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Research Article

Phylogenetic analysis and Predicted secondary structure of 5.8S gene in *Leptosphaerulina trifolii*

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ABSTRACT

Genomic DNA was isolated from the *Leptosphaerulina trifolii* using standard DNA extraction protocol. ITS region was amplified using universal primers ITS4 and ITS5 and then sequenced. 5.8S gene found to be highly conserved and length found 158 bp in addition, GC% was 48.1. In the present study the RNA secondary structure was predicted using program RNA structure software (version 5.6). The major domains of this structure are found to be highly preserved. Complementary base pairing forms hydrogen bonding which has created solid stems. The DG required for formation of the secondary structure of the 5.8S gene was -44.82 kcal/mol. At the DNA level, the motif M2 harbors an EcoRI restriction site, which is highly conserved in fungi and distinguishes between fungal and angiosperm. M2 motif which suggest that this motif play an important biological role in rRNA function. The nrITS region found to be strong phylogenetic marker. From phylogenetic tree it is infer that *Leptosphaerulina chartarum* and *Leptosphaerulina sp. Vega 582* are closely related and evolved from *Leptosphaerulina trifolli.*

Key words: Leptosphaerulina, nrITS, Phylogeny, RNA

INTRODUCTION

All living things can be classified into one of the five fundamental kingdoms of life, and the term fungus refers broadly to all members of the kingdom fungi. There are more than million species of fungi, but only about 400 cause diseases to man, animals and to the plants. Most fungi are associated with plants as saprotrophs and decomposers. These fungi break down organic matter of all kinds, including wood and other types of plant material. The term 'endophyte fungi' is defined as fungi which grow inside living plant tissues without causing disease symptoms (Petrini, 1991; Mostert et al, 2000; Stone et al, 2000; Sanchez-Marquez et al, 2007). *Leptosphaerulina trifolii* (Zaferanloo et al, 2013) is recognized as endophytic fungi and is very harmful and destroy Indian crops at great extent (Johansen, et al, 2000).

Leptosphaerulina trifolii is one of the disease causing fungal strain which has great adverse impact on Indian crops. As per International Crops Research Institute for the Semi -Arid Tropics (Andhra Pradesh, India), important crops grown in the Indo-Gangetic Plain region of South Asia are rice (*Oryza sativa L.)*, maize (*Zea mays L.*), wheat (*Triticumaestivum L.*), barley (*Hordeumvulgare L.*), black gram (*Vignamungo (L.) Hepper*), mung bean (*Vignaradiata (L.) Wilczek*), pea (*Pisumsativum L.*) and groundnut (*Arachishypogaea L.*). In other hand, most important cereals of India like Moong bean (*Vignaradiata (L.) Wilczek*), Black gram (*Vignamungo L.*) and Khesari (*Lathyrussativus L.;lathyrus; grass pea*), are greatly affected by *Leptosphaerulina trifolii*.(Johansen, et al, 2000).

Ribosomal RNA (rRNA) is by far the most predominant product of transcription, constituting 80-90% of the total mass of cellular RNA, in both prokaryotes and eukaryotes. It is an important component of ribosomes, the protein biosynthetic machinery. In eukaryotes the large subunit (60S) consists of three rRNA molecules (5S, 5.8S and 28S) and 50 proteins.

ITS1 and ITS2 were studied not only for phylogenetics and taxonomy, but also analyzed for development of diagnostic strategies for species identification in medicine and ecology (Pinto, et al 2004, Anderson et al 2007). Internal transcribed spacer region is found to be a good phylogenetic marker. So we have focused to reveal evolutionary history of *Leptosphaerulina trifolli*. In phylogenetic tree ITS 1 and ITS 2 can be used for distantly related

species whereas 5.8S gene can be used for closely related species.

