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Genotypic Diversity among Indian Isolates of *Sclerotium rolfsii* Sacc. [Teleomorph *Athelia rolfsii* (Curzi) Tu & Kimbrough] Based on ITS Region of Ribosomal DNA

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A B S T R A C T

Twenty isolates of *Sclerotium rolfsii* Sacc. collected from different hosts and locations of India was studied in relation to genomic DNA amplification through internal transcribed spacer (ITS-PCR) analysis. These isolates of *S. rolfsii* showed variation at rDNA level which was revealed through ITS1-5.8s-ITS 4 primer series. The consensus primers (ITS 1 and ITS 4) amplified a region of the rRNA gene repeat unit, which includes two non-coding regions designated as ITS 1 and ITS 2 and the 5.8s rRNA gene. It was carried out with ITS-PCR analysis based on their molecular size and a genetic distance was created using Rf value. Out of 20 isolates, six reproducible polymorphic bands were obtained using the above ITS1-5.8s-ITS 4 primer series. The ITS amplified region of 5.8s rRNA gene yielded an ITS fragment of 490−699 bp length in all the 20 isolates of *S. rolfsii*. Among 20 isolates, six isolates showed amplification of a double band whereas the remaining isolates showed amplification of a single band. These six isolates also showed a length variation in this region. Isolates of groundnut were same almost in their size. The results showed that the 'ITS types' within isolates were almost always phylogenetically distinct. There was no clear correlation between ITS-based phylogeny and isolate origin.

Introduction

Sclerotium rolfsii Sacc. (teleomorph *Athelia rolfsii* (Curzi) Tu & Kimbrough) is a devastating soil-borne plant pathogenic fungus with a wide host range due in part to its fast growth rate and the production of oxalic acid and cell-wall- degrading enzymes, distributed in both temperate and tropical regions (Aycock, 1966; Punja, 1985; 1988; Gazaway and Hagan, 1989). It forms differentiated sclerotia and sterile mycelia which can remain viable in the soil for 2-5 years. The fungus produces small tan to dark-brown or black spherical sclerotia and survives over a wide range of pH (1.4-8.8) with a temperature optimum of 27-30 °C. The disease occurrence by *S. rolfsii* is typically endemic and the

spatial distribution of the disease is clustered (Shew *et al.,* 1984). The fungus spreads by mycelial contact with healthy plants and over-winters as sclerotia in soil. The sclerotia serve as the primary source of inoculum and are capable of initiating infection with or without an additional food base (Punja, 1985). Collar-, seed-, stem- and pod-rot of groundnut, at different stages of crop growth are caused by *S. rolfsii* cause severe yield losses in India, especially in Saurashtra region of Gujarat.

The internal transcribed spacer (ITS) is a concept of molecular biology. The spacer is a sequence of RNA in a primary transcript that lies between precursor ribosomal sub units and is removed by splicing when the structural RNA precursor molecule is processed into a ribosome. These sequences are coded by ribosomal DNA. Eukaryotic organisms have two internal transcribed spacers; ITS 1 is located between the 18s gene and the 5.8s gene, and ITS 2 is located between the 5.8s and 28s gene. Ribosomal genes and spacers occur in tandem repeats that are thousands of copies long, each separated by an intergenic spacer (IGS) or non-transcribed spacer (NTS). The ITS region is widely used in taxonomy and molecular phylogenetics. The ITS region is now perhaps the most widely sequenced DNA region in fungi.

It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation among individual rDNA (for small- and large-subunit rRNA), variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions. In addition to the standard ITS 1+ITS 4 primers used by most labs, several taxon-specific primers have been described that allow selective amplification of fungal sequences (Gardes and Bruns, 1993). Harlton *et al.,* (1995) reported the genetic variation in internal transcribed spacer (ITS) regions of ribosomal DNA of *S. rolfsii* using restriction fragment length polymorphisms (RFLP). Okabe *et al.,* (1998) by the same method, classified Japanese isolates of *S. rolfsii* into five ITS-RFLP groups which had distinct geographical distribution patterns. The ITS region is well supported to molecular phylogenetics of fungi nowadays (Bruns, 2006) and also being used to know the genetic diversity among different strains of fungi by sequencing the ITS gene.

