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Phylogenetic and Secondary Structure analysis of rice false smut (*Ustilaginoidea virens*) fungal isolates using internal transcribed spacer ITS1, 5.8S and ITS2

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Abstract
False smut of rice is a fungal disease which converts florets in to yellow colored smut balls, once it was considered as minor disease, has of now it become a serious pathogen in almost all rice growing areas in the world. The disease incidence has been reported at many places in an alarming proportion. However, ambiguity in morphotyping of destructive pathogen which are of quarantine importance is impacted by a number of factors that negatively affect identification. Secondary structural information of the rRNA may be considered to be a conserved region which can be used as supplementary information for possible fungal identification. In the present study the known (four) and query sequences from NCBI (21) were subjected to sequence analyses for their ITS regions. Significant variation was observed for hairpins loops, exterior loop, interior loop, multi loop and bulged loops in all the sequences under investigation.

Keywords: Rice False smut, Phylogenetic, internal transcribed spacer

Introduction
The status of rice false smut (RFSm) as an emerging fungal disease (anamorph: *Ustilaginoidea virens* (Cooke) Takah.; teleomorph *Villosiclava virens* (Nakata) of rice has been recognized worldwide (Atia, 2004; Brooks *et al*., 2009; Ashizawa *et al*., 2010; Li *et al*., 2013; Singh *et al*., 2014; Nessa *et al*., 2015) [3, 5, 2, 13, 21, 15]. There is a inadequacies in conventional morphotyping of fungal systematics owing to the complicated life styles and discriminating morphological characters. However, correct identification is important for biosecurity and quarantine reasons, and for development of more targeted integrated disease management.
The ITS region is the most widely sequenced DNA region in molecular ecology of fungi (Peay *et al*., 2008) [18] and has typically been most useful for molecular systematics at the species level, and even within species (identify geographic races). Because of its higher degree of variation than other genic regions of rDNA (for small and large subunit rRNA), variation among individual rDNA repeats can sometimes be observed within the ITS regions. In addition to the standard ITS1+ITS4 primers (White *et al*., 1990) [23] used by most labs, several taxon specific primers have been described that allow selective amplification of fungal sequences (Gardes and Bruns 1993) [10].
Internal transcribed spacer (ITS) refers to the spacer DNA situated between the small subunit ribosomal RNA (rRNA) and large subunit rRNA genes in the chromosome. The fungal ITS gene cluster consists internal transcribed spacer 1 (ITS1), the 5.8S rDNA exon, internal transcribed spacer 2 (ITS2) (Figure 1). It is a multigene family with the potential for variation among the tandem repeats. Polymorphisms are not uniformly distributed across the ITS array. The 5.8S gene sequence is highly conserved but the ITS1 and ITS2 sequences are more variable and are highly polymorphic depending on the fungal species (Hillis and Dixon 1991; Coleman 2007; Nilsson *et al*. 2008) [12]. In addition to being widely used for phylogenetic inference and in systematics, the ITS region is the formal fungal barcode and is the primary choice for molecular identification of fungi from a number of sources (Schoch *et al*. 2012).
Secondary structure prediction is advantageous for species identification because it allows detection of sequencing errors, pseudogenes and genetic footprints indicative of past hybridization events (Coleman 2009) [7]. Accordingly, structural information can offer supplementary information for species identification (Coleman 2003, 2007) [8, 9].
This is the first study to investigate the potential use of ITS1, 5.8S and ITS2 secondary structure prediction towards identification of isolates of *U. virens*. The main objectives of this study was to assess the nature of polymorphisms that may accumulated in the ITS1, 5.8S and ITS2 region and predict the phylogenetic reconstruction.

