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# Virulence and genetic diversity of *Sclerotium rolfsii* Sacc., infecting groundnut using nuclear (RAPD & ISSR) markers

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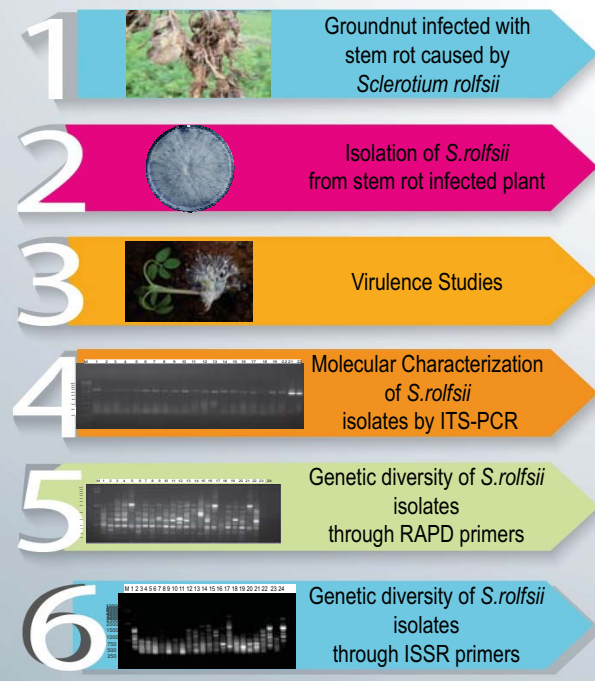
## Abstract

**Aim :** Assess the virulence and genetic diversity of *Sclerotium rolfsii* isolates causing stem rot disease in groundnut collected from different geographical regions of Tamil Nadu using nuclear markers viz., RAPD and ISSR.

**Methodology :** Survey was conducted in major groundnut growing areas of Tamil Nadu and twenty two cultures of *S. rolfsii* (18 from groundnut and 4 from other crops) were collected and isolated. Subsequently the virulence of *S. rolfsii* was tested. Genomic DNA was extracted from *S. rolfsii* isolates and assessed their genetic diversity using nuclear markers viz., RAPD and ISSR.

**Results :** Among the 22 isolates of *S. rolfsii*, the isolate from Udumalpet (SrUDM) showed higher per cent disease incidence (80.95%) followed by K.G. Savadi (SrKGS) and Tindivanam (SrTVM) isolates (61.90%), whereas the isolate from Madurai recorded lower per cent disease incidence (33.33%). ITS region of rDNA amplification with specific ITS-1 and ITS-4 universal primers produced the amplicon of approximately 650 to 700 bp and confirmed that all the 22 isolates belongs to the genus *Sclerotium*. Ten different oligonucleotide primers of RAPD and ISSR were used to assess the genetic diversity among 22 isolates of *S. rolfsii*. The dendrogram results indicated that all the *S. rolfsii* isolates from Tamil Nadu formed two distinct clusters. A total of 121 and 123 reproducible and scorable polymorphic bands recorded and ranging from 100 to 2500 bp and 250 to 2000 bp were generated with ten numbers of RAPD and ISSR primers, respectively. The ISSR marker recorded an average PIC of 0.87 as against 0.86 of RAPD and the effective multiplication ratio was also superior in ISSR (12.21) compared to RAPD (12.00).

**Interpretation :** The result of the present study indicated that ISSR markers were suited well for genetic diversity studies of *S. rolfsii* than RAPD and no correlation was observed between the virulence and genetic diversity of various *S. rolfsii* isolates using nuclear markers.



## Introduction

Groundnut (*Arachis hypogaea*, L.) is considered as a major oilseed crop of India and positioned first place among the oil seeds. It is cultivated in an area of 52.50 lakhs ha with an annual production and productivity of 94.72 lakh tonnes and 1804 kg ha<sup>-1</sup>, respectively in India. Among the top ten major groundnut growing states of India, Tamil Nadu accounts an area of 3.42 lakh ha, with an annual production of 9.62 lakh tonnes and productivity of 2813 kg ha<sup>-1</sup> (Anonymous, 2014). Despite the adaption of crop to all seasons, the production of groundnut is being challenged by the panorama of diseases viz., leaf spot, collar rot, stem rot, bud necrosis and other diseases. Among the major fungal diseases, the stem rot disease caused by *Sclerotium rolfsii* Sacc., is a major constraint for the production of groundnut (Johnson et al., 2008) and its attributes significant amount of yield loss in groundnut. Rodriguez et al. (1975) reported that normally groundnut stem rot causes pod yield loss of 10-25 % under regular condition, whereas in severe condition it accounts around 80 % of yield loss. *S. rolfsii* is a soil borne plant pathogen causing diseases on wide range of economically important agricultural and horticultural crops, including groundnut, cotton, soybean, sunflower, wheat, legumes, tomato, chilli, potato, crucifers, cucurbits, onion and others (Hossain, 2000).

A wide range of host, profuse mycelial growth, ability to produce persistent sclerotia (Asghari and Mayee, 1991; Kokub et al., 2007) and typical damaging symptom of yellowing and wilting of branches contribute to the greater economic loss. Integrated

management of *S. rolfsii* is considered as the most effective method for their management. In order to implement the effective integrated management strategy against *S. rolfsii*, the informations on current status and genetic variability is vital. Till date, limited work has been done on genetic variability of *S. rolfsii* isolates (Prasad et al., 2010). Molecular markers play a major role in analyzing the genetic variation of fungal pathogens (Punja and Sun, 2001; Adhipathi et al., 2013). Among the molecular markers, the nuclear DNA markers viz., RAPD and ISSR are the markers of choice for genetic variation studies because of their high level of polymorphism (Moulin et al., 2012). Hence, the present study focused on genetic variation of *S. rolfsii* isolates of groundnut collected from different geographical regions of Tamil Nadu using RAPD and ISSR markers.

## Materials and Methods

**Collection of samples :** Survey and collection of stem rot infected groundnut samples were done in major groundnut growing areas of Tamil Nadu (Table 1). The stem rot infected groundnut, plants were pulled out with intact roots showing the presence of white mycelial mat with small round brown sclerotia and gently tapped to remove the soil adhering on the root region. The samples were bagged with butter paper and then placed in polythene cover. The field collected samples were brought to the laboratory, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore for further isolation of *S. rolfsii*.

