almost about 228 species of *Trichoderma* and various species which are not taxonomically characterized are being distinguished [10]. It can help to select suitable specific strain of biocontrol agents for agricultural and horticultural use. Keeping in view of importance the present investigations

were carried out on molecular characterization of the native isolates of *Trichoderma* species using ITS sequencing.

Materials and Methods

The experiments were conducted at Department of Plant Pathology, Uttar Banga Krishi Viswavidyalaya during 2019-2020. The details of experimental procedures adopted, materials used and techniques followed during the course of present investigation are described below.

Media Used

Potato dextrose agar (PDA, 1936) [11]

It is a semisynthetic growth media for routine culture of the test bio control agent *Trichoderma* spp.

Compositions

	g/L
Potatoes, infusion from	200.0
Dextrose anhydrous	20.0
Agar agar	20.0
Distilled water	1L
Final pH (at 25°C)	5.6±0.2

Native *Trichoderma* strains used

Two best strains of *Trichoderma* i.e. UBT-18 and T21 were used. These strains were isolated from soil of Cooch Behar (vegetable plots, tea plantation), West Bengal.

Pure culture of *Trichoderma*

Single spore of *Trichoderma* strains was taken to get pure culture from the previous available culture. The spore is placed on fresh PDA medium on sterile petriplate. The Petriplates were properly wrapped and incubated in BOD at 28±1°C for 5-7 days. The process can be repeated after 15-20 days to maintain the culture for future use.

Transferon test tubes

From pure culture petriplates, *Trichoderma* was transferred on PDA slants that were then kept in BOD at 28±1°C for 5-7days and later maintained at 4°C as pure culture.

Molecular characterization of native *Trichoderma* isolates by ITS sequencing

- Genomic DNA was isolated from the culture using the method described [12]. Its quality was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA has been observed.

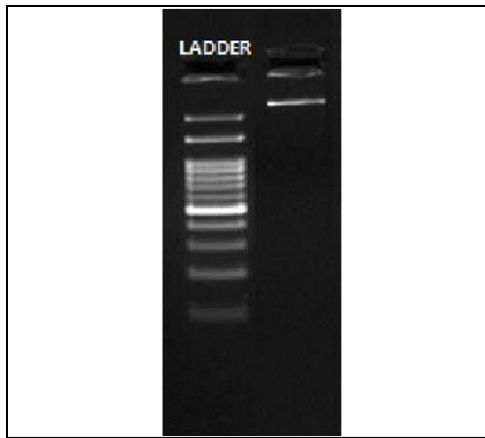


Fig 1

- Fragment of ITS region was amplified by PCR. A single discrete PCR amplicon band of ~700bp was observed when resolved on agarose.

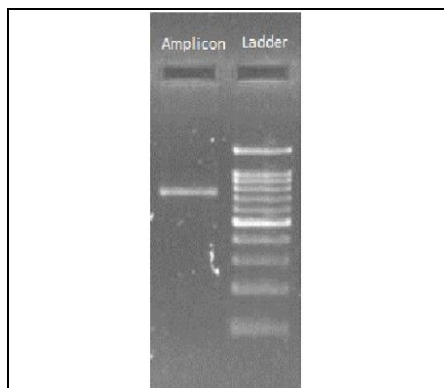


Fig 2

- The PCR amplicon was purified to remove contaminants.
- Forward and reverse reactions of PCR amplification of target DNA segment were carried out with ITS1(5'-TCCGTAGGTGAACTGCGG-3') and ITS4 primers(5'-TCCTCC GCTTATTGATATGC-3') described using BDTv3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer [13].
- The ribosomal DNA (rDNA) sequencing data were compared with the Gen Bank database (<http://www.ncbi.nlm.nih.gov/BLAST/>), using the nucleotide BLAST program to identify homology between the PCR fragments and sequences in the Gen Bank database. Hundred sequences were selected and distance trees for ITS 1 and ITS 4 sequences for both the strains were constructed with a maximum sequence difference of 75% using BLAST pair wise alignment and Neighbour joining method between a query (yellow marked) and data base searched.