Materials and method

Sampling and isolation of *U. virens*

The samples of typical false smut on rice were collected from different geographical regions *viz*., Paddy Breeding Station (Tamil Nadu Agricultural University, Coimbatore, 11.16ºN and 76.58ºE, 26.3 ºC), Hybrid Rice Evaluation Centre (HREC, Gudalur, 11.50ºN and 76.08ºE, 21.9 ºC) Farmers fields of Thanjavur, 10.47ºN and 79.8ºE, 30 ºC and Hyderabad (Indian Institute of Rice Research, 17.37ºN and 78.48ºE, 26.7 ºC) during 2016, site of sample collection depicted on map (Figure 2). Isolations were made from the diseased samples, initially the smutted balls were thoroughly washed with running tap water and surface sterilized with 0.1 per cent mercuric chloride solution for 1 minute and subsequently washed three times with sterile distilled water. Using a sterilized needle, the mass of chlamydospores was streaked onto Petri dishes containing potato sucrose agar (PSA) medium (peeled potato 200 g, sucrose 20 g and agar 20 g in 1000 ml distilled water) under complete sterile and aseptic conditions. To check the bacterial contamination, the medium was incorporated with streptomycin (100 ppm). The Petri dishes were incubated in BOD incubator at 25 ± 2^{0}C for 2 weeks for obtaining fungal growth. A single, well isolated colony of the fungus (arising from a single spore) was picked up using sterilised needle and transferred to the fresh PSA slants and maintained as a pure culture. Finally, freshly obtained 4 isolates were purified and maintained on PSA medium at 4 °C. Thus, the purified cultures were maintained by periodical transfers on PSA slants and used for further studies. The cultures were identified according to cultural descriptions given by Sharma and Joshi (1975) [20] and Verma and Singh (1987) [22] (Figure 3). The isolates were designated as TNUv1 (HYDERABAD), TNUv2 (COIMBATORE), TNUv4 (GUDALUR) and TNUv5 (THANJAVUR). These isolates were further subjected to molecular characterization.

Molecular identification, DNA sequence alignment and phylogenetic analysis.

Genomic DNA was extracted from the fungal isolates by the protocol described by Nakada *et al* (1994) [14]. Concentrations of DNA were estimated by measuring absorbance at 260 nm with Nanodrop (GENOVA NANO, UK). DNA samples were diluted to working solutions of 30 ng/ µl and stored at 4 °C until use. The primers ITS1 F (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 R (5′-TCCTCCGCTTATTGATATGC-3′) were used for specific amplification of ITS region of the fungal isolates. Amplifications were performed in a total volume of 25 µl containing 0.5 µl dNTPs mix (10mM), 1 µl primer (12.5 pmol), 2 ml of DNA (30 ng/ µl), 1.2 µl MgCl2, 1.5 µl reaction buffer (10X), 0.3 µl of *Taq* polymerase (1.5 U) and 18.5 µl deionized water. The DNA amplification was done in a thermal cycler (BioRad, USA) using the following PCR cycles. The first denaturation step of 2 minutes at 94 °C, followed by 35 cycles of denaturation for 30 sec at 94 °C, annealing for 1 min at 40 °C, extension at 72 °C for 2 min and final extension at 72 °C for 5 min with holding temperature at 4 °C for 10 min. Reaction products (8 µl) were resolved by electrophoresis in 1.2% agarose gels stained with ethidium bromide in 1X TBE buffer at 100 V for 90 min.

After electrophoresis the gel slice along with PCR fragments were excised with a clean, sharp scalpel. Then, they were purified using QIAquick Gel Purification Kit (QIAGEN, California) according to the manufacturer's protocol. The purified DNA from ITS-PCR was given for sequencing to Bioserve solutions, Hyderabad. The partial sequence of rDNA was obtained and it was submitted to the National Centre for Biotechnology Information (NCBI), Gene Bank, New York, USA and accession numbers were obtained (KX421100, KX421101, KX421102 and KX421103).

