Ribosomal DNA Gene Based Molecular Characterization of *Sclerotium rolfsii* causing Collar Rot in *Amorphophallus paeoniifolius* (Elephant Foot Yam)

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Abstract

Amorphophallus paeoniifolius, commonly known as elephant foot yam (EFY), is an aroid with high yield potential and culinary and medicinal properties. Collar rot disease caused *by Sclerotium rolfsii* is an economically important disease prevailing in all *Amorphophallus* growing areas. As an initial phase of the study, the morphological characters of the pathogen was studied on PDA medium. The isolates varied in their growth parameters viz. growth rate, sclerotia production, sclerotia color, sclerotial distribution pattern and sclerotial size. Although valuable, traditional methods of pathogen detection do possess limitations and these constraints could be overcome by culture-independent molecular approaches. The present study utilized PCR assay for molecular characterization of ten *S. rolfsii* isolates based on the rDNA genes ITS and LSU. PCR assay using universal primers for ITS and LSU yielded single amplicons of 700 bp and 650 bp respectively. Sequence analysis of the amplified products showed higher similarity (97-100 % similarity) to *S. rolfsii* sequences in the GenBank database whereas less similarity to other species belonging to the same genus, thereby revealing the identity of the fungal species. The study thus corroborated the significance of ITS and LSU regions in molecular characterization of the pathogen and also divulged the possibility of designing *S. rolfsii* specific primers based on rDNA.

Key words: Morphology, molecular characterization, *Sclerotium rolfsii*, *Amorphophallus*, elephant foot yam, PCR, rDNA

Introduction

Collar rot disease of *A. paeoniifolius* is caused by the soil borne Basidiomycete fungus *Sclerotium rolfsii*. Infection by the pathogen causes rotting of collar region which ultimately topples down and is responsible for heavy reduction in yield and qualitative degradation of the crop. The yield loss varies from 25 to 100% depending upon the nature of cultivars and various predisposing factors (Misra, 1997). The wide host range of the pathogen, heavy rainfall, warm and humid climate, excess organic matter, poor drainage, injuries caused by insects, fertilizers or during intercultural operations act as predisposing factors for disease incidence (Thankappan, 1994). Collar rot is generally observed during the later part of crop growth but the pathogen is capable of infecting the plant at any stages of development. The disease becomes more destructive during rainy season followed by warm dry weather. It also occurs in serious form if the clay content of the soil is high and water stagnates in the field.

Rapid and accurate detection of fungal pathogens to species or strain level is often essential for disease surveillance and in implementing a disease management strategy. Relying on morphological characterization alone is challenging because fungal pathogens can exist as multiple

species complexes in natural environments. Different molecular genotypes or varieties can also exist within species and may have different pathogenic profiles and virulence levels to the host (Balajee et al., 2009). Molecular methods have been widely applied in the identification of a large number of fungal species (Borman et al., 2008; Chen et al., 2000; Esteve-Zarzoso et al., 1999). PCR amplification followed by sequencing and pairwise alignment of amplicons has been widely accepted as the ''gold standard'' for fungal identification. PCR based sequencing demonstrates better specificity and accuracy compared with traditional phenotypic methods (Turenne et al., 1999 and Williams et al., 1995). In the present study the rRNA genes, ITS and LSU, were included for molecular characterization of the *Amorphophallus* collar rot fungus.

Materials and Methods

Culture conditions and colony morphology

The ten *S. rolfsii* isolates used in this study were isolated from infected samples collected from *Amorphophallus* fields located in diverse geographical regions in India *viz.* Andhra Pradesh, Bihar, Karnataka, Kerala, Maharashtra, Meghalaya, Orissa, Tamil Nadu, Tripura and Uttar Pradesh. The cultures were isolated from infected pseudostem or tuber samples by tissue segment method (Rangaswami and Mahadevan, 1999). Small tissue pieces of about 0.5 - 1 cm were excised with a sterile scalpel from the infected part. The excised fragments were subsequently disinfected using 1% sodium hypochlorite for 1 min, rinsed twice with sterile distilled water, dried using blotting paper and placed onto potato dextrose agar medium (PDA; 250 g L^{-1} potato, 20 g L^{-1} dextrose and 20 g L^{-1} agar). The plates were incubated at 30ºC for 5 to 7 days. The isolates were purified by growing single sclerotia from each colony onto plain agar (20 g agar^{-1}) plates. The growing hyphal tip was sub-cultured again on PDA after four days to ensure a completely pure culture.

