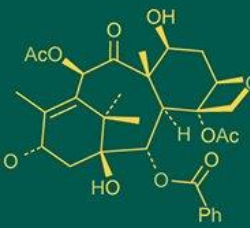
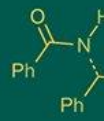


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Molecular characterization of *Colletotrichum gloeosporioides* associated with mango by using inter simple sequence repeat markers

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Abstract

Mango anthracnose, caused by *Colletotrichum gloeosporioides*, is one of the most destructive diseases affecting mango production. The present study aimed to assess the genetic diversity among eleven virulent isolates of *C. gloeosporioides* collected from different mango-growing regions of Maharashtra. The isolates, obtained from five mango cultivars (Alphonso, Kesar, Totapuri, Banganapalli and Amrapali), were characterized using Inter Simple Sequence Repeat (ISSR) markers. Ten ISSR primers generated a total of 67 bands, of which 61 were polymorphic, resulting in an overall polymorphism of 89.14%. The number of bands per primer ranged from 5 to 9, while PIC values varied from 0.19 to 0.42. Several primers exhibited 100% polymorphism, indicating their high efficiency in detecting genetic variation. Similarity coefficients among the isolates ranged from 0.5373 to 0.7761, revealing moderate to high genetic similarity. Cluster analysis based on ISSR data grouped the isolates into three major clusters, confirming the existence of considerable genetic diversity within the pathogen population. The results demonstrated substantial molecular variation among *C. gloeosporioides* isolates across different geographical regions of Maharashtra, highlighting the adaptability of the pathogen and emphasizing the importance of understanding pathogen diversity for effective disease management and resistance breeding programmes in mango.

Keywords: *Colletotrichum gloeosporioides*, mango anthracnose, ISSR markers, molecular characterization, polymorphism

Introduction

Mango (*Mangifera indica* L.) is one of the most important tropical fruit crops cultivated worldwide and is highly valued for its nutritional, economic and commercial significance. India is the largest producer of mango, contributing substantially to global production. However, mango cultivation is severely constrained by several diseases, among which anthracnose, caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., is considered one of the most destructive. The disease affects leaves, twigs, inflorescences and fruits both in the field and during post-harvest storage, resulting in significant yield losses and deterioration of fruit quality (Arauz, 2000) [1]. *Colletotrichum gloeosporioides* is a genetically diverse pathogen exhibiting considerable variation in cultural, morphological, pathogenic and molecular characteristics. The pathogen survives under diverse environmental conditions and possesses a broad host range, enabling its widespread distribution in mango-growing regions. Knowledge of the genetic variability and population structure of the pathogen is essential for understanding its epidemiology, evolution, virulence and for developing effective disease management strategies and resistant cultivars (Freeman *et al.*, 1998) [3].

Conventional identification of *C. gloeosporioides* based on morphological and cultural characteristics is often inadequate due to the high phenotypic plasticity and overlapping characteristics among species within the *Colletotrichum* complex. Molecular marker techniques have therefore become indispensable tools for accurate characterization and assessment of genetic diversity among pathogen populations. Among various molecular markers, Inter Simple Sequence Repeat (ISSR) markers have gained considerable attention because they are highly reproducible, cost-effective, require no prior sequence information,

and are capable of detecting high levels of polymorphism across the genome (Zietkiewicz *et al.*, 1994) ^[10]. ISSR markers amplify DNA regions located between microsatellite sequences and have been successfully employed in studies of genetic diversity, phylogenetic relationships, and population structure of several fungal pathogens. In *Colletotrichum* species, ISSR analysis has proven effective in differentiating isolates from diverse geographical regions and hosts, revealing significant genetic variation within pathogen populations (Sharma *et al.*, 2005; Mahmodi *et al.*, 2014) ^[6, 4]. Such information is valuable for understanding pathogen variability and designing sustainable disease management approaches.

