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Original Article

Molecular characterization of shisham dieback-associated fungal isolates across the Punjab Province, Pakistan

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Abstract

The Shisham (*Dalbergia sissoo*) population is threatened by the fungal pathogens causing dieback disease. Research over the past decade has shown the involvement of multiple fungal pathogens in causing the dieback disease. Here we reported the RAPD-PCR-based genetic diversity in three potential fungal pathogens including *Botrydiplodia theobromae*, *Fusarium solani*, and *F. oxysporum* isolated from diseased plants across the Punjab province in Pakistan. Twenty fungal isolates of three major fungal species were isolated from dieback-infected trees from eight districts in Punjab Pakistan to investigate the genetic diversity with a set of fifteen RAPD markers of OPB, OPK, and OPL series. Consequently, fifteen markers produced 166 loci with an average of 55.33 loci per population. Of these, 76.31% loci were polymorphic highlighting the presence of abundant genetic diversity in pathogens. Moreover, OPK-06 primer showed a higher PIC value of 0.416 along with higher heterozygosity contents of 0.49. However, population structure analysis of each fungal strain grouped similar and dissimilar ones based on their amplification into the same and different clusters respectively. The isolates of *B. theobromae* of the Ayub Agriculture Research Institute (AARI) and Attock district showed higher genetic distance (0.6812) and were found in different clusters. Similarly, isolates of *F. solani* from UAF formed a different group which further highlighted its degree of polymorphism. Moreover, the UAF isolate displayed a maximum of 0.6432 genetic distance from the AARI isolate. Conversely, the isolates of *F. oxysporum* were grouped into two main groups highlighting the limited genetic diversity. Further, isolates from UAF also exhibited a maximum of 0.7372 genetic distance from AARI. Consistent results of UAF and AARI isolates of *F. oxysporum* and *F. solani* are suitable grounds for further genetic studies.

Keywords: *Fusarium oxysporum*, RAPD, *Botrydiplodia theobromae*, *Fusarium solani*, Dieback, Pathogens

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Introduction

The genus *Dalbergia* of the *Fabaceae* family contains 250 species including shrubs, trees, and woody lianas. Out Of the total *Dalbergia* species, 15 are native to the Indo-Pak subcontinent (Bajpai, 2023). *D. sissoo* plant is a deciduous tree, and commonly known as shisham, sissoo, and tahli. It is mostly grown in forest plantations, along the way of railway lines, canal banks, riversides, and farmer's fields. It is a leguminous and deciduous tree that can attain up to 30 meters in height and up to 3 meters in girth and could also produce hardwood (Shah et al., 2021). It has good quality timber after the teak tree which makes it more important. Besides wood production, trees are essential for environmental stability. Its nitrogen-fixing ability makes it a more useful legume tree species, especially for large-scale industrial plantations (Haq et al., 2021). Moreover, it also enriches soil fertility through its fast-decomposing leaves and nitrogen fixation ability (Gupta et al., 2023). Shisham is a tree of tropical and subtropical environments, and it can be grown on high elevations of up to 1300 meters. It has a shallow root system that could prevent soil erosion. Trees of older ages can produce enough wood for making furniture but due to deadly dieback disease, shisham cannot live more than six years which causes great economic loss. Addressing the shisham dieback disease could lower the economic losses. Apart from wood production shisham trees also have some medicinal value *e.g.,* it is helpful for diabetic patients. Its leaf extract contains antioxidants and flavonoids (Sehra and Sharma, 2018). Its extract can also be used for the treatment of different infectious diseases (Hossen et al., 2023; Misganu, 2022).

Shisham population is susceptible to multiple stresses including biotic and abiotic. Dieback is at the top pushing its population to extinction. Dieback is a fungal disease that is caused by a single or complex group of fungi. Dieback is characterized by wilting of the crown part to drying of end branches, and progress from top to bottom as compared to the wilting which occurs from bottom to top. Ensuring the fatal damage to many *Dalbergia* species, the International Union for Conservation of Nature (IUCN) has enlisted several species of the *Dalbergia* genus as endangered ones (Arunkumar et al., 2022).

