Development of PCR based SSR markers for Wilsonomyces carpophilus: A PCR based diagnosis protocol for early detection of shot hole disease infecting stone fruit crops

Sehla Khursheed
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir
Mahiya Farooq
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir
Bilal A. Padder
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir
Imran Khan
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir
F U Khan
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir
Asha Nabi
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir
Rizwan Rashid
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir
Sana B. S.
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir
Tariq A Sofi
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir
Farooq A Ahanger
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir
Sumaira Hamid
Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir
Mehraj Ul Din Shah (mehraj547@rediffmail.com)
Sher-E-Kashmir University of Agricultural Sciences and Technology Kashmir https://orcid.org/0000-0003-3325-4229

Research Article

Keywords: Shot hole, stone fruits, molecular detection, SSR primers

Posted Date: January 3rd, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2354824/v1

Version of Record: A version of this preprint was published at Molecular Biology Reports on July 6th, 2023. See the published version at https://doi.org/10.1007/s11033-023-08636-6.

Abstract

Background

The conidial Ascomycota fungus *Wilsonomyces carpophilus* causing shot hole of stone fruits is a major constrain in the production of stone fruits worldwide. Shot hole disease symptoms appear on leaves, fruits, and twigs. Successful isolation of the pathogen from different hosts on synthetic culture medium is a time consuming and tedious procedure for identification of the pathogen based on morpho-cultural characterization.

Methods and Results

The current research was carried out to develop a successful detection protocol for shot hole disease of stone fruits *viz.*, peach, plum, apricot, cherry and almond using PCR based SSR markers that were designed from the *Wilsonomyces carpophilus* genome using Genome-wide Microsatellite Analysing Tool package (GMATA) software to detect the pathogen at early stages. Diseased leaf samples of stone fruits were collected and the pathogen isolated on potato dextrose agar (PDA) medium and maintained on Asthana and Hawkers' medium. For detection protocol, healthy and infected leaf samples of stone fruits were collected and DNA was extracted from the isolated pathogen cultures as well as from leaves infected shot hole disease along with the healthy control (DNA from healthy leaves of stone fruits). The successful amplification was observed in DNA extracted from all the pathogen isolates and from leaf samples with shot hole symptoms but was not observed in control (DNA from healthy leaves) thus confirming the detection of this disease from all the infected samples.

Conclusion

PCR based SSR makers were successfully designed for the for *Wilsonomyces carpophilus* causing shot hole disease in stone fruits and almond in nuts first time. PCR based detection protocol was successfully developed for the detection of pathogen directly from infected leaves of stone fruits such as peach, plum, apricot, cherry and almond among the nuts.

Introduction

The shot hole pathogen *Wilsonomyces carpophilus* belongs to phylum Ascomycota, class: Dothideomycetes, order: Pleosporales and family Dothidotthiaceae (1, 2). Shot hole disease is one of the most economically important diseases of stone fruits worldwide. Despite of the fungicidal management, shot hole disease of stone fruits has been reported to be responsible for huge yield losses. Stone fruits are prone to several plant diseases of fungal, bacterial and viral etiology that are the main limiting factors for their production. Out of various diseases caused by different plant pathogens, the fungal diseases such as brown rot (*Monilinia fructicola*), scab (*Cladosporium carpophilum*), powdery mildew

(*Podosphaera tridactyla*), green fruit rot (*Sclerotinia sclerotium*), anthracnose (*Colletotrichum* spp.), leaf curl (*Taphrina deformans*), Cercospora leaf spot (*Cercospora circumcissa*) and shot hole (*Wilsonomyces carpophilus*) are the major diseases responsible for huge economic losses (3, 4). Among these, shot hole blight or *Coryneum* blight caused by the *Wilsonomyces carpophilus* is a fungal disease having wide host range that infects peach, apricot, plum, cherry and almond. In India, shot hole disease on apricot, cherry and peach was reported from Kumaon (5) and from Kashmir (6). The disease incidence of 31, 25 and 37 per cent had been reported on almonds at Harparbath, Zawora and Tral areas, respectively, in Kashmir valley (3), and 60–80 per cent of disease incidence on cherry fruits and leaves in the Kashmir valley was reported (7).