Despite the economic importance of mango anthracnose, information on the genetic diversity of *C. gloeosporioides* populations associated with mango in Maharashtra remains limited. Therefore, the present investigation was undertaken to characterize isolates of *C. gloeosporioides* collected from different mango-growing regions of Maharashtra using ISSR markers and to assess the extent of genetic variability among the isolates.

Materials and Methods

Collection and isolation of pathogen

Mango leaves and fruits exhibiting typical anthracnose symptoms were collected from affected orchards locations of Maharashtra (Table 1) and brought to the laboratory for pathogen isolation. Small tissue bits (1-2 mm) were excised from the advancing margin between healthy and diseased tissues using a sterile scalpel. The tissue pieces were surface sterilized in 1% sodium hypochlorite solution for 3 min, rinsed twice with sterile distilled water, blotted dry on sterile filter paper and aseptically transferred onto potato dextrose agar (PDA) medium. The inoculated plates were incubated at 25±2 °C in a BOD incubator. Emerging fungal colonies were purified by the hyphal tip method and identified as *Colletotrichum gloeosporioides* based on cultural and morphological characteristics. Pure cultures were maintained on PDA slants and stored under refrigerated conditions for further studies.

Identification of the pathogen

The purified isolates of *Colletotrichum gloeosporioides* obtained from infected mango tissues were identified based on their cultural and morphological characteristics. Observations on colony morphology, mycelial growth pattern, colony colour and conidial characteristics were recorded from cultures grown on potato dextrose agar (PDA). Microscopic examination was carried out to study the shape and size of conidia, presence of acervuli and setae. The identification of the pathogen was made using standard taxonomic and mycological keys described by Barnett and Hunter (1972) ^[12]. The isolates exhibited characteristic features of *C. gloeosporioides*, including white to greyish aerial mycelium and hyaline, single-celled, cylindrical to oblong conidia with rounded ends. Based on these morphological characteristics, the pathogen was identified as *Colletotrichum gloeosporioides*.

Genomic DNA isolation

Genomic DNA was extracted from eleven isolates of *Colletotrichum gloeosporioides* using the Xploreagen Fungal gDNA Isolation Kit following the manufacturer's instructions with slight modifications. The isolates were

cultured in potato dextrose broth (PDB) and incubated under laboratory conditions until adequate mycelial growth was obtained. The mycelial mats were harvested by filtration through Whatman No. 1 filter paper and washed with sterile distilled water to remove residual culture medium. The harvested mycelium was frozen in liquid nitrogen and ground to a fine powder using a sterile mortar and pestle. The powdered mycelial tissue was transferred aseptically into sterile centrifuge tubes and genomic DNA was isolated according to the kit protocol. The quality and quantity of the extracted DNA were assessed and the DNA samples were stored at -20 °C until further molecular analysis.

DNA extraction protocol

Genomic DNA was extracted from fungal samples using the Xploreagen Fungal gDNA Isolation Kit following the manufacturer's protocol. Initially, 1.0 ml of Xploreagen gDNA Extraction Buffer™ 1 was added to a bead-containing vial and the fungal sample was transferred into the vial. The mixture was homogenized by horizontal vortexing at maximum speed for 10 min to ensure efficient disruption of fungal cells. Subsequently, 300 µl of Xploreagen gDNA Extraction Buffer™ 2 was added and the sample was vortexed horizontally for an additional 7 min. The homogenate was then centrifuged at 10,000 rpm for 3 min at room temperature and 800 µl of the supernatant was carefully transferred to a sterile 2 ml microcentrifuge tube. To the recovered supernatant, 200 µl of Xploreagen gDNA Extraction Buffer™ 3 was added and mixed thoroughly by vortexing for 5 s. The mixture was centrifuged at 10,000 rpm for 2 min, and 800 µl of the clarified supernatant was transferred to a fresh sterile tube. Thereafter, 1.0 ml of Xploreagen gDNA Extraction Buffer™ 4 was added and the solution was mixed thoroughly. Approximately 700 µl of the lysate was loaded onto a silica-based spin column and centrifuged at 10,000 rpm for 2 min. This step was repeated until the entire lysate had passed through the column. The DNA bound to the column membrane was washed sequentially with 600 µl of Xploreagen gDNA Extraction Buffers™ 5 and 6, each followed by centrifugation at 10,000 rpm for 2 min. After removal of the wash solutions, the spin column was centrifuged for 5 min to eliminate residual buffer. The column was then transferred to a sterile 1.5 ml microcentrifuge tube and genomic DNA was eluted by adding 30 µl of Xploreagen gDNA Extraction Buffer™ 7 directly onto the membrane. Following centrifugation at 10,000 rpm for 5 min, the purified DNA was collected and stored at -20 °C for subsequent molecular analyses.