Several reports are available on the association of different fungal species with shisham dieback. Of these species, *B. theobromae*, *F. solani*, and *F. oxysporum* are the most known and extensively reported. Genus *Fusarium* is related to numerous plant diseases; however, its diversity and distribution are very important. Similarly, *B. theobromae* is also considered a possible dieback-associated pathogen (Babu et al., 2021; Bakshi and Sharma, 2011; Dhiman et al., 2022).

Although, shisham is somehow studied at the molecular level to investigate the genetic causes of shisham dieback, however, molecular exploration of the pathogen is still unexplored. For the past few years, multiple reports have been published on the molecular characterization of plant-associated pathogens in India only. Some of these reports were focused on understanding the regional restricted genetic diversity within and between fungal pathogen groups (Dobhal et al., 2019).

RAPD markers may provide information that can help in distinguishing nature in addition to the phylogenetic relationships among the population. RAPD markers are very efficient in characterizing genetic connections at the population level. In most fungal species, RAPD markers are widely used to calculate genetic diversity as well as to estimate of population genetic studies (Mukhtar et al., 2014; Vishwakarma et al., 2016). Moreover, RAPD markers have also been used to approximate the genetic relationship among the various species of populations for the detection of clonal diversity (Ghazali et al., 2022).

In this study, we have characterized *B. theobramae*, *F. oxysporum*, and *F. solani* from eight different regions of Punjab, Pakistan with RAPD markers to explore the genetic diversity. We used 20 different decamers from three different series to compare the genetic diversity.

Material and Methods

Survey execution and population tagging

Shisham plantation sites in Punjab province, Pakistan, including UAF, Attock, Changa Manga, Bahawalpur, Fateh Jang, Chichawatni, Ayub Agricultural Research Institute (AARI), and Bhakkar were surveyed to collect diseased shisham stem and roots portions. Following the survey morphologically diseased plants were tagged accordingly. Finally, we collected 20 accessions from these districts (Table 1). After collecting the stem and root samples, a numerical number for each tree was designated to keep the record for further experimental investigations.

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Table-1: Population source/locations of each fungal species

Serial No.	Population District	Pop assigned number	Fungal isolates	Diseased source tissue
1	UAF	Pop1	*B. theobromae*	Diseased dry stems
2	AARI	Pop2	*B. theobromae*	Diseased dry stems
3	Bhakkar	Pop3	*B. theobromae*	Diseased dry stems
4	Fateh Jang	Pop4	*B. theobromae*	Diseased dry stems
5	Bahawalpur-I	Pop5	*B. theobromae*	Diseased dry stems
6	Chichawatni	Pop6	*B. theobromae*	Diseased dry stems
7	Changa Manga	Pop7	*B. theobromae*	Diseased dry stems
8	Attock	Pop8	*B. theobromae*	Diseased dry stems
9	UAF	Pop1	*F. oxysporum*	Diseased dry stems and roots
10	AARI	Pop2	*F. oxysporum*	Diseased dry stems and roots
11	Bhakkar	Pop3	*F. oxysporum*	Diseased dry stems and roots
12	Fateh Jang	Pop4	*F. oxysporum*	Diseased dry stems and roots
13	Bahawalpur-II	Pop5	*F. oxysporum*	Diseased dry stems and roots
14	Chichawatni	Pop6	*F. oxysporum*	Diseased dry stems and roots
15	UAF	Pop1	*F. solani*	Diseased dry root cuttings
16	AARI	Pop2	*F. solani*	Diseased dry root cuttings
17	Bhakkar	Pop3	*F. solani*	Diseased dry root cuttings
18	Chichawatni	Pop4	*F. solani*	Diseased dry root cuttings
19	Changa Manga	Pop5	*F. solani*	Diseased dry root cuttings
20	Attock	Pop6	*F. solani*	Diseased dry root cuttings

Isolation of the desired fungus from dieback-infected tissues

The diseased stems and root tissues showing the pathogen movement along with dieback symptoms were dissected to isolate the fungal pathogen. The stem chippings isolated from the stems and roots were washed with 50% sodium hypochlorite for 2 minutes and 70% ethanol for one minute, followed by rinsing 2 times with sterile water under sterile conditions. The sterile stem and root cuttings were additionally sliced into tiny pieces and placed on PDA solid media consisting of 250 g/L freshly boiled potatoes, 20 g/L dextrose, and 10g/L Agar Agar along with the selected antibiotics to avoid bacterial contamination and incubated at 25 ± 2 ℃ under the dark for 5 days. The growth of chippings was analyzed daily (Figure 1).

