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de Nova Whole Genome Sequencing of *Marssonina Juglandis* Causing Walnut (*Juglans regia* L.) Anthracnose and Development of PCR-Based Diagnosis Using Simple Sequence Repeat (SSR) Markers

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ABSTRACT

Walnut anthracnose, caused by *Marssonina juglandis*, is one of the economically important diseases of walnuts worldwide. The pathogen is a filamentous fungus belonging to the class Leotiomycetes. In this study, we isolated and purified the *Marssonina juglandis* from walnut leaves collected from the walnut orchard of SKUAST-Kashmir. The whole genome of the pathogen was sequenced using the Illumina HiSeq NGS platform. The whole genome of the pathogen was found to be 63.6354 Mb in size with 1916 scaffolds, 12086 genes, 205 total tRNAs, and 6884 SSRs containing 5861 SSRs with 150 flanking regions. A phylogenetic analysis using whole-genome alignment revealed that *Marssonina juglandis* is closely related to *Cryphonectria parasitica* and *Coniella lustricola*. The BlastP analysis using Uniprot, Pfam, and KOG showed 7612, 5515, and 5163 protein hits out of 12086 proteins. The total number of genes responsible for biological processes, molecular function, and cellular components were 1583, 1429, and 1843, respectively. In the *Venn* diagram, 3912 genes were found to be common in all three software, namely Uniprot, Pfam, and KOG including NR. In addition, we developed the SSR markers based on the whole genome and standardized them against 25 isolates of the *Marssonina juglandis* collected, isolated, and purified from the walnut-infected leaves in Jammu and Kashmir, India. Out of 30 randomly selected SSRs, 17 SSRs showed successful PCR amplification in 25 pathogen isolates that were found to be highly polymorphic. Further, a PCR-based detection protocol using SSR markers was also developed for early detection of the disease in the field for timely management. We found two primers that could successfully amplify the pathogen in DNA extracted directly from the infected walnut leaves.

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Introduction

Walnut (*Juglans regia* L.), belonging to the angiospermic family *Juglandaceae*, is a highly significant nutrient-rich nut tree. Walnuts are said to have originated from a wide region of Central Asia that stretches over Eastern Europe, encompassing Iran, Turkey, Iraq, Afghanistan, Southern Russia, and Northern India. In technical terms, walnuts are the seeds of drupes or drupaceous nuts rather than true botanical nuts (Fatima *et al*., 2018). India ranks eighth in the world's walnut production and sixth in the acreage under walnut plantations (Hassan *et al*., 2020). The main Indian states/ Union Territories that grow walnuts are Jammu and Kashmir, Uttarakhand, Himachal Pradesh, and Arunachal Pradesh.

China and the United States account for 47 and 31 percent of global walnut production, respectively (INC, 2017; INC, 2018; USDA, 2016). Walnuts are the most abundantly grown nuts and are crucial in India's horticulture sector. India is one of the major producers as well as exporters of walnuts. It enjoys good demand in the international market due to its superior quality. It has a special value in Indian foods and medicine as well as in traditional sweets (Uniyal *et al*. 2002). In India, walnuts are produced in states such as H.P., Uttarakhand, and the UT of J and K. Among them, the UT of Jammu and Kashmir alone contributes around 98 percent of the country's walnut produce (Sharma, 2014). The North-western Himalayan region, in general, and the Kashmir valley, in particular, are considered to be the bowl of walnut production (Verma *et al*., 2020).

Several biotic and abiotic factors influence walnut production. The biotic factors include diseases and pests, while abiotic factors include soil type, adverse climatic conditions, topography, etc. Among the major biotic factors, bacteria, fungi, viruses, and nematodes have been reported to be linked with the various diseases of walnuts worldwide. Diseases caused by fungi are prevalent in walnut-growing areas. The dominant fungal diseases include walnut anthracnose (*Marssonina juglandis* (Lib.) Magnus), root and crown rot [*Phytopthora cactorum* (Lebert and Cohn) Schrot], branch wilt [*Hendersonula toruloidea* Nattras], ring spot [*Ascochyta juglandis* Blotshauser.), downy leaf spot [*Microstroma juglandis* (Berenger) sacc.)], heart rot [*Polyporus squamosa* Huds. Ex Fr.) Fr.], powdery mildew [*Phyllactinia quttata* (Wallr. Ex Fr.) Lev., *Microsphaera extensa* (Cke and Peck)] besides stem canker and die-back [*Cytosperma leucosperma* (Pers. ex Fr.) Fr. and *Nectria galligena* (Bres.) and *Fusarium solani* (Mart. Sacc)] (Sharma and Sharma, 1999; Anonymous, 2013). Among the fungal diseases, anthracnose is the widespread foliar disease of walnuts (*Juglans* spp.), where the pathogen attacks leaves, nuts, and shoots of the current season growth (Berry, 1977; Belisario *et al.*, 2008). Walnut anthracnose initially appears on the leaves and fruits as uneven necrotic areas often surrounded by small chlorotic halos. The disease causes premature defoliation, sluggish plant growth, and reduced quantity and quality of nut produce, which leads to a striking drop in the economy in the walnut-growing areas of the world (Belisario *et al.*, 2001; Van-Sambeek, 2003; Kalkisim, 2012).

Accurate diagnosis of walnut anthracnose is the prerequisite for its management. The application of nucleic acid-based techniques for detection and diagnosis is of paramount importance. These studies are lacking in walnut anthracnose pathogen *M. juglandis* throughout the world. Microsatellite/simple sequence repeat (SSR) markers have been broadly exploited

given their high level of allelic variability, relative ease of use, transferability between mapping populations, and co-dominant expression (Hokanson *et al*., 1998; Pollegioni *et al*., 2009). SSRs are 1-6 nucleotide repeats that are highly polymorphic, informative, co-dominant, technically simple, reproducible, and relatively inexpensive when primer information is available in comparison with other molecular markers (Gupta *et al*., 1996; Weber and May, 1989; Rafalski *et al*., 1996). Microsatellites are widely distributed in eukaryotic genomes and highly polymorphic due to the variability of the number of repeat units (Ashley and Dow, 1994). Genome mapping and population genetics microsatellites are extensively used for genotypic identification as molecular markers (Groppe *et al*., 1995).

