International Journal of Advanced Biochemistry Research

ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2025): 5.29
IJABR 2025; 9(7): 1721-1726
www.biochemjournal.com
Received: 02-04-2025
Accepted: 07-05-2025

RG Matale
M.Sc. student, Vilasrao Deshmukh College of Agricultural Biotechnology, Latur, Maharashtra, India

DJ Wankhade
Senior Research Assistant, Vilasrao Deshmukh College of Agricultural Biotechnology, Latur, Maharashtra, India

KV Birajdar
M.Sc. student, Vilasrao Deshmukh College of Agricultural Biotechnology, Latur, Maharashtra, India

DV Tambe
M.Sc. student, Vilasrao Deshmukh College of Agricultural Biotechnology, Latur, Maharashtra, India

VR Hinge
Assistant Professor, Vilasrao Deshmukh College of Agricultural Biotechnology, Latur, Maharashtra, India

PK Jadhav
Junior Research Fellow, Vilasrao Deshmukh College of Agricultural Biotechnology, Latur, Maharashtra, India

AS Deshmukh
Junior Research Fellow, Vilasrao Deshmukh College of Agricultural Biotechnology, Latur, Maharashtra, India

YS Bhagat
Assistant Professor, Vilasrao Deshmukh College of Agricultural Biotechnology, Latur, Maharashtra, India

SJ Magar
Professor (CAS), College of Agriculture, Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani, Maharashtra, India

RL Chavhan
Associate Professor (CAS), Vilasrao Deshmukh College of Agricultural Biotechnology, Latur, Maharashtra, India

Corresponding Author:
RL Chavhan
Associate Professor (CAS), Vilasrao Deshmukh College of Agricultural Biotechnology, Latur, Maharashtra, India

Molecular characterization of *Sclerotium rolfsii* causing collar rot disease in soybean using ISSR markers

RG Matale, DJ Wankhade, KV Birajdar, DV Tambe, VR Hinge, PK Jadhav, AS Deshmukh, YS Bhagat, SJ Magar and RL Chavhan

DOI: https://www.doi.org/10.33545/26174693.2025.v9.i7v.5021

Abstract
Soybean (*Glycine max* [L.] Merrill) is a vital oilseed crop being cultivated globally. Its productivity in India remains below potential, partly due to the threat of soil-borne diseases like collar rot caused by *Sclerotium rolfsii*. This study aimed to assess the genetic diversity among *S. rolfsii* isolates collected from soybean-growing regions of Maharashtra using Inter Simple Sequence Repeat (ISSR) markers. A total of fifteen isolates were collected from Marathwada, Vidarbha, and Western Maharashtra and confirmed as *S. rolfsii* through ITS-based molecular identification. Seven ISSR primers were used for DNA fingerprinting analysis and generated 587 total amplicons, of which 337 were polymorphic, resulting in an overall polymorphism rate of 83.91%. Primers UBC-811 delineated 100% polymorphism and the highest PIC values (0.365), indicated high discriminatory power. UPGMA-based clustering grouped isolates based on moderate geographic association, revealing both inter- and intra-regional diversity. These results highlighted the effectiveness of ISSR markers in detecting genetic variability within *S. rolfsii*. The findings would help to provide a molecular basis for developing targeted breeding and disease management strategies to control collar rot in soybean across Maharashtra's key production zones.

Keywords: *Sclerotium rolfsii*, soybean, ISSR markers, genetic diversity, molecular characterization, Maharashtra, Collar rot

1. Introduction
Soybean (*Glycine max* [L.] Merrill) is one of the world's most vital oilseed crops, accounting for approximately half of global oilseed production (Fehr, 1989) [6]. Despite its agricultural significance, India's soybean productivity average is 991 kg/ha, markedly lowers than the global average of 2,880 kg/ha (Anonymous, 2022) [1]. This yield gap resulted due to several reasons among which soil-borne diseases, particularly collar rot caused by *Sclerotium rolfsii* Sacc., leading 10-30% yield losses annually (Hartman *et al*., 1999) [7] becoming the prominent cause. In Maharashtra's key soybean-growing regions of Vidarbha and Marathwada, where climatic conditions favour proliferation of *S. rolfsii*. The outbreak of *S. rolfsii* has become increasingly severe, often resulted in complete crop failure under heavy infestation.