Results and Discussion

Molecular characterization of native *Trichoderma* isolates by using ITS1 and ITS 4 primers were performed.

Characterization of UBT-18

The results of nucleotide sequences of UBT 18 obtained from PCR amplicon by using ITS1 and ITS 4 primers have been presented in Table 1.

Table 1: ITS 1 and ITS4 sequencing of ribosomal DNA of UBT 18

ITS 1 sequencing
GCGTAAGGTCTCGAAAGGTGAACCAGCGGAAGGATCA TTACCGAGTTTACAACCTCCCAAACCCAATGTGAACGTTA CCAAACTGTTGCCTCGGCGGGATCTCTGCCCGGGTGCG TCGCAGCCCCGACCAAGGCGCCCGGAGGACCAAC CAAACTCTTATTGTATACCCCTCGCGGGTTTTTTTATA ATCTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAA TGAATCAAACTTTCAACAACGGATCTCTTGTTTCTGGC ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA CATTCGCGCCGAGTATTCTGGCGGGCATGCCTGTCCG AGCGTCATTTCAACCTCGAACCCCTCCGGGGGGTCCGGC GTTGGGGATCGGCCCTGCCTTGGCGGTGGCCGTCTCCGA AATACAGTGGCGGTCTCGCCGAGCCTCTCCTGCGCAGT AGTTTGCACACTCGCATCGGGAGCGCGGCGTCCACAG CCGTTAAACACCCAACCTTCTGAAATGTTGACCTCGGATC AGGTAGGA ATACCCGCTGAACTTAAGCATATCAATAAGGCGGAGGAA AG
ITS 4 sequencing
ACCCCTGGTTCGTAAGTATCCGAGGTACATTTTCAGAAG TTGGGTGTTTAAACGGCTGTGGACGCGCCGCTCCCGAT GCGAGTGTGCAAACCTACTGCGCAGGAGAGGCTGCGGCG AGACCGCCACTGTATTTCGGAGACGGCCACCGCCAAGGC AGGGCCGATCCCCAACGCCGACCCCGGAGGGGTTTCGA GGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAGAAT ACTGGCGGGCGCAATGTGCGTTCAAAGATTTCGATGATC ACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTG CGTCTTTCATCGATGCCAGAACCAAGAGATCCGTTGTTG AAAGTTTGTATTCATTTTCGAAACGCCTACGAGAGGCGC CGAGAAGGCTCAGATTATAAAAAAACCCGCGAGGGGGT ATACAATAAGAGTTTTGGTTGGTCTCCGGCGGGCGCCT TGGTCCGGGGCTGCGACGCACCCGGGGCAGAGATCCCGC CGAGGCAACAGTTTGGTAACGTTTACA TTGGGTTTGGGAGTTGTAACCTCGGTAATGATCCCTCCGC TGTTTACCAACGAGACCTTGTACGACTTTTACTTCTCT CTAATTGACCACAGACTT

The above sequences were compared with the Gen Bank database using the nucleotide BLAST program to identify homology between the PCR fragments and sequences in the Gen Bank database and the distance tree were constructed which have been presented below.(Fig 1 and Fig. 2).

