

PHYLOGENETIC RELATIONSHIPS OF *TRICHODERMA HARZIANUM* ISOLATED FROM PADDY FIELD BASED ON SEQUENCE ANALYSIS

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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 paddy field. ITS 1 of the rDNA of *Trichoderma* isolates was amplified by polymerase chain reaction (PCR) using universal primers (ITS1). PCR products were purified and these purified products were used to amplify the ITS region of *Trichoderma* isolates. The amplified DNA was sequenced and aligned against ex-type strain sequences from Tricho BLAST and established *Trichoderma* taxonomy. 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.

KEYWORDS: *Trichoderma harzianum*, phylogenetic tree, rDNA, ITS, PCR

INTRODUCTION

Members of the genus *Trichoderma* (Ascomycota) are opportunistic fungi capable of rapidly colonizing diverse niches in both natural and artificial environments. In these habitats, *Trichoderma* spp. contribute to nutrient cycling by decomposing complex organic compounds, thus enhancing soil fertility and promoting overall ecosystem health (Druzhinina *et al.*, 2011). Beyond their ecological functions, *Trichoderma* spp. are used in agriculture as biocontrol agents to promote plant health by directly controlling phytopathogens or indirectly enhancing plant (Sood *et al.*, 2020). *Trichoderma* is recognized as a significant and extensively utilized fungal biocontrol agent. Fungi are significant pathogens in plants, capable of reducing agricultural yields by as much as 90% (Manimegalai *et al.*, 2011).

The genus *Trichoderma* has gained immense importance in past several decades due to its antagonistic ability against wide range of plant pathogens and growth promotion in crop plants. Some species of *Trichoderma* viz., *Trichoderma harzianum*, *T. viride*, *T. virens* and *T. koningii* are well known antagonists and are being utilized to control plant pathogens under field conditions (Manimegalai *et al.*, 2011). Promising *Trichoderma* isolates have different mechanisms or combination of direct parasitism, competition for nutrients, stimulators of plant health, or inducers of plant systemic resistance against various pathogens. A plethora of antagonistic *Trichoderma* isolates have been identified by several researchers from different places around the world, having history of varied climate, soil type, cropping system, etc., which differ in their innocuousness and efficacy as biocontrol agents (Yu and Luo, 2020).

Despite the commercial successes of these biocontrol agents, the major limitations remain their restricted efficacy and inconsistency under field conditions. Consequently, more efficient *Trichoderma* isolates with high antagonistic potential capabilities are needed for successful biological control systems (Modrzewska *et al.*, 2022). Due to the ecological importance of *Trichoderma* spp. and their application as a biocontrol agent in the field, it is important to understand their biodiversity. However, accurate species identification based on morphology is difficult due to the paucity and similarity of morphological characters and increasing numbers of morphologically cryptic species (Kullnig *et al.*, 2000). In recent years, the usefulness of molecular markers such as random amplified polymorphic DNA (RAPD) and repetitive-element polymerase chain reaction (REP-PCR) in resolving species differences among microbial species are also well documented. RAPD utilized PCR to amplify DNA segments with single primer of arbitrary nucleotide sequence generating fragments by hybridizing with compatible regions of DNA and amplifying the regions where the primers are in correct orientation and appropriately spaced (100–2500 bp). The diversity in the ecosystem, presenting the phylogenetic relationship between strains and discriminating between microorganisms those are genetically close to each other. This may be due to the

ubiquitous nature and seemingly random chromosomal distribution of random repeats in *Trichoderma* genome, giving rise to simultaneous PCR amplification of multiple genomic regions. The high genotypic variability among *Trichoderma* isolates could be associated with mutations in priming sites, rearrangements of chromosomal segments or recombination process in fungal genomes (Sun *et al.*, 2019).

Diversity studies have recently been undertaken to assess its ecological specialization. Several studies reported about a series of new isolates as well as new phylogenetic species of *Trichoderma* in a series of natural ecosystems (Degani and Dor, 2021). The accumulation of phenolic compounds in response to *Trichoderma* has been associated with biochemical mechanisms that enhance plant defense against diseases (Manimegalai *et al.*, 2024). On the other hand, only a few studies were focusing on agricultural environments. Recent evidences indicated the importance of the sterol biosynthetic pathway in inducing plant defense related gene expression in both the antagonistic fungus and the plant (Cardoza *et al.*, 2011; Malmierca *et al.*, 2013; Cardoza *et al.*, 2014). However, the results of these studies demonstrated that besides the natural ecosystems, the investigation of agricultural soils also reveals important information about *Trichoderma* biodiversity.