The genetic diversity of *S. rolfsii* populations remains poorly characterized in Indian soybean, despite its critical implications for disease management. Previous studies on chickpea-infecting strains of *S. rolfsii* in Maharashtra using SSR markers revealed 77.6% polymorphism among chickpea isolates (Swain *et al*., 2018) [17] and demonstrated the value of molecular diversity studies. Inter simple sequence repeat (ISSR) markers offer particular advantages for such studies, as they require no prior genome information while providing high reproducibility and multilocus detection capacity. These characteristics make ISSR as ideal marker for establishing baseline data on pathogen diversity, tracking strain evolution, and identifying regional adaptations knowledge which are essential for developing effective control strategies.

This study was conducted with 15 isolates of *S. rolfsii* collected from soybean growing fields of Vidarbha and Marathwada regions of Maharashtra. The study was conducted to reveal genetic diversity among these populations using seven ISSR primers with aiming to

determine the degree of polymorphism, identify potential geographic patterns in strain distribution, and identify molecular markers for future disease monitoring. The research findings would directly help in resistance breeding programs and implementation of regional disease management approaches, particularly in Maharashtra's vulnerable soybean-growing zones where collar rot incidence has risen alarmingly in recent years.

2. Materials and Methods

2.1 Sample collection, Pathogen isolation, and Identification

The collar rot infected diseased samples were collected from different soybean growing fields of Maharashtra. A total of 15 isolates were collected from infected plants exhibiting white, fan-like mycelial growth on the collar region. Adhesive soil was removed by rinsing under running tap water. The symptomatic collar tissues were cut into 0.5 to 1 cm segments and surface-sterilized in 1% sodium hypochlorite for 2 minutes. The segments were rinsed twice with sterile distilled water, dried on sterile filter paper, and plated on Potato dextrose agar (PDA) medium. Plates were incubated at 27±2 °C, and mycelial growth was observed after 3 days of incubation. Pure cultures were obtained by sub-culturing the leading edge of the white, fan-shaped colony onto fresh PDA plates. Cultures were maintained at 4 °C for further use. Identification of *Sclerotium rolfsii* was done based on morphological characteristics and molecular level using Internal Transcribed Spacer (ITS) sequence and BLASTn analysis (White *et al*., 1990; Kadam *et al.*, 2009) [18, 9].

2.2 Isolation of genomic DNA

The fungal mycelia were harvested from 5-day-old broth cultures grown in Potato Dextrose Broth (PDB) at 27 °C on a rotary shaker (100 rpm). Mycelial mats were filtered through cheesecloth, blotted dry, and utilized for immediate DNA extraction. Approximately 100 mg of fungal mycelium was ground to a fine powder using liquid nitrogen in a pre-chilled mortar and pestle. DNA extraction was performed using the CTAB method (Chavhan *et al*, 2015 & 2018) [4] with minor modifications. One milliliter of prewarmed extraction buffer (100 mM Tris-HCl pH 8, 20 mM EDTA, 2M NaCl, 3% CTAB, 2% β-mercaptoethanol) was added to the powdered sample and further homogenized. The mixture was transferred to 2 ml microcentrifuge tubes, and 5 µl of Proteinase K (10 mg/ml) was added. Tubes were incubated at 65 °C for 30 minutes with intermittent swirling. After incubation samples were centrifuged at 13,000 rpm for 10 minutes and the upper aqueous phase was transferred to fresh tubes. Further, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernant, mixed it gently and centrifuged at 13,000 rpm for 10 min. The supernant was extracted and DNA in each tube was precipitated with 0.6 volume of iso-propanol by incubating the mixture at -20˚C for 30 min. Further DNA was recovered by centrifugation of the mixture at 15000 rpm for 5 min at 4˚C. The pellet was rinsed with 70% ethanol, air dried briefly and resuspended in 40µl TE buffer. The DNA was further purified by giving *RNase* A (20µg/ml) treatment. The quality of DNA was assessed by resolving on 0.8% agarose gel. Quantification of DNA was done by spectrophotometer analysis and stored at -20˚ C until further use.