Disease management requires accurate detection and identification of plant pathogens to keep crops' high-yield potential alive. Hence, ongoing efforts are being made to provide a straightforward, dependable, quick procedure that is difficult for novice staff to execute and is restricted explicitly to diseases that affect the plant's aerial parts. Highly skilled taxonomists are needed for the favored approach of disease diagnosis, which involves microscopic inspection of infected tissues and diagnosis of the pathogen based on their morphological characteristics. Molecular approaches in pathogen identification and disease diagnosis can resolve numerous issues with morphological and microscopically plant pathogen detection. The most significant benefit of using DNA-based techniques is that they can be utilized for disease/ pathogen detection at any stage of disease development. With the aid of PCR, precise identification of the infections that would otherwise be challenging to identify morphologically can be achieved at the genus, species, or even race levels using DNA probes (Sharma, 2003). Considering the significance of molecular studies in this pathogen, the current study was proposed on the development and validation of SSR markers for accurate diagnosis of *Marssonina juglandis* causing walnut anthracnose with the following objectives.

Materials and Methods

The present research work on the development and validation of SSR markers for the pathogen *Marssonina juglandis* was carried out in the Plant Virology and Molecular Pathology (PVMP) Laboratory of Division of Plant Pathology, Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar, Srinagar during the year 2022-2024.

Collection of diseased samples, isolation, and purification of the pathogen

Collection of diseased leaves of walnuts infected with anthracnose disease caused by *M. juglandis* was done from the university walnut orchard. The infected leaves were collected and brought to the laboratory to isolate the pathogen.

Isolation of the pathogen isolates and their purification

Isolates of the pathogen were isolated and maintained on a potato dextrose agar (PDA) medium. PDA medium was prepared using peeled potato (200 g), dextrose (20 g), and agar-agar (20 g) per liter of water. The potato extract was obtained by boiling potatoes until potatoes became soft. Filtration of this mixture was done using a muslin cloth to obtain the extract in a separate container, followed by a thorough mixing of dextrose and agar-agar powder. Agar-agar

was added slowly to avoid any lump formation. The final volume of the medium was made to one liter by adding water and continued to boil until all components were mixed properly, especially agar-agar. PDA medium of about 200 ml was placed in a 250 ml capacity Erlenmeyer flask and test tubes (18×150 mm), followed by their sealing using non-absorbent cotton plugs. These flasks and test tubes containing PDA medium were sterilized in an autoclave at 15 lbs psi for 25-30 minutes. After autoclaving, the flasks and test tubes containing the medium were taken out after releasing the pressure of the autoclave. Test tubes were placed in a slanting position for solidification of the medium. Then, under aseptic conditions of laminar airflow, the medium of flaskg was poured into a sterilized Petri plate after slight cooling. Petri plates containing PDA medium were allowed to solidify in the laminar airflow and used for pathogen isolation.

A single spore technique was adopted for the purification of the pathogen. Water agar was prepared by adding dextrose (20g) and agar-agar powder (20g) to one liter of distilled water. The prepared medium was poured into Erlenmeyer flasks (250 ml) and autoclaved for 20-25 minutes at 15 lbs psi pressure. The lukewarm medium in the flasks was poured into Petri plates (15-20 ml) under an aseptic environment. After solidification, the plates were used to purify pathogen isolates and stored in the refrigerator at 4°C for further use.

The isolation of the pathogen cultures was carried out on an already prepared water agar medium. The infected leaf samples collected from the orchard were surface sterilized with 0.1 percent mercuric chloride. Later, these leaves were kept in a moisture chamber and incubated at 22±1°C for 24 hrs to make the acervuli present in the samples swollen. After incubation, a small bit of the sample containing bulged acervuli was added to a sufficient quantity of sterilized distilled water in a test tube with the aid of a sterilized needle under a stereoscopic microscope (Jamshidi *et al*., 2012). The test tube was thoroughly perturbed to release conidia. The conidial suspension was spilled onto the water agar plates and evenly distributed. The plates were kept under incubation for 48 hrs at 22±1°C. The germinated spores were spotted and marked using a color marker under a compound microscope. The water agar from a marked spot was excised using a 3 mm sterilized cork borer and shifted to Petri plates holding PDA medium under aseptic conditions. The plates were incubated at 22±1°C (Belisario *et al*., 2008).

Morpho-cultural studies of the pathogen

The pathogen isolates from the host were used for the morpho-cultural studies. From the 30-day old cultures of the pathogen, semi-permanent slides were prepared with lactophenol and examined under a microscope (400X) for the colony color and shape, mycelial growth, color, breadth, septation, and branching and conidial color, shape, length, width, and septation. These characters of the pathogen isolates were compared with those available in the literature to confirm the identity of the isolated pathogen (Hedwigia, 1906; Sogonov *et al*., 2008; Holliday, 1989; Dastjerdi and Hassani, 2009; Seremi and Amiri, 2010; Kochman, 1973; Anonymous, 2013).

Molecular characterization of the pathogen using PCR-based markers

DNA extraction. The various pathogen isolates were grown in Petri plates containing potato dextrose agar medium for 25 to 30 days. The total genomic DNA was extracted from each

pathogen isolate using the CTAB (Cetyl Trimethyl Ammonium Bromide) method given by Murray and Thompson (1980) with slight modifications. The mycelial layer from the Petri plates of each isolate was scrapped, and the mixture was crushed in liquid nitrogen using a sterilized pestle and mortar. The fine mycelial powder formed after crushing was transferred to 1.5 ml microfuge tubes. CTAB buffer already kept in a water bath at 65℃ was added to these microfuge tubes @ 700 µl per tube using a micropipette. These tubes were incubated in a thermo-mixer for one hour at 65℃ at 500 rpm. At room temperature, these centrifuge tubes were allowed to cool, and 700 µl of pre-chilled chloroform: isoamyl alcohol in 24:1 ratio was added, followed by centrifugation at 12,000 rpm for 20 minutes. With a micropipette, an equal volume of supernatant was taken out after centrifugation and transferred to another centrifuge tube. 700 µl pre-chilled isopropanol was added to these tubes containing supernatant and kept overnight at -20℃ or -80℃ for two hrs. The next day (after 12 hrs), the centrifuge tubes were thawed, followed by centrifugation at 12,000 rpm at room temperature for 20 minutes. The centrifuged tubes were decanted to remove the supernatant, and the pellet formed at the bottom was washed with pre-chilled 700 µl of 70 percent ethanol twice, followed by centrifugation at 10,000 rpm for 10-15 minutes at room temperature. After centrifugation, 70 percent ethanol was decanted, and the tubes holding the DNA pellet were kept inverted for drying till the ethanol smell vanished. Finally, 1X TE buffer was added to the pellets according to the pellet size to dissolve the DNA and kept at -4℃ overnight. *Rnase* treatment @ 1 µl/ml was given at 37℃ in a thermo mixer for 1 hr and stored at -80℃ for future use.