MATERIALS AND METHODS

Sample collection

The sample of pathogenic fungi of *Bipolaris oryzae* and biocontrol agent of *Trichoderma* are isolated from paddy soil at Thanjavur dt. The soil particles attached to roots were carefully collected after uprooting plants, stored at 4 °C and processed within 24 h of collection. Root adhered soil (10 g) was suspended in 90 ml of sterile distilled water and dilution plate technique was used for the isolation of *Trichoderma* spp. The suspensions from all samples were serially diluted (up to 10⁻⁵) and 100 µl of each dilution was added to sterile Petri dishes, in triplicates of each dilution, containing sterile Potato Dextrose Agar (PDA) medium. Streptomycin solution (1%) was added to the medium for preventing bacterial growth, before pouring into Petri plates. The plates were then incubated at 28 ± 1 °C. The isolates were characterized based on the monograph of Gams and Bissett (2002).

For morphological analysis, isolates were grown on PDA at 28 ± 1 °C for 5–7 days. Radial growth was measured at 24 h intervals until colony covered the whole Petri dish. Growth rate was calculated as the 7 day average of mean daily growth (mm day⁻¹). All micro morphological data were examined from cultures grown on PDA for 5 days at 28 ± 1 °C. Microscopic observations were done using trinocular microscope (Axio Imager M2 microscope, Carl Zeiss, Germany). For examination of conidial morphology, cultures were washed with sterile water and drops of the suspension were placed on microscope slides and mixed with lactophenol/cotton blue to stain the conidia. Length and width were measured for 30 conidia per isolate. Conidial morphology and size were recorded after 7 days of incubation.

Extraction of antifungal compound

The fungus which showed promising activity against the pathogen was cultured in liquid potato dextrose medium at 25°C in darkness for three weeks. After incubation, the culture was filtered twice through Whatman No.1 filter paper and Seitz filter (G.5). To 100 ml of culture filtrate, 10 ml of ethyl acetate was added in a separation funnel (250 ml), shaken well for 3 min. and the solvent and aqueous layer were separated. The ethyl acetate layer of the culture filtrate was used for further analysis.

Molecular characterization of *Trichoderma harzianum*

Isolation of chromosomal DNA

The DNA extraction was performed by the methods of Melo *et al.*, (2006). Approximately 200mg of washed mycelia *Trichoderma harzianum* cultures was transferred in to a micro centrifuge tubes and suspended in 200 µl buffer (2% TritonX-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM Na₂EDTA). When DNA was extracted from *T. harzianum* that had been grown in liquid culture with shaking, the mate (upper layer) that had formed were washed three times with cold-sterile distilled water and the DNA extraction buffer had 10 – fold EDTA, 200 µl phenol chloroform – alcohol isoamyllic (25:24:1) mixture and 0.3 g sterile glass beads (Sigma, G1277). The suspension was at top setting for 5 min. To each tube, 200 µl Tris EDTA, (pH 8.0), was added mixed and the suspension was centrifuged for 5 min at 13,500 rpm. The supernatant was transferred in to a new micro centrifuge tube and the nucleic acids were precipitated by adding 1 ml absolute ethanol. Suspensions were mixed and centrifuged (13,500 rpm) for 2 min and the pellet was resuspended in 400 µl Tris- EDTA, (pH 8.3) µl RNAase (10 mg/ml) and incubated for 5 min. Then, it was centrifuged again with 10 µl ammonium acetate (4 M) for 3 min at 13,500 rpm and the supernatant was discarded. The DNA pellet was dried in airflow for 15 min and finally resuspended in 40 µl sterile distilled water. The genomic DNA was verified by 1.2% agarose gel electrophoresis, a single band of high molecular weight DNA was allowed to develop.

PCR amplification of 18S rRNA

The extracted DNA was used for PCR, which was performed in 25 µl reaction volumes containing 20 mg genomic DNA, 100 µM dNTPs, 1 mM MgCl₂, 2.5 µl 10 x PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, pH 8.3), 0.2 µM of each primer pair and 1U *Taq* DNA polymerase (Invitrogen®); distilled water was added to complete the final volume of the reaction cycling were initial denaturation step at 95°C for 5 min followed by 30 cycles, each consisting of 94°C for 30sec, annealing temperature 55°C for 30sec and 72°C for 45sec with a final extension at 2°C for 10 min. *T. harzianum* specific action primers(D forward: 5'-TCC GTA GGT GAA CCT G CG G-3': D Reverse: 5'- TCC TCC GCT TAT