**Table 1 :** List of *S. rolfsii* isolates collected from different groundnut growing regions of Tamil Nadu

Isolates	Source	Location	District
SrPLC	Groundnut	Pollachi	Coimbatore
SrKGS	Groundnut	K.G.Savadi	Coimbatore
SrCBE	Groundnut	Coimbatore	Coimbatore
SrKNK	Groundnut	Kinathukadavu	Coimbatore
SrDGL	Groundnut	Dindigul	Dindigul
SrBTG	Groundnut	Batlagundu	Dindigul
SrUSP	Groundnut	Usilampatti	Madurai
SrMDU	Groundnut	Madurai	Madurai
SrTHN	Groundnut	Theni	Theni
SrANP	Groundnut	Andipatti	Theni
SrVLR	Groundnut	Vellore	Vellore
SrVAL	Groundnut	Vallanadu	Tuticorin
SrTVM	Groundnut	Tindivanam	Villupuram
SrMDT	Groundnut	Madurantakam	Kanchipuram
SrTRM	Groundnut	Thiruvannamalai	Thiruvannamalai
SrUDM	Groundnut	Udumalpet	Tiruppur
SrERP	Groundnut	Erisanampatti	Tiruppur
SrKAN	Groundnut	Kaniyur	Tiruppur
SrBLG	Blackgram	Madurai	Madurai
SrSUN	Sunflower	Coimbatore	Coimbatore
SrJAS	Jasmine	Nilakottai	Dindigul
SrTOM	Tomato	Kinathukadavu	Coimbatore

**Isolation of *S. rolfsii* from infected groundnut :** The *S. rolfsii* was isolated from the diseased groundnut plants by tissue segment method (Rangaswami, 1996). The infected stem portion of groundnut, collected from different area, were cut into small pieces (1.0 cm size) using sterilized scalpel blade and surface sterilized with 0.1 % mercuric chloride for one minute. Subsequently the pieces were washed in sterile distilled water thrice and then placed in a Petri plate at equidistance onto previously poured and solidified Potato Dextrose Agar (PDA) medium. In order to prevent the contamination of bacteria, the bacterial antibiotic (Streptomycin sulphate) was added at 1000 ppm into the sterile PDA medium at 60°C. These plates were incubated at 28 ± 2 °C room temperature and 65±5 % relative humidity for five days and observed for fungal growth.

The pathogen was identified based on their cultural and morphological characters (Punja, 1985). A loop full of fungal culture grown on PDA plates were taken on a glass slide and observed with image analyzer (LABOMED iVu5100, Labo America Inc, USA) under light microscope. After confirming the sclerotia, the cultures were purified and maintained for further studies (Ricker and Ricker, 1936). The pathogen from other hosts viz., tomato, jasmine, sunflower and black gram were also isolated, purified and maintained to compare the genetic variability of *S. rolfsii* isolates with groundnut isolates.

**Virulence of isolates of the pathogen :** A pot culture experiment was conducted to test the virulence of different *S. rolfsii* isolates collected from various groundnut growing areas of Tamil Nadu. Mass multiplication of *S. rolfsii* was followed in sand maize medium. Two hundred grams of sand maize mixture was taken in polybags and mixed with 30 per cent of distilled water and they were sterilized at 15 lb pressure for 20 min. The pure culture of each *S. rolfsii* isolate was inoculated to the polybags under aseptic conditions and incubated at 28 ± 2°C for 20 days. The polybags were shaken periodically to get uniform growth of the pathogen. Subsequently the mass cultures thus obtained were used for further studies.

**Assessing the virulence of *S. rolfsii* isolates by pot culture method :** The potting mixture was prepared by mixing clay loam soil, sand and farm yard manure in the ratio of 1:1:1 ratio. Potting mixture was sterilized in an autoclave at 1.4 kg cm<sup>-2</sup> pressure for 2 hr on two successive days. Earthen pots with uniform size of 30cm diameter were used for filling the soil and mass cultured inoculum. The inoculum of each isolate of *S. rolfsii* grown on sand maize medium (20 days old) was mixed thoroughly at 5 % (w/w basis) level, and applied on top two centimeter of the soil. Then groundnut seeds were sown in the inoculated pots. Each isolate was considered as treatment and three replications were maintained for each treatment. The seeds sown in pots without inoculums served as control. Soil moisture was maintained at moisture holding capacity of soil by adding sterilized water on weight basis throughout the period. Seed infection started within

three days of sowing. After 20 days of sowing, plants showing typical seedling rot symptoms were observed. Reisolation was made from the affected portion of the plant tissue and compared with that of original isolate for conformity. Per cent disease incidence (Kokalis-Burelle *et al.*, 1992) was measured.

**Molecular confirmation of *S. rolfsii* isolates with ITS primers :**

For molecular confirmation, the genomic DNA from *S. rolfsii* was obtained using CTAB method (Zolan and Pukkila, 1996). The Internal Transcribed Spacer (ITS) of rDNA was used to amplify different *S.rolfsii* isolates using universal primers ITS-1 (5' - TCCGTAGGTGGACCTGCGG - 3') as forward primer and ITS-4 (5'-TCCTCCGCTTATTGATATGC- 3') as reverse primer (White *et al.*, 1990). PCR amplification was carried out as 25 µl reaction mixture containing 2.5 µl of 10x Taq buffer, 0.5 µl of 25 mM MgCl<sub>2</sub>, 2.0 µl of ITS-1 forward primer (0.6 picomolar µl<sup>-1</sup>), 2.0µl of ITS-4 reverse primer (0.6 picomolar µl<sup>-1</sup>), 0.5 µl of 100 mM dNTP mix, 0.125 µl of Taq polymerase (0.5 u µl<sup>-1</sup>) and 14.375 µl of sterile PCR water (Genei, Bangalore) and 3 µl (50-60 ng) of DNA sample. Reactions were performed using a Eppendorf PCR master cycler. PCR cycling conditions consist of 35 cycles, of which denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1.5min with initial denaturation at 94°C for 4 min before cycling and final extension at 72°C for 6 min after cycling. The size of the PCR product was estimated by comparison with known DNA marker of 100 bp DNA ladder. The banding profiles of ITS-PCR products were documented by gel documentation system .

**Genetic diversity analysis of *S. rolfsii* isolates using RAPD Primers :**

Total volume of 20 µl PCR reaction mixture containing DNA template 25ng, 10x Taq buffer, 2.5mM each of dNTP mixture, 2.5 mM of MgCl<sub>2</sub>, 30 picomole of random primer, and 3 units of Taq DNA polymerase (Genei, Bangalore). PCR amplification was performed in a thermocycler using the following conditions: initial denaturation at 94°C for 5min, 30 cycles of denaturation at 94°C for 1min, annealing at 36°C for 1min, extension at 72°C for 2 min, and a final elongation at 72°C for 5 min (Gaitan *et al.*, 2002). A set of 10 random primers used in this study; OPA-01 (CAGGCCCTTC), OPA-02 (TGCCGAGCTG), OPA-03 (AGTCAGCCAC), OPA-04 (AATCGGGCTG), OPA-05 (AGGGGTCTTG), OPA-06 (GGTCCCTGAC), OPA-07 (GAAACGGGTG), OPA-08 (GTGACGTAGG), OPA-09 (GGGTAACGCC) and OPA-10 (GTGATCGCAG). The amplified PCR products were screened on 1.5 per cent agarose gel.