Successful detection protocols have been developed in many plant pathogens by various researchers using different molecular markers (8, 9) but the work on the detection and development of protocol for shot hole disease infecting stone fruit hosts has not been carried out till date. The current research was carried out to develop a detection protocol for shot hole disease infecting stone fruits using PCR based SSR markers for the detection of disease at early stages of infection.

Materials And Methods

Isolation of the pathogen isolates

Diseased leaf samples were collected from different stone fruits *viz*., peach, plum, apricot, cherry and almond from Srinagar and Ganderbal districts during year 2021 (20 isolates) that were isolated on potato dextrose agar (PDA) medium. Pure culture was obtained by single spore technique and maintained on Asthana and Hawker's medium at 24 ± 1°C in BOD. Diseased and healthy leaf samples (control check) of stone fruits *viz*., peach, plum, apricot, cherry and almond were used for developing detection protocol.

Genomic Dna Extraction Of The Pathogen Isolates And Leaf Samples

Genomic DNA extraction of the pathogen isolates and leaf samples
Total genomic DNA of the pathogen isolates, infected and healthy leaf samples of five stone fruit host (peach, plum, apricot, cherry and almond) was extracted using CTAB method (10). The samples were cut and ground to fine powder in liquid nitrogen using sterilised pre-chilled pestle and mortal. The fine powder was transferred to 1.5 ml microfuge tubes containing 700µl of CTAB (Cetyl Trimethyl Ammonium Bromide) buffer maintained at 65°C in water bath. The samples were thoroughly mixed and incubated at 65°C for one hour in thermomixer at 500 rpm. After cooling of the microfuge tubes to room temperature, 700 µl of pre-chilled isoamyl alcohol:chloroform in 24:1 ratio was added followed by centrifugation at 12,000 rpm for 20 minutes. After centrifugation, equal volume of supernatant was taken out with the help of micropipette and placed in new centrifuge tubes. Pre-chilled isopropanol of about 700 µl was then added to these tubes containing supernatant and kept overnight at -20°C or -80°C for two hours. Next day (After 12 hours), 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 per cent ethanol twice followed by centrifugation at 10,000 rpm for 10–15 minutes. After centrifugation, 70 per cent ethanol was decanted and the tubes containing DNA pellets were kept inverted for drying till 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℃ for 2 hours in thermomixer (Eppendorf, Germany). The genomic DNA from the healthy leaves was also obtained in similar manner as control. The genomic DNA isolated from all the samples in similar way was stored at -80℃ for further use.

Dna Quantification And Quality Check

Genomic DNA extracted by CTAB method was quantified by agarose gel electrophoresis. In this method, 1.0 per cent agarose gel was prepared (1 g of agarose powder in 100ml of 1X TAE buffer in 250 ml flask). The solution was allowed to cool followed by addition of ethidium bromide @ 2µl to this mixture as fluorescent dye. The solution was poured into the cast and allowed to solidify for 15–20 minutes. Loading dye bromophenol blue (6X) was placed on Para film in drop manner and the DNA approximately 2 µl from each sample was mixed with this loading dye using micropipette followed by loading into wells of already prepared gel. The gel was allowed to run for 20 minutes at 80 Volt in gel electroporation system (Consort, Belgium) and the DNA was visualized under 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. Quality of each sample was checked on the basis of whether single intact high molecular weight band was formed (good quality) or a smear was formed (poor quality). The quantification of DNA was also carried out using 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.

Designing and development of simple sequence repeat (SSR) markers for polymerase chain reaction (PCR) amplification of Wilsonomyces carpophilus

Draft genome of 29.9 Mb size of *Wilsonomyces carpophilus* (GenBank Accession No. PRJNA791904) distributed on 130 Scaffolds with 2851 SSR's was used for SSR mining using Genome-wide Microsatellite Analysing Tool package (GMATA) software. After designing and custom synthesis of SSR markers, 30 SSR primers were initially screened on isolates collected from different hosts *viz*., peach, plum, apricot, cherry and almond (Supplementary Table 1). After standardization, 10 primers showing polymorphism were selected for further analysis (Table 1). Successful amplification was obtained by selected 10 primers on all pathogen isolates but only one SSR namely ShotholeSSR9 showed positive amplification in all the five stone fruits of infected leaf samples.