DNA quantification and quality check. DNA extracted from the different pathogen isolates by the CTAB method was quantified by agarose gel electrophoresis. This method prepared 1.0 percent agarose gel (1 g of agarose powder in 100 ml of 1X TAE buffer in a 250 ml flask) using a microwave oven until the solution became utterly transparent. The solution was allowed to cool, followed by adding ethidium bromide @ 2 µl as a fluorescent dye. On the other hand, the gel cast was placed on a cast holder, and combs were inserted into the cast. The solution was allowed to solidify at room temperature for 15-20 minutes after pouring into the cast. Loading dye bromophenol blue (6X) was placed on Para film in a drop manner. Then approximately 2 µl DNA from each isolate was mixed with this loading dye using a micropipette and loaded into wells of the already prepared gel. The gel was allowed to run for 20 minutes at 80 Volt in a gel electroporation system (Consort, Belgium). The DNA bands were visualized under a gel documentation system (Alpha Imager EC, Protein Simple, USA). The fluorescence intensity of each sample (band) was compared with the standard marker (ladder) to ascertain the quantity of DNA. The quality of each sample was checked based on whether a single high molecular weight intact band was formed (good quality) or a smear was formed (poor quality). The quantification of DNA was also ascertained by a Nanodrop Bio-spectrophotometer (Eppendorf, Germany). After quantification, the DNA of each isolate was diluted to a final concentration of 20-25ng/µl using 1X TE or nuclease-free water (NFW).

Molecular confirmation of the pathogen isolates. Molecular confirmation of the representative isolate of the pathogen was carried out using PCR-based Internal Transcribed Spacer primers (ITS), and the ITS region was sequenced to confirm the pathogen identity.

Polymerase chain reaction (PCR) amplification of Marssonina juglandis using ITS primers. Polymerase chain reaction (PCR) amplification was performed in 0.2 ml PCR tubes in a thermal cycler (Eppendorf, Germany) using 40-50 ng of genomic DNA of *Marssonina juglandis* isolate in a final volume reaction mixture of 25 µl. The stock and final concentration of different components used in PCR for ITS are given in Table 3.1. The reaction mixture in the PCR tubes was given a short spin or vortexed in a microfuge (Thermo Scientific, Thermo Electron Corporation) and placed in a 96-well thermal cycler. PCR amplifications of the ITS region were performed using ITS1 (5'TCCGTAGGTGAACCTGCG3') and ITS4 (5'TCCTCCGCTTATTTGATATGC3') primers in thermal cycler programmed for initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, primer annealing for 2 minutes at 56 °C, extension for 3 minutes at 72°C and final extension at 72°C for 10 minutes and hold at 4°C.

Table 1: Stock and final concentration of various components of polymerase chain reaction (PCR) mixture for ITS and SSR markers

Components	**Stock Conc.**	**Reaction volume (µl)**	**Final Conc.**
PCR buffer*	10X	2.5	1.0X
$Mgcl_2$	25 mM	1.5	1.5 mM
DNTPs	2.5 mM	2.0	0.2 mM
Primer (Forward and Reverse)	10 pmol	1.0	0.4 pmol
Taq polymerase	5 U/µl	0.2	1.0 Unit
DNA template	20-25 ng	2.0	50.0 ng
Sterilized distilled water	--	15.8	--
Total		**25.0**	

Visualization and sequencing of ITS region of *Marssonina juglandis*. After successful PCR amplification using ITS primer in a 25 µl reaction mixture, 5 µl of the PCR product of *Marssonina juglandis* isolate was electrophoresed after adding 2 µl of the 6X loading dye. The products were resolved on 1.2 percent agarose gel. The gel was run at 80 Volt for 20 minutes, visualized under UV light, and photographed using a gel documentation system (Alfa Imager EC, Protein Simple, USA) to ensure successful amplification, and the remaining product was sent for custom sequencing (Unipath Lab, Gujarat, India).

Whole genome sequencing of the *Marssonina juglandis* using Illumina HiSeq next-generation sequencing platform. The customized whole genome sequencing of *Marssonina juglandis* was done using Illumina HiSeq next-generation sequencing platform from Unipath (Ahmadabad) India, and the data was obtained in fastaq format.

Mining of *Marssonina juglandis*-specific SSR markers from the pathogen genome. Simple sequence repeats (SSR) markers were developed using the whole genome of *Marssonina juglandis* available in FASTA formats at Plant Virology and Molecular Pathology Laboratory (Major Advisor) of the Division of Plant Pathology, FOH, SKUAST-K, Shalimar, Srinagar. The robust SSR markers were filtered using Genome-wide Microsatellite Analysing Tool package

(GMATA) software (Wang and Wang, 2016). The software recorded the number of repetitions, motif type, and position of the sequence, and the results obtained were recorded in the output files

Validation of the identified SSR markers on Marssonina juglandis isolates under in-situ condition.

PCR amplification of *Marssonina juglandis* isolates using SSR markers. The PCR amplification was carried out in thermocycler (Eppendorf, Germany) in a PCR reaction mixture of 25 µl containing 2 µl (20-25 ng/µl) of genomic DNA of each *Marssonina juglandis* isolate, 1X PCR buffer, 2.5 mM dNTPs, 1µl each reverse and forward SSR primer (10 pmol), 1.5 mM $Mgcl_2$, 1 unit of *Taq* polymerase enzyme and 15.8 µl of nuclease-free water (NFW) (Table 3.1). The PCR amplification was initially standardized using 5 DNA samples and different SSR primers. SSR primers showing positive results were further utilized for amplification of the whole pathogen isolates with PCR profile of initial denaturation at 94°C for 2 minutes followed by 35 cycles with denaturation at 94°C for 40 seconds, annealing at 53-56 °C for 40 seconds and extension at 72°C for 40 seconds followed by a final extension at 72°C for 10 minutes and hold at 4°C. The PCR amplified products were resolved on 2.5 percent agarose gel (2.5 g of agarose powder dissolved in 100 ml of 1X TAE buffer) using gel electrophoresis run for one hour at 80 Volt and photographs captured using gel documentation system (Alpha Imager EC, Protein Simple, USA).