2.3 Molecular Confirmation of *Sclerotium rolfsii* Isolates

Molecular confirmation of the isolates was performed using nuclear rDNA-based sequence analysis. The ITS primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATG-3') as described by Chavhan *et al*. (2008) [3] were utilized in PCR reactions. PCR reaction was set up in 25 µl volumes containing 2 µl (50 ng) template DNA, 2.5 µl 10X PCR buffer, 0.5 mM dNTPs, 1 µL each of ITS1 and ITS4 primers, 2 µL MgCl2, 0.3 µl *Taq* polymerase (5 U/µL), and 16.37 µL sterile distilled water. The PCR program included initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 45 sec, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. PCR amplified products were separated by electrophoresis on 1.2% agarose gels, stained with ethidium bromide and visualized under UV light using a gel documentation system. The gel purified fragments were submitted for sequencing by M/s. Eurofin Genomics India Pvt. Ltd.

2.4 Molecular characterization using ISSR Primer

Amplification of genomic DNA was carried out using ISSR primers. PCR was performed in 25 µl volumes in 0.2 ml PCR tubes containing 2.5 µl 10X PCR buffer, 0.3 µl dNTPs mix, 0.33 µl *Taq* DNA polymerase (5 U/µl), 1.5 µl 25 mM MgCl2, 1 µl primer, 2 µl template DNA and 16.37 µl distilled water. Amplification was conducted using a Thermal Cycler with the program comprising: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 1 min 30 sec, final extension was set at 72 °C for 10 min and final reaction was hold at 4 °C.

Amplified products were resolved on 2% agarose gels in 1X TAE buffer, stained with ethidium bromide, and visualized using a Gel Doc system. Molecular weight of amplified bands was estimated using 3000 bp DNA ladder. The presence of band was scored as '1' and absence as '0'. Jaccard's coefficient was used to compute similarity matrix for pairwise comparison of isolates. Dendrogram based on ISSR data was constructed using NTSYS-pc version 2.2. The informativeness of ISSR markers was evaluated through polymorphism percentage, polymorphic information content (PIC), genotypic diversity as described by Bhardwaj *et al*. (2010) [2].

3. Results and Discussion

3.1 Sample Collection, Pathogen Isolation, and Morphological Identification

A total of fifteen isolates of *Sclerotium rolfsii* were isolated from collar rot-infected soybean plants collected from different agro-climatic regions of Maharashtra. The infected plants were characterized by typical white, fan-like mycelial growth on the collar region, which is a diagnostic feature of *S. rolfsii*. The infected collar tissues, after proper surface sterilization, consistently yielded fungal colonies when cultured on PDA medium. After 72 hours of incubation at 27±2 °C, the pathogen showed rapid radial growth forming dense, white, fan-shaped mycelium, which is consistent with the morphological features (Figure 1) previously reported for *S. rolfsii* (Kadam *et al*., 2009) [9]. Sub-culturing of the leading edge of colonies was utilized to prepare pure cultures, which were maintained and further utilized for molecular characterization. These morphological and

microscopic observations provided a preliminary base for the identification of the species *S. rolfsii*.

3.2 Molecular Confirmation Using nuclear rDNA sequences

All fifteen isolates were subjected to molecular confirmation using amplification by ITS1 and ITS4 primers targeting the Internal Transcribed Spacer region of nuclear ribosomal DNA. PCR amplification yielded a single amplicon of approximately 650-700 bp for all isolates, which was clearly visualized on 1.2% agarose gels stained with ethidium bromide.

The amplified nuclear rDNA after purification were submitted for sequencing at Eurofins Genomics India Private Limited, Bengaluru, India. The Sanger dideoxy sequencing method was employed, and the consensus nuclear rDNA sequences were annotated using bioinformatics tools. The resulting sequences were confirmed using BLASTn analysis. The BLASTn analysis showed high similarity (≥99%) with known sequences of *Sclerotium rolfsii*, available in public domain and confirmed the identity of the isolates of *Sclerotium rolfsii* at molecular level. The ITS-nuclear rDNA sequences were significantly utilized by several researchers for accurate identification of several fungal pathogens (White *et al*., 1990; Chavhan *et al*., 2008; Kadam *et.al*., 2009) [18, 3, 9].