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Figure-1. Collection and tagging of diseased plants, identification, isolation, purification and multiplication of desired fungal strains; a) crown of infected tree at earlier stage, b) stem of diseased tree at earlier stage of infection d) crown of infected tree at advanced stage of infection showing the wilting of crown e) external view of stem bark at advanced stage of infection showing the appearance of disease establishing and pathogen progress c, f) dissection of diseased bark to observe the cross-sectional view of pathogen progress n infected tree (g, h, i) infection on younger trees showing the pathogen progress in cross-section of bark; j) incubation of infected tissues on PDA media to isolate the desired fungus by visual observation and also grow on the SNA (Spezeiller Nahrstoffarmer agar) for sporulation, (o) showing the diseased tree roots growth on the PDA media;(p, q, plates showing the different stages of fungus purification steps (k, l, m, n) cultures of fungal mycelia for DNA isolation, (r, s, t, u, v) visualization of fungal spores under light microscope.

Mass multiplication of fungal isolates

The grown fungus mycelium was further analyzed for the different types through microscopy and incubated on PDA media at 25 ± 2 ℃ under the dark. After 5 days, the isolated fungus was sub-cultured to further purify through repeated incubation cycles and again

incubated on SNA (KH_2PO_4 1 g/L, KNO_3 1 g/L, $MgSO_4$* $7H_20$ 0.5 g/L, KCl 0.5 g/L, Glucose 0.2 g/L, Sucrose 0.2 g/L, and Agar 20 g/L) for two weeks under the same incubation conditions for better sporulation. After two weeks, the conidial formation at 100 x magnification was observed under a light microscope to confirm the fungal type.

Spore collection and culture initiation

Following the multiplication of the desired isolated fungi, spores from each fungal strain were collected by swirling the nucleases free water on the plate of conidia. Later, the spore's concentration was maintained at 3 x 10^6 with the help of a hemocytometer.

Culture media of fungal and DNA extraction

Genomic DNA isolation

Spores of each fungus with the same concentrations were added to the PDA broth. The DNA isolation was carried out with a lab-based unpublished DNA isolation protocol. Cultures were placed on a rotary shaker at 180 RPM at 26±2℃ for two weeks under dark conditions. After two weeks the bulk of mycelia was collected by filtering the broth culture with sterile filter paper. Finally, the 50mg of mycelia from each fungal source were collected and dried for 10 minutes and ground in autoclaved sand to make a fine mixture. Then 0.3µl of Proteinase K and 2/10 volume of lysozyme were added to the collected mixture and incubated at 37℃ for 60 minutes followed by 40 minutes of incubation in a water bath to ensure a homogenized mixture. Later, the liquid was centrifuged at 14500RPM for 10 minutes and the supernatant was collected in the new Eppendorf. An equal volume of modified CTAB buffer was added and again incubated at 42℃ for 15 minutes to confirm homogenization and centrifuged at 14000 RPM for 10 minutes to collect the supernatant. The collected supernatant was treated with 2µl RNase for 30 minutes at 37 ℃ in each reaction tube. The 2 volumes of Chloroform Isoamyl alcohol were further added to each tube and centrifuged to separate the supernatant (repeat the step). The supernatant was saved, and an equal volume of chilled isopropanol and 0.1 volume of Na-acetate was added to support the sufficient precipitation and incubated the samples at -30 ℃ for 2 relationships among the population (Jena and Chand, 2021).

hours. Centrifuged the mixture at 14000 rpm for 10 minutes at 4 ℃ to get the final pellet. The final pellet was then washed by adding 75% chilled ethanol and centrifuged at 14000 RPM for 10 minutes. The pellet was air-dried and dissolved in pre-heated sterile water. Isolated DNA was checked on Nanodrop-2000 to check the nucleic acid ratio and other contamination. Samples fulfilling the quality parameters were selected for PCR reactions.