Development of PCR-based detection protocol using SSR markers for the identification of walnut anthracnose disease under field conditions

Genomic DNA extraction from the infected and healthy leaves of walnut. Total genomic DNA was extracted using the CTAB method from the anthracnose-infected and healthy walnut leaves (Murray and Thompson, 1980). The infected and healthy leaves were collected from the walnut trees and brought to the laboratory. The infected and healthy portions of leaves were cut and ground to a fine powder in liquid nitrogen using a sterilized pestle and mortar. The fine powder was transferred to 1.5 ml microfuge tubes containing 700 µl of CTAB (Cetyl Trimethyl Ammonium Bromide) buffer maintained at 65°C in a water bath. The samples were thoroughly mixed and incubated at 65°C for one hr in a thermo mixer at 500 rpm. After cooling the microfuge tubes to room temperature, 700 µl of pre-chilled chloroform: isoamyl alcohol in a 24:1 ratio was added, followed by centrifugation for 20 minutes at 12,000 rpm. The supernatant was taken out after centrifugation with the help of a micropipette and transferred to new centrifuge tubes. Pre-chilled isopropanol of about 700 µl was added to these tubes containing supernatant and kept overnight at -20°C or -80°C for two hrs. The next day (after 12 hrs), the centrifuge tubes were thawed, followed by centrifugation at 12,000 rpm for 20 minutes at room temperature. The centrifuged tubes were decanted to remove the supernatant, and the pellet formed at the bottom was washed with pre-chilled 700 µl of 70 percent ethanol twice, followed by centrifugation at 10,000 rpm for 10-15 minutes at room temperature. After centrifugation, 70 percent of the ethanol was decanted, and the tubes containing the DNA pellet were kept inverted for drying until the smell of ethanol vanished. Finally, 1X TE buffer was added to the pellets according to the pellet size for dissolving the DNA and kept at 4°C overnight. *Rnase* treatment @ 1 µl/ml was given at 37 °C for 1 hr in a thermo-mixer (Eppendorf, Germany). The

genomic DNA from the healthy leaves was also obtained similarly and used as a control. The genomic DNA isolated from all the samples in a similar way was stored at -80°C for further use.

DNA quantification and quality check. Genomic DNA extracted by the CTAB method was quantified by agarose gel electrophoresis. This method prepared 1.0 percent agarose gel (1 g of agarose powder in 100 ml of 1X TAE buffer in a 250 ml flask) using a microwave oven until the solution became completely transparent. The solution was allowed to cool, followed by adding ethidium bromide @ 2 µl to this mixture as a fluorescent dye. On the other hand, the gel cast was placed on a cast holder, and a comb was inserted into the cast. For 15-20 minutes, the solution was allowed to solidify after being poured into the cast. Loading dye bromophenol blue (6X) was placed on parafilm in a drop manner, and the DNA of approximately 2 µl from each sample was mixed with this loading dye using a micropipette, followed by loading into wells of already prepared gel. The gel was allowed to run for 20 minutes at 80 Volt in a gel electroporation system (Consort, Belgium). The DNA was visualized under a gel documentation system (Alpha Imager EC, Protein Simple, USA), and photographs were captured. The fluorescence intensity of each sample was compared with a standard marker (ladder) to ascertain the quantity of DNA in different samples. The quality of each sample was checked based on whether a single intact high molecular weight band was formed (good quality) or a smear was formed (poor quality). The DNA quantification was also done using a Nanodrop Bio-spectrophotometer (Eppendorf, Germany). After quantification, the DNA of each isolate was diluted to a final concentration of 20-25 ng/µl using 1X TE or nuclease-free water.

Polymerase chain reaction (PCR) amplification using SSR primers. The PCR amplification to detect the pathogen directly from the infected samples was carried out in a thermal-cycler in a PCR reaction mixture of 25 µl containing 2 µl (20-25 ng/µl) of genomic DNA of the infected as well as healthy leaf samples of walnut, 1X PCR buffer, 2.5 mM dNTPs, 1 µl each reverse and forward SSR primer (10 pmol), 1.5 mM $Mgcl_2$, 1 unit of *Taq* polymerase enzyme and 15.8 µl of sterilized nuclease-free water (NFW). PCR-based SSR markers were further utilized for amplification of the pathogen in infected samples with a PCR profile of initial denaturation at 94°C for two minutes followed by 35 cycles of denaturation at 94°C for 40 seconds, annealing at 60°C for 40 seconds and extension at 72°C for 40 seconds followed by a final extension at 72°C for 10 minutes and held at 4°C.

Data Analysis

The polymerase chain reaction (PCR) amplified products were resolved on 2.5 percent of agarose gel (2.5 g of agarose powder dissolved in 100 ml of 1X TAE buffer) using gel electrophoresis system (Consort, Belgium) run at 80 Volt for one hr and photographs captured by gel documentation system (Alpha Imager EC, Protein Simple, USA). The specific amplified alleles (band) were compared with the 100 bp ladder to ascertain the expected amplified fragment size compared with the healthy check, and conclusions were drawn.

Results

Collection of diseased samples, isolation and purification of the pathogen isolates and their morpho-cultural characterization

Diseased samples from the walnut orchards of SKUAST-Kashmir, Shalimar, and Srinagar were collected from 2022-2023. The diseased samples comprising infected leaves from various walnut trees were collected and brought to the laboratory to isolate the pathogen on Petri plates containing potato dextrose agar (PDA) medium. The spore transfer methods were isolated (Belisario *et al*., 2008). The spore transfer method was found to be comparatively better for the isolation of pathogens due to the minimum contamination rate compared to the tissue bit method, and it also aided in the development of pure culture at a faster rate. A total of 25 isolates were isolated, purified, and maintained on PDA medium and stored at 4℃ in the refrigerator of Plant Virology and Molecular Pathology Laboratory of Division of Plant Pathology, FOH, SKUAST-K, Shalimar, Srinagar for further use.

Morpho-cultural and molecular characterization of the pathogen isolates

The pathogen isolates were grown/ sub-cultured on a PDA medium and observed critically for colony characters and growth behavior (Table 2). The pathogen isolates appeared as circular, whitish glabrous aerial mycelial tufts after 15 days of incubation at 22±1℃, which later turned creamy white, surrounded by flat white mass after 30 days and finally greyish white with the appearance of characteristic concentric zones, well defined lobate margins, and radial furrows after 45 days of incubation. The 25 pathogen isolates showed a radial growth of 56.00- 60.00 mm with an average radial growth of 57.40 mm on PDA medium after 30 days of incubation at 22±1℃ (Plate. 4.2). The cultural characters of different isolates showed a slight variation in colony growth and color. Microscopic observations revealed that the pathogen produced smooth, compact, branched septate and hyaline hyphae measuring 3.20-6.50 µm in width with an average size of 4.43 µm. The acervuli developed in concentric rings were discoid pitch dark in color. Conidia were crescent-shaped, hyaline with single septation, ranging from 19.25-29.75 x 7.00-10.50 µm in size with an average size of 24.85 x 9.10 µm on a total of 25 isolates (Plate 4.3). The morphological characteristics of different pathogen isolates were compared with the available literature, which led to the identification of the pathogen as *Marssonina juglandis* (Lib.) Magnus (Hedwigia, 1906; Sogonov *et al*., 2008; Holliday, 1989; Dastjerdi and Hassani, 2009; Seremi and Amiri, 2010; Kochman, 1973; Anonymous, 2013).

