Comparative Alignment of Ribosomal Sequence of the Floral Waste Degrading Fungi: A Way to Establish the Evolutionary Correlation

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Abstract: This experiment was done to recognize the phylogenetic relationships among vermicomposting fungi by comparing the sequences of internal transcribed spacer regions (ITS) and 18S ribosomal DNA (rDNA) repeat unit. The ITS sequences of Aspergillus flavus, A. fumigatus, Alternaria alternata and A. terreus were amplified, determined and compared with each other. The sequence alignment was done to revealed the distance between the fungi. In the phylogenetic tree, the fungal strains generally divided into four groups. Result illustrate that fungal strain showed functional similarity which indicate their evolutionary closeness.

Keywords: Vermicomposting, fungi, evolutionary relationship, comparative analysis, MSA

1. INTRODUCTION

The most of the decomposing organisms are belong to group fungi and have long been used for decomposition of several types of organic wastes (Suberkropp & Klug, 1976; Pope et. al. 1963). Specially Aspergillus, Rhizopus, Penicillium, Alternaria species have been regarded as commercial decomposer for degrading municipal solid waste and agricultural waste. These fungi are known to secrete several types of enzymes (Abdullah & Taj-Aldeen, 1989; Zemek et.al., 1985). Several investigators have been reported that enzymes of fungi are commercially used in detergent industry, medicinal industry and paper industry. In view of floral waste degradation, Aspergillus and Alternaria species play an important role to decompose it and turn into manure. The commercial use of these fungi required their correct identification. The taxonomy and accurate detection of the decomposing fungi is a typical task. Generally, the classification of these fungi is based on the morphology of the fruiting body and their mycelia structure. Besides morphological traits of fruiting bodies, other special characteristics have also been investigated for the systematics of the fungi. A various culture studies were conducted to classify the fungi. Recently, molecular techniques such as DNA/DNA hybridization, electrophoretic karyotyping, RFLP, and DNA sequencing have been used for phylogenetic analysis of various kinds of organisms (Sasamua et al., 1998). PCR direct sequencing method reported by White et al. is an excellent method applicable to the fungi because it can be carried out using a small amount of starting material (White et al., 1990). Hirata et al. improved the method to determine the rDNA sequences of fungi using tiny amounts of material (Hirata and Takamatsu, 1996). Eukaryotic rDNA is composed of tandemly repeated clusters of 18S, 5.8S, and 28S rRNA genes, which are transcribed as a precursor molecule by RNA polymerase I (Raue and Planta, 1995). The external and internal spacer molecules are then removed in nucleolus before escaping for cytoplasm. The nucleotide sequences of the conservative rRNA coding regions have been widely used for phylogenetic analysis among families or distantly related genera (Berbee and Taylor, 1993; White et al., 1990). However, the variable ITS regions have an advantage of the phylogenetic analysis and identification of the closely related fungal species (Kim et al., 1999).

In this work, we studied 16S rDNA and ITS regions to infer their applicability for the systematics of floral decomposing fungi. The objective is to construct the phylogenetic relationship among the regions of ITS1 and ITS2, and 16S ribosomal RNA gene of Aspergillus and Alternaria species to compare the evolutionary correlation regarding to their function. To address these aims, we amplified
and sequenced the 16S rDNA, and ITS. Our results could reveal the detailed phylogenetic relationship among the closely related *Aspergillus* and *Alternaria* and related taxa.

2. **MATERIALS AND METHODS**

2.1. **Preparation of Fungal Isolates**

In the present study, four isolates i.e. *Aspergillus flavus*, *A. fumigatus*, *Alternaria alternata* and *A. terreus* were obtained from floral waste vermicomposting process done in Ujjain (M.P.), India. The test strains were cultured by shaking flask method done in 100 ml medium of Czepex dox broth medium at 28°C for seven days. The fungal mycelia were harvested by filtration, and stored at low temperature until they were used.