TGA TAT GC-3') were added to the extracts of the few different fungi and single reaction were performed in an Eppendorf Master cycleris® thermocycler. The quantity of the PCR reaction was monitored in 1% Trisacetate-EDTA-agarose gel and bands were visualized by staining with ethidium bromide. Image were made and stored with the Kodak-EDAS system. Full length D2 region of 18S rRNA gene fragment was amplified by PCR from the above isolated genomic DNA. A single discrete PCR amplification band of 466 bp was observed when resolved on agarose gel. To identify the fungus for phylogenetic analysis either ITS (Internal Transcribed spacer) region of 18S rRNA region (LSU: Large subunit) with the D2 set of primer were used.

18S rRNA sequencing of fungal isolate

Consensus sequence of 18S rRNA gene was generated from forward and reverse sequence data using aligner software and sequential using the facility available at UniBiosys Biotec Research Labs, Cochin.

Nucleotide sequence accession

The 18S rRNA sequence for the *T. harzianum* has been deposited in Gene Bank <http://www.ncbi.nlm.nih.gov/genbank>.

Phylogenetic analysis

The D2 region of 18S rRNA gene sequence was used to carried out BLAST with the NR database of NCBI genbank database (URL <http://www.ncbi.nlm.nih.gov>). Based on maximum identity scores first ten sequence were selected and Global pair wise sequence similarity between the sequence were performed using Needleman and Wunsuh algorithm available with the emboss sequence analysis suite. Multiple sequence analysis was performed using alignment program of CLUSTAL W.

The evolutionary history was inferred using the neighbour - joining method of Saitou and Nei (1987). The bootstrap consensus tree inferred from 500 replicated was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicated tree in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (next to the branches). The evolutionary distance were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitution gaps substitutions per site codon position included were 1st + 2nd + 3rd + Noncoding. All positions gaps and missing data were eliminated from the dataset (complete deletion option). They have a total of 663 positions were occur in the final dataset. Phylogenetic analyses were conducted in MEGA 4 software (Tamura *et al.*, 2007).

18S rRNA secondary structure prediction of fungal isolate

The secondary structure of *Trichoderma harzianum* was predicted using the bioinformatics tools available in online www.genebee.msu.su/service/rna2-reduced.html.

Restriction site analysis of 18S rRNA of fungal isolate

The restriction sites in 18S rRNA of *Trichoderma harzianum* were analyzed by using NEB cutter programme version 2.0 in online www.neb.com/NEBcutter2/index.php.

RESULTS AND DISCUSSION

The DNA extracted from *T. harzianum* grown in PDA liquid medium (Plate I). The full-length D2 region of 18S rRNA gene fragment was amplified by PCR from the above isolated genomic DNA. A single discrete PCR amplicon band of 466 bp was observed when resolved on agarose gel (Plate II). The secondary structures of *T. harzianum* JX277056.1 were predicted by using Bio-information tools. The secondary structure of 18S rRNA showed 22 stems, 15 bulge loops and 6 hairpin loops in their structure. The free energy structure of 18S rRNA secondary structure of *T. harzianum* was -133.2 kkal/mol respectively (Fig 1). Isolation of chromosomal DNA was described from fungal mycelia. Since, the currently available DNA extraction protocols are rather costly and time consuming (Whitney and Mortimore, 1961).

Fungal genetic studies require a rapid method of isolating DNA from a large number of samples for restriction enzyme analysis. These steps consist of DNA extraction of a variable region of 18S rRNA gene with universal primers and identification by probe hybridization or DNA sequencing under the condition of identical for all fungi (Pani *et al.*, 2021; Manimegalai *et al.*, 2011b). Hermosa *et al.*, (2000) studied the most common biological control agents (BCAs) of the genus *Trichoderma*. Since *Trichoderma* BCAs use different mechanisms of biocontrol, it is very important to explore the synergistic effects expressed by different genotypes for their practical use in agriculture. Previously identified as *Trichoderma harzianum* Rifai is one of the best biocontrol strain, was carried out by using several molecular techniques. Sanna *et al.*, (2022) studied the seven *Trichoderma* species (33 strains) were classified using secondary metabolite profile-based chemotaxonomy.