Secondary structure prediction, phylogenetic analysis

Secondary structures were predicted for 21 query (downloaded from genbank NCBI) and 4 known isolates for ITS region using Mfold programme (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) with default conditions (linear RNA sequence, folding temperature: 37 °C, 1M NaCl (no divalent ions) ionic conditions, 5% sub-optimality, upper bound number of the folding: 50, maximum interior/bulge loop size: 30, maximum asymmetry of an interior/bulge loop: 30, maximum distance between paired bases: no limit). The inferred structure was subsequently examined manually for the total number of paired bases, number of total hairpins loops, exterior loop, interior loop, multi loop and bulged loops. GC content is known to influence structural energy, since GC percentage was determined. Energy levels of presumptive secondary structures were then calculated with Mfold. ITS sequences of our isolates and query sequences were used for phylogenetic analysis (Neighbor-joining method with 1000 bootstrap replication) using MEGA 7.0.18.

Results

Molecular characterization and phylogenety analysis

The sequence data of ITS1, 5.8S and ITS2 of all the isolates (Query and isolates collected from different geographical locations) were used to construct dendrogram using MEGA 7.0.18. Phylogenetic profile was constructed based on the sequence data sets which resulted in well separated clades in both the method of neighbor joining and maximum parsimony. In both the methods the dendrogram topology is similar and clades are formed in the similar pattern. The isolates *viz*., TNUv1, TNUv4, and TNUv5 were grouped in the same clade in both the method of phylogenety analysis, whereas TNUv2 falls under different clade (Fig. 5). The analysis revealed that, the clade relationship existed among the isolates collected from different geographical origin and query registered at NCBI.

Secondary structure

In this present investigation, ITS1, 5.8S and ITS2 region of four *U. virens* isolates were amplified using ITS1 and ITS4 region specific markers, and blast analysis showed that 91-99% of sequence similarity with sequences of *U. virens* available at NCBI. The ITS1, 5.8S and ITS2 sequences were retrieved from NCBI for other 21 *U. virens* sequences. The length of the region varied from isolate to isolate and it was ranged from 610 to 678. Using sequence data, secondary structure of rRNA was constructed with the help of Mfold programme, mean of secondary structure was 22.48 with the maximum of 27 and the minimum of 18 respectively. The mean for lowest energy structure for the region was -242.77 kcal/mol, the maximum and minimum of lower energy was -231.5 kcal/mol and 262.6 kcal/mol respectively (Table. 1). Among conserved loops in secondary structure *viz*., interior loop, exterior loop, bulge loop, multi loop and hairpin loops (Fig. 6) hairpin loops ranged from 7 to 13, interior loops ranged from 11 to 21, bulged loops varied from 4-13, multi loops varied from 4-11 and exterior loop did not showed any variation (Fig 4). Hence, the information reported by the

secondary structure of ITS region would help to resolve the relationship among the races in the same species of *U. virens* under studied.

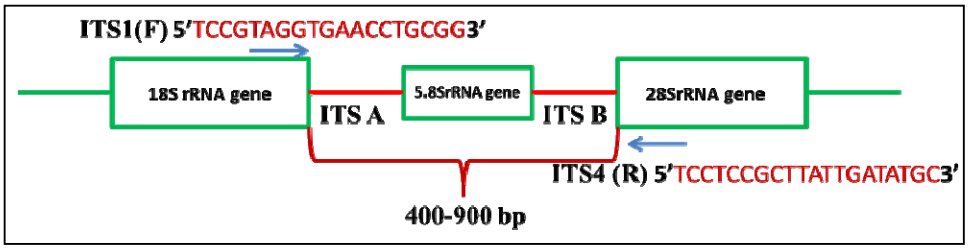

Fig 1: Diagrammatic representation of fungal rDNA gene cluster: the internal transcribed sequences ITS A and ITS B separates the 18S, 5.8S and 28S ribosomal RNA subunits, which are sliced after transcription.