Molecular characterization of *S. rolfsii* through ITS series is based on the polymerase chain reaction (PCR) to detect genotypic diversity within a species (Almeida *et al.,* 2001). It is a very rapid and accurate technique was performed as detailed here in.

Materials and Methods

Isolation and Purification of Fungal Genomic DNA

For isolation of fungal genomic DNA, mycelia of 20 isolates of *S. rolfsii*, collected from different hosts and locations of India (Table 1) were grown on 100 ml potato dextrose broth (PDB) at 26±1 °C for 7-15 (1-2 wk) days, then filtered through Whatman filter paper No. 1 and washed with sterilized water. Mycelia were harvested and either used immediately for DNA extraction or stored at – 60 °C until use. Sterilized and chilled mortar and pestles were used for the grinding of mycelium. The required mycelium was washed twice in distilled water and DNA was extracted according Murray and Thompson (1980) by using the CTAB extraction buffer (50 mM Tris-HCl, pH 8.0, 700 mM NaCl, 10 mM EDTA, 10 % hexa-decyl tri-methyl ammonium bromide) with slight modification, followed by phenol-chloroform purification and precipitation with ethanol. DNA of the samples were quantified as per Sambrook and

Russell (2001) by using Nano drop spectrophotometric analysis and stored at – 20 °C.

ITS (internal transcribed spacer) PCR (polymerase chain reaction) Amplification

PCR was performed with all the DNA samples (already accessed qualitatively and quantitatively) by using 50 picomoles (for each) primers ITS 1 (reverse) and ITS 4 (forward). For ITS-PCR amplification, in a sterile 0.2 ml thin wall PCR tube, the components were added and mixed as given below.

Four µl of diluted DNA was added (containing about 25 ng/µl) and 21 µl master mixture were added to each PCR tube and quick spinning was performed for few seconds at 10000 rpm. The samples were ready for amplification. The PCR was carried out in a Eppendorf thermal-cycler (model AG 22331, Hamburg, Made in Germany) with the following programme.

Sequences of ITS primers synthesized for use

The internal transcribed spacers (ITS) primer used for amplification was generated by Oligo Synthesis Department in salt free status (Bagalore Genei, Bangalore, India).

Detection of ITS-PCR Amplified Products

After the completion of PCR reaction, the products were run on 1.5 per cent (w/v) agarose gel prepared in 1 X TAE containing 10 µl of ethidium bromide (1 mg/ml). Ten µl of PCR product was mixed properly with 3 µl of tracking dye (6X, Bangalore Genie, India) and loaded on to the well. The standard DNA size marker (100 bp, fermentas, catalog SM 1153) was also loaded in to the last well and run along with the samples. The gel was run at 80 V (constant) for 30 minutes to separate the amplified bands properly for give better resolution of each and every band of varying molecular weight amplified during PCR. Separated bands were seen under UV and photographed by gel documentation system, Alpha Imager^{TM} 2200 Documentation and Analysis System (Alpha Innotech Corporation).

Analysis of ITS-PCR Data

For analysis of ITS-PCR amplification, the data were recorded on the basis of presence or absence of band with a particular Rf value and their molecular weight size. The data scored by automated tool version programme by Alpha Imager^{TM} 2200 Documentation and Analysis System (Alpha Innotech Corporation). The presence of an amplified band (amplicon) in each position in each lane was recorded. The polymorphism was recorded to determining genetic distances between the isolates. On the basis of comparison of one or more band size (molecular weight) presence in each lane, the ITS-PCR amplified data was prepared. Previously published ITS sequences from *S. rolfsii* strains were included for references (Almeida, *et al.*, 2001) was used as an outgroup.