Table 1
Simple sequence repeat (SSR) markers along with their sequences developed from *Wilsonomyces carpophilus* genome using GAMATA software

S. No.	Primer Name	Sequence
1	ShotholeSSR1F	GCGTGGTGTTACATGGTGAG
	ShotholeSSR1R	AAGATGGACGTGTGTGTGGA
2	ShotholeSSR2F	GCCGAGTTTCTTCAAAGTGC
	ShotholeSSR2R	ACCAATAACAAACCCCACCA
3	ShotholeSSR3F	ATCCAGCATAACATGGCACA
	ShotholeSSR3R	CTGATCGAAGGGATCGAGAG
4	ShotholeSSR4F	GTAGGGATTTACGGGCGTTT
	ShotholeSSR4R	CGTGGTAACACAGCACTCGT
5	ShotholeSSR6F	GACAGACGGCTGAAGAGGAG
	ShotholeSSR6R	TCACAAAACCACATGGGCTA
6	ShotholeSSR9F	GGGATGAGGGGTTAGTAGGG
	ShotholeSSR9R	TGGGGAGTTTTGATGCTTTT
7	ShotholeSSR17F	TGAAGTTCGACCGTGGTGTA
	ShotholeSSR17R	ATTCTTTCCCTCCCTCCAGA
8	ShotholeSSR18F	CTGGAGGCTTTCGTATTCCA
	ShotholeSSR18R	TGCACAAAACACAATGCAGA
9	ShotholeSSR22F	GCCCCGAGGTACATATAGCA
	ShotholeSSR22R	GCTGAAAAGGGTAGCTGTCG
10	ShotholeSSR30F	GCCCCGAGGTACATATAGCA
	ShotholeSSR30R	GCTGAAAAGGGTAGCTGTCG
*Annealing temperature for SSR markers was 60°C		

Polymerase chain reaction (PCR) amplification of Wilsonomyces carpophilus **directly from infected leaves using SSR primers**

The PCR amplification was carried out in thermocycler in a PCR reaction of 25µl containing 2µl (20–25 ng/µl) of genomic DNA of pathogen isolates and from the infected leaves along the control comprised of DNA from healthy leaf samples of different stone fruits, 1X PCR buffer, 2.5 mM dNTPs, 1µM 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 used for amplification of the pathogen from infected samples with PCR profile of initial denaturation at 94℃ for two minutes followed by 35 cycles of denaturation at 94℃ for 40s, annealing at 60℃ for 40s and extension at 72℃ for 40s followed by a final extension at 72℃ for 10 minutes and hold at 4℃.

Data analysis

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

Results

Genomic DNA extraction and quality check

The total genomic DNA was isolated from the pathogen isolates and the infected as well as healthy leaves of five stone fruit hosts (peach, plum, apricot, cherry and almond) using CTAB method (10). The quantification and quality of DNA was analysed on 1.0 per cent agarose gel and photographed using gel documentation system. DNA extracted from leaves resulted in single intact high molecular weight bands indicating a good quality DNA of the pathogen isolates. In addition, the quantification was also carried out using Nanodrop Bio-spectrophotometer (Eppendorf, Germany) and maintained at 20-25ng/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 using PCR based SSR primers. The amplified PCR product was resolved on 2.5 per cent agarose gel using gel electrophoresis system and photographed using gel documentation system. The successful amplification was shown by all pathogen isolates using different primers, whereas successful PCR amplification was only shown by leaf samples infected with shot hole symptoms but PCR amplification was not successful in healthy leaf samples using SSR primer ShotholeSSR9 (Fig. 2). However, other primers failed to show amplification in all the five stone fruits. Therefore, primer ShotholeSSR9 can be used to ascertain and identify the shot hole disease from infected samples directly in field for early detection of the disease.