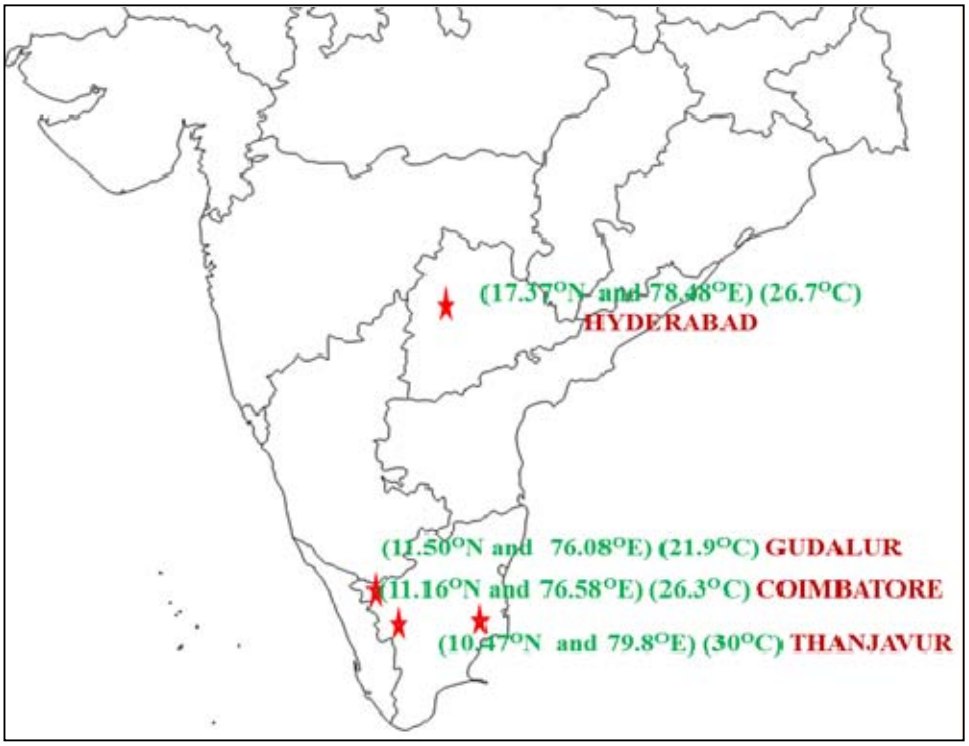

Fig 2: Sites of sample collection, geographical location and annual mean temperature

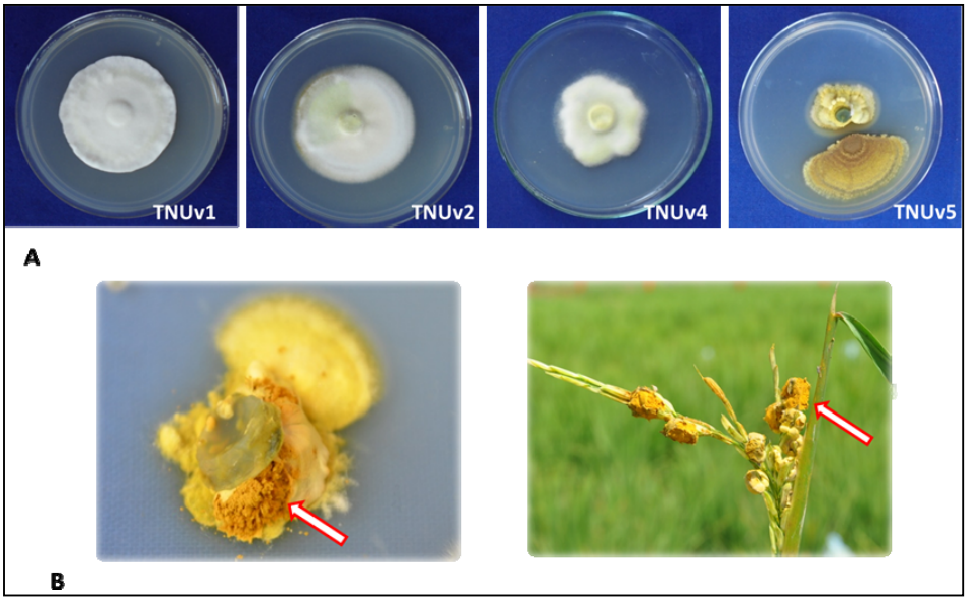

Fig 3: A) Morphology of isolates from different geographical location at 25th day. B) Showing typical and similar pattern of burst opening of clamydospores both in lab and field condition.