Results and Discussion

Twenty isolates of Sclerotium *rolfsii* Sacc. collected from different hosts and locations of India was studied in relation to genomic DNA amplification through internal transcribed spacer (ITS-PCR) analysis. The results obtained in the present investigation are narrated herein.

ITS (Internal transcribed spacers) PCR Based Studies

The present study was aimed to assess the genetic diversity of different isolates of *S.*

rolfsii using rDNA region variations. The genomic DNA was extracted from 20 isolates of *S. rolfsii*, using standard protocol as described earlier. Internal transcribed spacers (ITS) region was amplified using the universal primers previously described. The forward and reverse primers *viz*., ITS 1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS 4 (5' TCCTCCGCTTATTGATATGC 3') were based on conserved 18s and 28s coding regions of the nuclear rDNA sequence analysis, including 5.8s gene (White *et al.,* 1990). *Sclerotium* specific primer sets, which was previously published by (Harlton *et al.,* 1995; Okabe *et al.,* 1998; Okabe *et al.,* 2001; Okabe and Matsumoto, 2003), specific to target species (*S. rolfsii*) were tested on a set of present isolates collection. PCR conditions were set as described by various authors to produce a single band diagnostic of *Sclerotium spp*. with primers sets of ITS1 and ITS 4 (specific for *S. rolfsii*, amplification length approximately 700 bp).

The above consensus primers (ITS 1 and ITS 4) were used to amplify a region of the rRNA gene repeat unit, which includes two non-coding regions designated as ITS 1 and ITS 2 and the 5.8s rRNA gene. The genetic diversity was carried out with ITS-PCR analysis based on their molecular size & Rf value and genetic distance was calculated. All the isolates of *S. rolfsii* loaded in lanes 1-10 and 11-20 were amplified along with a 100 bp molecular weight marker (Fermentas, catalog SM1153). A total of 26 reproducible polymorphic bands were obtained using the above ITS 1-5.8s-ITS 4 primer series (Plate 1A & 1B). PCR amplification of ITS region of 5.8s rRNA gene yielded an ITS fragment of 490−699 bp length with 0.631 to 0.790 Rf value in all the 20 isolates of *S. rolfsii* (Table 2).

Among 20 isolates; I 4630, I 4679, I 4723, I 4724, I 4725, and I 4737 amplified double band (Plate 1A) and remaining isolates with a single band (Plate 1A & 1B). These six isolates showed a length variation in this region, in which isolates I 4630_A, I 4679_A, I 4723_A, I 4724_A, I 4725_A, I 4737_A showed 561 to 643 bp in size.

In these isolates, a second band of low molecular weight size 490 to 561 bp was also scored designated as I 4630_B, I 4679_B, I 4723_B, I 4724_B, I 4725_B, I 4737_B.

The minimum size of band *i.e*. 490 bp was scored in two isolates *viz*., I 4630_B, I 4679_B with 0.690 Rf value; whereas, the maximum size of band was scored in isolate I 5146 with 699 bp size and 0.767 Rf value. Isolates J 2004 I and M 1999 were confirmed to be from groundnut as their size was 654 and 665 bp with their respective Rf values as 0.783 and 0.779. Among twenty isolates, a few isolates displayed bands with similar molecular weight. Isolates no. I 4737_A, I 4972 and I 5061 were same in their molecular size (643 bp). Likewise, isolates I 5068, I 5226, I 2782 and M 1999 were same in their length (665 bp). Isolates I 5543 and J 2004 II were also scored with similar molecular weight of 676 bp. In the present studies, results indicated that all the isolates of *S. rolfsii* amplified single and double bands approximately 650 bp molecular weight in their size.

Almeida *et al.,* (2001) studied genotypic diversity among Brazilian isolates of *S. rolfsii* using the same primer sets and found genetic variation, amplified with two fragments containing ITS1 region, 5.8s rDNA gene and ITS 2 region that were present in all the isolates with molecular sizes of 739 and 715 bp. Okabe and Matsumoto (2003) reported a relationship of *S. rolfsii* and *S. delphinii* based on ITS sequences. They also found a single band amplified with approximately 700 bp using the primer sets (ITS1+ITS4) which consisted of ITS 1, 5.8s rDNA, and ITS 2 regions.