3.3 Molecular Characterization using ISSR Primers

Seven ISSR primers were employed for PCR amplification of genomic DNA of 15 *isolates of S. rolfsii*. All primers successfully amplified the target regions and generated distinct, reproducible banding patterns **(**Figure 2a-c). A total of 587 bands were produced, of which 337 were polymorphic, resulting in an overall polymorphism rate of 83.91%. Each ISSR primer exhibited a distinct level of informativeness, as reflected in its polymorphism percentage and Polymorphic Information Content (PIC) values as described in Table 1. The primer UBC-810 exihibited100% polymorphism with PIC value of 0.236, indicating that while all loci were polymorphic. While primer UBC-811 revealed 100% polymorphism with a PIC of 0.365, making it one of the most informative primers. The primer efficiently discriminates isolates from Western Maharashtra and Vidarbha regions. The primer UBC-857 exhibited highest polymorphism after UBC-811 and UBC-810 (85.71%) and showed PIC value of 0.328. The primer enabled to discriminate the isolates based on geographic patterns and abled to identify intra-regional variability among the isolates.

This high level of polymorphism indicated significant genetic diversity among the studied isolates. The study has validated the usefulness of ISSR markers for studies of *S. rolfsii* population.

These findings are consistent with previous reports described by Muthulekshmi *et al*. (2008) [13] and Singh *et al.* (2019) [16] wherein they observed similar levels of variability within *S. rolfsii* populations using ISSR markers. The results ensured that, ISSR marker is a powerful tool to detect inter- and intra-population genetic diversity in soil-borne *S. rolfsii* fungal pathogens.

3.4 Phylogenetic analysis based on ISSR fingerprint pattern

Cluster analysis based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Jaccard's similarity coefficient revealed wide genetic variation among the isolates. All 15 isolates were categorized into three major groups with several subclusters (Figure 3). The genetic similarity among members of S. rolfsii isolates was ranged between 35% to 83% (Table 2). The highest genetic similarity i.e. 83% was observed between isolate SR10 (Latur) and SR11 (Amravati), possibly due to similar ecological pressures or genetic backgrounds. While isolate SR10 (Latur) and SR14 (Chakur), both were from Marathwada region of Maharashtra exhibited lowest i.e. 35% genetic similarity (0.35) suggesting presence of intra-regional diversity among the member isolates of *S. rolfsii.* The overall molecular characterization and clustering pattern expressed that, geographical origin plays an determining role in shaping the genetic structure of *S. rolfsii* populations. However, complete regional separation was not evident, suggesting higher evolutionary rates, gene flow through factors like agricultural practices, alternate hosts, shared seed materials, or environmental adaptations etc. Similar kind of intra-species variability patterns among isolates of several other fungal pathogens were reported by Muthulekshmi *et.al.* (2008) [13], Zhong *et al*. (2014) [19], and Sharma *et al*. (2015) [15], who also reported regional genetic clustering with overlapping population boundaries in fungal pathogens. The high resolution and reproducibility of ISSR markers in this study suggested their strong potential for population genetics studies, monitoring population shifts of *S. rolfsii*, assisting in the breeding of resistant soybean varieties and developing location-specific disease management practices.

4. Conclusion

This study provides the first comprehensive molecular characterization of *Sclerotium rolfsii* populations infecting soybean in Maharashtra, India, using ISSR markers. The ISSR markers has identified as robust, cost-effective tools and investigated substantial genetic diversity within member isolates of *S. rolfsii*. The primer UBC-811 identified as significant markers for screening the regional population of *S. rolfsii.* UPGMA clustering demonstrated partial geographic structuring, with vidarbha isolates showing tighter genetic cohesion compared to Marathwada's diffuse clustering. The research findings suggested presence of differential evolutionary pressures across the studied agroclimatic zones. The study would help to bridge the knowledge gap on *S. rolfsii* population structure, and development of future strategic plan to mitigate collar rot disease in soybean.