The typical morphology of the isolates was studied by growing on PDA medium. The morphological characteristics such as mycelial growth rate, mycelial colour, mycelial growth pattern, sclerotial production, sclerotial size, sclerotial distribution and sclerotial occurrence pattern were considered for the study. Circular disc of 5 mm diameter from the margin of 5-7 days old culture of each isolate were placed on centre of PDA plates

under aseptic conditions and incubated at 30ºC in a BOD incubator. Daily increments in radial colony growth were measured along two diameters (colony diameter in mm) at right angles and colony growth was noted as the mean of the two measurements. The observations were continued until full growth occurred in the plate. The texture, colour and growth pattern of the colony were recorded 3 - 7 days after inoculation. To get mature sclerotia, the plates were further incubated for 20 days. The number of days taken for sclerotial formation was noted and the total number of sclerotia produced per square cm was counted after 20 days. The colour, diameter, distribution and occurrence pattern of sclerotia were also recorded 20 to 25 days after inoculation.

DNA extraction and PCR amplification

Genomic DNA was isolated using the standardized protocol of Jeeva et al. (2008). The universal primer pairs ITS1 and ITS4 and LROR and LR5 were used to amplify the ribosomal ITS and LSU gene regions, respectively. Each 25 ìl PCR reaction consisted of 50 ng of template DNA, 100 ìM each deoxyribonucleotide triphosphate, 0.2 ìM of each primer, 1.5 mM MgCl$_2$, 1 X Taq buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.01 % gelatin) and 1U of Taq DNA polymerase (Bangalore GeNei, India). Amplifications were performed in Eppendorf thermal cycler (Eppendorf AG, Hamburg, Germany). Amplification with the primer pairs ITS1 and ITS4 was performed using a denaturation step of 94°C for 2 min, followed by 35 cycles of 94°C for 30s, 52°C for 1 min and 72°C for 1 min 30 s. A final extension step at 72°C for 8 min was then employed. For the primer pair LROR and LR5 the PCR cycling regime was denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30s, 55°C for 1 min and 72°C for 1 min 30s. A final extension step at 72°C for 8 min was then used. The amplified products were resolved on a 1.2 % agarose gel containing 0.5 µl (10 mg ml^{-1} ethidium bromide) ethidium bromide and photograph was scanned through Gel Doc System (Alpha imager, Alpha Innotech, USA).

DNA sequencing and analysis of sequence data

Among the hundred isolates, ten isolates corresponding to different geographic area were selected for amplification and subsequent sequence analysis. The single amplified PCR products of each primer set was eluted using QIAquick Gel extraction kit (QIAGEN, Tokyo, Japan)

and cloned into the pGEM-T vector (Promega, WI, USA). The DNA clones were transformed and amplified in *E. coli* DH5α cells. The positive transformants were selected by blue/white colony screening and sequenced using T7 and SP6 promoter primers (Applied Biosystems 3500 Genetic Analyzer, Life Technologies). Prior to NCBI-BLAST (National Centre for Biotechnology Information - Basic local alignment search tool) analysis, the vector sequences were removed from the obtained sequences with the aid of BioEdit tool (Hall, 1999). Nucleotide BLAST (Zhang et al., 2000) search was then performed against the Genbank database to confirm the authenticity of isolates. The fungal ITS and LSU gene sequences determined in this study were deposited in GenBank database. The sequences were further aligned using the Clustal W module (Thompson et al., 1994) included in the MEGA 6 software (Tamura et al., 2013). Sequences of *S. rolfsii* strains reported from different regions of the world (downloaded from Genbank) and other species belonging to the same genus were also included in the study. A phylogenetic tree was constructed based on UPGMA method using MEGA 6 software. For all analysis, 1000 bootstrap replicates were performed to evaluate the node support of the generated trees.