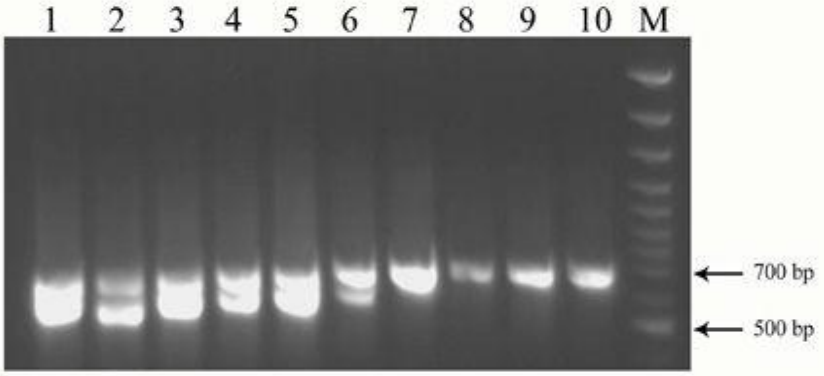

Plate 1A: Internal transcribed spacer region (ITS) of *Sclerotium rolfsii* isolates. Lanes 1-10 represent isolates I 4630, I 4679, I 4723, I 4724, I 4725, I 4737, I 4743, I 4877, I 4972 and I 5061, respectively. M indicates the molecular weight marker 100 bp ladder.

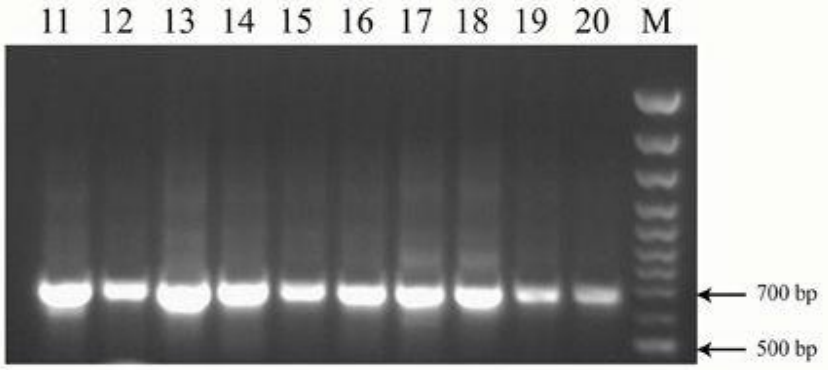

Plate 1B: Internal transcribed spacer region (ITS) of *Sclerotium rolfsii* isolates. Lanes 11-20 represent isolates I 5068, I 5146, I 5220, I 5226, I 5518, I 5543, I 2782, J 2004 I, J 2004 II and M 1999, respectively. M indicates the molecular weight marker 100 bp ladder.