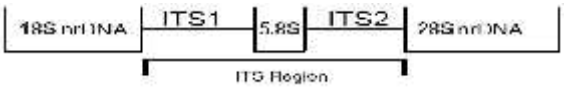

Fig 1: Internal transcribed spacer region of ribosomal RNA

rRNA genes are mainly common targets for identifying and quantifying phylotypes in medical and environmental samples and have been widely used in systematic studies in fungi and beyond. Literature survey reveals that secondary structures of the initial transcript play crucial roles in ribosome assembly, and putative secondary structures have been recognized and archived for the coding regions of rDNA and recently for the internal spacers as well (Wolf et al. 2005). Characters from nuclear ribosomal gene sequences have been used to hypothesize phylogenetic relationships among even distantly related organisms.Molecular phylogenetic analyses require the alignment of homologous sequence characters, guidance from secondary structure information may aid in the alignment of homologous regions for phylogenetic analysis among plant and animal species (Jobes & Thien 1997), even between genomes as evolutionarily distant as of eukaryotic nuclei, prokaryotes, and eukaryotic organelles. A further application of structure information to phylogenetics is recoding structure into new ITS regions have been used for phylogenetic analyses at the species to generic level.

MATERIALS AND METHOD

Pure culture of *Leptosphaerulina trifolii* was obtained from National Fungal Culture Collection of India, Agharkar Research Institute, Pune, India. The isolate cultures were maintained on potato dextrose agar (PDA). Fungal cultures were raised by using actively growing fungal plugs from mother cultures with a sterile scalpel and then placed on fresh PDA media (39 g of PDA/ 1 liter of distilled water). The cultures were then placed on laboratory benches and left to grow for about 7-10 days or until there was enough mycelia to harvest for DNA extraction.

Whole-cell DNA was isolated from *Leptosphaerulina trifolii* by the Chelex method (Walsh et al., 1991; Hirata and Takamatsu, 1996). Primer pairs ITS1 (50-TCC GTA GGT GAA CCT GCG- 30) and ITS4 (50-TCC TCC GCT TAT TGA TAT GC-30) were used for PCR amplification of rDNA containing the internal transcribed spacer (ITS) 1, the 5·8S gene and the ITS2 regions (Jasalavich*et al.*, 1995). For amplification of ITS region, the composition of the PCR reaction mixture is as follows. The reaction mixture (25 µl) contained 20 mM Tris-HCl (pH 8.8), 10 mMKCl, 10 mM (NH4)2SO4, 2 mM MgSO4, 0.1 % TritonX-100, 0.2 mM of each dNTP, 0.2 µM of each oligonucleotide primer, 1.0 Unit of Taq DNA polymerase and 25 ng of genomic DNA. The basic thermal cycling conditions for the above-mentioned regions were similar: One step of initial denaturation at 94^{o}C for 5 min, 35 cycles of denaturation at 94^{o}C for 1 min, annealing (55^{o}C) for 1 min and extension at 72^{o}C for 1 min, followed by final extension of 10 min at 72^{o}C. The PCR product was subjected to preparative electrophoresis in 1.5% agarose gel in TAE buffer. The amplified product was checked on the ethidium bromide-stained gel. ITS sequencing was carried out on ABI Sequencer (Chromous Biotech, Bangalore) with minor manual adjustments. The sequence of ITS region of *Leptoshaerulina trifolii* was submitted to NCBI genbank. The sequence was accepted by the organization and accession number allotted is KJ190940. ITS sequence was compared by using the BLAST alignment program with data available from GenBank at the National Institutes of Health.

RESULTS

The 5.8S gene of *Leptosphaerulina trifolii* found to be 158 bp and GC% was calculated and found to be 48.1%. The length of ITS 1 and ITS 2 region was found 204 bp and 156 bp respectively in *Leptosphaerulina trifolii* whereas GC% found 55.4% and 63.5% respectively.

In ITS1 and ITS2 regions of *Leptosphaerulina trifolii*, gaps and dots were found which indicates that there is an insertion and deletion of the sequences (Indels).The entire ITS region was used to find identical sequences in NCBI genbank using BLAST (Basic Local Alignment Search Tool). In BLAST, 100% of 5.8S gene sequence was found identical to *Leptosphaerulina chartarum*whereas 96% of entire ITS region of *Leptosphaerulina trifolii* was found identical with *Leptosphaerulina chartarum*, which has proved that 5.8S gene sequence can be used for exact identification of fungal species which causes disease to important crop plants, food products and ultimately affect country's economy. In *Leptosphaerulina trifolii*, the length of 5.8S gene was found 158 bp. Out of these 158 residues only at two residues variation was found, all other 156 residues found highly conserved.