**RAPD scoring and data analysis :** The banding patterns obtained from RAPD primers were analyzed based on the presence and absence of each band, coded as 1 and 0 respectively. The scores were used to create a data matrix to analyze genetic relationship using the NTSYS-pc program version 2.02 (Exeter Software, New York, USA) described by Rohlf (1993). A dendrogram was constructed based on Jaccard's similarity coefficient (Jaccard, 1908) using the marker data from

*S. rolfsii* isolates with unweighted pair-group method on arithmetic average (UPGMA) cluster analysis to group the isolates based on their similarities. The relationships among the isolates were examined and presented as dendrogram by using UPGMA.

**PCR details for amplification of ISSR primers :** A set of ten ISSR primers were used for assessing the genetic diversity of *S. rolfsii*. The list of primers and PCR conditions used were given in Table 2. For ISSR, the amplifications were run in a final volume of 20  $\mu$ l cocktail mixture including, Tris-HCl (10 mmol l<sup>-1</sup>), pH 8.3, KCl (50 mmol l<sup>-1</sup>), MgCl<sub>2</sub> (2.4 mmol l<sup>-1</sup>), dNTP (100  $\mu$ mol l<sup>-1</sup> of each), Oligonucleotide primers (0.4  $\mu$ mol l<sup>-1</sup>), Genomic DNA (50 – 100 ng), and Taq DNA polymerase (0.75 units). Agarose gel electrophoresis and data analysis was done as in RAPD.

**The efficacy of molecular markers viz., RAPD and ISSR for assessing the genetic diversity of *S. rolfsii* isolates :** Two marker systems viz., RAPD and ISSR were compared for their efficiency in assessing the genetic diversity of *S. rolfsii* isolates. The parameters viz., total number of bands, the number of monomorphic and polymorphic bands, bands per primer, per cent polymorphism and monomorphism were documented. In addition, Polymorphic Information Content (PIC) was calculated by the formula developed by Anderson *et al.* (1993).

Marker Index (MI) was determined as the product of PIC

and the number of polymorphic bands per assay unit and EMR (E) is the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay (Powell *et al.*, 1996).

## Results and Discussion

Stem rot disease caused by *S. rolfsii* isolates ranged from 33.33 to 80.95 % disease incidence. Among the twenty two isolates tested in the present study, the *S. rolfsii* isolate collected from Udumalpet (SrUDM) showed higher per cent disease incidence (80.95%), followed by K.G. Savadi (SrKGS) and Tindivanam (SrTVM) isolates (61.90 %). However, the isolate collected from Madurai recorded low per cent disease incidence (33.33%). The *S. rolfsii* isolated on groundnut samples collected from Kinathukadavu (SrKNK), Andipatti (SrANP), Maduranthagam (SrMDT), Tiruvannamalai (SrTRM), Erisanampatti (SrERP) and the pathogen isolated on blakgram (SrBLG), jasmine (SrJAS) and tomato (SrTOM) from Madurai, Nilakottai and Kinathukadavu, respectively showed 50 to 60 per cent, whereas the remaining samples from Pollachi (SrPLC), Coimbatore (SrCBE), Dindigul (SrDGL), Butlagundu (SrBTG), Usilampatti (SrUSP), Theni (SrTHN), Vellore (SrVLR), Vallanad (SrVAL) and Kaniyur (SrKAN) recorded 30 to 40 % disease incidence (Table 3).

The field collected *S. rolfsii* isolates were amplified with ITS universal primers viz., ITS-1 (forward) and ITS-4 (reverse)

**Table 2 :** ISSR primers and PCR conditions used in the study

Nucleotide sequence 5' to 3'	PCR conditions	Duration	No of Cycles	Reference
1. (CAG) <sub>5</sub>	Initial denaturation	95°C; 5 min.	1	Gupta and Filner, 1991
2. (GTG) <sub>5</sub>	Denaturation	95°C; 30 sec	30	
3. (AGG) <sub>5</sub>	Annealing	60°C; 30 sec		
	Extension	72°C; 1.5 min.		
	Final extension	72°C; 10 min.	1	
4. (GACA) <sub>4</sub>	Initial denaturation	95°C; 5 min.	1	Weising <i>et al.</i> , 1989
	Denaturation	95°C; 30 sec	30	
	Annealing	48°C; 30 sec		
	Extension	72°C; 1.5 min.		
	Final extension	72°C; 10 min.	1	
5. (TCC) <sub>5</sub>	Initial denaturation	95°C; 5 min.	1	Gupta and Filner, 1991
	Denaturation	94°C; 1 min.	30	
	Annealing	46°C; 1min.		
	Extension	72°C; 1.5 min.		
	Final extension	72°C; 10 min.	1	
6. (CAC) <sub>5</sub>	Initial denaturation	95°C; 5 min.	1	Gupta <i>et al.</i> , 1994
	Denaturation	94°C; 1 min.	40	
	Annealing	51°C; 1min.		
	Extension	72°C; 2 min.		
	Final extension	72°C; 10 min.	1	
7. (AG) <sub>8</sub> T	Initial denaturation	94°C; 2 min.	1	Ratanacherdchai <i>et al.</i> , 2007
8. (AG) <sub>8</sub> C	Denaturation	94°C; 1 min.	35	
9. (GA) <sub>8</sub> T	Annealing	52°C; 1min.		
10. (TG) <sub>8</sub> A	Extension	72°C; 1.5 min.		
	Final extension	72°C; 6 min.	1	

**Table 3:** Virulence of *S. rolfsii* isolates in groundnut

<i>S. rolfsii</i> Isolates	Disease Incidence (%)
SrPLC	47.62 (43.63) <sup>e</sup>
SrKGS	61.90 (51.89) <sup>b</sup>
SrCBE	42.86 (40.89) <sup>f</sup>
SrKNK	52.38 (46.36) <sup>d</sup>
SrDGL	42.86 (40.89) <sup>f</sup>
SrBTG	38.10 (38.11) <sup>g</sup>
SrUSP	38.10 (38.11) <sup>g</sup>
SrMDU	33.33 (35.26) <sup>h</sup>
SrTHN	47.62 (43.63) <sup>e</sup>
SrANP	52.38 (46.36) <sup>d</sup>
SrVLR	42.86 (40.89) <sup>f</sup>
SrVAL	38.10 (38.11) <sup>g</sup>
SrTVM	61.90 (51.89) <sup>b</sup>
SrMDT	57.14 (49.11) <sup>c</sup>
SrTRM	52.38 (46.36) <sup>d</sup>
SrUDM	80.95 (64.20) <sup>a</sup>
SrERP	52.38 (46.36) <sup>d</sup>
SrKAN	47.62 (43.63) <sup>e</sup>
SrBLG	52.38 (46.36) <sup>d</sup>
SrSUN	47.62 (43.63) <sup>e</sup>
SrJAS	57.14 (49.11) <sup>c</sup>
SrTOM	52.38 (46.36) <sup>d</sup>
Control	0