Discussion

Molecular detection using PCR based SSR marker protocol for the identification of shot hole disease infecting stone fruit hosts namely peach, plum, apricot, cherry and almond was successfully developed for the detection of disease at early stages of infection. Healthy and infected leaves along with the

pathogen isolates from different stone fruit hosts (peach, plum, apricot, cherry and almond) were used for the genomic DNA extraction using CTAB method. Successful PCR amplification was carried out using primer ShotholeSSR9. Other SSR markers failed to show any amplification in all five stone fruit hosts therefore, a successful detection protocol for shot hole disease at early stages of infection was developed using PCR based SSR marker ShotholeSSR9 for the first time. Successful detection protocol have been developed in many plant pathogens using DNA extracted directly from the infected samples. Successful detection protocol for *Monilinia* species infecting stone fruits using DNA extracted from the infected stone fruits and amplified by PCR based Random Amplified Polymorphic DNA (RAPD) markers using multiplex PCR has been reported (8). Detection protocol for *Taphrina deformans* causing peach leaf curl from the DNA of infected and uninfected leaves using TDITS-1 PCR detection and probe TDE634 is documented (9). Multiple DNA markers for identifying *Xanthomonas arbicola* pv. *juglandis* causing brown apical necrosis (BAN) and vertical oozing canker (VOC) in walnut by isolating DNA from the infected fruits and leaves has been reported (11) and in case of scab disease caused by *Venturia* species on different pome and stone fruit plants (12) but detection protocol for shot hole disease infecting stone fruits have been developed for the first time using PCR based SSR markers.

Conclusion

Molecular detection protocol for various diseases using different molecular markers have been reported by many researchers but the detection protocol for shot hole disease infecting stone fruits has not been reported till date. This research has resulted in successful development of detection protocol for the shot hole disease that will help in detecting the pathogen at even early stages of infection and hence can help in reducing the crop loss in advanced stages of infection.

Declarations

Acknowledgements: The authors are thankful to Professor and Head, Division of Plant Pathology, SKUAST-Kashmir for providing necessary help and support, and SERB-DST, GOI and SKUAST-Kashmir for financial support.

Author contributions: S.K, M.F., M.D.S; carried out most of the experiments; M.D.S, B.A.P. and S.K analysed the data; S.K, M.D.S drafted the manuscript; S.K, M.D.S, I.K., R.R., F.U.K., A.N., M.F., S.B.S; reviewed the manuscript; T.A.S., F.A.A., S.H. isolated and purified the pathogen cultures. All the authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest: there was no involvement of animals in this study. All authors declare that there are no conflicts of interest.

Informed consent: Informed consent was received from all individual participants included in this study

References

1. Ye S, Jia H, Cai G, Tian C and Ma R (2020) Morphology, DNA Phylogeny, and Pathogenicity of Wilsonomyces carpophilus Isolate Causing Shot-Hole Disease of Prunus divaricata and Prunus armeniaca in Wild-Fruit Forest of Western Tianshan Mountains, China. Forests 11, 319
2. Marin-Felix Y, Groenewald J, Cai L et al (2017) Genera of phytopathogenic fungi: GOPHY 1. Studies in mycology 86, 99-216
3. Nabi A, Shah M-U-D, Padder B, Dar M and Ahmad M (2018) Morpho-cultural, pathological and molecular variability in Thyrostroma carpophilum causing shot hole of stone fruits in India. European Journal of Plant Pathology 151, 613-627
4. Rasool RS, Wani AA, Masoodi KZ et al (2020) Study on Cross Infectivity of Different Isolates of Thyrostroma carpophilum on Stone Fruits in Kashmir Valley. Int. J. Curr. Microbiol. App. Sci 9, 616-623
5. Singh U (1943) Some diseases of fruits and fruit trees in Kumaon-IICAR. Misc. Bull. 51, 16
6. Munjal R and Kulshreshta D (1968) Some Dematiaceae hyphomycetes from India. Indian. Phytopath 21, 309-314
7. Puttoo B and Razdan V (1991) Stigmina blight of cherry: A new record from India. Plant Disease Research 6, 60
8. Côté M-J, Tardif M-C and Meldrum AJ (2004) Identification of Monilinia fructigena, M. fructicola, M. laxa, and Monilia polystroma on inoculated and naturally infected fruit using multiplex PCR. Plant Disease 88, 1219-1225
9. Tavares S, Inácio J and Oliveira C (2004) Direct detection of Taphrina deformans on peach trees using molecular methods. European journal of plant pathology 110, 973-982
10. Murray M and Thompson W (1980) Rapid isolation of high molecular weight plant DNA. Nucleic acids research 8, 4321-4326
11. Fernandes C, Albuquerque P, Sousa R, Cruz L and Tavares F (2017) Multiple DNA markers for identification of Xanthomonas arboricola pv. juglandis isolates and its direct detection in plant samples. Plant Disease 101, 858-865
12. Dar, M.S., Ahmad, M., Nabi, A., Shah, M.D. and Padder, B.A. (2022). Multiplex PCR based detection method for *Venturia* species infecting pome and stone fruits. Indian Phytopathology, pp.1-10.