RAPD-assisted genotyping

A minimum of 15 random decamers oligonucleotide RAPD primers of different series like OPB, OPK, and OPL were applied to each DNA sample. For their application on each DNA sample, a working dilution containing 20ng/µl was prepared for the PCR reaction. Before genotyping each primer was optimized using gradient PCR. Afterward, each PCR was carried out in a final 10µl volume including 1µl DNA (20ng DNA), 2 µl Taq buffer, 2µl decamer, 3µl DNTPs, 0.25µl Taq polymerase, and 2.75 µl D_3H_2O against the PCR conditions 95℃ for 5 minutes, 40 cycles of 95℃ for 1minute, variable annealing temperature for 30s, 72℃ for 1minute and final extension at 72℃ for 15 minutes. The PCR products were isolated on 3% gel electrophoresis.

Use of primers

In this study, we have characterized the *B. theobramae*, *F. oxysporum,* and *F. solani* collected from eight different regions of Punjab, Pakistan with RAPD markers to explore the genetic diversity. We used 20 different decamers from three different series to compare the genetic diversity.

RAPD primers, especially of series OPB (Oligonucleotide Primer B), OPK (Oligonucleotide Primer K), and OPL (Oligonucleotide Primer L) were used. RAPDs have already been used for the molecular characterization of plant-associated pathogens. These primers can generate diverse banding patterns by making it easier to differentiate between genetically diverse fungal pathogen strains (Singh et al., 2022). The combination of OPB, OPL, and OPK primers enhances the resolution and sensitivity of genetic diversity studies. The use of RAPD markers in this study may provide information that could help in distinguishing nature in addition to the phylogenetic

Gel scoring

Subsequently, the gel electrophoresis and the resulting banding patterns were analyzed, and data sheets of binary codes were prepared. For that, each amplified product was scored as either present (1) or absent (0) based on the observed banding pattern. Faint and low-quality bands were excluded from the analysis to ensure accurate and satisfactory results. Later, this sheet was used as input to carry out further analysis (Tewari et al., 2022).

Data analysis

Genetic differences among the diverse sources of the fungal pathogens data set were created in a Microsoft Excel sheet for further analysis. A similarity matrix was however created by using the recorded binary banding patterns. The Jaccard (1908) similarity coefficient, introduced by was therefore employed to calculate and investigate the genetic similarity. The genetic similarity among each fungal population along with the set the 15 primers was evaluated by using the unweighted paired group method UPGMA with an arithmetic average (UPGMA). This method is a hierarchical clustering technique that groups similar genotypes according to their genetic similarity. The discriminatory power of the marker was assessed by evaluating the PIC (Polymorphism information content) value through the calculation of PICi =(2fi(1-fi)) where Fi is the frequency of marker fragments that were present and (1-fi) is the frequency of absent markers (Sakhraoui et al., 2024).

Results

In this study, 20 fungal isolates from eight different locations were genotyped with RAPD-based genotyping by using a set of 15 distinct 10 bp oligonucleotide RAPD primers. Among these species, *F. oxysporum* showed the highest amplification (151) followed by *B. theobromae* and *F. solani* which displayed 141 and 129 amplifications respectively. Likewise, variations are also supported by the percentage of polymorphic loci. Like, *F. oxysporum* possessed 83.73% polymorphic loci while the lowest polymorphic percentage was showed by *F. solani* (68.67%).

Table-2. RAPD-based basic parameters of primers used in the current study

Serial No.	Marker Name	Sequence	No. of Loci	Ht	I	Size range	PIC
1	OPL1	CATTCGAGCC	7	0.12	0.20	300-1000	0.190
2	OPL03	GTCTCCGCAA	12	0.19	0.30	300-500	0.298
3	OPL05	CCAGCTTAGG	10	0.31	0.45	300-700	0.390
4	OPL14	CCGCCCAAAC	11	0.30	0.45	350-1500	0.430
5	OPK01	TCTGTCGAGG	11	0.27	0.41	300-700	0.432
6	OPK02	CACCTTTCCC	8	0.28	0.41	300-1000	0.410
7	OPK03	AGCGAGCAAG	11	0.21	0.33	300-1500	0.331
8	OPK05	AATGCCCCAG	11	0.32	0.47	300-700	0.414
9	OPK06	TGGCCCTCAC	10	0.33	0.49	350-1000	0.416
10	OPK11	GTTTCGCTCC	11	0.26	0.40	350-1000	0.416
11	OPK12	TGATCCCTGG	6	0.29	0.43	500-1000	0.421
12	OPB01	GGACTGGAGT	10	0.33	0.47	300-1000	0.343
13	OPB03	TGCGCCCTTC	5	0.23	0.35	300-700	0.360
14	OPB08	TGCTCTGCCC	7	0.27	0.40	500-700	0.384
15	OPB09	GGTGACGCAG	8	0.28	0.42	500-1000	0.374