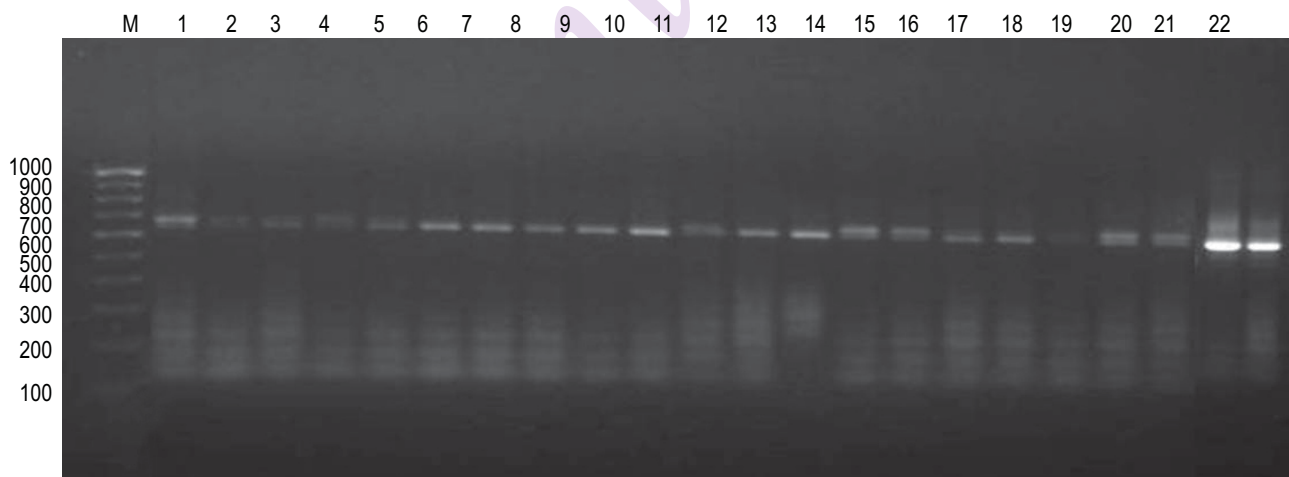
Values are mean of three replicates; Figures in parentheses represent arcsine transformation; Means in a column followed by same superscript letters are not significantly different according to DMRT at  $P \leq 0.05$

and the amplicon size recorded were 650-700bp (Fig. 1). It showed that the isolates collected from different locations confirmed as the genus *Sclerotium*. This is in confirmation with the earlier report of Harlton *et al.* (1995) who screened worldwide collection of *S. rolfsii* revealing variation in ITS regions of 12 sub-groups of *S. rolfsii*. Similar findings were also observed by Almeida *et al.* (2001) who reported genetic confirmation of *S.*

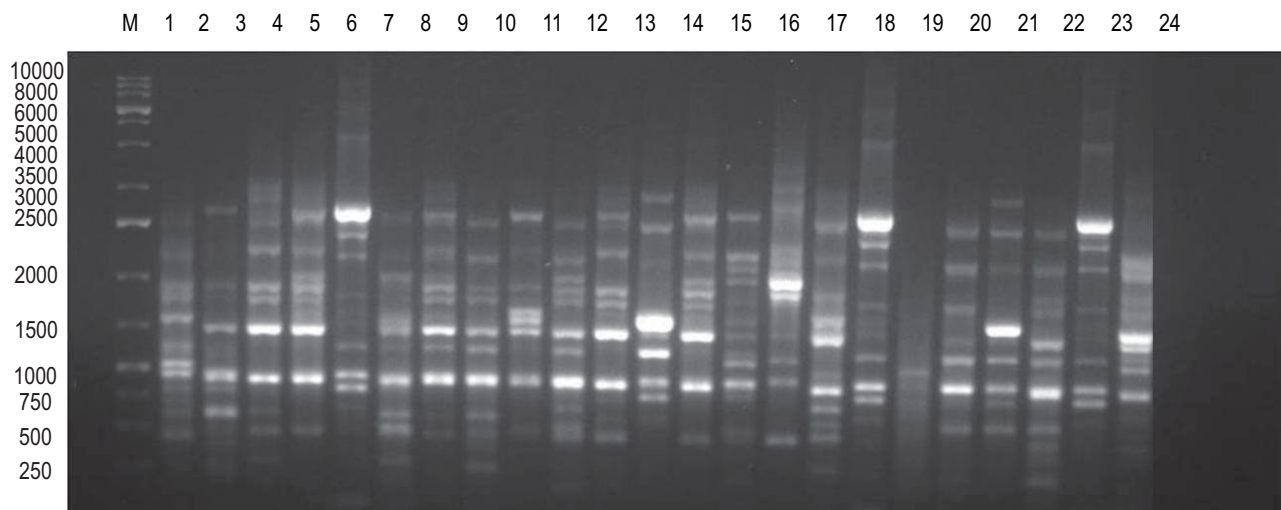
*rolfsii* with ITS primers. In addition, the present study is in concordance with Prasad *et al.* (2010) who studied the molecular variability of *S. rolfsii* isolates using ITS-PCR primers and found molecular confirmation of *S. rolfsii* isolates and significant genetic variations among the isolates. The results of the present study also reported that the ITS – PCR was an effective tool for the identification of fungal pathogens, including genus *Sclerotium*.

Genetic diversity of fungal pathogens is essential for the assessment of characteristics and diversity of fungal pathogens and it would be helpful for the development of effective integrated disease management strategy. Nuclear markers play a major role in genetic study of living organisms particularly the efficiency of RAPD, as a arbitrary DNA marker has been extensively reported as efficient marker for genetic diversity studies. The researchers from phytopathology enunciated the potential of RAPD markers for assessing the diversity of fungal and other pathogens (Thilagavathi *et al.*, 2013; Kumar *et al.*, 2014; Mallaiah and Muthamilan, 2015). Thus, the present study tested the field collected *S. rolfsii* isolates with ten RAPD primers viz., OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, OPA-06, OPA-07, OPA-08, OPA-09 and OPA-10. All the primers were amplified with *S. rolfsii* isolates showed polymorphic and distinguishable banding pattern. A total of 121 reproducible and scorable polymorphic bands with 100 to 2500 bp were generated among the 22 isolates. Among the primers, the primer OPA-09 yielded maximum of 17 total bands, followed by OPA-05 that showed 15 bands. In polymorphism, most of the primers showed cent percent polymorphism, except for the primer OPA-03 and OPA-09 (Table 4; Fig. 2).