Table 2. Morphological characteristics of Marssonina juglandis isolates

Isolate	Conidial width (µm)	Conidial length (µm)	Hyphal width (µm)	Radial* growth (mm)
MJ1	10.50	28.00	3.20	58.00
MJ2	7.00	31.50	4.30	57.00
MJ3	8.75	22.75	3.20	60.00
MJ4	10.50	24.50	4.30	58.00
MJ5	10.50	26.25	4.30	56.00
MJ6	10.50	28.00	6.50	58.00
MJ7	8.75	24.50	4.30	57.00
MJ8	7.00	29.75	4.30	56.00
MJ9	7.00	28.00	4.30	60.00

MJ10	10.50	21.00	3.20	60.00
MJ11	7.00	24.50	3.20	57.00
MJ12	10.50	22.75	4.30	57.00
MJ13	8.75	21.00	6.50	58.00
MJ14	8.75	28.00	6.50	56.00
MJ15	10.50	24.50	4.30	58.00
MJ16	10.50	31.50	4.30	60.00
MJ17	10.50	19.25	4.30	56.00
MJ18	7.00	24.50	6.50	57.00
MJ19	10.50	21.00	4.30	57.00
MJ20	7.00	26.25	4.30	56.00
MJ21	8.75	21.00	3.20	56.00
MJ22	7.00	22.75	3.20	58.00
MJ23	8.75	24.50	4.30	56.00
MJ24	10.50	24.50	6.50	56.00
MJ25	10.50	21.00	3.20	57.00
MEAN**	**9.10**	**24.85**	**4.43**	**57.40**

* After 30 days of observation at 22±1°C; ** Mean of 25 isolates

Molecular characterization of the pathogen using polymerase chain reaction (PCR) based internal transcribed spacer (ITS) and simple sequence repeat (SSR) markers

DNA extraction from *Marssonina juglandis* isolates, quantification, and quality check of DNA. The isolates were grown on potato dextrose agar medium, and the total genomic DNA was isolated from all 25 isolates of *M. juglandis* by CTAB method (Murray and Thompson, 1980).). The extracted DNA from different isolates resulted in single high molecular weight intact bands, indicating a good quality DNA on 1.0 percent agarose gel using a gel electroporation system (Consort, Belgium) (Plate 4.5). The quantification was also done using a Nanodrop Bio-spectrophotometer (Eppendorf, Germany) and maintained the DNA concentration at 20-25 ng/µl using 1X TE buffer or nuclease-free water (NFW).

Molecular characterization of the pathogen causing walnut anthracnose using ITS markers and sequencing of ITS region. The molecular characterization of the representative isolate of *Marssonina juglandis* was carried out using PCR-based ITS-1 and ITS-4 markers (White *et al.*, 1990). PCR amplification was carried out in a 25 µl PCR reaction mixture (Table 3.1). After successful PCR amplification, 5 µl of the amplified PCR product was analyzed on a 2.5 percent agarose gel using gel electrophoresis to confirm the successful PCR amplification. The remaining 20 µl of PCR amplified product was sent for custom sequencing (Unipath, Gujarat, India). The sequence result was obtained as a chromatogram for both forward (ITS-1) and reverse (ITS-4) primers. The forward and reverse sequences were retrieved from the chromatograms received, and sequence alignment was performed using BioEdit version 7.0 software (Hall, 1999) to obtain the consensus sequences. The obtained sequence was analyzed through the BLASTn program http://www.ncbi.nlm.nih.gov/BLAST to compare the present

sequence of the pathogen with the available sequences of *Marssonina juglandis*. The present sequence showed maximum similarity (more than 98%) with *Gnomonea liptostyla* which is the perfect stage of *Marssonina juglandis,* and therefore confirmed the identity of the pathogen as *Marssonina juglandis* (Lib.) Magnus.

Whole genome sequencing of *Marssonina juglandis* (Lib.) Magnus using Illumina HiSeq NGS sequencing platform. The whole genome of the pathogen using Illumina HiSeq NGS sequencing platform revealed that the pathogen genome was found to be 63.6354 Mb in size with 1916 scaffolds, 12086 genes, 205 total tRNAs, and 6884 SSRs containing 5861 SSRs with 150 flanking regions. A phylogenetic analysis using whole-genome alignment revealed that *Marssonina juglandis* is closely related to *Cryphonectria parasitica* and *Coniella lustricola*. The BlastP analysis using Uniprot, Pfam, and KOG showed 7612, 5515, and 5163 protein hits out of 12086 proteins. The total number of genes responsible for biological processes, molecular function, and cellular components were 1583, 1429, and 1843, respectively. In the *Venn* diagram, 3912 genes were found to be common in all three software namely Uniprot, Pfam, and KOG including NR.

Development of simple sequence repeat (SSR) markers for *Marssonina juglandis* (Lib.) Magnus. The whole genome of the fungus *Marssonina juglandis,* having 63.84 Mbps size with 1916 scaffolds, was obtained from PVMP Lab and used to develop SSR markers specific to the pathogen using GMATA software. The GMATA software yielded 6884 SSR markers specific to *M. juglandis* distributed on different scaffolds of the pathogen genome.

Validation of SSR markers specific to *Marssonina juglandis* under *in-silico* conditions. Validation of SSR markers specific to *Marssonina juglandis* under *in-silico* conditions was done using electronic polymerase chain reaction (*e-PCR*) using whole genome sequence of *M. juglandis* and 6884 SSR markers obtained from GMATA software as input files, resulting in 1474 successfully amplified primers under *in-silico* PCR condition. Among them, 30 SSRs were randomly selected and custom synthesized based on contig size and repeat numbers of the SSRs on scaffolds chosen (Table 3). These markers were standardized on five pathogen isolates and used for molecular characterization of different isolates of *M. juglandis* obtained from walnut tree leaves.