2.2. **Extraction of Fungal DNA**

Fungal DNA was extracted from each sample according to the miniprep protocol described by Cenis (1992) and Abd-Elsalam (2003). Fungal mycelium were filtered and suspended with 500 μl Tris-EDTA buffer. The mat was then transferred in 300 μl of extraction buffer for few min. 150μl of 3 M sodium acetate (pH 5.2) was added, and the mixture was cooled to 20°C for 10 min. After the lyses of mycelium, fungal debris was pelleted by centrifugation at 10,000 rpm for 5 min. The supernatant was taken into a fresh tube, and an equal volume of ice-cold isopropanol was added. DNA was then pelleted by centrifugation at 10,000 rpm for 10 min. Excess salt was removed by washing with 70% ethanol, and DNA was resuspended in Tris-EDTA.

2.3. **PCR Amplification of rDNA**

The ITS and the inverting 5.8S coding rDNA were amplified by PCR using the primers ITS described by White et al. (1990). The PCR profile was prepared at 95°C for 2 min, followed by 30 cycles of 94°C for 1 min, 54°C for 30 s, and 72°C for 1 min. The bands of interest were separated on agarose gel by electrophoresis. The isolated DNA excised from agarose gels and re-amplified by PCR using the same primer pair that was used for generating the ITS bands (Fig. 1).

2.4. **Fungal rDNA Sequencing**

The nuclear 18S rDNA region containing ITS gene was amplified by polymerase chain reaction from each strain. Primers were derived from the conserved region of 18S and 28S rDNA, respectively. PCR and sequencing was carried out on the behalf of BioAxis DNA research centre private limited. (MH.). The PCR products from the amplification were subjected to preparative electrophoresis in a agarose gel in buffer. All PCR products yielded only a single visible band. The PCR products were excised from the ethidium bromide-stained gel and purified using a gel elution kit. Direct sequencing of PCR products was done by a sequencer according to the standard protocol (Gyllensten, 1989; Hiraishi, 1992; Smith et al., 1986). Four primers, were used for sequencing in both directions and the DNA sequences were edited and assembled with the help of program.

2.5. **Molecular Phylogeny Analysis**

Multiple sequence alignment was obtained by using Clustal- W 2.1 program. CLUSTAL W is desired to provide an adequate alignment of a large number of more closely related sequences and a reliable indication of the domain structure of those sequences. Clustal W also has options for adding one or more additional sequences with weights or an alignment to an existing alignment (Higgins et al. 1996). It was used to generate the phylogenetic tree. Molecular phylogeny analysis (MPA) was carried out using the data set of complete genome of *Aspergillus fumigates* strain ATCC42826 YLL034C, *Aspergillus flavus* strain AF12, *Alternaria alternata* sp.L2785 18s, *Aspergillus terreus* strain MF12 using neighbor joining (NJ), Maximum likelihood (ML) and Maximum persimony (MP) method and trees are shown in fig. 2.

3. **RESULT AND DISCUSSION**

The obtained product of rDNA by agarose gel electrophoresis and DNA sequencing of PCR product revealed that amplified DNA was pure expected rDNA (Fig. 1). The alignment data of the DNA sequences of ITS, and 18S rDNA using CLUSTAL W were shown in Fig. 2. There is significant sequence variation in the ITS sequences and regions of the 18S rDNA. The sequence difference of different species in *Aspergillus* and *Alternaria* genus is supposed as the variation of cultural, geographical, environmental conditions, and gene variability. The sequence number 3 with 4 showed...
higher alignment score that was 62.31 while sequence 1 with 4 showed lowest score that was 39.77. By using Clustal W the following result were obtained in Fig.3 and Fig.4.