Results and Discussion

The traditional phenotypic identification methods for pathogenic fungal species are based on morphological and biochemical characteristics of different species. It is widely acknowledged that morphological characteristics alone are insufficient for accurate identification of species. Molecular approaches especially sequence-based strategies which are being more and more widely used, show the potential to be a powerful supplement for the phenotypic identification (Balajee et al., 2007; Balajee et al., 2009; Ecker et al., 2010 and Lenc et al., 2008). Due to sufficient intra-species conservation and interspecies specificity, the 5' end of the large subunit (LSU D1/D2 region) rRNA gene and the internal transcribed spacer 1 and 2 (ITS1 and ITS2 regions) between 18 s and 28 s rRNA genes are most frequently used as targets for discriminating species (Ciardo et al., 2010; Fell et al., 2000; Kurtzman and Robnett, 1997; Rakeman et al., 2005 and Scorzetti et al., 2002). Single-copy conserved genes are also being used as targets for taxonomic studies when multi-copy segments from the rDNA complex lack variability. The major objective of this study was to determine the effectiveness of using PCR techniques to amplify the ITS and LSU sequence regions of fungal DNA for the purpose of sequencing and eventually species identification.

The morphological characteristics of *S. rolfsii* isolates grown on PDA medium revealed variations in their growth parameters. All the isolates produced silky white mycelia with linear or apical growth in PDA medium and attained full growth by 3 to 10 days (Fig. 1.A). Based on the growth rate the isolates were clustered into three groups namely fast growers (81-90 mm in 2 to 4 days), intermediate growers (51 to 80 mm in diameter in 5 to 7 days) and slow growers (less than 50 mm in 8 to 10 days). The time taken for sclerotial production ranged from 4 to 15 days after inoculation. The sclerotia produced were small and uniformly round but the sclerotial size varied from 1.43 mm to 2.70 mm. The colour of sclerotia was generally brownish black at maturity, while sclerotia were light brown in some isolates. The isolate differed significantly with respect to the sclerotial production also. The number of sclerotia produced per square cm ranged from 5 to 30. Three different sclerotial distribution patterns were observed among the isolates namely peripheral, throughout plate and centre (Fig. 1.B). Besides these, the sclerotia were also found to occur either individually or in groups. The technique proved successful as its application led to the identification of all the ten representative isolates to the species level. PCR amplification of the ITS and LSU regions of the representative isolates yielded single amplicons of 700 bp and 650 bp respectively (Fig. 2 and 3). The sequencing of cloned fragments consistently produced good reads. Alignment of sequences of representative isolates using Clustal W revealed 98 to 100% nucleotide sequence similarity with each other. Taxonomic correlation of the isolates upon NCBI BLAST analysis proved that the isolates were highly similar to the *S. rolfsii*
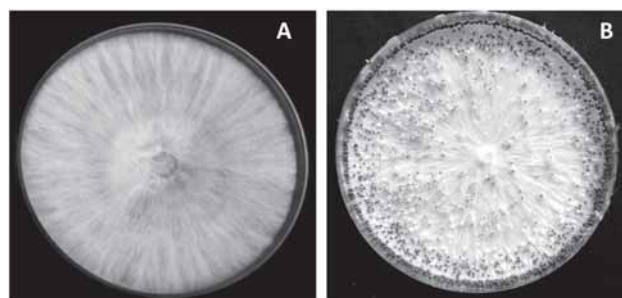

Fig. 1. Growth of *S. rolfsii* in PDA medium. (A) Compact mycelial growth (B) Sclerotial production throughout the plate

Fig. 2. Amplification of *S. rolfsii* isolates using universal primer (ITS 1/ ITS 4). Lane M: 1 kb plus DNA ladder, Lane 1-10: *S. rolfsii* isolates

Fig. 3. Amplification of *S. rolfsii* isolates using universal primer (LROR/ LR5). Lane M: 1 kb DNA ladder, Lane 1-10: *S. rolfsii* isolates