5.8S ribosomal RNA (5.8S rRNA) is a non-coding RNA component of the large subunit of the eukaryoticribosome and so plays an important role in protein translation. It is transcribed by RNA polymerase I as part of the 45S precursor that also contains 18S and 28S rRNA. Its function is thought to be in 5.8S rRNA ribosome translocation. It is also known to form covalent linkage to the p53 tumour

suppressor protein. 5.8S rRNA is also found in archaea.The internal transcribed spacer regions and the 5.8S rDNA were defined based on the conserved sequence at the 3'end of the 18S gene, the 5' and 3' ends of the 5.8S gene, and the 5' end of the 26S gene. Major domains of 5.8S gene structure in *Leptosphaerulina trifolli* is found to be highly preserved. Complementary base pairing forms hydrogen bonding which has created solid stems. The dG (sequence/folding energy) was found -44.82 kcal/mol. The secondary structure for a functional 5.8S gene has single large central loops from which four helices emerge. Conserved motifs for the 5.8S gene are poorly described in fungi. However, three motifs of the 5.8S gene are conserved among angiosperms are M1 (5'-CGAUGAAGAACGUAGC-3'), M2 (5'-GAAUUGCAGAAUCC-3') and M3 (5'-UUUGAACGCA-3') (Harpke et al 2008). In *Leptosphaerulina trifolli* one motif was found as highly conserved that is M2 (5'-GAATTGCA**GAATTC**-3'). The motif M2 has an EcoRI restriction site (highlighted) is highly conserved in fungi which distinguishes between fungal and angiosperm based on M2 motif (Jobes et al 1997), which suggest that this motif play an important biological role in rRNA function.

To construct phylogenetic tree ClustalW online software was used. Other ITS sequences of genus Leptosphaerulina were retrieved from NCBI genbank. From phylogenetic tree it is infer that *Leptosphaerulina chartarum* and *Leptosphaerulina sp. Vega 582* are closely related and evolved from *Leptosphaerulina trifolli.* These three species has form one monophyletic group. Other monophyletic group infer that *Leptosphaerulina americana* and *Leptosphaerulina trifolii isolate 572* are closely related whereas *Leptosphaerulina australis* and *Leptosphaerulina arachidicola* are also closely related. These four species has evolved from *Leptosphaerulina isolate KK-76-2.*

DISCUSSION

As fungi is uncertainly the best studied kingdom in eukaryotic organism. Hence there is enormous potential to combine molecular and morphological data for accurate identification of fungal species and also to resolve any type of controversy in species identification. Plant and fungi kingdoms have some common characteristics. First, fungi and plants are both eukaryotic, meaning they belong to the Eukaryotic organism with cells contain a nucleus and membrane-bound organelles. Organism belongs to both Kingdoms are having cell walls, are stationary, and are typically multicellular, which means they are made up of multiple cells. Hence in earlier studies plants and fungi used to be grouped together. However, three motifs of the 5.8S gene are conserved among angiosperms are M1 (5'-CGAUGAAGAACGUAGC-3'), M2 (5'-GAAUUGCAGAAUCC-3') and M3 (5'-UUUGAACGCA-3') (Harpke et al 2008). In *Leptosphaerulina trifolli*, one motif was found as highly conserved that is M2 (5'-GAATTGCA**GAATTC**-3'). The motif M2 has an EcoRI restriction site (underlined) is highly conserved in fungi which clearly distinguishes between fungal and angiosperm based on M2 motif (Jobes et al 1997), which gives very strong support in systematic classification and also suggest that this motif play an important biological role in rRNA function.

ITS is a section of non-functional RNA located on a common transcription precursor between rRNAs (structural ribosomal RNAs). This ITS region contains ITS1 and ITS2 informative regions that are located between the 18S and 28S ribosomal subunits and the ITS1 and ITS2 regions are separated by the 5.8S ribosomal subunit. By the use of amplicon around 400 to 700 bp in length, the ITS region can be amplified from fungal genome with primers ITS-1 and ITS-4, which could be recovered in a single PCR.