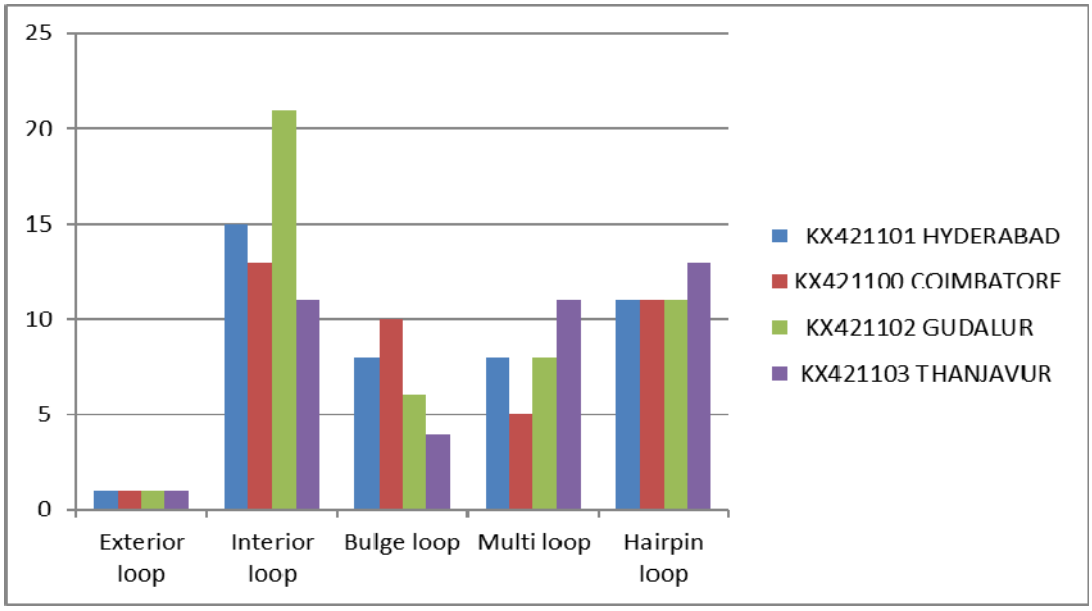

Fig 4: Variation in formation of loops in secondary structure prediction of ITS region among four isolates of *U. Virens*