Standardization of PCR-based SSR markers. Initially, 30 SSR primers were screened on only five randomly selected isolates of *Marssonina juglandis*. Out of 30 SSRs, only 17 SSR primers (Table 4) showed successful amplification of five randomly selected pathogen isolates and were selected for further amplification of all the 25 *M. juglandis* isolates collected from different walnut trees.

Table 3. Simple sequence repeat (SSR) markers along with their sequences developed from Marssonina juglandis genome using e- PCR

	Primer name*	Sequence	Motif (Repetitions)	No. of base pairs	Tm (°C)
1	MJSSR01F	5′CATCTGCTTTCCTTCCACAGC3′	(CCTGT)11	21	55.7
	MJSSR02R	3′TTACTCTGACTCACCTATATGGGG5′		24	
2	MJSSR02F	5′CTTGTACTTGCGATTGTACTGGAT3′	(CA)27	24	55.55
	MJSSR02R	3′TTTTGCGAGTGTGCTCAAACTTT5′		23	
3	MJSSR03F	5′CGAGCAGGGAAAAGAAAAGAAAAG3′	(TTTCT)22	24	55
	MJSSR03R	3′GTGTATACTCGGCCAGTTTCTTTT5′		24	
4	MJSSR04F	5′GTGATGTGATCAGCATGGGTTC3′	(TAG)16	22	55.5
	MJSSR04R	3′ATGACAACCCAACAGTTAGTTAGG5′		24	
5	MJSSR05F	5′GATGGTGATGGTGATGGTGATG3′	(CATCAC)13	22	55.2
	MJSSR05R	3′ACTAACGGACAAACTTCTACAGTG3′		24	
6	MJSSR06F	5′TTGCTGTTGCTGTTGCTGTT3′	(CAGCAA)21	20	56.4
	MJSSR06R	3′AACGCCCGAGTCTATCACTTTTT5′		23	
7	MJSSR07F	5′CATCTCACTTCCCTTTGATTCTCC3′	(GGAT)14	24	55.6
	MJSSR07R	3′GTTGGTGGTTGATAAGTTGGCTAG5′		24	
8	MJSSR08F	5′GATCTCCAGCAGGCGAATGTC3′	(AAG)26	21	56.55
	MJSSR08R	3′GAAGGTTCTTTAGTCTTCCTCGTG5′		24	
9	MJSSR09F	5′GGGATGAGGGGTTAGTAGGG3′	(ACA)15	23	55.6
	MJSSR09R	3′TGGGGAGTTTTGATGCTTTT5′		24	
10	MJSSR10F	5′GCGTGGTGATATTGTCTGGA3′	(CATTCA)7	24	55.05
	MJSSR10R	3′TCATGGAGCTCATTGCAATA5′		24	
11	MJSSR11F	5′CGATTTTCTAGAGGGCGATG3′	(TCAC)14	23	55.15
	MJSSR11R	3′GGTTTGGGGAGGGTTAGAGA5′		24	

12	MJSSR12F	5′GGGGATGCCTAAACTGCATA3′	(TCCCAG)12	24	55.6
	MJSSR12R	3′AGGAAGTTTTCGGTCGTTTG5′		24	
13	MJSSR13F	5′CGCACTTGCAAATACCCTTT3′	(TCA)35	24	
	MJSSR13R	3′CGGGAAGGTGTGTGGAGT5′		24	55.1
14	MJSSR14F	5′CAAAGGGAAGTCGAGGATGA3′	(GAGTGT)18	24	54.55
	MJSSR14R	3′CGAGATGTCGTGTACCTTGC5′		24	
15	MJSSR15F	5′CTTTTCACTTCGCCGTTTCT3′	(AATGGA)20	24	54.3
	MJSSR15R	3′GCGGGGATGTAGTGAAGTGT5′		24	
16	MJSSR16F	5′CCAAACTGAATACCCACCACTTAT3′	(AATGGA)20	24	54.25
	MJSSR16R	3′TGGAAATGGAAATGGAAATGGAAA5′		24	
17	MJSSR17F	5′GGAGATATGTCCTGTCAGTCATGA3′	(GT)47	24	55.75
	MJSSR17R	3′CTGCTGTAAGTTGCTCTCTACCTA5′		24	
18	MJSSR18F	5′CTGTCACTTGGGCTGTATTTGTTT3′	(CGACTG)6	24	55.7
	MJSSR18R	3′ATCCACCACCCTTACAGCTATTAT5′		24	
19	MJSSR19F	5′CCCATCCCGAAAGTTAAGTTAGTA3′	(GTAG)10	24	54.7
	MJSSR19R	3′CCCATCCCGAAAGTTAAGTTAGTA5′		24	
20	MJSSR20F	5′CGAAAAGTGGTAAGAGATAGCCTC3′	(GCT)16	24	55
	MJSSR20R	3′TCAAGCGGTTGAAGATTGTTAGTT5′		24	
21	MJSSR21F	5′CACTCATCATCTAAACTCCATCGG3′	(GTCGCA)8	24	55.05
	MJSSR21R	3′GTACACAAGACAAAGTACCCGTAG5′		24	
22	MJSSR22F	5′AAGCTTTTAAACGCCGTAATGAAC3′	(AATAA)5	24	54.7
	MJSSR22R	3′CCAATTATAAGGCTTTTTAGCGCC5′		24	
23	MJSSR23F	5′TTCAAGTAGGACATAGTGGTACCT3′	(GAGT)6	24	54.85
	MJSSR23R	3′TGTTGATGGACTTGATTGGACAAT5′		24	
24	MJSSR24F	5′ACCCTCATAAAACCCCTTCCTC3′	(GGTGCT)5	22	56.5

	MJSSR24R	3′GTTGGAATTGGAATTGGGAAGGG5′		23	
25	MJSSR25F	5′GGTAGTAGCACAAAATAAGCCAGT3′	(TCACTG)6	24	54.9
	MJSSR25R	3′ACAAGACTTTGTTGTACTTGGGAA5′		24	
26	MJSSR26F	5′TGAGGATGATGAAGATGTTGTTCG3′	(GAAAA)5	24	54.95
	MJSSR26R	3′TGTGACGAGAAAAGAAGAGAAAGG5′		24	
27	MJSSR27F	5′GTGCACCGTTACTTGGTAGTG3′	(CGGTG)10	21	55.85
	MJSSR27R	3′GTCGGGTTAATTATGGGCAGTTAT5′		24	
28	MJSSR28F	5′TAAGGCTCTCGCAAGGTAAGG3′	(CCTTCG)5	21	55.75
	MJSSR28R	3′GACTGCTGTGTTTAATTTTGACCC5′		24	
29	MJSSR29F	5′GCCGTCTTCTTCTTCTTCTTCTTC3′	(CCTGC)5	24	56.4
	MJSSR29R	3′CATGGTGTTGACGTGTGGTGTAT5′		23	
30	MJSSR30F	5′GTAATGAAGCACCCTTTCCCTTTC3′	(TCCCAG)12	24	56.65
	MJSSR30R	3′TTTGACCTTAGGAACAATAACGGG5′		24	

