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A Species-Specific PCR Based Assay for Rapid Detection of Mango Anthracnose Pathogen *Colletotrichum gloeosporioides* Penz. and Sacc.

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Abstract

Mango (*Mangifera indica* L.) a fruit of nutraceutical value is accepted as the most eatable fruit crop worldwide. Mango production has been severely affected by several biotic stress mainly diseases and anthracnose is the major post-harvest disease of mango results in heavy losses. The present investigation describes PCR based assay for rapid and sensitive detection of *Colletotrichum gloeosporioides* causing mango anthracnose. Genus specific universal primer pair ITS1 and ITS4 was employed to amplify *Colletotrichum* genus which shows ~560 bp amplicon. The *Colletotrichum gloeosporioides* species-specific sequences for conserved domains were retrieved from the NCBI Genbank (sequence HM10205) and specific primers were designed. In order to validate the species-specific designed primer, a sensitive nested PCR assay was carried out using designed primer-pair MKCgF 5` TTGCTTCGGCGGGTAGGGTC 3` (forward) and MKCgR 3`ACGCAAAGGAGGCTCCGGGA 5` (reverse) produced an amplicon size of 380 bp as specific. Our investigation revealed that *C. gloeosporioides* causal agent of mango anthracnose was discriminated on the basis of species as specific for mango in comparison to other *Colletotrichum* spp. viz. *C. acutatum, C. falcatum* and *C. capsici* causing anthracnose in other crops.

Keywords: PCR; Internal transcribed spacer region; Species-specific; Diagnosis

Introduction

Mango (*Mangifera indica* L.) is considered as one of the most popular fruits grown throughout the tropics and subtropics worldwide [1]. India is the world`s largest producers, shares around 56% of total global production [2]. Production of mango is affected by a large number of fungal pathogens. The genus *Colletotrichum* contains many morphologically similar taxa comprising endophytic, saprobic and plant pathogenic fungi [3]. Of which, *C. gloeosporioides* an incitant of mango anthracnose is the most important biological constraint which restricts mango production in Southeast Asia [4]. *C. gloeosporioides* affects mango production both in the pre and post harvest stages particularly when attempting to extend storage life resulting in huge economic losses about 5-20% in the form of damage on the stems, leaves, fruit decay and damage [1,5]. Anthracnose disease is clearly identified by morphological symptoms but sometimes the symptoms are masked as this disease survived in the form of latent infection in absence of congenial environment. Symptom based identification and characterization is not accurate and reliable due to incipient infection. The diagnostic techniques, thus will assist in the monitoring the spread and distribution of pathogens. PCR technology can provide very accurate quantitative data required for control and quarantine decisions. The ability to design PCR primers to target specific regions of DNA has led to rapid, accurate, and sensitive detection which is a greater understanding for managing *Colletotrichum* diseases.

The development of species-specific primers has provided a powerful tool for the detection of plant pathogens. The identification of fungal pathogens based on polymerase chain reaction (PCR) using species-specific primers is now widely used, especially for economically important plant pathogens such as quarantine listed fungi or those that are difficult to isolate or cause symptomless [6,7]. The internal transcribed spacer regions (ITS1 and ITS4) within the nuclear ribosomal gene clusters are particularly attractive loci for the design of PCR-based detection assays since they are readily accessible using universal primers [8] typically present in high copy number increasing PCR sensitivity and often exhibiting sufficient inter-specific sequence divergence for the designing of species-specific primers [9]. In recent years, molecular tools have been employed to infer the evolutionary relationships of *Colletotrichum* species. Based on nu-rDNA ITS sequence data and morphological characteristics, some species have been segregated from the *Colletotrichum gloeosporioides* complex. Although ITS sequence data may help in *Colletotrichum* species identification, it cannot alone be used to adequately address species delimitation for closely related species [10]. Researchers have recently tried to examine multiple genes sequence data to distinguish species in *Colletotrichum* [10,11-16].

These regions can be further exploited for diagnostic purpose and the primers can be designed against species specific regions from rDNA. Therefore, a more contemporary approach aimed to develop a molecular diagnostic assay for rapid and accurate diagnosis against mango anthracnose pathogen for species-specific identification.

Materials and Methods

Thirty isolates of *Colletotrichum gloeosporioides* were collected from different agro-climatic mango growing regions of the India and reference pure culture were maintained and collected from leaf tissue and fruits affected with anthracnose. For further PCR assay

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standardization the 11 most pathogenic isolates of *C. gloeosporioides* collected from mango were procured. The details of *colletotrichum gloeosporioides* isolates collected and place of origin is given in Table 1.