In this study, three series of RAPD markers including OPL, OPK, and OPB were used to genotype the fungal pathogens. Overall, 15 primers produced 166 amplifications across three fungal species, with an average of 11 amplifications per decamer. The amplification size ranged from 300bp to 1400bp. Out of these primers, OPL14, OPK11, OPK01, OPK03, OPK05, and OPK11 each produced 11 loci and OPB03 gave 05 loci only. The Shannon index (I) content detected by each primer ranged from 0.20 (OPL03) to 0.49 (OPB03) with an average of 0.4, whereas the heterozygosity content (Ht) varied from 0.12 (OPB01) to 0.33 (OPK06) with the mean value of 0.27. Also, the PIC value has been measured to select the most effective RAPD marker. PIC ranged from 0.190 (OPL01) to 0.432 (OPK01) with an average of 0.374. Furthermore, displaying the highest PIC value along with the higher Heterozygosity Ht contents would be the most advantageous primer to explore the genetic diversity within or between shisham populations. Here OPK06 possessed the highest PIC and Ht contents (Table 2).

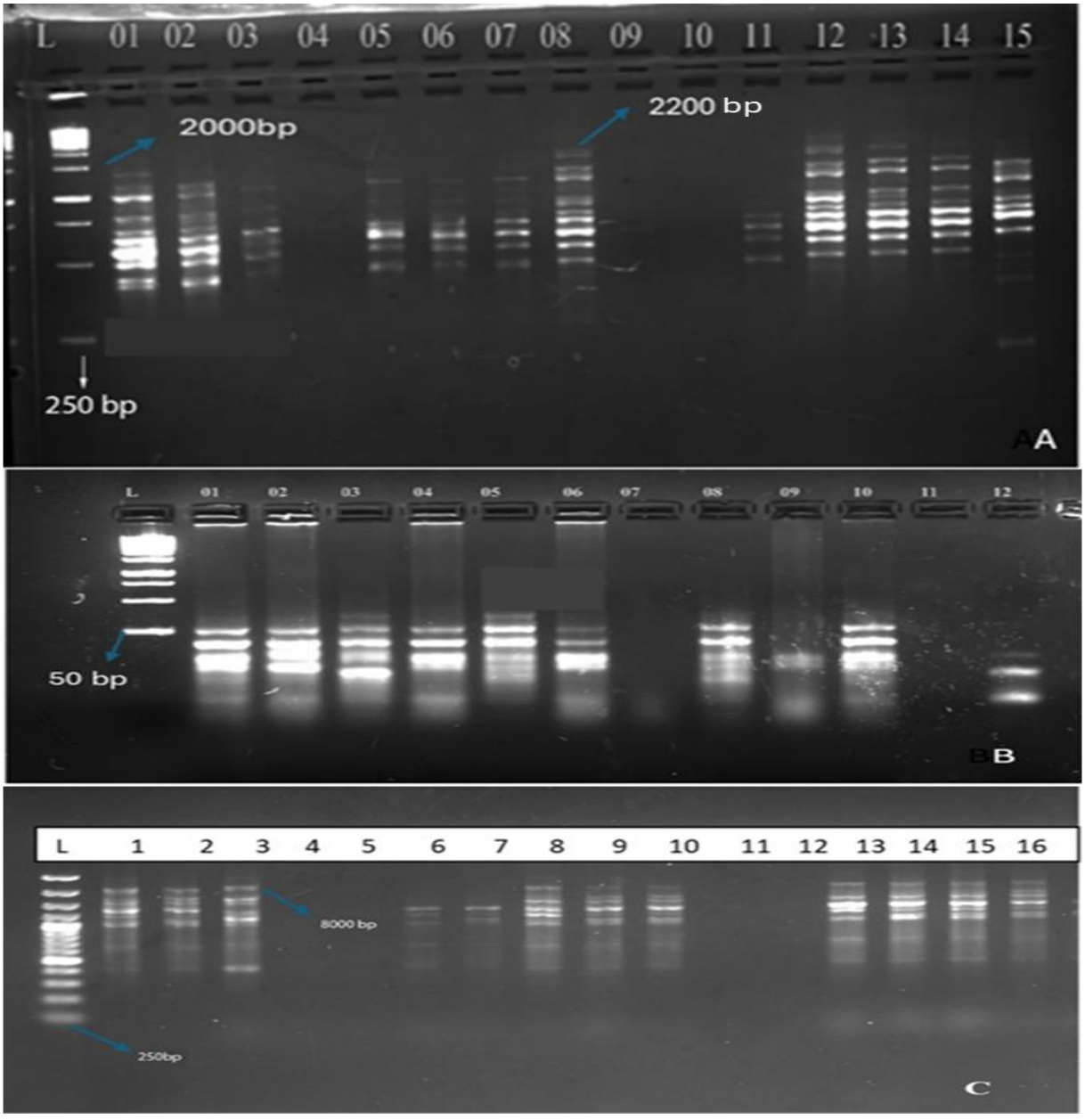

Figure-2. RAPD PCR-based amplifications were obtained from fifteen decamers

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Population structure analysis of *B. theobromae*

Pathogen populations are distantly discrete to each other; therefore, they were processed separately to avoid the effect of one fungus on the other fungus.

Table-3. Nei's Unbiased Measures of Genetic Identity and Genetic Diversity in *B. theobromae* population

Pop ID	UAF	AARI	Bhakkar	Fateh Jang	Bahawalpur I	Chichawatni	Changa Manga	Attock
UAF	****	0.7048	0.741	0.6928	0.6867	0.5904	0.5361	0.5964
AARI	0.3498	****	0.7108	0.6506	0.5843	0.5964	0.5663	0.506
Bhakkar	0.2998	0.3413	****	0.7229	0.6928	0.6807	0.6506	0.6867
Fateh Jang	0.3671	0.4299	0.3245	****	0.8133	0.7771	0.6867	0.6747
Bahawalpur District I	0.3758	0.5373	0.3671	0.2067	****	0.759	0.6446	0.6807
Chichawatni	0.527	0.5169	0.3846	0.2522	0.2757	****	0.7289	0.7651
Changa Manga	0.6234	0.5687	0.4299	0.3758	0.4392	0.3162	****	0.7952