Relationship among the isolates was evaluated by cluster analysis of data based on similarity matrix. The dendrogram was generated by using UPGMA package based on Ward's Squared Equalidean Distance method (Fig. 3). All the 22 isolates of *S. rolfsii* were grouped into two main clusters. Cluster I had 20



**Fig. 1 :** Molecular characterization of *S.rolfsii* isolates by ITS-PCR. Isolates numbers are shown as 1 to 22. M-100bp molecular marker



**Fig. 2 :** RAPD of *S. rolfsii* isolates using OPA-03 primer. Isolates numbers are shown as 1 to 22. 23 - Positive control. 24 - Negative control. M-1Kb molecular marker.

**Table 4 :** Polymorphism, PIC and EMR of *S. rolfsii* isolates revealed by RAPD

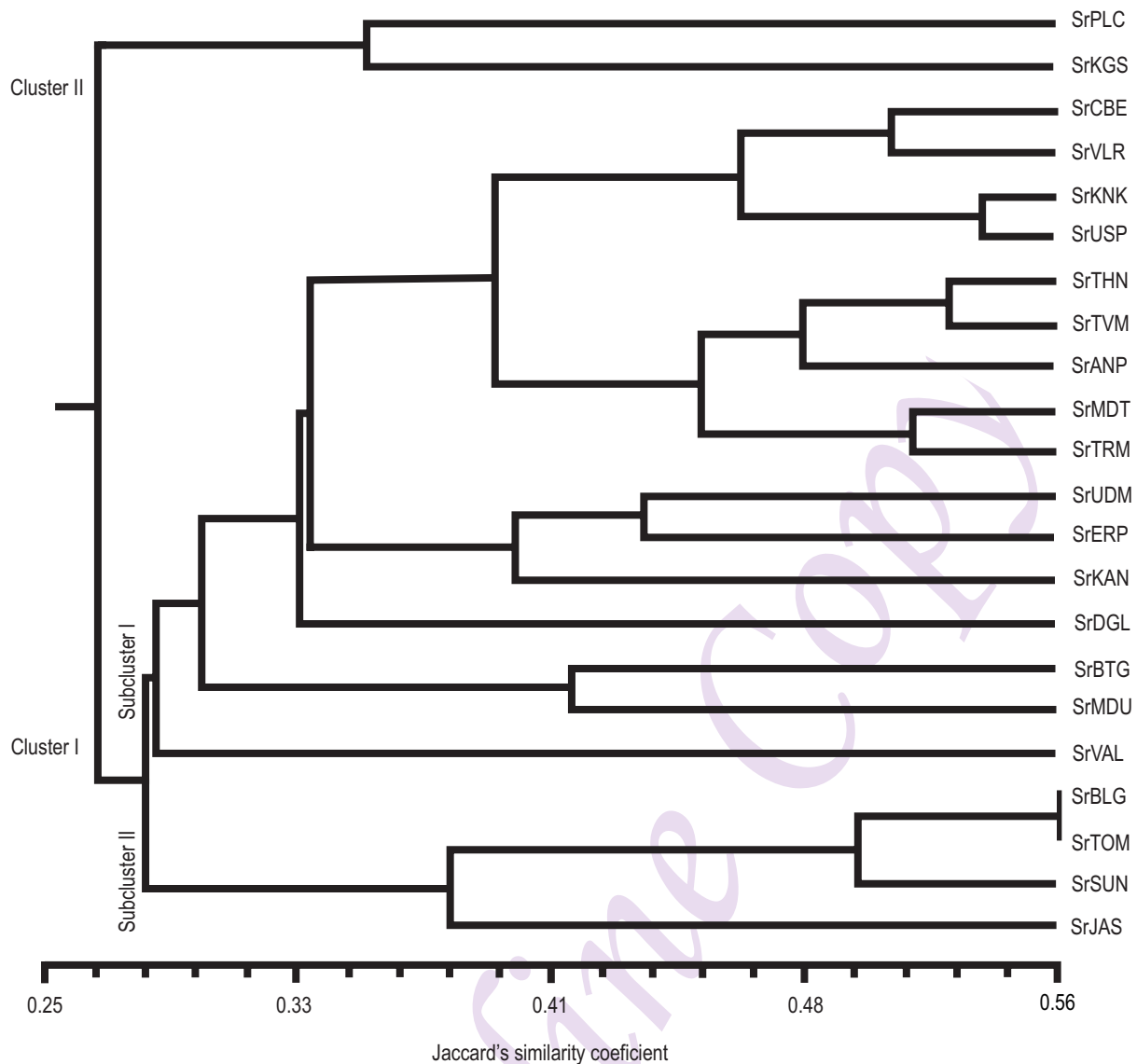
Primers	Total of bands	No. of Polymorphic bands	No. of Monomorphic bands	Percent Polymorphic bands	Percent Monomorphic bands	PIC Value	EMR
OPA1	11	11	0	100	0.00	0.902	11.00
OPA2	09	09	0	100	0.00	0.836	09.00
OPA3	13	12	1	99.41	0.59	0.912	12.85
OPA4	10	10	0	100	0.00	0.845	10.00
OPA5	15	15	0	100	0.00	0.906	15.00
OPA6	09	09	0	100	0.00	0.822	09.00
OPA7	09	09	0	100	0.00	0.762	09.00
OPA8	14	14	0	100	0.00	0.895	14.00
OPA9	17	16	1	99.65	0.35	0.906	16.88
OPA10	14	14	0	100	0.00	0.871	14.00
Total	121	119	2	-	-	-	-

PIC-Polymorphic Information Content; EMR- Effective Multiplication Ratio

isolates and shared similarity of 27 % and cluster II contained two isolates (SrPLC and Sr KGS). The cluster I had two subclusters viz., subcluster I with sixteen isolates of *S. rolfsii* collected from groundnut and subcluster II with four isolates from black gram, sunflower, tomato and jasmine at 28 % similarity. The results showed genetic variability of *S. rolfsii* with host level.

The binary data from the polymorphism were used for computing the similarity indices. The similarity coefficient values obtained for each pair wise comparison is presented in Table 5. The similarity coefficients ranged from 0.14 to 1.00. Among the 22 isolates of *S. rolfsii*, the highest similarity matrix index (0.56) was observed between black gram (Madurai) and tomato (Kinathukadavu). The lowest similarity matrix index (0.14) was observed in the groundnut *S. rolfsii* isolates from Butlagundu and Erisanampatti (Table 5).

