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PHYLOGENETIC ANALYSIS AND PREDICTED SECONDARY STRUCTURE OF 5.8S GENE IN *PUCCINIA GRAMINIS F. SP. TRITICI*

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ABSTRACT

Genomic DNA was isolated from the *Puccinia graminis f.sp.tritici* 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 155 bp in addition, GC% was 35.5. 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 -32.40 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 *Puccinia graminis f. sp. tritici* is closely related to *Puccinia helianthi isolate 1* and strain *NM-1*. These three species has evolved from *Puccinia caricina.*

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). *Puccinia graminis f. sp. tritici* (Zaferanloo et al, 2013) is recognized as endophytic fungi and is very harmful and destroy Indian crops at great extent (Johansen, et al, 2000).

In India, *Puccinia graminis f. sp. tritici* affects North West plain zone which is most important wheat growing area in India. Around 10 million hectares land is under wheat cultivation in this zone. It is considered as back-bone of the country's food security system. Any production threat in North West plain zone will have serious implications on Indian crop production (Ahlawat et al, 2007). Same way barley and ground nut are also affected by *Puccinia graminis f. sp. tritici* (Olivera et al, 2012).

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.

In fungi, ITS1 and ITS2 were studied not only with regard to phylogenetics and taxonomy, but also in connection to 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 *Puccinia graminis f.sp.tritici*. 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.

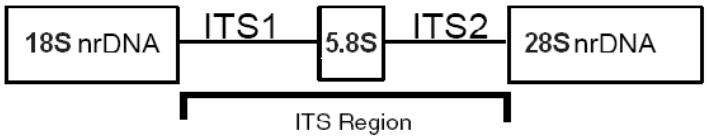

Fig 1: Internal transcribed spacer region of ribosomal RNA

rRNA genes have been widely used in systematic studies in fungi and beyond, and are common targets for identifying and quantifying phylotypes in medical and environmental samples. The 'coding' (coding as coding for RNA) SSU rRNA and LSU rRNA genes are

highly conserved. Evidence suggests that secondary structures of the initial transcript play important roles in ribosome assembly, and putative secondary structures have long 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 for hypotheses of phylogenetic relationships among even distantly related organisms. Molecular phylogenetic analyses require the alignment of homologous sequence characters, and 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.

MATERIAL AND METHOD

Pure culture of *Puccinia graminis f.sp.tritici* 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 *Puccinia graminis f. sp. tritici* 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 mM KCl, 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 *Puccinia graminis f.sp.tritici* was submitted to NCBI genbank. The sequence was accepted by the organization and accession number allotted is KJ190941. 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 *Puccinia graminis f.sp.tritici* found to be 155 bp and GC% was calculated and found to be 35.5%. The length of ITS 1 and ITS 2 region was found 183 bp and 196 bp respectively in *Puccinia graminis f.sp.tritici* whereas GC% found 30.6% and 31.1% respectively.

Table 1: Length and GC% of ITS1, 5.8S gene and ITS2 in *Puccinia graminis f. sp. tritici*

Species Name		***ITS 1***	***5.8S gene***	***ITS 2***
Puccinia graminis f. sp. tritici	**Length**	183 bp	155 bp	196 bp
	GC%	30.6%	35.5%	31.1%

In ITS1 and ITS2 regions of *Puccinia graminis f. sp. tritici*, 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, 98% of 5.8S gene sequence was found identical to *Puccinia helianthi* whereas 88% of entire ITS region of *Puccinia graminis f. sp. tritici* was found identical with *Puccinia helianthi*, 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 *Puccinia graminis f. sp. tritici*, the length of 5.8S gene was found 155 bp. Out of these 155 residues only at three residues variation was found, all other 152 residues found highly conserved.

5.8S ribosomal RNA (5.8S rRNA) is a non-coding RNA component of the large subunit of the eukaryotic ribosome 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.