Table.1 Collection of different isolates of *Sclerotium rolfsii*

Sr. No.	Isolates* No.	Host	Common Name	Location and State
1.	I 4630	-	Straw	Raipur (Chattisgarh)
2.	I 4679	*Plantago ovata* Forsk.	Blonde psyllum	Bhubneshwar (Orissa)
3.	I 4723	*Tagetes minuta* L.	-	Nainital (Uttaranchal)
4.	I 4724	*Solanum tuberosum* L.	Potato	Nainital (Uttaranchal)
5.	I 4725	*Canavalia gladiata* (Jacq.) DC.	Sword bean	Nainital (Uttaranchal)
6.	I 4737	*Catharanthus roseus* (L.) G. Don.	Periwinkle	New Delhi
7.	I 4743	*Dioscorea alata* L.	White yam	New Delhi
8.	I 4877	*Allium cepa* L.	Onion	Dharwad (Maharashtra)
9.	I 4972	*Tagetes sp*.	Marigold	Navasari (Gujarat)
10.	I 5061	Unknown	-	IARI, New Delhi
11.	I 5068	*Lagerstroemia sp.*	-	Hyderabad (Andhra Pradesh)
12.	I 5146	*Vigna unguiculata* (L.) Walp.	Cowpea	Unknown
13.	I 5220	*Eleusine coracana* (L.) Gaertn.	Finger millet	Vellayani (Kerala)
14.	I 5226	*Pogostemon cablin* (Blanco) Benth.	Patchouli	New Delhi
15.	I 5518	*Nicotiana sp.*	Tobacco	Lucknow (U.P.)
16.	I 5543	Unknown	-	Thiruvanantpuram (Kerala)
17.	I 2782	*Capsicum annum* L.	Chilli	Unknown
18.	J 2004 I	*Arachis hypogea* L	Groundnut	Junagadh (Gujarat)
19.	J 2004 II	*Capsicum annum* L.	Chilli	Junagadh (Gujarat)
20.	M 1999	*Arachis hypogea* L	Groundnut	Udaipur (Rajasthan)

*Source: 01-17: Indian Type Culture Collection, IARI, New Delhi
18: National Research Centre for Groundnut, ICAR, Junagadh
19: Dept. of Plant Pathology, College of Agril., JAU: Junagadh
20: Dept. of Plant Pathology, Maharana Pratap University of Agriculture & Technology, Udaipur, Rajasthan.

Table.2 ITS-PCR amplification of genomic DNA of 20 isolates of *S. rolfsii*

Sr. No.	Isolate No.	Mol. Wt. (bp)	Rf
1 A	I 4630$_A$	561	0.665
1 B	I 4630$_B$	490	0.690
2 A	I 4679$_A$	601	0.652
2 B	I 4679$_B$	490	0.690
3 A	I 4723$_A$	587	0.656
3 B	I 4723$_B$	512	0.681
4 A	I 4724$_A$	614	0.648
4 B	I 4724$_B$	537	0.673
5 A	I 4725$_A$	601	0.652
5 B	I 4725$_B$	531	0.675
6 A	I 4737$_A$	643	0.640
6 B	I 4737$_B$	561	0.665
7	I 4743	623	0.644
8	I 4877	673	0.631
9	I 4972	643	0.640
10	I 5061	643	0.640
11	I 5068	665	0.779
12	I 5146	699	0.767
13	I 5220	638	0.790
14	I 5226	665	0.779
15	I 5518	688	0.771
16	I 5543	676	0.775
17	I 2782	665	0.779
18	J 2004 I	654	0.783
19	J 2004 II	676	0.775
20	M 1999	665	0.779

Preparation of PCR reaction master mixture (for 10 reactions)

10 x PCR buffer	2 µl ×10	20.00 µl
2.5 m M dNTP mix	1.25 µl ×10	12.50 µl
Taq Polymerase (3 U/µl)	1 µl ×10	10.00 µl
R (Reverse) Primer	1 µl ×10	10.00 µl
F (Forward) Primer	1 µl ×10	10.00 µl
DDW (Sterilized)		147.50 µl
Final Volume	21 ×10	210.00 µl

ITS-PCR amplification programme

For	Temp.	Time	Cycle
Initial denaturation	92 °C	60 Sec.	1
Denaturation	94 °C	60 Sec.	44
Anealing	58 °C	60 Sec.	44
Extention	72 °C	120 Sec.	44
Final Extention	72 °C	300 Sec.	1
Final Hold	4 °C	300 Sec.	1

Sequences of ITS primers synthesized for use

Sr. No.	Target species	Primer Designation	Sequence (5'to 3')	Author
1.	*Sclerotium rolfsii*	ITS 1 (4R)	TCCGTAGGTGAACCTGCGG	White *et al.*, 1990
2.	*Sclerotium rolfsii*	ITS 4 (5F)	TCCTCCGCTTATTGATATGC	White *et al.*, 1990