The results of genetic diversity between the *Sclerotium* isolates of groundnut and other hosts were elucidated, indicating the influence of host factor on the genetic makeup of the pathogen. Further, genetic diversity revealed by RAPD primers was not in accordance to the geographical origin of the isolates. Thus, RAPD can be used efficiently to differentiate *S. rolfsii* groundnut with other hosts. The results of the present study was in accordance with the findings of Almeida *et al.* (2001), they studied the genetic variability of *S. rolfsii* isolates collected from different hosts in Brazil and found significant genetic variability among isolates using RAPD analysis. During same period, Punja and Sun (2001) studied the genetic diversity of *S. rolfsii* collected from more than ten countries and found high level of variations from the samples collected from different regions. The findings of Perez-Moreno *et al.* (2002) are also in agreement with the present study and found that genetic polymorphism among the isolates of



**Fig. 3 :** RAPD dendrogram showing genetic relationship between twenty two *S.rolfsii* isolates.

*Sclerotium cepivorum* collected from Mexico, based on RAPD analysis.

Inter simple sequence repeats (ISSR) is an effective and alternative tool for studying the genetic variability of any living organism due to the presence of microsatellites regions throughout genomes (Raina *et al.*, 2001; Wang, 2002 and Pharmawati *et al.*, 2005). In the present study, ISSR markers were used to investigate the genetic diversity of *S. rolfsii* isolates collected from different geographical regions. All the field samples collected were amplified with ten ISSR primers and produced a total of 123 bands and the amplicon size ranged from 250 - 2000 bp (Fig. 4). Of the ten primers used in the study, most of the primers showed cent per cent polymorphism. However, the primer (GACA)<sub>4</sub> yielded maximum bands of 17 and all were polymorphic (Table 6). Genetic similarity matrix was constructed

from binary data of markers using Jaccards algorithm index. The genetic similarity coefficient ranged from 16 to 78 %. Maximum similarity of 78 % was observed between SrKGS and SrCBE isolates, and least similarity matrix index of 16 % was observed between SrTVM and SrKGS isolates (Table 7).

A dendrogram resulting from cluster analyses showed two main distinct groups, designated as A and B exhibiting overall genetic relationship among the isolates. Four isolates (SrBLG, SrSUN, SrJAS, SrTOM) were grouped together as group "A" and all other isolates were grouped in "B" at 26 % similarity. Group B was subdivided into two subgroup, I and II. Among the eighteen isolates from group B, five isolates were present in subgroup I and the remaining thirteen isolates formed the subgroup II (Fig. 5). The results of the present study with ISSR primer showed high level of polymorphism and genetic diversity, not only at host level

Table 5 : Similarity matrix for Nei and Li's coefficient of *S. roffsigii* isolates revealed by RAPD

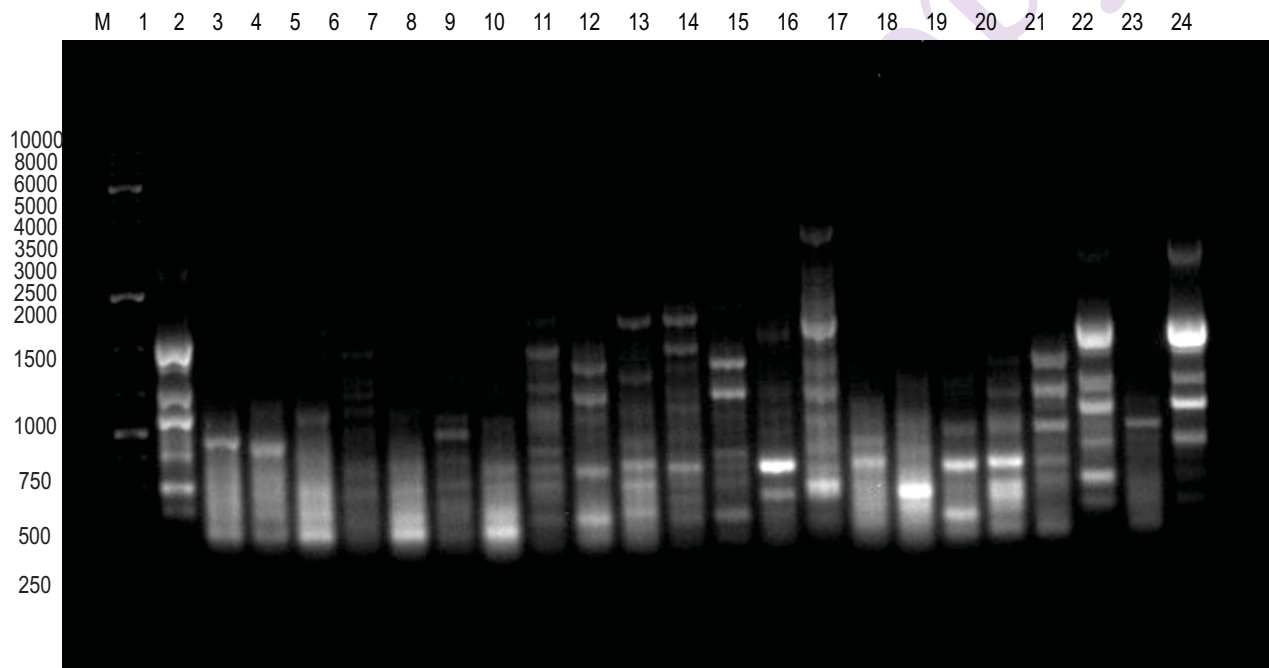
Isolate	SrPLC	SrKGS	SrCBE	SrKKNK	SrDGL	SrBTG	SrUSP	SrMDU	SrTHN	SrANP	SrVLR	SrVAL	SrTVM	SrMDT	SrTRM	SrJUDM	SrERP	SrKAN	SrBLG	SrSUN	SrJAS	SrTOM	
SrPLC	1																						
SrKGS	0.348	1																					
SrCBE	0.431	0.413	1																				
SrKKNK	0.303	0.326	0.509	1																			
SrDGL	0.250	0.239	0.285	0.384	1																		
SrBTG	0.241	0.277	0.312	0.333	0.357	1																	
SrUSP	0.245	0.283	0.406	0.537	0.363	0.359	1																
SrMDU	0.152	0.224	0.209	0.363	0.288	0.410	0.368	1															
SrTHN	0.322	0.206	0.349	0.416	0.400	0.369	0.442	0.355	1														
SrANP	0.267	0.260	0.278	0.392	0.296	0.322	0.396	0.403	0.482	1													
SrVLR	0.377	0.300	0.509	0.481	0.309	0.354	0.456	0.293	0.440	0.471	1												
SrVAL	0.173	0.232	0.259	0.259	0.250	0.161	0.245	0.369	0.237	0.320	0.307	1											
SrTVM	0.293	0.264	0.344	0.438	0.345	0.303	0.491	0.327	0.526	0.481	0.464	0.320	1										
SrMDT	0.254	0.269	0.306	0.327	0.280	0.268	0.400	0.288	0.365	0.462	0.446	0.277	0.509	1									
SrTRM	0.246	0.200	0.328	0.291	0.307	0.259	0.352	0.294	0.441	0.421	0.388	0.306	0.507	0.516	1								
SrJUDM	0.312	0.333	0.378	0.421	0.360	0.266	0.362	0.303	0.371	0.348	0.421	0.338	0.453	0.415	0.424	1							
SrERP	0.259	0.224	0.293	0.271	0.340	0.144	0.258	0.186	0.269	0.237	0.363	0.285	0.283	0.433	0.333	0.433	1						
SrKAN	0.245	0.234	0.303	0.280	0.274	0.166	0.266	0.259	0.238	0.245	0.303	0.326	0.315	0.345	0.303	0.400	0.387	1					
SrBLG	0.210	0.270	0.266	0.310	0.236	0.230	0.196	0.267	0.173	0.275	0.255	0.254	0.238	0.262	0.219	0.318	0.267	0.352	1				
SrSUN	0.280	0.272	0.314	0.392	0.285	0.209	0.275	0.245	0.245	0.301	0.244	0.255	0.303	0.263	0.252	0.344	0.269	0.306	0.522	1			
SrJAS	0.183	0.235	0.258	0.344	0.186	0.261	0.350	0.327	0.317	0.407	0.392	0.269	0.355	0.362	0.338	0.308	0.280	0.245	0.396	0.380	1		
SrTOM	0.270	0.261	0.283	0.307	0.250	0.220	0.245	0.211	0.258	0.320	0.283	0.272	0.272	0.254	0.246	0.295	0.369	0.326	0.560	0.475	0.346	1	