Figures

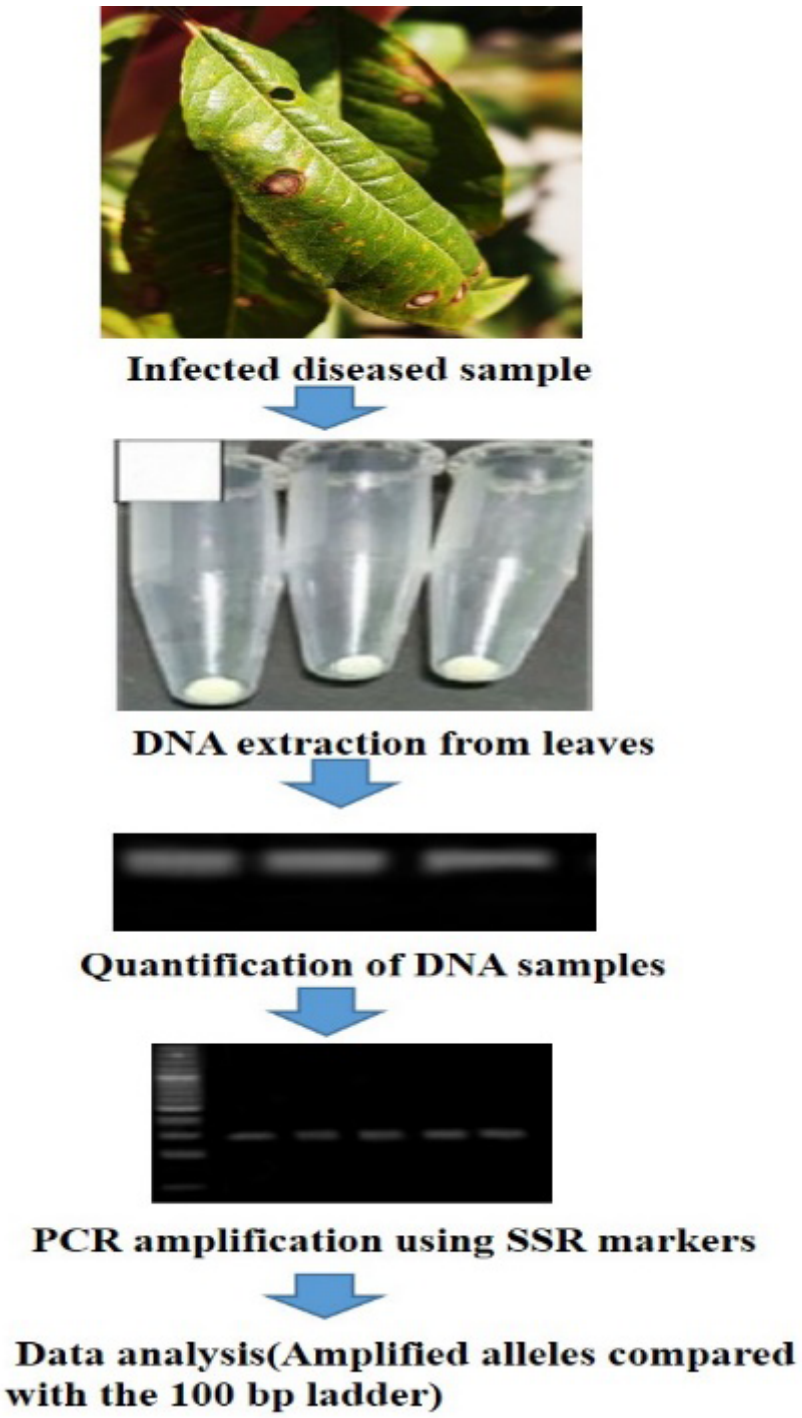

Figure 1

Flowchart of detection protocol using PCR based SSR markers for the detection of shot hole disease infecting stone fruits