PCR amplification

Polymerase chain reaction (PCR) was performed to amplify the target DNA region using specific forward and reverse primers. The PCR reaction was carried out in a final volume of 50 µl containing 1 µl template DNA, 2 µl each of forward and reverse primers, 4 µl dNTPs (2.5 mM each), 10 µl of 10× Taq DNA polymerase assay buffer, 1 µl Taq DNA polymerase (3 U ml⁻¹) and 30 µl nuclease-free water. The reaction mixture was mixed gently and briefly centrifuged before amplification.

PCR amplification was carried out in a programmable thermal cycler with an initial denaturation at 94 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 45 s, annealing at 45 °C for 1 min and extension at 72 °C for 90 s. A final extension step was performed at 72 °C for 7 min.

The amplified PCR products were separated by electrophoresis on 1.0-1.2% agarose gel prepared in 1× TAE buffer and stained with ethidium bromide. The DNA bands were visualized under a UV transilluminator and documented using a gel documentation system. A suitable DNA ladder was used to estimate the size of the amplified fragments.

Gel electrophoresis

Amplified PCR products (10 µl) mixed with loading dye were electrophoresed on a 1% agarose gel along with a 1 kb DNA ladder. The gel was run at 70 V for 1-2 h, visualized under UV light and documented using a gel documentation system. Amplicon sizes were estimated by comparison with the DNA ladder and banding patterns were analyzed to assess genetic similarity and polymorphism among the isolates.

Data analysis

The gel images were captured and visualized under UV/fluorescence light in gel documentation system. The amplified profiles for all the primers were compared with each other and bands of DNA fragments will be scored as '1' for presence and '0' for absence, generating a '0' and '1' matrix. The percent polymorphism were calculated by using the formula.

$$\text{Per cent polymorphism} = \frac{\text{Number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

Pair wise genetic similarity coefficient between the test isolates were estimated by the Jaccard's coefficient using NTSYS-PC version 2.0 (Williams, 1990) [9].

Results and Discussion

A total of eleven virulent isolates of *Colletotrichum gloeosporioides* causing mango anthracnose were collected from different mango-growing regions of Maharashtra representing diverse agro-climatic zones (Table 1). The isolates were obtained from five mango cultivars, namely Alphonso, Kesar, Totapuri, Banganapalli and Amrapali were designated as Cg-1 to Cg-11. The geographical distribution of the isolates indicated a wide occurrence of *C. gloeosporioides* across the state. The isolates were collected from the Konkan region (Ratnagiri, Sindhudurg, Raigad and Palghar), Western Maharashtra (Solapur, Sangli, Kolhapur, Pune and Satara) and Marathwada region (Jalna and Dharashiv). The latitude of collection sites ranged from 16.05°N (Kolhapur) to 20.05°N (Jalna), while longitude ranged from 73.17°E (Ratnagiri) to 76.19°E (Dharashiv), indicating substantial geographical variation among the sampling locations. The occurrence of anthracnose in all surveyed locations suggests the widespread prevalence and adaptability of *C. gloeosporioides* under varying environmental conditions.