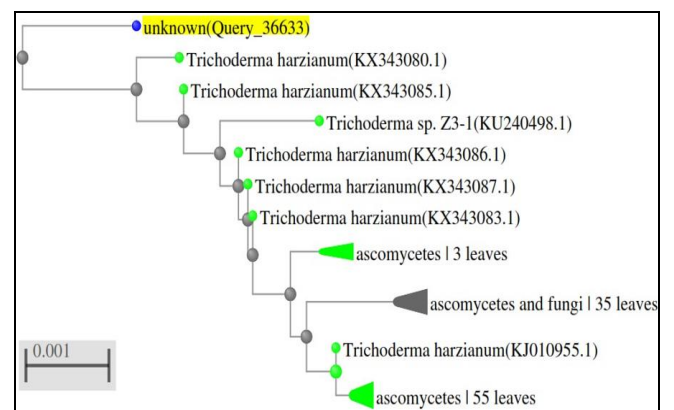


Fig 3: Distance tree of nucleotide sequence of UBT18 using ITS 1 primer

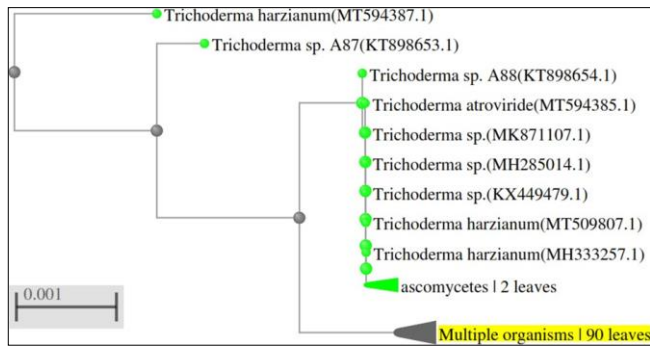


Fig 4: Distance tree of nucleotide sequence of UBT 18 using ITS 4 primer

The above figures 1 and 2 suggest that the test isolate belongs to *Trichoderma harzianum*. The ITS1 sequence data was submitted to NCBI GeneBank and the accession no. MT876632 has been obtained.

Characterization of T-21

The results of nucleotide sequences of T 21 obtained from PCR amplicon by using ITS1 and ITS 4 primers have been presented in Table 2.

Table 2: ITS 1 and ITS4 sequencing of ribosomal DNA of T21

ITS 1 sequencing
GGGTCGGTCTCGTTGGTGAACCAGCGGAAGGATCATTAC CGAGTTTACAACCTCCAAACCCAATGTGAACGTTACCAA ACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCGCA GCCCGGAACCAGGCGCCCGGAGGAACCAACCAAAA CTCTTTCTGTAGTCCCTCGCGGACGTATTTCTTACAGCT CTGAGCAAAAATTCAAATGAATCAAACTTTCAACAAC GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA TCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGG CGGGCATGCCTGTCCGACGCTCATTCAACCCTCGAACC CTCCGGGGGATCGGCGTTGGGGATCGGGACCCCTCACA CGGGTGCCGGCCCCGAAATACAGTGGCGGTCTCGCCGCA GCCTCTCCTGCGCAGTAGTTTGCAAACTCGCACCGGGA GCGCGGCGCGTCCACGTCCGTA AACACCCAACCTTTCTG AAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAAC TTAA GCATATCAATAGCCGGAGGAAAA
ITS 4 sequencing
AGGAGCGGGTTCTACTGATCCGAGGTCACATTTTCA AAGTTGGGTGTTTTACGGACGTGGACGCGCCGCGCTCCC GGTGCAGAGTTGTGCAAACACTGCGCAGGAGAGGCTGCG GCGAGACCGCCACTGTATTTCCGGGGCCGGCACCCGTGTG AGGGGTCCCGATCCCAACGCCGATCCCCCGGAGGGGTT CGAGGGTTGAAATGACGCTCGGACAGGCATGCCCGCCAG AATACTGGCGGGCGCAATGTGCGTTCAAAGATTTCATGA TCACTGAATTCTGCAATTCACATTAATTATCGCATTTTCG CTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTG TTGAAAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTG TAAGAAATACGTCCGCGAGGGGACTACAGAAAGAGTTT GGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCG ACGCACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGG TAACGTTACATTTGGGTTTGGGAGT TGTAACCTCGGTAATGATCCCTCCGCTGGTTTCCCAACG GAGACCTTGTACGACTTTTACTTCCCTCAATTCAGACCAA GAAG

Characterization of T-21

The results of nucleotide sequences of T 21 obtained from PCR amplicon by using ITS1 and ITS 4 primers have been presented in Table 2.