**Table 6 :** Genetic characterization of *S. rolfsii* isolates revealed by ISSR

Primer	Total of bands	No. of Polymorphic bands	No. of Monomorphic bands	Per cent Polymorphic bands	Per cent Monomorphic bands	PIC Value	EMR
(CAG) <sub>5</sub>	11	11	0	100	0.00	0.884	11.00
(GTG) <sub>5</sub>	9	9	0	100	0.00	0.867	9.00
(AGG) <sub>5</sub>	10	10	0	100	0.00	0.853	10.00
(GACA) <sub>4</sub>	17	17	0	100	0.00	0.902	17.00
(TCC) <sub>5</sub>	11	10	1	90.91	9.09	0.857	10.82
(CAC) <sub>5</sub>	11	11	0	100	0.00	0.883	11.00
(AG) <sub>8</sub> T	15	15	0	100	0.00	0.885	15.00
(AG) <sub>8</sub> C	12	12	0	100	0.00	0.894	12.00
(GA) <sub>8</sub> T	8	5	3	62.50	37.50	0.788	07.29
(TG) <sub>8</sub> A	19	19	0	100	0.00	0.922	19.00
<b>Total</b>	<b>123</b>	<b>119</b>	<b>4</b>				

PIC-Polymorphic Information Content; EMR- Effective Multiplication Ratio



**Fig. 4 :** DNA banding profiles generated by ISSR-PCR with the primer (GACA)<sub>4</sub>. Isolates numbers are shown as 1 to 22. 23 - Positive control. 24 - Negative control. M-1Kb molecular marker

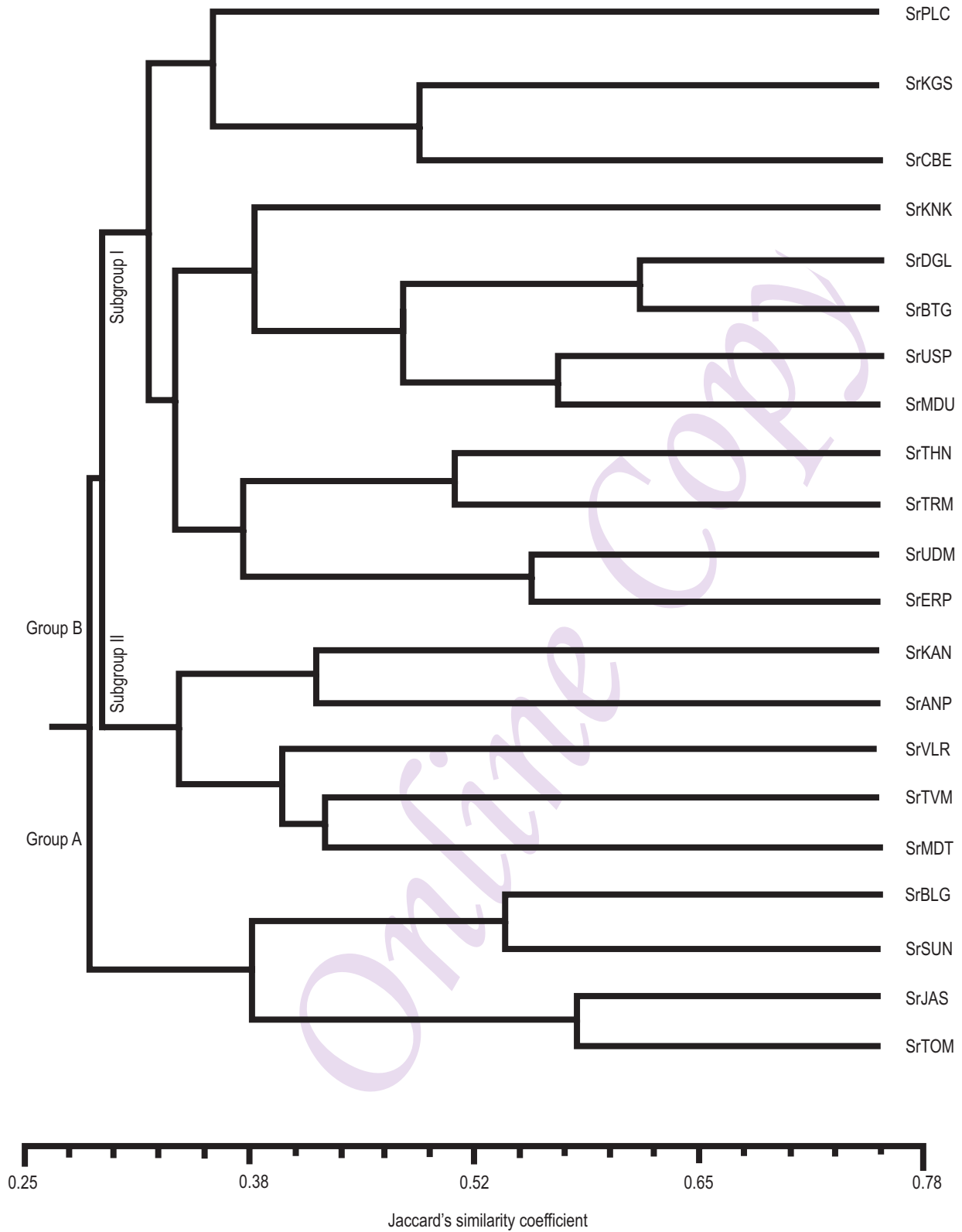
but also at sampling regions. The results of the present study is in agreement with the previous reports of Gupta *et al.* (1994); Rathour *et al.* (2004); Misra *et al.* (2005) where significant level of genetic variations of fungal pathogens were found.