Ten ISSR primers were used to assess the genetic variability among eleven isolates of *Colletotrichum gloeosporioides* causing mango anthracnose. A total of 67 bands were amplified, of which 61 were polymorphic, resulting in an overall mean polymorphism of 89.14 percent (Table 2). The number of bands generated per primer ranged from 5 to 9, with an average of 6.7 bands per primer. Among the primers tested, TriCAC'RC, TriGTG, TriGTG3'yC, TriGTG5'Cy,

TriTGT5'Cy, TriTCA3'RC and TriTCC3'RC exhibited 100 percent polymorphism, indicating their high efficiency in detecting genetic variation among the isolates. Primer TriCGA3'RC and B1 showed 80.0 and 71.43 percent polymorphism, respectively, whereas DiGA5'CR recorded the lowest polymorphism (40.0%). The PIC values ranged from 0.19 to 0.42, with TriGTG3'yC showing the highest PIC value (0.42), indicating its superior discriminatory power. The high level of polymorphism observed suggests the presence of considerable genetic diversity among the *C. gloeosporioides* isolates collected from different geographical regions of Maharashtra.

The results are in close agreement with the findings of Parashar *et al.* (2017) [5], who reported highly polymorphic ISSR banding patterns with 59 polymorphic bands out of 64 total bands, an average polymorphism of 93.04 percent and high PIC values among *C. gloeosporioides* isolates. Similarly, Sornakili *et al.* (2020) [8] reported 100 percent polymorphism for several ISSR primers while assessing genetic diversity in mango anthracnose pathogens. The PIC values obtained in the present study (0.19-0.42) were also comparable with those reported by Silva Neto *et al.* (2022) [7], who observed PIC values ranging from 0.215 to 0.460 in *Colletotrichum* populations.

The similarity coefficients ranged from 0.5373 to 0.7761, indicating moderate to high genetic similarity among the isolates. The highest similarity coefficient (0.7761) was observed between isolates Cg-2 and Cg-9 (Table 3), suggesting that these isolates were genetically the most closely related. High similarity values were also recorded between Cg-2 and Cg-3 (0.7164), Cg-8 and Cg-9 (0.7164), Cg-8 and Cg-10 (0.7164) and Cg-5 and Cg-11 (0.7164). In contrast, the lowest similarity coefficient (0.5373) was observed between isolates Cg-3 and Cg-6, indicating maximum genetic divergence between these isolates. Other relatively low similarity values were recorded between Cg-4 and Cg-8 (0.5522), Cg-5 and Cg-9 (0.5522) and Cg-6 and Cg-11 (0.5522). Overall, the similarity coefficients indicate the presence of considerable genetic variability among the *C. gloeosporioides* isolates collected from different mango-growing regions of Maharashtra.

These findings are consistent with those of Parashar *et al.* (2017) [5], who reported moderate to high ISSR similarity coefficients among *Colletotrichum* isolates, indicating the presence of genetically related as well as divergent isolates within the same population. Similarly, Sornakili *et al.* (2020) [8] observed comparable ISSR similarity values in *C. gloeosporioides* isolates causing mango anthracnose and reported that isolates exhibiting higher similarity coefficients clustered together in dendrogram analysis. The present results therefore support the effectiveness of ISSR markers in revealing genetic relationships and population structure among *C. gloeosporioides* isolates.

The dendrogram based on ISSR marker data grouped the eleven *Colletotrichum gloeosporioides* isolates into three major clusters at similarity coefficients ranging from 0.60 to 0.78, indicating moderate genetic diversity within the population (Fig.1). Cluster I comprised isolates Cg-1, Cg-2 and Cg-9, which showed high genetic similarity. Cluster II included isolates Cg-8 and Cg-10, indicating moderate genetic relatedness. Cluster III was the largest group and consisted of isolates Cg-3, Cg-5, Cg-11 and Cg-7, forming distinct sub-clusters. Isolates Cg-4 and Cg-6 were associated with Cluster III at lower similarity levels, suggesting greater

genetic divergence. The clustering pattern confirmed the presence of genetic variability among *C. gloeosporioides* isolates collected from different mango-growing regions of Maharashtra.