Table 2: ITS 1 and ITS4 sequencing of ribosomal DNA of T21

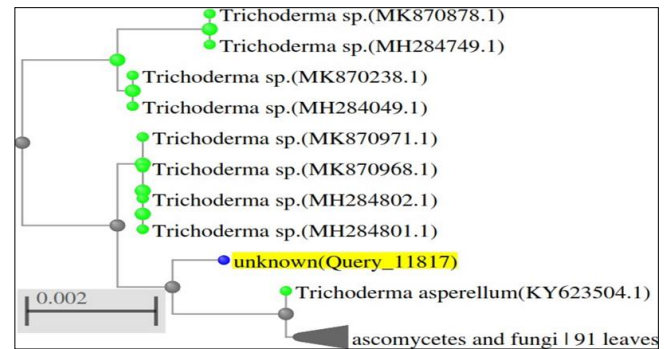


Fig 5: Distance tree of nucleotide sequence of T21 using ITS1 primer

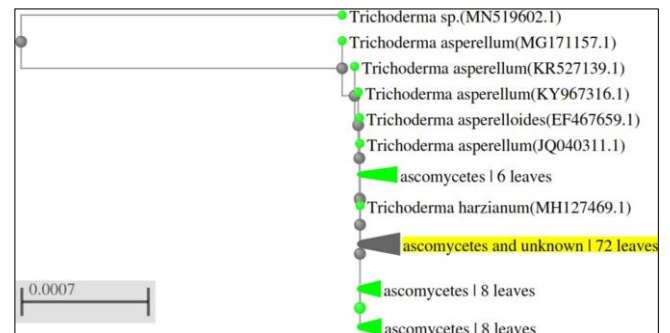


Fig 6: Distance tree of nucleotide sequence of T 21 using ITS4 primer

The above figures 3 and 4 suggest that the test isolate belongs to *Trichoderma asperellum*. The ITS1 sequence data was submitted to NCBI Gene Bank and the accession no. MT951635 has been obtained.

Species of the *Trichoderma* genus are all-pervasive in nature and normally found as soil-borne or wood-decaying fungi. Some species are economically significant because of having capability to produce industrial enzymes (cellulase and hemicellulose) and antibiotics or act as biocontrol agents [14, 15]. For that reason, accurate findings of *Trichoderma* at the species level is necessary. Morphological identification of species has proven unreliable because of their high degrees of similarity [16, 17]. By the reason of sensitivities to environmental components, recognition depending on host inclination and morphological distinction is not dependable. Hence, molecular methods have been introduced recently for accurate identification. Molecular patterns in genomic DNA are hypothetically not affected by these variations and can be useful for species identification [18, 19]. The ribosomal RNA genes (rDNA) possess characteristics that are appropriate for 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 [20]. They also occur in multiple copies with up to 200 copies per haploid genome arranged in tandem repeats with each repeat consisting of the 18S small subunit, the 5.8S, and the 28S large subunit genes.

Conclusions and Recommendations

Two best strains of *Trichoderma* (UBT-18 and T21) isolated from soil of Cooch Behar, West Bengal were used. Both strains showed different visual appearance i.e. UBT-18 was light yellowish green in colour with low sporulation rate and on the other hand T21 exhibited dark green spores, high growth rate and marginal mycelial growth. Molecular characterization of native *Trichoderma* isolates was done by ITS sequencing using ITS1 and ITS4 primers. The obtained nucleotide sequences of the strains were compared with the GeneBank database using the nucleotide

BLAST program to identify homology between the PCR fragments and sequences in the GeneBank database and the distance tree was constructed. The distance tree obtained suggest that the test isolate UBT-18 belongs to *Trichoderma harzianum* and the test isolate T21 belongs to *Trichoderma asperellum*.

DNA Barcoding forms one of the most rapid and accurate method for fungal species characterization and is applied to a standardized and small DNA region having unique pattern where ITS region is used within species level for species identification. This region is non-coding and polymorphic having taxonomic portions which helps to isolate sequences at species level. It helps to better understand the various complicated connections of *Trichoderma* species to provide a base for future studies and its effective use as a biocontrol agent.

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