Table 1
Length and GC% of ITS1, 5.8S gene and ITS2 in *Leptosphaerulinatrifolii.*

Species Name		***ITS 1***	***5.8S gene***	***ITS 2***
Leptosphaerulina trifolii	**Length**	204 bp	158 bp	156 bp
	GC%	55.4%	48.1%	63.5%

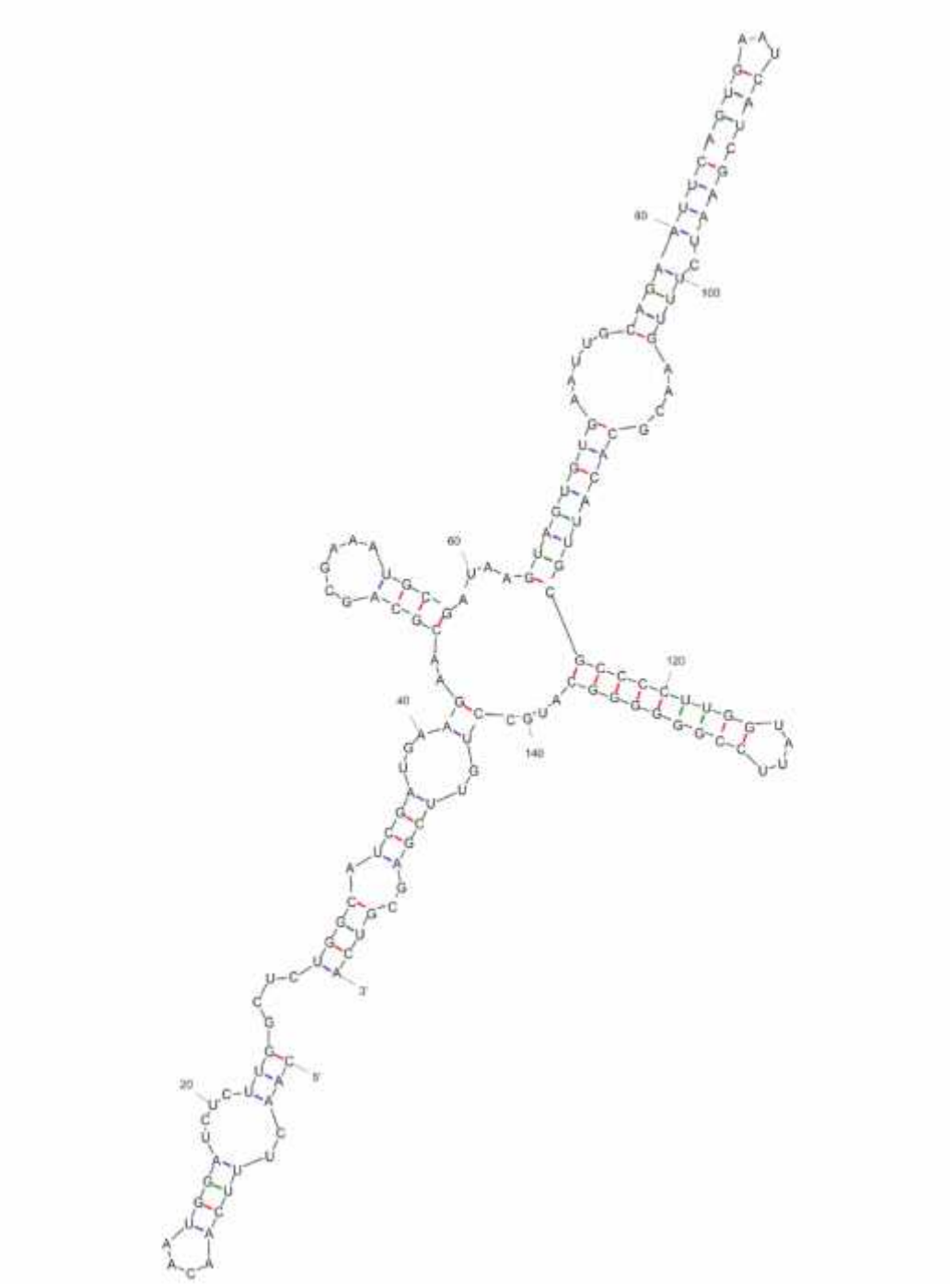

Fig 2
Predicted Secondary structure of 5.8S gene of *Leptosphaerulina trifolli.*

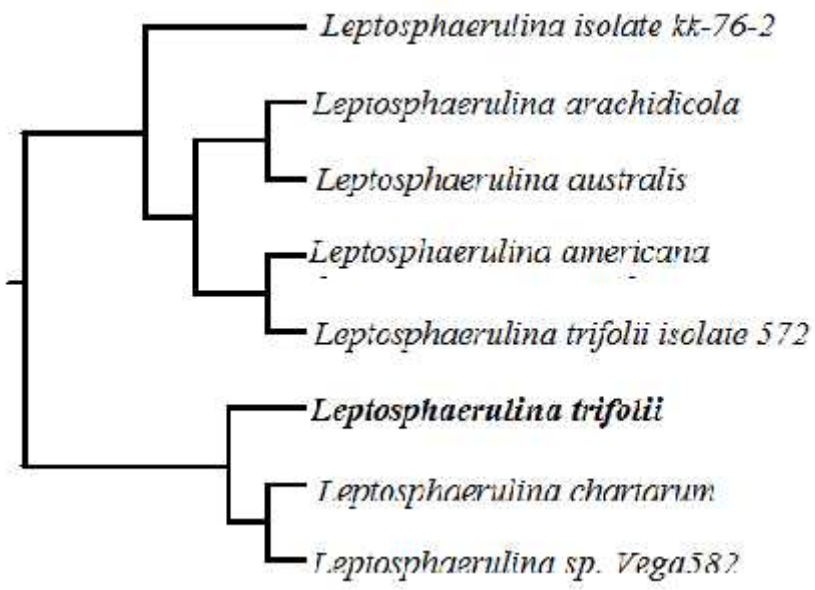

Fig 3
Phylogenetic analysis of *Leptosphaerulina trifolii*

The universal primers were first reported by Trost and colleagues, who further developed a method based on the enzyme restriction of PCR fragments in order to identify a set of fungal pathogens (Trost et al., 2004). Molecular characterization of 5.8S gene is very important to understand the biochemical pathways as this gene is concerned with ribosome production and associated with protein synthesis. For this bioinformatics approach and computational softwares illustrate potential benefits for sequence analysis, sequence alignment, to locate mutations in gene (if any) and also to predict secondary structure of genes involved in biochemical pathway. The secondary structure (stem loop structure) of 5.8S gene of both Leptosphaerulina trifolii and Puccinia graminis f.sp.tritici was estimated online using RNA structure software (version 5.6) (Stern et al 2013). Here in the present study phylogeny of *Leptosphaerulina trifolli* has been carried out. In which, *Leptosphaerulina chartarum* and *Leptosphaerulina sp. Vega 582* are closely related and evolved from *Leptosphaerulina trifolli*, which has given accurate additional support to the research work in identification of fungal species. The technique is cost-effective since it only requires essentially PCR components and DNA isolation reagents. Furthermore, no DNA probes or expensive restriction enzymes are needed. Taking all these parameters into account, It has been proposed that ITS region along with 5.8S gene sequence is the standard barcode for fungal identification. Hence ITS region has been recommended as the universal barcode sequence for fungal study because it is the most widely sequenced DNA region in molecular phylogeny of fungi. Also, it is the most useful method in systematic identification of species or even within species at a molecular level, for example in identifying geographic races.

CONCLUSION

The study reveals that ITS sequencing is a very fast, accurate and sensitive method for pathogenic fungal identification. As sequencing method required only short term incubation time, it is more convenient and better than the traditional phenotypic method. Due to the large amount of fungal genome copies and species variation, the sensitivity and specificity of ITS regions is good enough to identify fungal isolates to species level. ITS region along with 5.8S gene sequence can act as the DNA barcode for pathogenic fungi which affects the important crops of the nation. The rapid and accurate identification of fungal isolates helps shorter the waiting time on treatments for patients. Hence it will benefit to the clinical diagnosis, medical care and in accurate identification of fungi which causes disease to the human being.

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