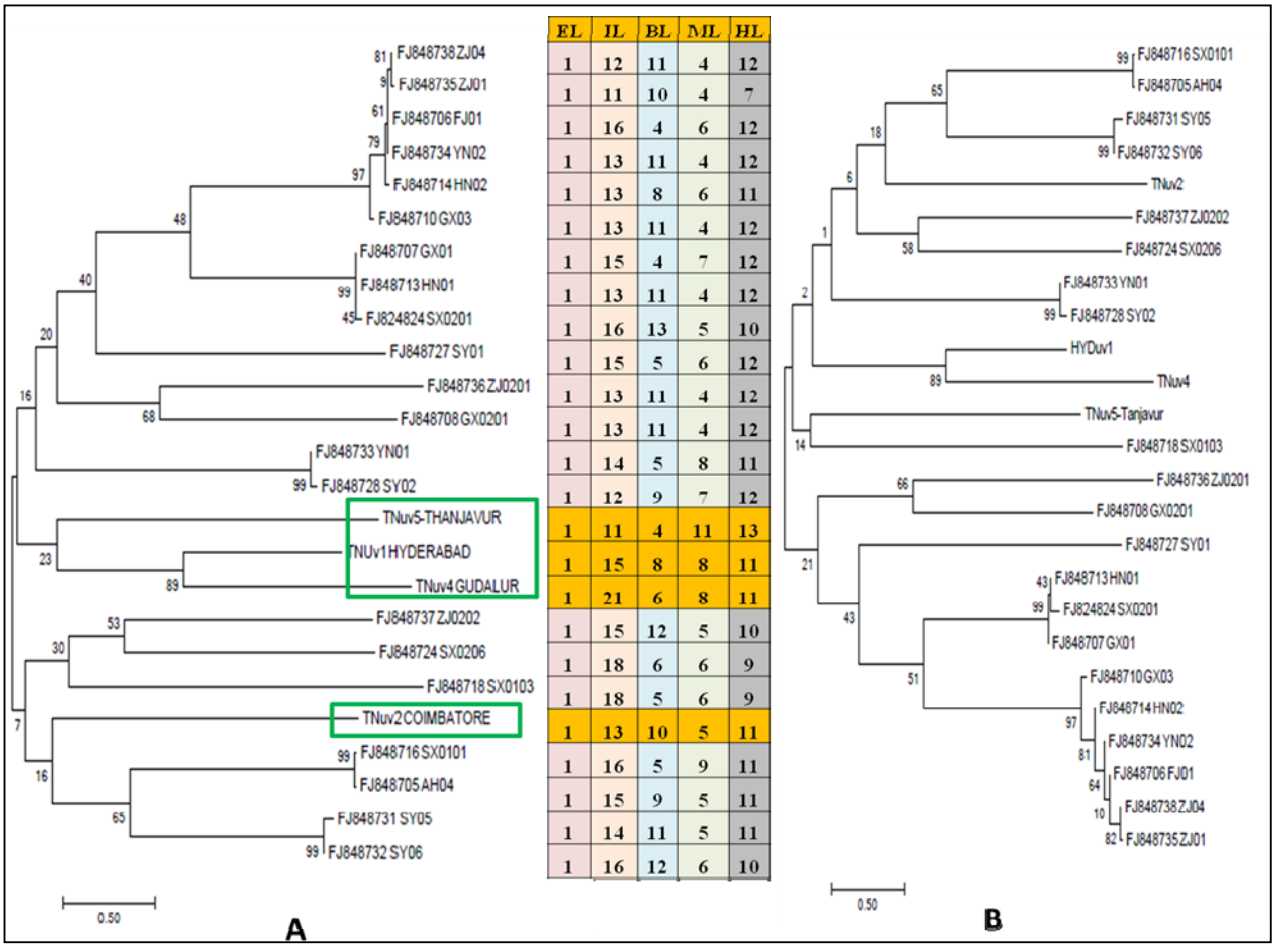

Fig 5: A) Phylogenetic tree inferred using Neighbor-Joining method for query (present outside the green box) and known ITS sequence (present inside the green box). B) Maximum parsimony method.