The separation of certain isolates at lower similarity levels suggests genetic differentiation possibly driven by mutation,

recombination or ecological adaptation. Similar clustering patterns and levels of genetic diversity in *C. gloeosporioides* populations using ISSR markers have been reported earlier by Parashar *et al.* (2017)^[5], Sornakili *et al.* (2020)^[8] and Silva Neto *et al.* (2022)^[7], who emphasized the usefulness of ISSR markers in resolving intra-specific variability.

Table 1: Different isolates of *Colletotrichum gloeosporioides* collected from different places and geographical locations

Sr. No.	Isolate	Variety	Location	District	Geographical locations	
					Latitude	Longitude
1	Cg-1	Alphanso	Dapoli	Ratnagiri	17.752359°	73.17843°
2	Cg-2	Alphanso	Malvan	Sindhudurg	16.059683°	73.504035°
3	Cg-3	Alphanso	Dhagdewadi	Raigad	18.4246336°	73.2903468°
4	Cg-4	Kesar	Madha	Solapur	18.030467°	75.21047°
5	Cg-5	Totapuri	Palghar	Palghar	17.828086°	73.34387°
6	Cg-6	Totapuri	Bagalwadi	Sangli	17.480262°	75.089768°
7	Cg-7	Kesar	Nesari	Kolhapur	16.0566715°	74.3270546°
8	Cg-8	Banganapalli	Bedshinge	Pune	18.059389°	75.054159°
9	Cg-9	Amrapali	Phaltan	Satara	17.998388°	74.585796°
10	Cg-10	Kesar	Pokhari	Jalna	20.059237°	75.950497°
11	Cg-11	Kesar	Dharashiv	Dharashiv	18.05951°	76.19052

Cg-*Colletotrichum gloeosporioides*

Table 2: Percent polymorphism observed in ISSR primers

Sr. No.	Primer name	Primer sequence (5'-3')	TB	PB	MB	P (%)	PIC
1	DiGA5'CR	CRGAGAGAGAGAGAGAGA	5	2	3	40	0.19
2	TriCAC'RC	CACCACCACCACCACRC	8	8	0	100	0.38
3	TriGTG	GTGGTGGTGGTGGTG	7	7	0	100	0.34
4	TriGTG3'yC	GTGGTGGTGGTGGTGyC	6	6	0	100	0.42
5	TriGTG5'Cy	CyGTGGTGGTGGTGGTG	6	6	0	100	0.36
6	TriTGT5'Cy	CyTGTGTGTGTGTGTGT	6	6	0	100	0.33
7	TriTCA3'RC	TCATCATCATCATCARC	9	9	0	100	0.39
8	TriTCC3'RC	TCCTCCTCCTCCTCCRC	8	8	0	100	0.33
9	TriCGA3'RC	CGACGACGACGACGARC	5	4	1	80	0.20
10	B1	CTCTCTCTCTCTCTTG	7	5	2	71.43	0.23
Total			67	61			
Mean			6.7	6.1		89.143	

(TB = Total bands, PB = Polymorphic bands, MB = Monomorphic bands, P = Percent polymorphism and PIC = Polymorphism information content)

Table 3: Genetic similarity coefficient matrix for *C. gloeosporioides* isolates based on ISSR marker