MJSSR- *Marssonina juglandis* simple sequence repeats; F- Forward, R- Reverse; Tm- Melting temperature; bp- base pairs

Table 4: Standardization of PCR amplification for Marssonina juglandis isolates using SSR Markers

S. No.	Primer Name**	Sequence	Annealing Temperature (℃)*
1	MJSSR01F	5′CATCTGCTTTCCTTCCACAGC3′	53/ 56
	MJSSR01R	3′TTACTCTGACTCACCTATATGGGG5′	
2	MJSSR02F	5′CTTGTACTTGCGATTGTACTGGAT3′	55/ 53
	MJSSR02R	3′TTTTGCGAGTGTGCTCAAACTTT5′	
3	MJSSR04F	5′GTGATGTGATCAGCATGGGTTC3′	53
	MJSSR04R	3′ATGACAACCCAACAGTTAGTTAGG5′	
4	MJSSR05F	5′GATGGTGATGGTGATGGTGATG3′	53
	MJSSR05R	3′ACTAACGGACAAACTTCTACAGTG5′	
5	MJSSR08F	5′GATCTCCAGCAGGCGAATGTC3′	53/ 56
	MJSSR08R	3′GAAGGTTCTTTAGTCTTCCTCGTG5′	
6	MJSSR09F	5′GGGATGAGGGGTTAGTAGGG3′	53/ 56
	MJSSR09R	3′TGGGGAGTTTTGATGCTTTT5′	
7	MJSSR10F	5′GCGTGGTGATATTGTCTGGA3′	53
	MJSSR10R	3′TCATGGAGCTCATTGCAATA5′	
8	MJSSR11F	5′CGATTTTCTAGAGGGCGATG3′	55/ 53
	MJSSR11R	3′GGTTTGGGGAGGGTTAGAGA5′	
9	MJSSR12F	5′GGGGATGCCTAAACTGCATA3′	55
	MJSSR12R	3′AGGAAGTTTTCGGTCGTTTG5′	
10	MJSSR13F	5′CGCACTTGCAAATACCCTTT3′	55/ 53
	MJSSR13R	3′CGGGAAGGTGTGTGGAGT5′	
11	MJSSR20F	5′CGAAAAGTGGTAAGAGATAGCCTC3′	53/ 55
	MJSSR20R	3′TCAAGCGGTTGAAGATTGTTAGTT5′	
12	MJSSR21F	5′CACTCATCATCTAAACTCCATCGG3′	53/ 55
	MJSSR21R	3′GTACACAAGACAAAGTACCCGTAG5′	
13	MJSSR23F	5′TTCAAGTAGGACATAGTGGTACCT3′	55
	MJSSR23R	3′TGTTGATGGACTTGATTGGACAAT5′	
14	MJSSR24F	5′ACCCTCATAAAACCCCTTCCTC3′	53/ 56
	MJSSR24R	3′GTTGGAATTGGAATTGGGAAGGG5′	
15	MJSSR25F	5′GGTAGTAGCACAAAATAAGCCAGT3′	55/ 53
	MJSSR25R	3′ACAAGACTTTGTTGTACTTGGGAA5′	
16	MJSSR29F	5′GCCGTCTTCTTCTTCTTCTTC3′	55
	MJSSR29R	3′CATGGTGTTGACGTGTGGTGTAT5′	
17	MJSSR30F	5′GTAATGAAGCACCCTTTCCCTTTC3′	55
	MJSSR30R	3′TTTGACCTTAGGAACAATAACGGG5′	

*Primers showed amplification at both annealing temperatures

**MJSSR- *Marssonina juglandis* simple sequence repeats; F- Forward, R- Reverse

Development of PCR-based detection protocol using SSR markers for *Marssonina juglandis* isolates under field conditions

Genomic DNA extraction using the CTAB method directly from the infected walnut leaves

The total genomic DNA from the infected and healthy walnut leaves was isolated using the CTAB method (Murray and Thompson, 1980). The quantification and quality of DNA were analyzed on 1.0 percent agarose gel and photographed using a gel documentation system (Alpha Imager EC, Protein Simple, USA). DNA extracted from the leaves resulted in single intact high molecular weight bands, indicating suitable quality DNA. In addition, quantification of the extracted DNA was also carried out on a Nanodrop Bio-spectrophotometer (Eppendorf, Germany) and maintained at 20-25 ng/µl concentration by 1X TE buffer or nuclease-free water (NFW) and stored at -80 ℃ for further studies.

Polymerase chain reaction (PCR) amplification using SSR markers

PCR amplification was carried out for the DNA samples extracted directly from healthy and infected leaves using SSR primers in a reaction mixture of 25 µl containing 2 µl (20-25 ng/µl) of genomic DNA of the infected as well as healthy leaf samples of walnut trees, 1X PCR buffer, 2.5 mM dNTPs, 1µl each reverse and forward SSR primers (10 pmol), 1.5 mM $Mgcl_2$, 1 unit of *Taq* polymerase enzyme and 15.8 µl of sterilized nuclease-free water (NFW). PCR-based SSR markers were further used for amplification of the pathogen from the infected samples with a PCR profile of initial denaturation at 94℃ for two minutes followed by 35 cycles of denaturation at 94℃ for 40 seconds, annealing at 60℃ for 40 seconds and extension at 72℃ for 40 seconds followed by a final extension at 72℃ for 10 minutes and held at 4℃. The amplified PCR products were resolved on 2.5 percent agarose gel using a gel electrophoresis system and photographed using a gel documentation system (Plate 4.7). After separation, the specific PCR amplified products resulted in the amplification of 230 and 240 bp fragment sizes from the infected leaf samples by primer MJSSR10 and MJSSR23, respectively. However, other primers and the DNA from healthy leaf samples failed to show any amplification. Therefore, primers MJSSR10 and MJSSR23 can be used to identify the anthracnose disease from the infected walnut samples directly in the field at the early stages of infection.