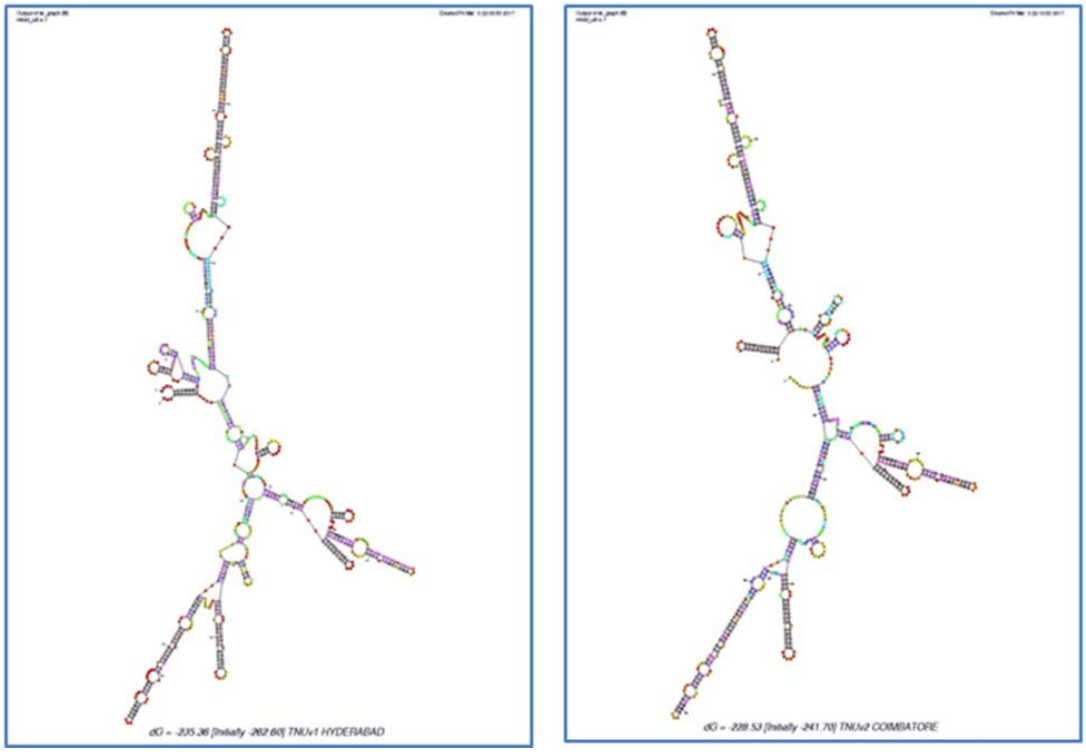

Fig 6: Schematic representation of ITS1, 5.8S and ITS2 rRNA predicted secondary structure for TNUv1 Hyderabad and TNUv2 Coimbatore**.**

Table 1: Summary of secondary structures information modelled with RNA folding form 3.0

ISOLATES	Length	LES	TNS	A	C	G	T/U	G+C	GC%	EL	IL	BL	ML	HL
FJ848738 ZJ04	612	-241.6	22	135	182	161	134	343	56.05	1	12	11	4	12
FJ848735 ZJ01	611	-239.7	18	134	182	161	134	343	56.14	1	11	10	4	7
FJ848733 YN01	612	-238.2	18	137	182	160	133	342	55.88	1	14	5	8	11
FJ848728 SY02	613	-241	20	136	182	161	134	343	55.95	1	12	9	7	12
FJ848716 SX0101	627	-239.7	19	146	183	162	136	345	55.02	1	16	5	9	11
FJ848736 ZJ0201	610	-243.2	26	134	182	160	134	342	56.07	1	13	11	4	12
FJ848734 YN02	617	-243.2	22	137	182	162	136	344	55.75	1	13	11	4	12
FJ848731 SY05	629	-243.9	23	144	183	163	139	346	55.01	1	14	11	5	11
FJ848713 HN01	614	-243.2	26	136	182	161	135	343	55.86	1	13	11	4	12
FJ848708 GX0201	611	-243.2	27	135	182	160	134	342	55.97	1	13	11	4	12
FJ848705 AH04	629	-248.3	22	145	183	164	137	347	55.17	1	15	9	5	11
FJ848706 FJ01	611	-237.5	23	135	182	159	135	341	55.81	1	16	4	6	12
FJ824824 SX0201	614	-240.9	26	136	182	162	134	344	56.03	1	16	13	5	10
FJ848732 SY06	631	-242.4	26	146	183	163	139	346	54.83	1	16	12	6	10
FJ848710 GX03	615	-248.4	23	136	183	161	135	344	55.93	1	13	11	4	12
FJ848707 GX01	611	-237.7	21	135	182	159	135	341	55.81	1	15	4	7	12
FJ848714 HN02	612	-236.5	24	135	182	160	135	342	55.88	1	13	8	6	11
FJ848727 SY01	611	-237.5	23	136	182	159	134	341	55.81	1	15	5	6	12
FJ848718 SX0103	610	-231.5	20	137	182	155	136	337	55.25	1	18	5	6	9
FJ848737 ZJ0202	616	-231.9	22	140	184	156	136	340	55.19	1	15	12	5	10
FJ848724 SX0206	628	-237.9	22	147	186	160	135	346	55.10	1	18	6	6	9
KX421101 HYDERABAD	678	-262.6	21	167	207	169	135	376	55.46	1	15	8	8	11
KX421100 COIMBATORE	612	-241.7	22	134	182	158	138	340	55.56	1	13	10	5	11
KX421102 GUDALUR	674	-257.2	23	156	190	183	145	373	55.34	1	21	6	8	11
KX421103 THANJAVUR	649	-251.8	23	143	176	200	130	376	57.94	1	11	4	11	13
MIN	610	-262.6	18	134	176	155	130	337	54.834	1	11	4	4	7
MAX	678	-231.5	27	167	207	200	145	376	57.935	1	21	13	11	13
MEAN	622.28	-242.43	22.48	140	184	163.2	135.5	346.68	55.712	1	14	8.5	5.9	11