Figures

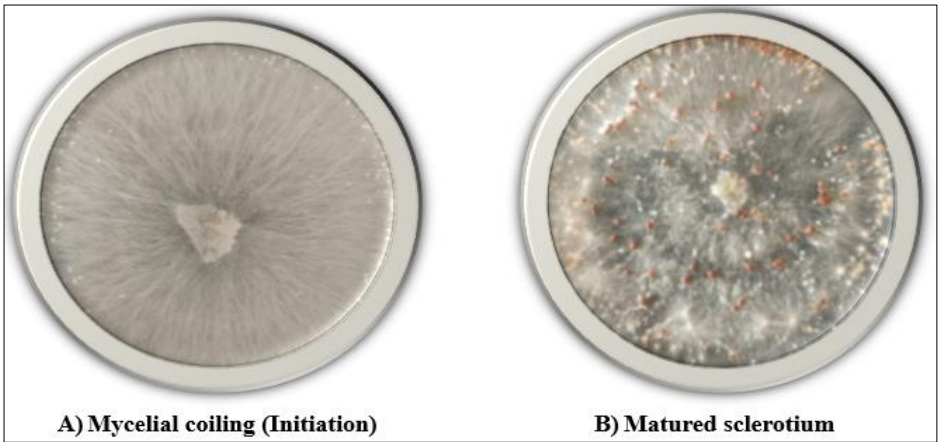

Fig 1: Morphological characterization of *Sclerotium rolfsii* on PDA medium; a, mycelial coiling; b, matured sclerotium