In the present study the genomic DNA was isolated from *T. harzianum* and PCR amplification was performed. The 18S rRNA gene sequencing was made by using ITS (internal transcribed spacer) method. The phylogenetic tree was constructed using MEGA 4 software. Based on the neighbour joining tree analysis the isolate *T. harzianum* has been found closely related to already existing species of *Hypocrea lixii* at 99 per cent of similarity. The 18S rRNA gene sequences were submitted to Genebank (NCBI) and accession number JX277056.1. *T. harzianum* were independently clustered based

on both ITS (Internal Transcribed Spacer) sequence and secondary metabolite analyses. *T. harzianum* formed three sub clusters in the ITS-based phylogenetic tree and two sub clusters in the metabolite based dendrogram.

Restriction site map in 18S rRNA of *T. harzianum* JX277056.1 was analyzed. The restriction sites of *T. harzianum* found in the rRNA are in 52 ambiguous sites. A large number of restriction enzyme sites was observed in the fungal isolates. The GC content of this species was 56%. Similarly, the AT content of *T. harzianum* was 44%, were determined using NEB cutter program V 2.0 in online [www.neb.com/NEB cutter +2/index php](http://www.neb.com/NEB_cutter_+2/index_php) (Fig 2). Isolation of chromosomal DNA was described from fungal mycelia. Since, the currently available DNA extraction protocols are rather costly and time consuming (Hajiehhrari *et al.*, 2008; Gupta *et al.*, 2019). Fungal genetic studies require a rapid method of isolating DNA from a large number of samples for restriction enzyme analysis. In present investigation, isolated genomic DNA from fungi using the QIA amp® DNA minikit. These procedures have been successfully used for the isolation of genomic DNA from *Aspergillus*, *Candida* species and several other fungal cultures (Garcia *et al.*, 2018; Pfordt *et al.*, 2020).

These steps consist of DNA extraction of a variable region of 18S rRNA gene with universal primers and identification by probe hybridization or DNA sequencing under the condition of identical for all fungi (Tomah *et al.*, 2020; Erazo *et al.*, 2021). Hermosa and Grondona *et al.*, (2000) studied the most common biological control agents (BCAs) of the genus *Trichoderma*. Since *Trichoderma* BCAs use different mechanisms of biocontrol, it is very important to explore the synergistic effects expressed by different genotypes for their practical use in agriculture. Previously identified as *Trichoderma harzianum* Rifai is one of the best biocontrol strain, was carried out by using several molecular techniques (Tyskiewicz *et al.*, 2022; Al-Rubaiey and Al-Juboory, 2020).

The 18S ribosomal RNA gene, partial sequences of *T. harzianum* were compared with sequences obtained Gene bank using BLAST and per cent similarity was assessed. The results revealed that the close relative to the isolates of *T. harzianum* JX277056.1 was 99 % similarity of *Hypocrea lixii*. Phylogenetic tree related of the *T. harzianum* was analyzed by neighbour joining methods (Fig 3). The PCR amplified 18S rRNA genes of *T. harzianum* was partially sequenced using specific 18S rRNA sequence primer and deposited in Genebank (NCBI), accession number JX277056.1 (Fig 4).

In the present investigation the 18S rRNA of *T. harzianum* fragment was amplified by PCR. A single discrete PCR amplicon band of 466 bp was observed when resolved on agarose gel. The PCR amplified 18S rRNA genes of *T. harzianum* have been deposited in Genebank <http://www.ncbi.nlm.nih.gov/genbank> (NCBI) accession number JX277056.1. A phylogenetic tree was also constructed by neighbour- joining method. The present investigation concludes that the culture of *T. harzianum* closely related to *Hypocrea lixii* (at 99 % level) based on nucleotide homology and phylogenetic analysis. The secondary structure of 18S rRNA showed 22 stems, 15 bulge loops and 6 hairpin loops in their structure. The free energy structure of 18S rRNA was -133.2 kkal/mol respectively. The restriction sites of *T. harzianum* (JX277056.1) found in the rRNA are in 52 ambiguous sites and GC content 56%. Similarly, the AT content of *T. harzianum* was 44% which were determined using NEB cutter programme.