	Cg-1	Cg-2	Cg-3	Cg-4	Cg-5	Cg-6	Cg-7	Cg-8	Cg-9	Cg-10	Cg-11
Cg-1	1.0000	0.7015	0.7015	0.6716	0.5672	0.6119	0.6418	0.6119	0.7164	0.6567	0.6418
Cg-2	0.7015	1.0000	0.7164	0.6119	0.6269	0.5821	0.6716	0.7015	0.7761	0.7164	0.6418
Cg-3	0.7015	0.7164	1.0000	0.5970	0.7015	0.5373	0.6866	0.5672	0.7015	0.6119	0.6866
Cg-4	0.6716	0.6119	0.5970	1.0000	0.5672	0.6716	0.5821	0.5522	0.6567	0.5970	0.7015
Cg-5	0.5672	0.6269	0.7015	0.5672	1.0000	0.5672	0.6269	0.5672	0.5522	0.6119	0.7164
Cg-6	0.6119	0.5821	0.5373	0.6716	0.5672	1.0000	0.5522	0.7015	0.6567	0.5672	0.5522
Cg-7	0.6418	0.6716	0.6866	0.5821	0.6269	0.5522	1.0000	0.6119	0.6567	0.6567	0.6418
Cg-8	0.6119	0.7015	0.5672	0.5522	0.5672	0.7015	0.6119	1.0000	0.7164	0.7164	0.6119
Cg-9	0.7164	0.7761	0.7015	0.6567	0.5522	0.6567	0.6567	0.7164	1.0000	0.7015	0.6866
Cg-10	0.6567	0.7164	0.6119	0.5970	0.6119	0.5672	0.6567	0.7164	0.7015	1.0000	0.6269
Cg-11	0.6418	0.6418	0.6866	0.7015	0.7164	0.5522	0.6418	0.6119	0.6866	0.6269	1.0000

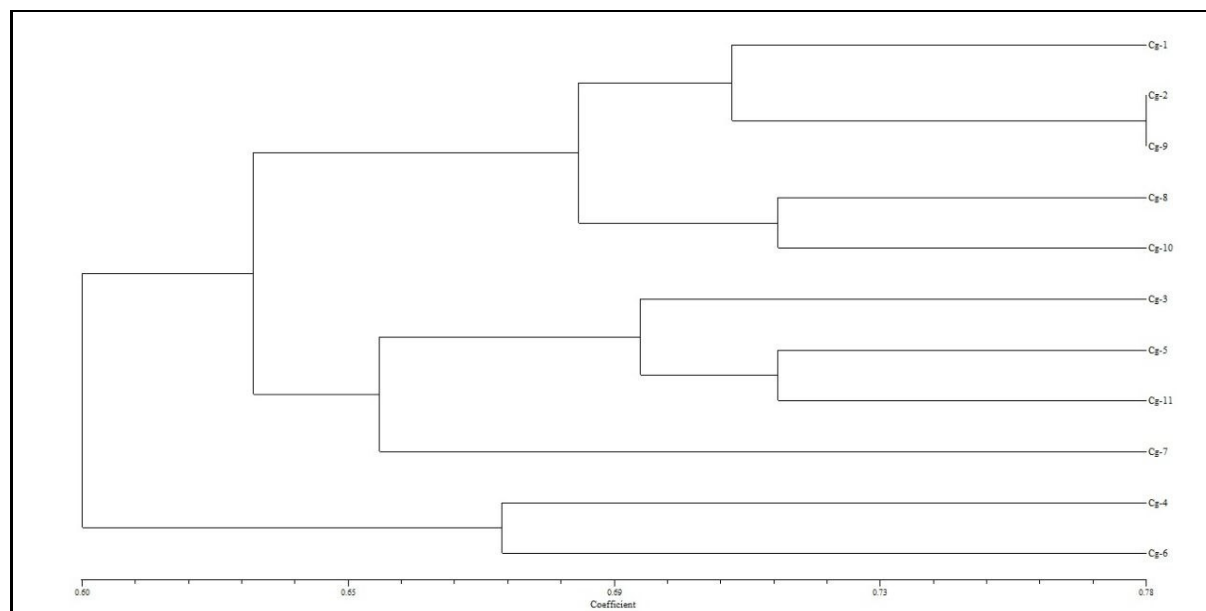


Fig 1: Dendrogram generated by UPGMA analysis based in ISSR marker data showing genetic relationship among 11 isolates of *C. gloeosporioides*

Conclusion

The present study revealed considerable genetic diversity among eleven isolates of *Colletotrichum gloeosporioides* causing mango anthracnose collected from different regions of Maharashtra. ISSR analysis showed high polymorphism (89.14%), indicating substantial genetic variation within the pathogen population. Similarity coefficient and cluster analyses grouped the isolates into three major clusters, confirming moderate genetic diversity.

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