species of genus *Sclerotium* reported in GenBank database (97–100 % similarity with *S. rolfsii* sequences available in the GenBank database). The representative sequences were deposited in GenBank NCBI and the accession numbers assigned are JN811674, JN811673, GQ148561, JN811675, JN811676, KJ598085, KJ598086, KX434524, KX434525, KX434526, KX434527, KX643346, KX643347, KX643348, KX643349, KX643350, KX643351, KX643352 and KJ598087. The phylogenetic tree constructed by UPGMA algorithm showed that the ITS and LSU sequences from the present study shared a common clade with the *S. rolfsii* sequences from GenBank thus confirming the pathogen's identity (Fig. 4 and 5). The phylogenetic analysis showed clear homology of isolate with respective reference species and also their evolutionary relationships with other species in the same genus. The robustness of the clustering was further supported by the high bootstrap values.
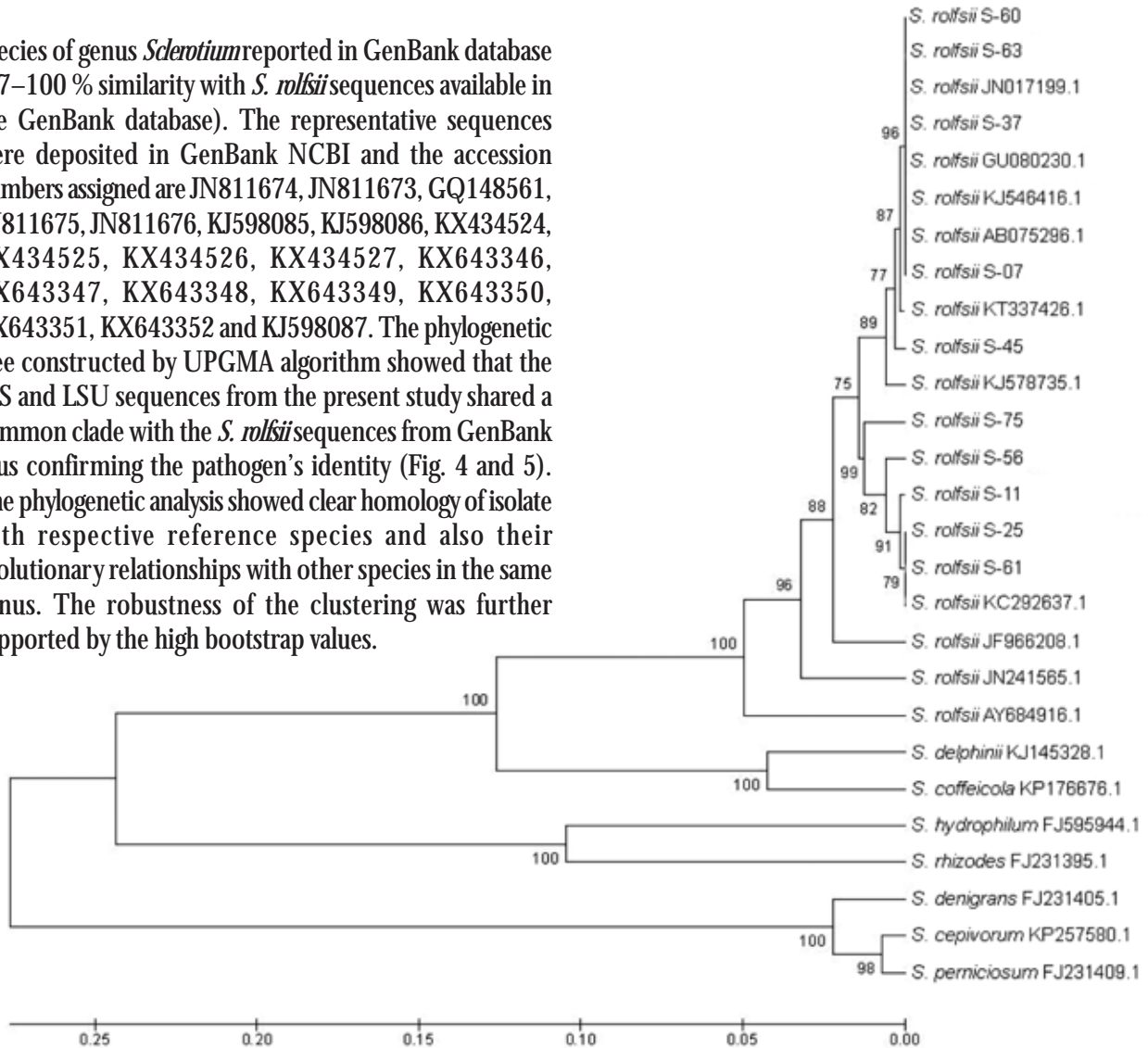