CONCLUSION

In conclusion, the present study revealed the scientific validation and proof for the usage of *Trichoderma harzianum* as best eco-friendly and cost effective alternate fungicide. Further, combined studies including biological, biochemical and molecular technologies, are essential to select indigenous antagonistic *Trichoderma* isolates that can be used under different environmental conditions. Correct identification will provide information on understanding the inter parasitic relationship with target pathogens and the subsequent environmental fate of the antagonist needed for effective application. Molecular markers can be definitive in confirming the correctness of any morphological taxonomic system and provide additional tool to characterize fungal genotypes. The DNA technology has opened several new avenues of investigation. The molecular data is also useful for taxonomic purposes and identification of unknown isolates with morphological identification which is time consuming and difficult and some species are asexual or heterothallic, making identification without sexual structures difficult. The aim of molecular studies in biodiversity has four steps (1) phylogenetic studies, i.e., tracing the most probable course of evolution and the historic coherence among the groups at higher taxonomic ranks; (2) taxonomic studies, mostly at the level of genera and species (3) diagnostic applications i.e., recognition of defined taxonomic entities and (4) epidemiology and population genetics i.e., monitoring-outbreaks of sub specific entities with respect to the analysis of population and their mode of reproduction. Each of these broad aims and levels of diversity has its own set of optimal techniques.