3.1. Fungal rDNA Sequencing

Sequence 1:
The given Fungal culture was identified Bacillus as Aspergillus fumigatus strain ATCC42826 YLL034C
Length: 615
Score: 1136 bits (615)

Sequence 2:
The given Fungal culture was identified as Apergillus flavus isolate AF13C
Length: 1216
Score: 2340 bits (1267)
Sequence 3:
The given Fungal culture was identified as Alternaria sp. L2745 18S ribosomal RNA gene, partial sequence
Length: 573
Score: 736 bits (398)

Sequence 4:
The given Fungal culture was identified as Aspergillus terreus strain MF12
Length: 535
Score: 976 bits (528)

3.2. CLUSTAL 2.1 Multiple Sequence Alignment
CLUSTAL 2.1 multiple sequence alignment

Comparative Alignment of Ribosomal Sequence of the Floral Waste Degrading Fungi: A Way to Establish the Evolutionary Correlation

<table>
<thead>
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<td>Alternaria alternata</td>
<td>--------------AACA------------------------------- 12</td>
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3.3. Show Multiple Sequence Alignments

Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: Alternariaalternata 398 bp
Sequence 2: Aspergillustereus 528 bp
Sequence 3: Aspergillusflavus 1216 bp
Sequence 4: Aspergillusfumigates 615 bp
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score: 71.36
Sequences (1:3) Aligned. Score: 71.86
Sequences (1:4) Aligned. Score: 59.80
Sequences (2:3) Aligned. Score: 68.75
Sequences (2:4) Aligned. Score: 47.35
Sequences (3:4) Aligned. Score: 62.93
Guide tree file created: [clustalw2-I20150415-073811-0437-26135157-oy.dnd]

There are 3 groups
Start of Multiple Alignment

Aligning...
Group 1: Sequences: 2  Score: 5498
Group 2: Sequences: 3  Score: 5178
Group 3: Sequences: 4  Score: 4472
Alignment Score 7341

CLUSTAL-Alignment file created [clustalw2-I20150415-073811-0437-26135157-oy.aln]

3.4. Phylogenetic Tree

Fig4. This is a Neighbour-joining tree with Branch length

The rRNA genes, act as most conservative sequence of the genome hence it is commonly used in identification and taxonomic studies. There were several researchers carried out study on different aspect to carry out study about evolutionary relations ship among similar functional group. Nazar et al. (1991), specifically worked within species of plant pathogens. O'Donnell (1992) found a surprising level of divergence for ITS sequences within the species of F. sambucinum. In our study we amplified the ITS primers and 18S rDNA gene. The amplified DNA was sequenced with sequencing primers to develop a genus/species specific PCR assay for the rapid identification of Aspergillus and Alternaria genuses isolated from floral vermicomposting wastes and also find out alignment score.
4. CONCLUSION

The nucleotide sequence analysis of rDNA region has been widely accepted to have phylogenetic significance, and is therefore useful in taxonomy and the study of phylogenetic relationships (Hibbett, 1992). This approach, designing primers from the rDNA region has far superior reliability compared to the use of random non-defined probes or primers. After using Clustal-W for multiple sequence alignment of fungal sequences we come to know that there were good correlations among all the four fungi because if alignment score is 40 or more then 40 then this score is called significant to each other and in our result the pair wise alignment score is more creditable. We got 7341 score in multiple sequence alignment which is a good result which means there is a characterization similarity between the species, it also shows good relationship among them and the evolutionary distance between them is also very less. Good MSA score also indicates that there are higher numbers of conserved regions which means less evolutionary distance between them and if number of conserved regions are very less than the evolutionary distance will increase.

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REFERENCES

PMID: 17846036


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Currently working as an Assistant Professor (Zoology), co-ordinator of Bioinformatics Govt. Madhav Science P.G. College, Ujjian (M.P.), having 30 year of teaching experience. She has received National and C.S.I.R fellowship. More than 25 research papers have been published in reputed national and international Journals. The field of research is Toxicology, Environmental Microbiology and Entomology. She has also attended the National and International conferences at Nepal, Bangkok, Mauritius and also received best paper presentation award in ICSTS(international conferences on Science and Technology for society) 2014 held at Bangkok. Recently she has completed her major Project Entitled “Comparative study of fungal DNA isolated from floral vermicomposting using DNA sequencing methods” Funded by MPCOST Bhopal.