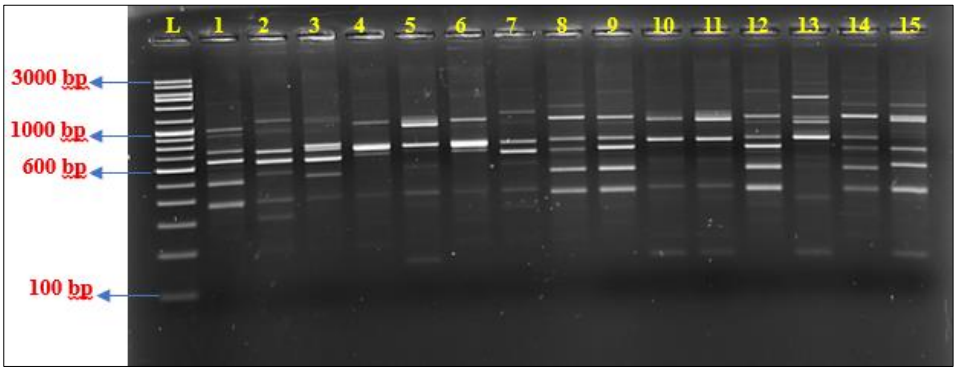

Fig 2.a: ISSR Banding Profile of Sclerotium Isolates using Primer UBC-823

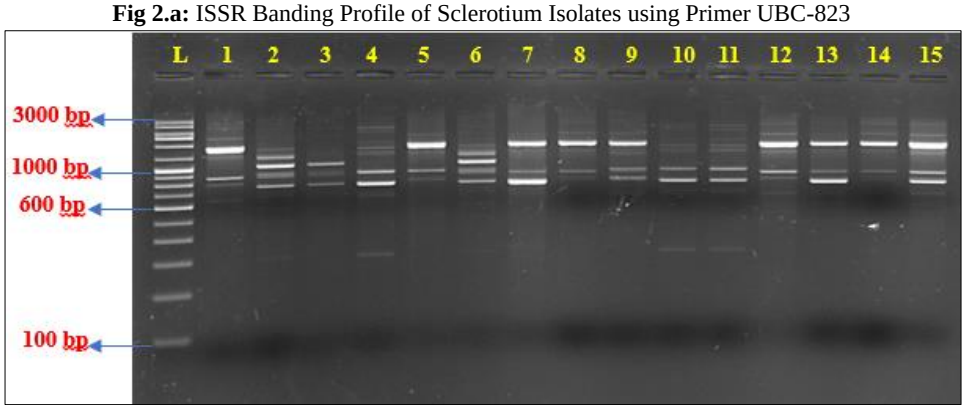

Fig 2.b: ISSR Banding Profile of Sclerotium Isolates using Primer UBC-826

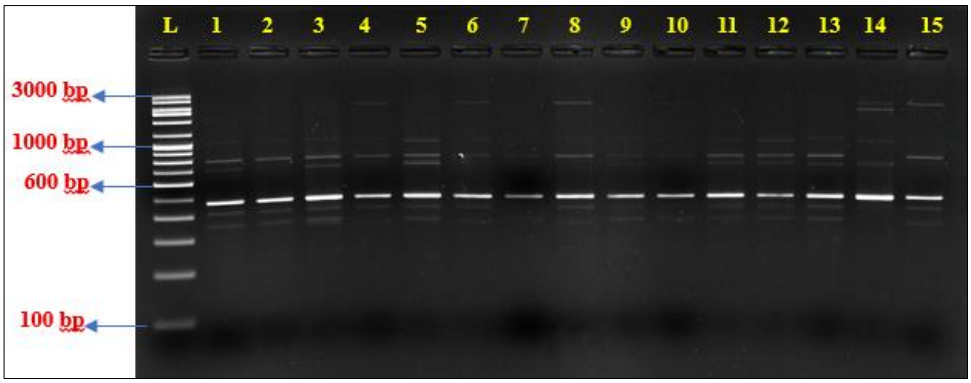

Fig 2.c: ISSR Banding Profile of Sclerotium Isolates using Primer UBC-840

Fig 2a-c: DNA fingerprint profile generated by ISSR Markers; a, UBC-823, UBC-826 and UBS- 840
1. SR01, 2. SR02, 3.SR03, 4.SR04, 5. SR05, 6. SR06, 7. SR07, 8.SR08, 9. SR09, 10. SR10, 11. SR11, 12. SR12, 13. SR13, 14. SR14, 15. SR15