Several workers have reported that the molecular markers are effective for assessing the genetic diversity of fungal pathogens than the conventional methods (Sharma, 2003; Lal and Dutta, 2012; Adhipathi *et al.*, 2013). Hence, the present study focused to assess the genetic variability of *S. rolfsii* using nuclear

markers viz., RAPD and ISSR and also validate their efficacy. The parameters including percent polymorphism, polymorphic information content (PIC) and effective multiplication ratio were used to compare the efficiency of marker systems. The total number of bands produced was more in ISSR (123) compared with RAPD (121) (Table 4 and 6). However, the percent polymorphism was almost same in both cases as percent polymorphism was recorded in many primers in both markers. The PIC value varied significantly between both markers. The ISSR marker had an average PIC of 0.87 as against 0.86 of

Table 7 : Similarity matrix for Nei and Li's coefficient of *S. roffsi* isolates revealed by ISSR

Isolate	Sr PLC	Sr KGS	Sr CBE	Sr KNK	Sr DGL	Sr BTG	Sr USP	Sr MDU	Sr THN	Sr ANP	Sr VLR	Sr VAL	Sr TVM	Sr MDT	Sr TRM	Sr UDM	Sr ERP	Sr KAN	Sr BLG	Sr SUN	Sr JAS	Sr TOM	
Sr PLC	1																						
Sr KGS	0.403	1																					
Sr CBE	0.372	0.780	1																				
Sr KNK	0.242	0.423	0.530	1																			
Sr DGL	0.228	0.238	0.295	0.403	1																		
Sr BTG	0.230	0.309	0.280	0.345	0.418	1																	
Sr USP	0.246	0.303	0.345	0.339	0.410	0.622	1																
Sr MDU	0.290	0.358	0.351	0.345	0.278	0.440	0.520	1															
Sr THN	0.327	0.277	0.296	0.339	0.363	0.380	0.521	0.568	1														
Sr ANP	0.322	0.320	0.339	0.263	0.266	0.296	0.365	0.296	0.395	1													
Sr VLR	0.220	0.230	0.250	0.375	0.206	0.333	0.300	0.306	0.355	0.409	1												
Sr VAL	0.298	0.222	0.240	0.333	0.309	0.320	0.196	0.269	0.285	0.306	0.380	1											
Sr TVM	0.212	0.161	0.177	0.345	0.322	0.358	0.258	0.241	0.301	0.228	0.361	0.404	1										
Sr MDT	0.200	0.183	0.180	0.363	0.207	0.288	0.255	0.288	0.309	0.244	0.388	0.368	0.414	1									
Sr TRM	0.205	0.290	0.285	0.366	0.211	0.355	0.285	0.333	0.327	0.238	0.285	0.233	0.311	0.375	1								
Sr UDM	0.240	0.349	0.323	0.359	0.281	0.370	0.323	0.393	0.322	0.202	0.283	0.253	0.268	0.290	0.500	1							
Sr ERP	0.246	0.327	0.321	0.388	0.316	0.351	0.275	0.377	0.320	0.163	0.274	0.264	0.237	0.282	0.372	0.535	1						
Sr KAN	0.169	0.220	0.265	0.260	0.313	0.270	0.319	0.326	0.260	0.180	0.177	0.145	0.150	0.236	0.210	0.321	0.550	1					
Sr BLG	0.218	0.206	0.290	0.241	0.266	0.260	0.245	0.228	0.218	0.259	0.192	0.230	0.186	0.191	0.278	0.257	0.339	0.439	1				
Sr SUN	0.274	0.290	0.354	0.280	0.203	0.245	0.220	0.245	0.236	0.254	0.260	0.250	0.183	0.239	0.274	0.253	0.333	0.333	0.533	1			
Sr JAS	0.186	0.169	0.166	0.230	0.192	0.265	0.235	0.240	0.229	0.250	0.317	0.272	0.240	0.333	0.250	0.271	0.340	0.307	0.276	0.452	1		
Sr TOM	0.240	0.282	0.275	0.326	0.181	0.311	0.224	0.282	0.217	0.239	0.378	0.325	0.255	0.406	0.313	0.333	0.395	0.297	0.325	0.414	0.580	1	



**Fig. 5 :** ISSR dendrogram showing genetic relationship between twenty two *S.rolfsii* isolates.

RAPD, and the effective multiplication ratio was also superior in ISSR (12.21) compared with RAPD (12.00). Thus, considering all parameters studied and also the reproducibility of ISSR markers, it was concluded that ISSR markers are suited well for diversity studies compared to RAPD. The results of the present study is in agreement with the findings of Sonia and Gopalakrishna (2007), who compared the efficacy of ISSR and REMAP for genetic diversity study of *Pyricularia oryzae*. Among the nuclear markers, the ISSR markers are considered as co-dominant and may help to assess the heterozygosity nature of the samples and also produce high polymorphic information content (PIC). Thus, it was considered as the marker of choice for the genetic diversity of fungal pathogens. The present study also found the superiority of ISSR than RAPD hence, it was demonstrated that the ISSR marker is suitable for genetic diversity of *S. rolfsii*.

The *S. rolfsii* isolates collected from Udumalpet, K.G. Savadi and Tinidvanam recorded higher per cent disease incidence, and the isolate from Madurai showed lower percent disease incidence. All other isolates showed medium level of per cent disease incidence. Further confirmation with the effective nuclear marker (ISSR) showed not much more variation between the isolates based on percent disease incidence (Virulence). At though, the host wise variation was observed in the present study, the isolates from groundnut formed separate group (Group B) from all other isolates from blackgram, sunflower, jasmine and tomato (Group A), and the variation was also observed within the isolates (Group A & B) (Fig. 5). The present results showed that there were no correlation between virulence (per cent disease incidence) and genetic diversity of *S. rolfsii* isolates collected from major groundnut growing areas of Tamil Nadu. The genetic variability was observed between the *S. rolfsii* isolates of groundnut and the *S. rolfsii* isolates from blackgram, jasmine, tomato and sunflower. This enunciated that the nature and type of host may influence genetic variation between the isolates.

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