Discussion

The sequence data of ITS region of four isolates of *U. virens* and data obtained from NCBI were subjected to phylogenetic analysis in two methods i.e., neighbor joining and maximum parsimony. The results showed that, both method of phylogenetic structuring represents the same pattern of clade formation. These was supported by the results from phylogenetic analysis of the *Dactylogyroides longicirrus* based on the 18S and ITS1 ribosomal genes reported that phylogenetic analysis of endophytic fungal isolates using ITS 2region based on neighbor joining method resulted in well separarated clades, apart from the sequence based phylogenetic structuring reconstruction, the secondary structure of ITS region in the difference races of fungal

pathogen *U. virens* provides the better understanding of relationship among the races of *U. virens.*

Traditionally, molecular characterization and systematic phylogenetic evaluation approaches relaid on comparision of limited number of orthologus sequenced to obtain genus or species or races relationship in organism. Nowadays, the advent of polymerase chain reaction and DNA sequencing promotes early identification of sequence variation among the species. In general, internal transcribed spacer (ITS) region of rRNA gene clusters are the most commonly uased marker for estimating species relationships. Due to nonfunctional and neutrally evolving property, the rDNA exons regions are highly conserved regions across eukaryotes, but the ITS regions are variable in length due to point mutations and indels, resulting in a size variation ranging from 500 to 700 bp across angiosperms (Baldwin *et al*., 1995; Nilsson *et al* 2012) [4, 17] and from 1500 to 3700 bp in gymnosperms (Alvarez *et al*., 2003) [1]. There are several evidences suggest that significant variation among ITS sequences is found only within organisms that are diploid or polyploids and disparet parents (Buckler 1997) [6]. In this study ITS region was amplified using ITS1 F and ITS4 R primers and the sequencing results reported that the sequence variation ranged from 612 to 678 bp among the four isolates of false smut pathogen *U. virens.* Hausner and Wang (2005) [11] reported that ITS1 and ITS2 sequence may vary significantly at the sequence level, but still high level of conservation at the structural level. Therefore secondary structure prediction is more advantages for detection of variation among the species or races level (Coleman 2009) [7].

Conclusions

This is the first study to weigh up the predicted secondary structures for the rRNA gene cluster sequences of *U. virens* isolates from different geographical locations of south India. The results provide novel hold up from immense analysis of ITS1, 5.8S and ITS2. The proposed ITS based phylogenetics has clearly distinguished the isolates with greater precision than any other existing methods. This is the first report from elsewhere on ITS1, 5.8S and ITS2 sequence-structure analysis of *U. virens* fungus.

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