Fig. 4. UPGMA dendrogram showing clustering of *S. rolfsii* ITS sequences from the present study with Genbank submitted *S. rolfsii* sequences and other species belonging to the genus *Sclerotium*
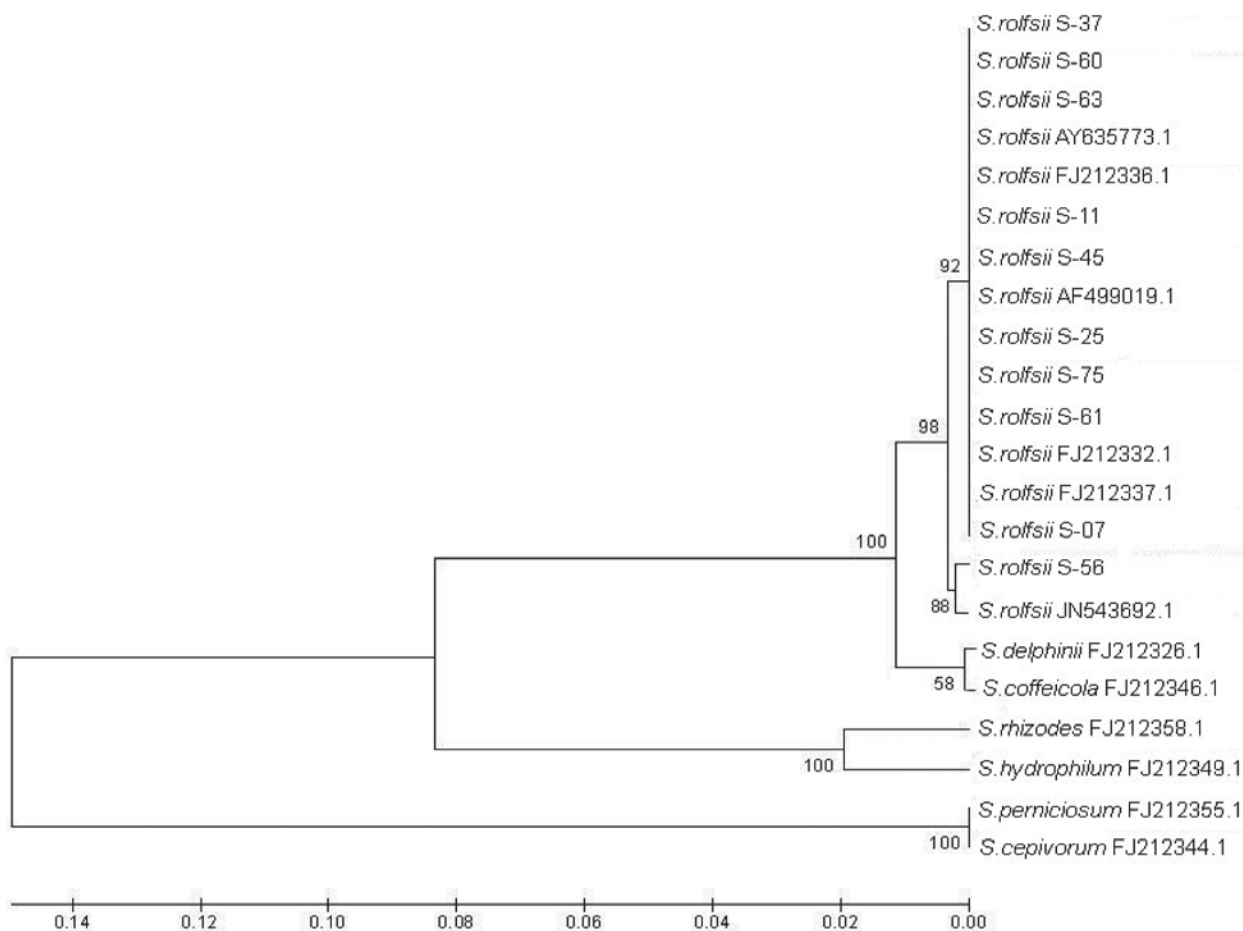
Fig. 5. UPGMA dendrogram showing clustering of *S. rolfsii* LSU sequences from the present study with Genbank submitted *S. rolfsii* sequences and other species belonging to the genus *Sclerotium*

Conclusion

It could be concluded from the study that the rDNA ITS and LSU regions do possess sufficient genetic divergence to distinguish *Sclerotium rolfsii* from other species. Besides these the sequence information obtained from the study could be further utilized for the development of species specific primers or probes for diagnosis of *S. rolfsii.*

Acknowledgment

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