Plate I: Pure culture and microscopic view of *Trichoderma harzianum*

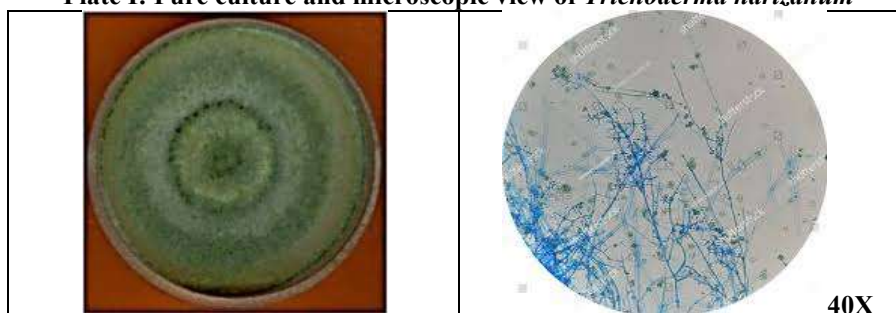


Fig 1: Identification of DNA from *T. harzianum*

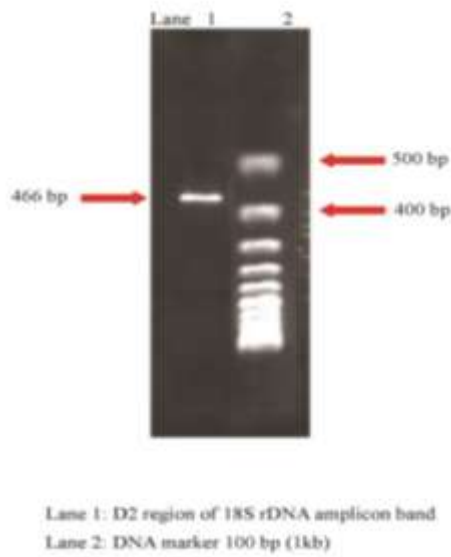


Fig 2: Phylogenetic tree of 18 rRNA of *T. harzianum* JX277056.1

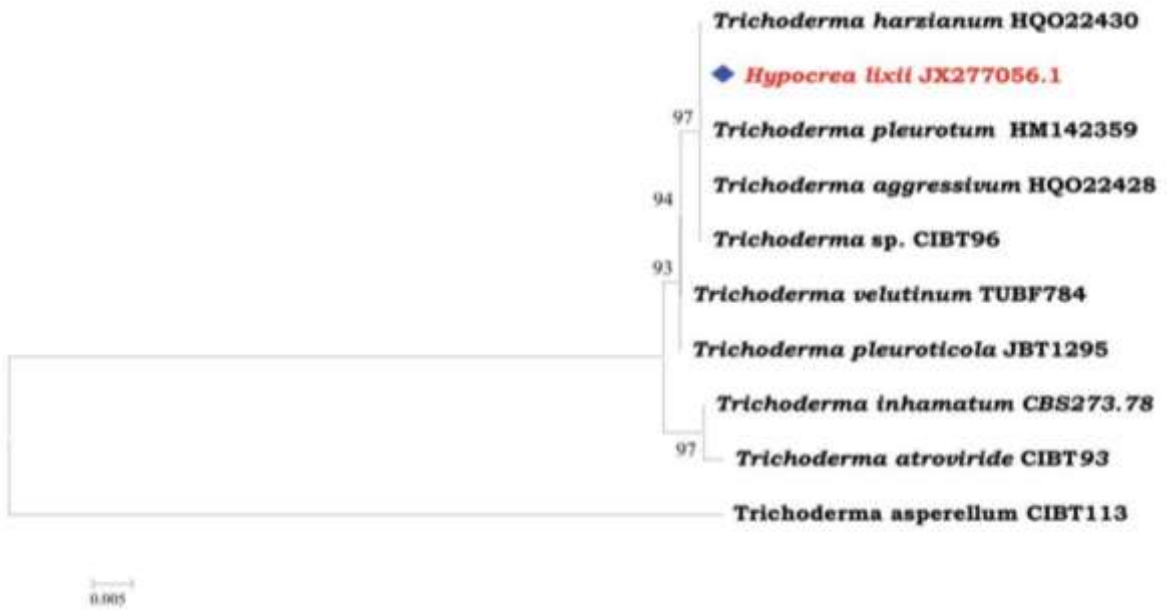


Fig 3: NCBI submission of *T. harizianum*



Fig 4: Secondary structure of 18S rRNA of *T. harzianum*

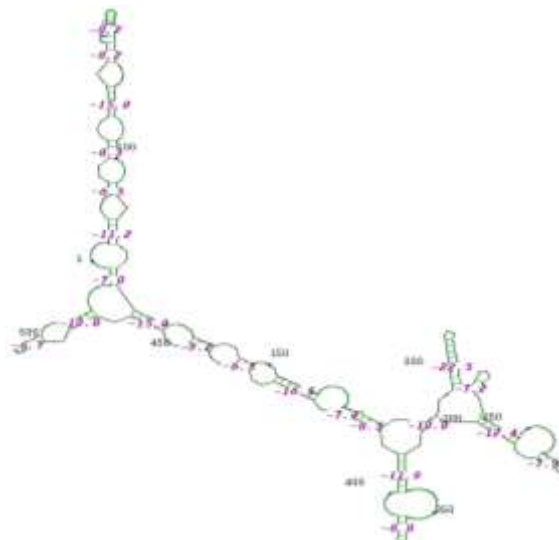
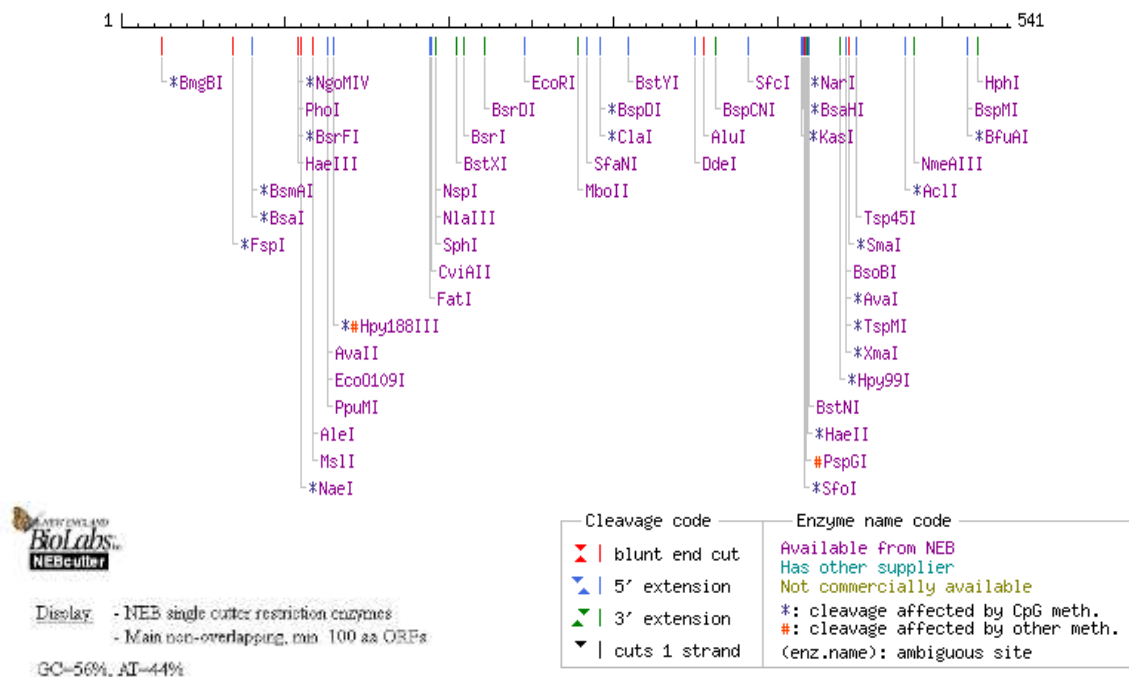


Fig 5: Restriction site analysis of 18S rRNA of *T. harzianum*



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