F = Forward primer, R = Reverse primer

Above results indicated that amplification with species-specific primers was obtained with all the isolates. Further, no inter- or intra-species ITS length diversity was detected. Since the fact is that 5.8s rRNA gene is known to be highly conserved at genus level. The study revealed that all the isolates belonged to a single genus. In case of groundnut isolates *viz.*, J 2004 I and M 1999, the fragment size was observed between 654−665 bp in length and the ITS length diversity was minimum within groundnut isolates. Functionally and evolutionarily, conserved rRNA gene blocks contain both highly non-conserved sequences which have been used in various studies to determine phylogenic relationships (Olsen *et al.*, 1986; Gaudet *et al.*, 1989; Forster *et al.*, 1990; Sreenivasaprasad *et al.*, 1994). A further analysis of the molecular differences among the isolates can help in complimenting to the specification based on morphology. Also, wherever, in case of any ambiguity in morphological classification, molecular tools could be handy to clear the taxonomic position of the genotypes. Re-classification of *S. rolfsii* isolates with DNA fingerprinting has been reported previously. To resolve disagreement, the isolates can be fingerprinted using AFLPs and can be compared with isolates of respective species. The PCR assays validated in this study can be used for identification of *Sclerotium spp.* causing stem rot of groundnut. PCR assays validated will also allow rapid diagnosis of *Sclerotium spp.*, which will help in developing appropriate disease management strategies.

Different clades were inferred from the phylogenetic analysis, yielding diverse associations. There was no apparent clustering of isolates according to host or origin, although isolates of the same country grouped together. Isolates from peanut and other hosts were in the same clade, ITS-PCR analysis showed that all isolates grouped together with *S. rolfsii*, though isolates one to

six showed two different molecular weight size band; whereas, remaining isolates had shown only one band in the current study. Therefore, results in the present study indicated that there is a close affinity among *S. rolfsii* isolates. However, the isolates showed diversity with no specificity based on host and geographic origin. In this study, significant insight into the variability among isolates of *S. rolfsii* from India was obtained. More isolates would be required to explore the complete genetic diversity among *S. rolfsii* isolates and *Sclerotium spp*. However, based on the phylogenetic study of the isolates used in this study, a possibility could be that the isolates may represent a new pathovar and this needs to be investigated in future. For more confirmation of the results we should go to DNA sequencing to detect the diversity among isolates of *S. rolfsii*.

Stevens (1931) differentiated *S. delphinii* and *S. rolfsii* based on sclerotial morphology and host range with *S. delphinii* producing the largest sclerotia. In an early ITS study, a close relationship was detected between *S. delphinii* and *S. rolfsii* (Boerema and Hamers, 1988; Harlton *et al.*, 1995). They reported that *S. rolfsii* and *S. delphinii* grouped together but separately from *S. coefficola*. Based on their similarity, *S. rolfsii* was designated as *S. rolfsii* var *rolfsii* and *S. delphinii* as *S. delphinii* var *delphinii*. Moreover, a phylogenetic tree was constructed based on ITS-RFLP analysis and found a close relationship between *S. rolfsii* and *S. delphinii* (Okabe *et al.*, 2000; Okabe and Matsumoto, 2003). Therefore, results in the current study indicated that there is a close affinity of the isolates of *S. rolfsii* but showed diversity among isolates without any specificity to host and origin. Almieda *et al.*, (2001) studied 30 isolates of *S. rolfsii* from different host and regions in relation to morphology, mycelial compatibility, analysis of genomic DNA through random amplified polymorphic DNA (RAPD), variation within the nuclear rDNA [internal transcribed spacers (ITS)] and sequencing of ITS fragments and its role in the phylogeny. The results showed that the 'ITS types' within isolates were almost always phylogenetically distinct. There was no clear correlation between ITS-based phylogeny and isolate origin; of course this may be due to different environmental conditions, host specification and geographical distribution.

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