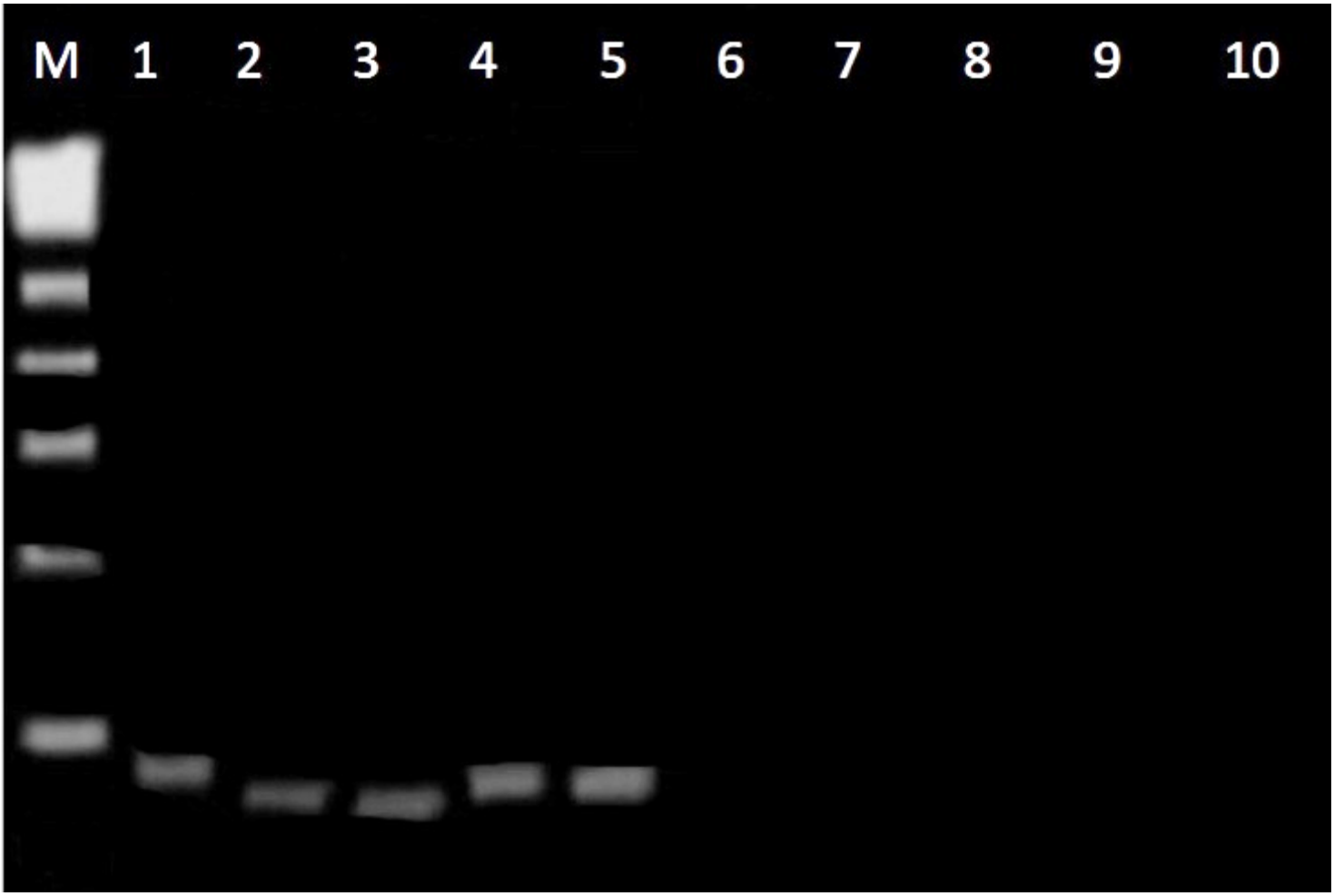

Figure 2

Molecular detection of *Wilsonomyces carpophilus* causing shothole of stone fruits using PCR based SSR marker ShotholeSSR9

M: 100 bp ladder

Lane : 1) Peach, 2) Plum, 3) Apricot, 4) Almond, 5) Cherry 6-7) Control (peach, plum, apricot, almond and cherry)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytablefile.docx