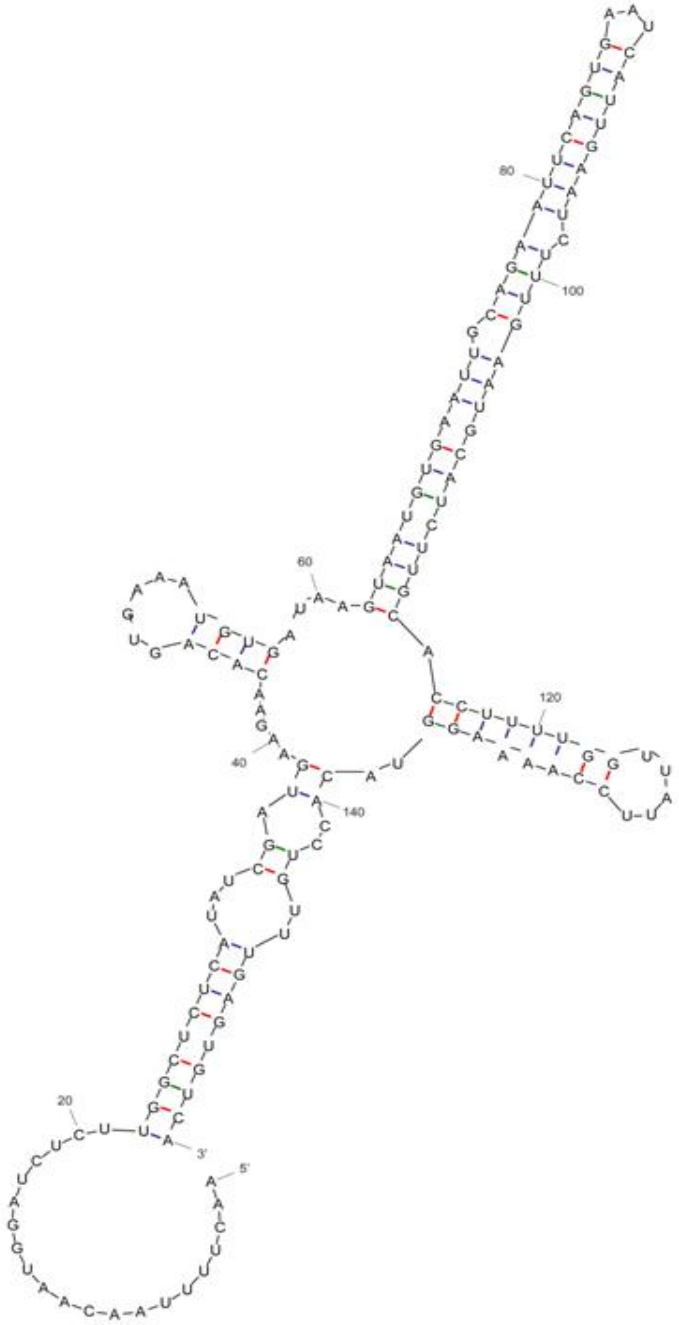

Fig 2: Predicted Secondary structure of 5.8S gene of *Puccinia graminis f.sp.tritici.*

Major domains of 5.8S gene structure in *Puccinia graminis f.sp.tritici* is found to be highly preserved. Complementary base pairing forms hydrogen bonding which has created solid stems. The dG (sequence/folding energy) was found -32.40 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 *Puccinia graminis f.sp.tritici* 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.

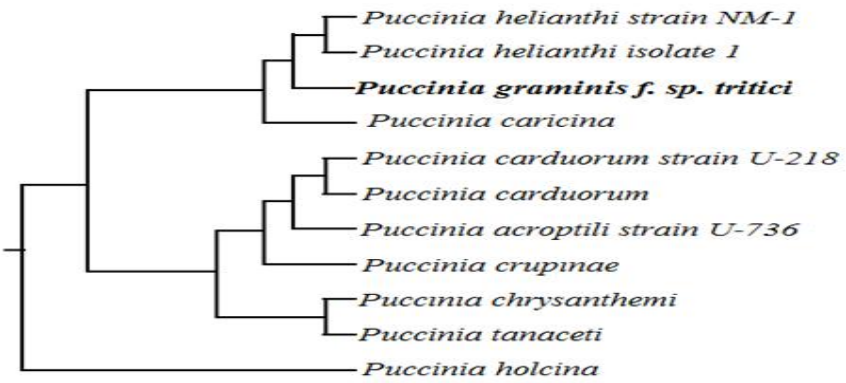

Fig 3: Phylogenetic analysis of *Puccinia graminis f. sp. tritici*

To construct phylogenetic tree ClustalW online software was used. Other ITS sequences of genus Puccinia were retrieved from NCBI genbank. From phylogenetic tree it is infer that *Puccinia graminis f. sp.* tritici is closely related to *Puccinia helianthi isolate 1* and strain *NM-1*. These three species has evolved from *Puccinia caricina. Puccinia carduorum, Puccinia acroptili and Puccinia crupinae* has form one monophyletic group whereas *Puccinia chrysanthemi* and *Puccinia tanaceti* has form other monophyletic group. All species has evolved from *Puccinia holcina.*

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, they are both eukaryotic, meaning they belong to the Eukarya domain and that their cells contain a nucleus and membrane-bound organelles. Both of them also have cell walls, are stationary, and are typically multicellular, which means they are made 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 *Puccinia graminis f.sp.tritici*, 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.

Here in the present study phylogeny of *Puccinia graminis f.sp.tritici* has been carried out. In which, *Puccinia graminis f. sp. tritici* is closely related to *Puccinia helianthi isolate 1* and strain *NM-1*. These three species has evolved from *Puccinia caricina.* 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.

CONCLUSION

To conclude, 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 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 on 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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