Discussion

Walnut is an economically important dry fruit crop mainly cultivated in temperate regions globally. Walnut crops are prone to various diseases which inflict considerable yield losses. Walnut anthracnose caused by *Marssonina juglandis* (Lib.) Magnus is one of the most severe diseases worldwide. This disease retards the size and mass of nuts, causes failure in metabolic processes in leaves, and change in biochemical indices (Shirnina and Kotljarova, 2000), indicating the necessity to ascertain the evolving nature and variability of the pathogen in different pathogen populations for successful management of the disease. Hence, current research was conducted to develop and validate SSR markers for *Marssonina juglandis* (Lib.)

Magnus causes anthracnose of walnut (*Juglans regia* L.) to aid future studies related to the evolving character of the pathogen over time and for the early stage disease diagnosis of the crop for timely management.

A survey of walnut orchards of SKUAST-Kashmir, Shalimar, Srinagar was carried out for the collection of diseased leaf samples from different walnut trees which were infected with anthracnose disease during the year 2022-2023. The diseased samples were brought to the laboratory for isolation of the pathogen on potato dextrose agar (PDA) medium using the tissue bit method (Cline and Neely, 1984) and spore transfer method (Belisario *et al*., 2008). The spore transfer method was more successful in pathogen isolation due to the comparatively lesser possibility of contamination by other pathogens. A total number of 25 isolates were isolated, followed by utilizing the single spore technique (Tuite, 1969) for purification and maintained on PDA medium at Plant Virology and Molecular Pathology Laboratory of Division of Plant Pathology, FOH, SKUAST-K, Shalimar, Srinagar.

Marssonina juglandis, the causal organism for walnut anthracnose disease, was isolated from the infected leaves collected from the university orchard on potato dextrose agar medium and maintained for further studies. After 5 days of incubation at 22±1°C PDA medium, a circular whitish fungal colony was developed with a glabrous aerial mycelial tuft. After 15 days, the color of the colony gradually turned creamy white. Characteristic concentric zones, well-defined lobate margins, and radial furrows were also developed for further colony growth. Black-colored discoid acervuli were observed in and around the concentric rings after 20 days of colony growth. Microscopic examination disclosed that the pathogen produced smooth, compact, branched septate and hyaline hyphae measuring 3.20-6.50 µm in width with an average width of 4.43 µm in 25 isolates of the pathogen. Conidia were crescent-shaped hyaline with single septation, ranging from 19.25-29.7x7.00-10.50 µm in size with an average of 24.85 x 9.10 µm. The morphological characteristics observed are in agreement with the findings of Sharma and Sharma (1999), Saremi and Amiri (2010), Sogonov *et al*. (2008), Hassan *et al*. (2012), Hedwigia (1906), Holliday (1989); Dastjerdi and Hassani (2009); Kochman (1973) and Anonymous (2013) who also observed and reported the similar findings.

The DNA from all the 25 pathogen isolates was extracted by the CTAB method quantified, and its quality was ascertained per the procedure given by Murray and Thompson (1980) with slight modification. The DNA of the representative pathogen isolate of *Morssonina juglandis* was used for PCR amplification using ITS-1 and ITS-4 primers, and custom sequencing was performed on the amplified products. The consensus sequence of the ITS region was blasted in the NCBI database, showing maximum similarity (more than 98%) with *Marssonina juglandis* (*Gnomonia leptostyla*), confirming the pathogen identity. The methodology used here was similar to the pathogen confirmation using ITS1 and ITS 4 markers by White *et al.* (1990). Mejia *et al.* (2008) and Green and Castlebury (2007) also amplified ITS-1 and ITS-2 regions of nuclear ribosomal DNA in their studies on *Gnomoniaceae* phylogeny.

The whole genome of 63.84 Mbps size of *Marssonina juglandis* with a scaffold number of 1916 was used to develop 6884 SSR markers by GMATA software (Wang and Wang, 2016). These SSR markers were validated under in-silico conditions using an electronic polymerase chain reaction (e-PCR), resulting in *M. juglandis* specific 1474 successfully amplifying SSRs in the output file. Depending upon the contig and scaffold size, 30 SSR markers were selected randomly and custom synthesized. These 30 SSR primers developed were standardized on five randomly selected pathogen isolates under *in-situ* PCR conditions. Out of 30 SSR primers, only 17 polymorphic SSR markers were further utilized for the molecular characterization of 25 isolates of the pathogen *M. juglandis*. Data analysis of these 17 SSR primers was done by calculating polymorphism information content (*PIC*) values that ranged between 0.07-0.37 for each primer were computed using a binary data set, and the polymorphic alleles number also varied from 02-05. The highest *PIC* value of 0.37 was observed in primer MJSSR08, indicating that this primer was found to be highly polymorphic and the most efficient SSR marker among all to ascertain the genetic diverseness of the pathogen with several polymorphic alleles 03 followed by primer MJSSR04 with *PIC* value of 0.35 amplifying 02 polymorphic alleles. The lowest *PIC* value of 0.07 was obtained in primer MJSSR01 with only 02 polymorphic alleles. Jamshidi *et al*. (2012) researched the genetic diversity of Iranian *Ophiognomonia leptostyla* (Fr.) populations using RAPD and ISSR markers. Among the 30 RAPD primers, only four primers could produce considerable bands with sufficient polymorphism, comprising RAPD1, RAPD12, RAPD211, and RAPD213. RAPDs revealed more polymorphism than ISSRs.

A PCR-based detection protocol using SSR markers for *Marssonina juglandis* isolates under field conditions was successfully developed to identify anthracnose infection of walnuts at the early stages of the disease. Healthy and infected leaves from walnut trees were used for the genomic DNA extraction using the CTAB method. Successful PCR amplification was carried out using primer MJSSR10 and MJSSR23, which amplified different product sizes of 230 and 240 in infected walnut samples compared to control DNA from healthy leaves that showed no amplification. A successful detection protocol for anthracnose disease at the early stages of infection was developed using PCR-based SSR markers MJSSR10 and MJSSR23 for the first time. Successful detection protocols have been developed for many plant pathogens using DNA extracted directly from the infected samples. Similar outcomes were reported by Khursheed *et al.* (2023), who also created a molecular detection protocol for the identification of shot hole disease infecting stone fruit hosts, namely peach, plum, apricot, cherry, and almond at early stages of infection using PCR-based SSR markers. Dar *et al*. (2020) also developed a PCR-based detection protocol for scab disease infecting different crops.

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