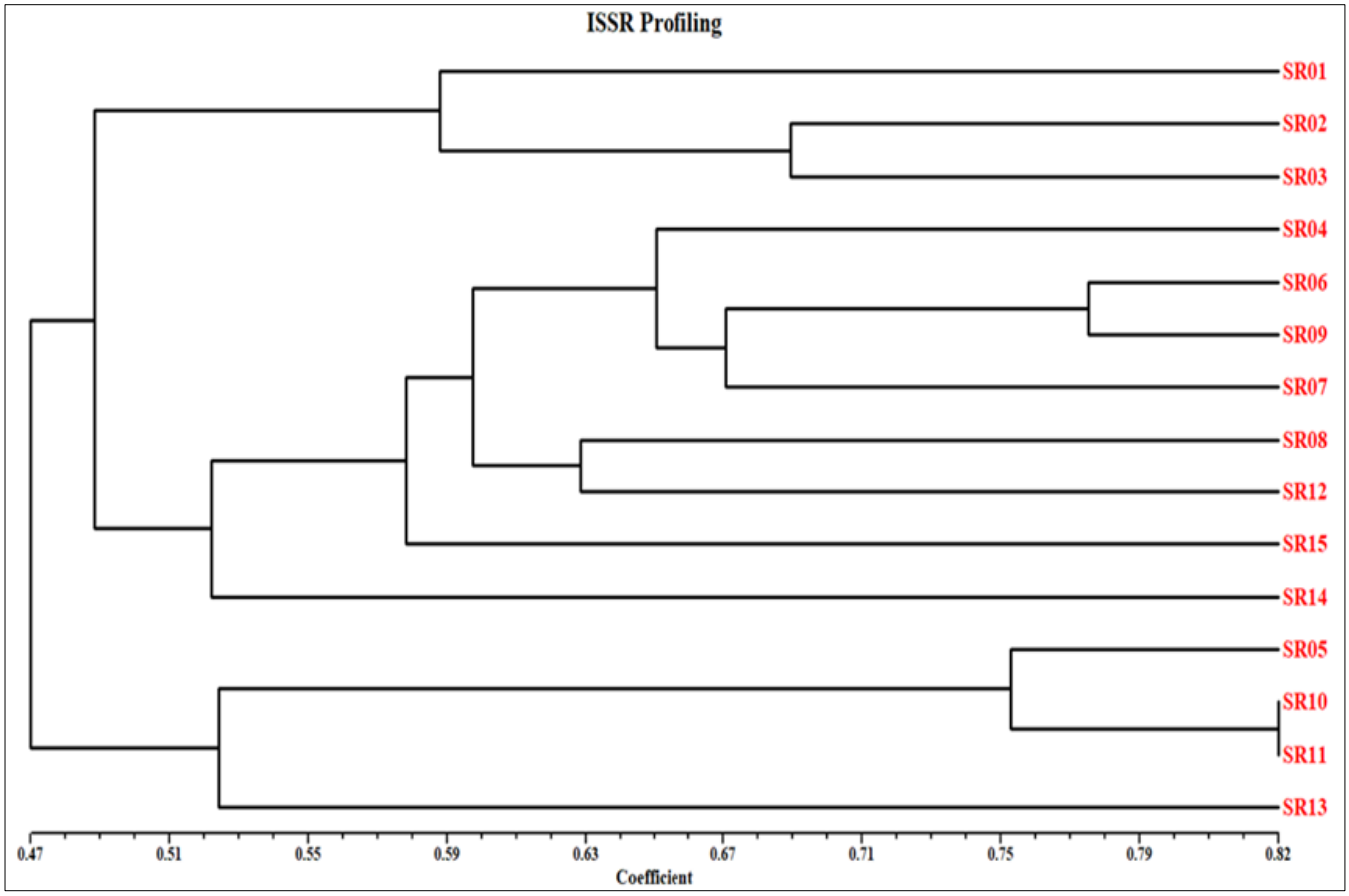

Fig 3: Dendrogram generated by UPGMA analysis based on ISSR Markers.

Table 1: Polymorphism depicted using Inter Simple Sequence Repeat (ISSR) markers analysis in Sclerotium isolates.

Sr. No	Primer Code	Total No. of Amplicons	Total No. Polymorphic Fragments	Total No. Loci (T)	Total No. Polymorphic Loci (P)	% Of Polymorphism P/T × 100	PIC 2f (1−f) (f- Frequency)
01	UBC-16	69	24	9	6	66.66	0.272
02	UBC-810	28	28	6	6	100	0.236
03	UBC-811	74	74	12	12	100	0.365
04	UBC-823	144	84	17	13	76.47	0.255
05	UBC-826	120	75	14	11	78.57	0.350
06	UBC-840	80	50	10	8	80	0.249
07	UBC-857	72	42	14	12	85.71	0.328
Total		587	337	82	68	83.91	-
Average				11.71	9.71	83.91	0.293

Table 2: Similarity matrix of *Sclerotium rolfsii* isolates based on NTSYSSs-pe UPGMA clustering method similarity coefficient value obtained from ISSR markers analysis.

	SR01	SR02	SR03	SR04	SR05	SR06	SR07	SR08	SR09	SR10	SR11	SR12	SR13	SR14	SR15
SR01	1.00														
SR02	0.68	1.00													
SR03	0.50	0.69	1.00												
SR04	0.49	0.49	0.49	1.00											
SR05	0.45	0.46	0.49	0.41	1.00										
SR06	0.54	0.47	0.46	0.69	0.52	1.00									
SR07	0.59	0.50	0.50	0.63	0.45	0.63	1.00								
SR08	0.51	0.48	0.47	0.58	0.53	0.67	0.56	1.00							
SR09	0.65	0.55	0.56	0.63	0.57	0.77	0.71	0.61	1.00						
SR10	0.36	0.45	0.49	0.59	0.73	0.49	0.49	0.47	0.50	1.00					
SR11	0.40	0.50	0.50	0.49	0.77	0.47	0.47	0.42	0.51	0.83	1.00				
SR12	0.51	0.48	0.44	0.53	0.50	0.61	0.55	0.63	0.68	0.44	0.45	1.00			
SR13	0.40	0.38	0.40	0.50	0.50	0.59	0.51	0.45	0.56	0.50	0.58	0.46	1.00		
SR14	0.41	0.39	0.38	0.44	0.42	0.52	0.49	0.50	0.58	0.35	0.37	0.60	0.37	1.00	
SR15	0.51	0.51	0.41	0.57	0.56	0.61	0.55	0.59	0.60	0.53	0.54	0.55	0.52	0.53	1.00