Attock	0.5169	0.6812	0.3758	0.3935	0.3846	0.2678	0.2292	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

The genetic identity ranged from 0.5060 to 0.8133 as compared to the genetic distance which varied from 0.2067 to 0.6234. The Bahawalpur district I and Fateh Jang were the most related as they showed the highest genetic identity of 0.8133. Therefore, AARI and Attock were found to be different from each other with the highest genetic distance of 0.6234. Moreover, we can get further understanding of the relatedness between these fungal populations from cluster analysis. Cluster analysis divided the eight populations into two main groups and five subgroups. The isolates of UAF and Attock were the most diverse fungal strains. Furthermore, isolates of Changa Manga and Attock, Fateh Jang and Bahawalpur districts, UAF, and Bhakkar were grouped in the same subgroup to highlight the minimum differences among them (Table 3, Figure 3).

Population structure analysis of *F. oxysporum*

Genetic identity content ranged from 0.5385 to 0.7372 compared to the genetic distance which ranged from 0.3049 to 0.6190. The maximum genetic distance (0.6190) was found between UAF and Bahawalpur whereas the highest genetic identity 0.7372 was observed for UAF and AARI isolates. Moreover, cluster analysis divided the populations into two and three main groups and subgroups, respectively. Interestingly, all six populations were grouped into three subgroups UAF and AARI, Bhakkar and Fateh Jang, and Bahawalpur districts, and Chichawatni were grouped into the same subgroups (Table 4).

Table-4. Nei's Unbiased Measures of Genetic Identity and Genetic Diversity

Pop ID	UAF	AARI	Bhakkar	Fateh Jang	Bahawalpur I	Chichawatni
UAF	****	0.7372	0.6987	0.6859	0.5385	0.5897
AARI	0.3049	****	0.7179	0.6923	0.5962	0.6731
Bhakkar	0.3585	0.3314	****	0.7179	0.6731	0.5577
Fateh Jang	0.3770	0.3677	0.3314	****	0.6474	0.5962
Bahawalpur I	0.6190	0.5173	0.3959	0.4347	****	0.7051
Chichawatni	0.5281	0.3959	0.5839	0.5173	0.3494	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