Maximum: 0.83, **Average:** 0.59, **Minimum:** 0.35

5. References:

1. Anonymous. Annual Report 2021-22. Ministry of Agriculture and Farmers Welfare, Government of India; 2022.
2. Bhardwaj P, Sharma P, Singh R, Srivastava M, Kharwar RN. Assessment of genetic diversity among *Trichoderma* spp. using molecular markers. Folia Microbiol (Praha). 2010;55(5):443-453. https://doi.org/10.1007/s12223-010-0071-2
3. Chavhan RL, Chakrabarty PK, Patil FS, Patil HB, Singh SK, Khadi BM. Association of new fungal species with leaf spot and blight of sunflower and cloning of their ribosomal RNA genes. Indian Phytopathol. 2008;61(1):70-74.
4. Chavhan RL, Hinge VR, Kadam US, Kalbande BB, Chakrabarty PK. Real-time PCR assay for rapid, efficient and accurate detection of *Paramyrothecium roridum*, a leaf spot pathogen of *Gossypium* species. J Plant Biochem Biotechnol. 2018;27(2):199-207.
5. Chavhan RL, Hinge VR, Wankhade DJ, Deshmukh AS, Mahajan N, Kadam US. Bioinformatics for molecular breeding and enhanced crop performance: applications and perspectives. In: Bioinformatics for Plant Research and Crop Breeding. 2024. p. 21-74.
6. Fehr WR. Principles of cultivar development: Theory and technique. New York: Macmillan Publishing Company; 1989.
7. Hartman GL, Sinclair JB, Rupe JC. Compendium of soybean diseases. 4th ed. St. Paul (MN): APS Press; 1999.
8. Hidayat I. Identification and phylogenetic relationship of fungi based on ITS region. Hayati J Biosci. 2012;19(4):145-150. https://doi.org/10.4308/hjb.19.4.145
9. Kadam US, *et al*. 2009
10. Kwon JH, Jee HJ, Park CS, Choi HW. Molecular identification and genetic diversity of *Sclerotium rolfsii* isolates using ITS and RAPD markers. Plant Pathol J. 2011;27(1):10-16. https://doi.org/10.5423/PPJ.2011.27.1.010
11. Meena PD, Chattopadhyay C, Meena SS, Kumar A. Evaluation of genetic diversity in Indian isolates of *Sclerotium rolfsii* through ISSR markers. Indian J Biotechnol. 2013;12:373-377.
12. Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 1980;8(19):4321-4326. https://doi.org/10.1093/nar/8.19.4321
13. Muthulekshmi M, Malathi P, Nakkeeran S. Genetic variability among *Sclerotium rolfsii* isolates causing stem rot disease in groundnut using ISSR markers. Indian Phytopathol. 2008;61(3):317-322.
14. Prasad Y, Naik MK, Ramesh B, Basavaraj A. Characterization of *Sclerotium rolfsii* isolates from sunflower using molecular techniques. Ann Plant Prot Sci. 2010;18(2):414-417.
15. Sharma P, Sankaralingam A, Srivastava AK. ISSR analysis reveals inter and intra-population genetic diversity in *Sclerotium rolfsii*. Biotechnol Lett. 2015;37(2):393-400. https://doi.org/10.1007/s10529-014-1707-7
16. Singh RK, Chaudhary RC. Genetic structure analysis of *Sclerotium rolfsii* causing collar rot in chickpea through molecular markers. Legume Res. 2019;42(2):248-253. https://doi.org/10.18805/LR-4187
17. Swain E, Gadekar AA, Mane SS, Meher J. Determination of genetic diversity among *Sclerotium rolfsii* isolates causing collar rot of chickpea using simple sequence repeat (SSR) markers. Int J Curr Microbiol Appl Sci. 2018;7(6):1190-1197. https://doi.org/10.20546/ijcmas.2018.706.142
18. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: A guide to methods and applications. San Diego: Academic Press; 1990. p. 315-322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1
19. Zhong S, Sun Y, Zhuang JY, Li M, Wang B. Genetic variation and differentiation of *Sclerotium rolfsii* in southern China as determined by ISSR analysis. Plant Dis. 2014;98(4):436-441. https://doi.org/10.1094/PDIS-05-13-0454-RE