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Table-5. Nei's Unbiased Measures of Genetic Identity and Genetic Diversity

Pop ID	UAF	AARI	Bhakkar	Fateh Jang	Bahawalpur II	Chichawatni
UAF	****	0.526	0.5449	0.5449	0.5705	0.5641
AARI	0.6431	****	0.7115	0.6603	0.6474	0.5769
Bhakkar	0.6072	0.343	****	0.6667	0.7051	0.6346
Fateh Jang	0.6072	0.411	0.4055	****	0.641	0.6603
Bahawalpur II	0.5612	0.437	0.3494	0.4447	****	0.6474
Chichawatni	0.5725	0.55	0.4547	0.4151	0.4347	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Population structure analysis of *F. solani*

The genetic identity ranged from 0.5256 to 0.7115, whereas the genetic distance varied between 0.3403 to 0.6431. Isolates of Bhakkar and AARI were the most similar ones as they displayed the highest genetic identity. Moreover, UAF and AARI were the most dissimilar as per the highest genetic distance between these populations. Unlike other species, the results of cluster analysis are quite different for *F. solani*. The populations were divided into two main groups and three subgroups. Group I contained only UAF whereas the second group was further subdivided into three subgroups containing other populations (Table 5).

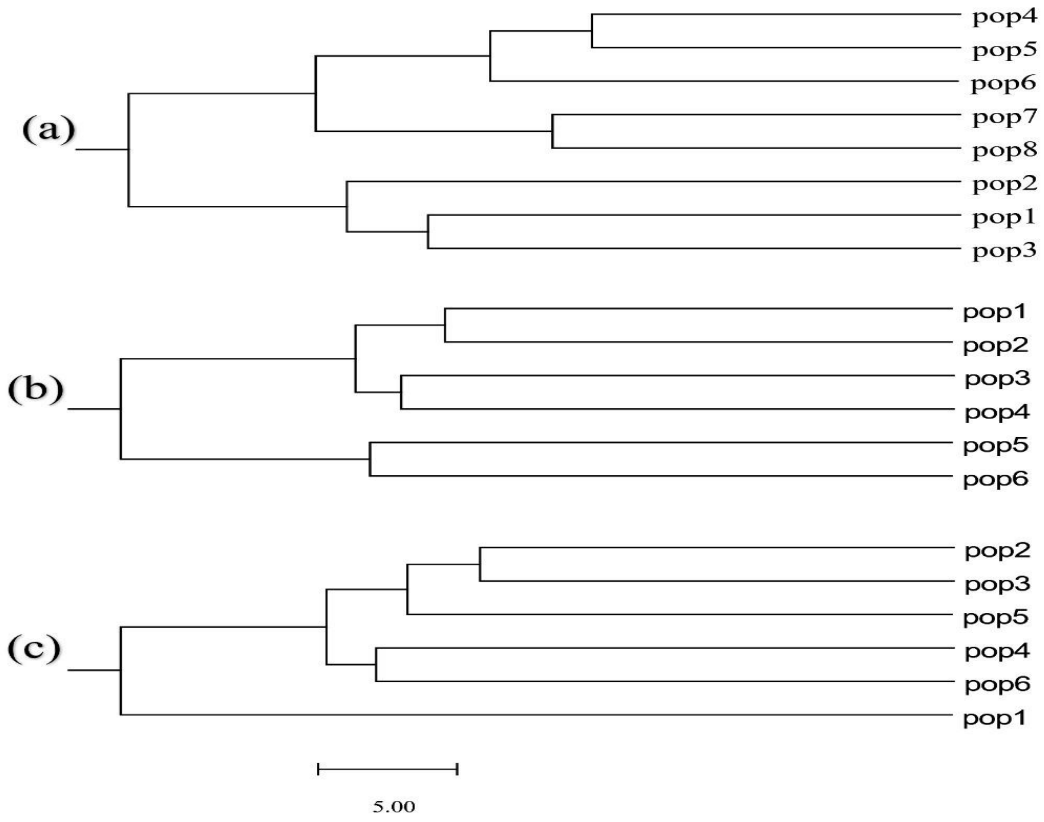

Figure-3. Cluster analysis of isolates of fungal pathogens of various locations. (a) Cluster analysis of isolates of *B. theobromae* isolates of distinct locations (b) Cluster analysis of isolates of *F. oxysporum* isolates of various locations (c) Cluster analysis of isolates of *F. solani* isolates of 8 different locations.

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Discussion

D. sissoo also known as Indian rosewood is an important leguminous timber tree of the Indian subcontinent due to its efficiency in nitrogen fixation and drought tolerance (Srivastava et al., 2020). For the last 2-3 decades shisham dieback disease has eliminated many *D. sissoo* trees from these countries. Various fungal pathogens like *B. theobromae*, *F. solani*, and *F. oxysporum* have been associated with this disease. The current study will provide valuable insights into the genetic diversity among fungal isolates of these species collected from various districts of Punjab, Pakistan. These isolates exhibited a high degree of polymorphism by producing a significant percentage of polymorphic amplifications. (Mukhtar et al., 2014) reported a similar higher degree of variability in *F. solani* collected from different districts based on RAPD amplifications. Isolates of unalike *Fusarium* species have been genotyped with RAPD primers in different crops to assess the genetic diversity including citrus, passion fruit, and guava (Jamil et al., 2010; Aruga et al., 2012; Mishra et al., 2013; Gupta, 2012).

In this study, we have used 3 series of RAPD primers which produce 166 loci across the 3 fungal pathogens and with an average of 9 amplifications per primer. Amplification ranges between 60-2200bp. Higher differences among populations proposed that there is sufficient variability in pathogens. Moreover, the presence of such variability might be associated with pathogenicity. However, this argument needs further in-depth investigations and research at both the genomic and the transcriptomic levels. Moreover, an association of dieback-linked symptoms with different localities may yield fruitful results. (Shah et al., 2010) have reported the possible pathogenicity in different *Botrydiplodia* isolates based on molecular characterization.

Since *Fusarium* and *Botrydiplodia* are indistinguishably related to each other, we conducted separate population structure analysis for each fungal pathogen strain. For *B. theobromae*, isolates of Fateh Jang and Bahawalpur district's locations have a higher level of similarities while the isolates of UAF and Chichawatni are distantly related to each other. Further, genetic distance results are also supported with cluster analysis where isolates of Fateh Jang and Bahawalpur isolates are grouped into the same clusters. These findings are in line with the conclusions of Shah et al. (2010) where they have found varying degrees of variability within the isolates of distinct locations in India. Moreover, Mohali et al. (2005) also showed lower variability between populations of different localities. For *F. solani*, unpredictably UAF isolates formed a separate cluster showing its degree of variability to others. The isolates of this region could be a suitable candidate for pathogenicity-related investigations. For instance, in-depth, transcriptomic and genomic studies could help us to find the genetic basis of that unrelatedness species and thereby, these findings could be associated with symptoms later.

Furthermore, isolates of AARI and Bhakkar showed higher degree similarities and grouped into the same cluster. *F. oxysporum* populations were grouped into one main (group) and three subgroups showing the presence of lower genetic variability. Each subgroup had two populations. Like subgroups, I had UAF and AARI isolates, subgroups II and III had Bhakkar and Jhang and Bahawalpur districts and Chichawatni respectively. The formation of one main cluster is also an indication of a lower degree of variation.

RAPD markers are useful tools to explore genetic diversity or polymorphism among different fungal pathogens. Similarly, OPB-01 showed its usefulness in genetic diversity studies by exhibiting higher levels of PIC and Ht Heterozygosity contents. This primer could be used to address genetic diversity in future studies.

Conclusion

This study concludes that genetic diversity is present within species most reported to be responsible for the dieback disease of shisham. Pathogens collected from various locations differ genetically from each other which indicates their independent evolution. Our results guide us to explore further and identify the diversity with respect to virulence. Furthermore, the role of each pathogen in dieback disease development should be fixed. RAPDs seem to be competent enough to fix the genetic diversity in fungal pathogens linked to the dieback disease of shisham.

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Conflict of Interest: None.
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Use of Generative AI Tools Statement

None of the AI tools have been used in the experiment except to format the references and citations.

Contribution of Authors

Zafar UB: Experimentation, writeup and validation.
Khan SHU & Atif RM: Reviewed and improved the manuscript.
Rana IA: Conceptualization, validation, reviewed the manuscript and overall supervision of the experiment.

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