Fungal DNA isolation

DNA extraction: The fungal mycelium and conidia from pure cultures, grown on one potato dextrose agar (PDA) (Himedia) petridish for 2 weeks at 25°C in the dark, were scraped and mechanically disrupted by grinding to a fine powder using a mortar and pestle. Total DNA was extracted with the Fast DNA Extraction Kit (MP Bio) following the manufacturer's instructions. Genomic DNA isolated was visualized under UV light on 0.8% (wt/v) agarose gel stained with ethidium bromide and stored at –20°C.

Species-specific primers designing

Species-specific primers were designed using advanced bioinformatics tools and the sequence retrieved from the NCBI database (http//:www.ncbi.nlm.niv.gov.in) from accession no. HM102505 of *Colletotrichum gloeosporioides* [17] and the conserved sequences were then picked up through Genmark software and the multiple sequences were aligned through Clustal W. The primer designed through the software Primer 3 and confirmed through the repeated NCBI-Primer BLAST and synthesized by Metabion International Pvt. Inc. A sequence based species-specific primer were designed according to Kamel et al. [18] (Figure 1) for the identification of *Colletotrichum gloeosporioides* and validated with other species of *Colletotrichum* (Table 2).

ITS–PCR

The ITS rDNA region including ITS1, 5.8S and ITS4 were amplified in 11 isolates of *C. gloeosporioides* selected from different mango growing regions of India (Figure 2). The isolates were PCR amplified using the universal primers ITS1/ITS4 [8]. Each PCR reaction contained 1×PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each primer, 0.7 U of Taq DNA polymerase (Biochem), and 1 µl of template DNA. The PCR reaction mix was adjusted to a final volume of 25 µl with nuclease free water. PCR amplifications were performed on a Thermal Cycler (Eppendorf, India Limited). The program consisted of an initial step of 1 min at 94°C for 1 min, followed by 30 cycles of 60 s at 94°C, 2 min at 58°C, and 60 s at 72°C; and a final extension step of 5 min at 72°C. PCR products were analyzed in 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. 100-bp DNA ladder plus was used as a molecular weight marker (Biochem).

Results and Discussion

Nuclear rDNA, including the small and large subunits, 5.8 S, and the Internal Transcribed Spacer (ITS) region, proved an ideal target for specific PCR primers, as each sequence is variable at the family, genus, or species level [8]. Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species [19]. Amplification of target DNA through PCR with taxon-specific primers is a potentially more sensitive and accurate approach than conventional microscopic techniques [20-22]. Unlike identification based on culture techniques, PCR does not

S. No.	Culture No.	Region of isolate	Symptom	Pathogen isolated	Sporulation	Metabolite colour
1	Cg1	Nallangandu-chittoor	100%	*C. gloeosporioides*	+++	White black
2	Cg2	chittoor	100%	*C. gloeosporioides*	+++	Yellow white black
3	Cg3	Totapur	100%	*C. gloeosporioides*	+++	Yellowish white
4	Cg4	Totapur	40%	*C. gloeosporioides*	+++	Yellowish white
5	Cg5	Bagampalli chittoor	30-50%	*C. gloeosporioides*	+++	Yellowish pink white
6	Cg6	Assam	50%	*C. gloeosporioides*	+++	Pink yellow white
7	Cg7	Guahati	100%	*C. gloeosporioides*	+++	(Rounded) black white
8	Cg8	Guahati	70%	*C. gloeosporioides*	+++	Black brown white
9	Cg9	Jorhat	50%	*C. gloeosporioides*	+++	White brown dots
10	Cg10	Jorhat	100%	*C. gloeosporioides*	+++	Brown orange dots
11	Cg11	Rewa (M.P.)	100%	*C. gloeosporioides*	+++	black white
12	Cg12	Rewa (M.P.)	50%	*C. gloeosporioides*	+++	White browm dots
13	Cg13	Akbarpur (U.P.)	100%	*C. gloeosporioides*	+++	White orange
14	Cg14	Akbarpur (U.P.)	50%	*C. gloeosporioides*	+++	White orange
15	Cg15	CISH Lucknow, (U.P.) (mallika)	50%	*C. gloeosporioides*	++	White rounded
16	Cg16	Siliguri	100%	*C. gloeosporioides*	+++	White round
17	Cg17	Siliguri	55%	*C. gloeosporioides*	++	Pink yellow white
18	Cg18	Tanda, (U.P.)	80%	*C. gloeosporioides*	++	(Rounded) brown white
19	Cg19	Tanda (U.P.)	100%	*C. gloeosporioides*	+++	(Rounded) Yellowish white
20	Cg20	Dasehri leaf Lucknow	100%	*C. gloeosporioides*	+	(Rounded) pink yellow white
21	Cg21	Lucknow	100%	*C. gloeosporioides*	Nill	(Rounded) yellow white
22	Cg22	Lucknow	100%	*C. gloeosporioides*	+++	(Rounded) pink white
23	Cg23	Lucknow	50%	*C. gloeosporioides*	+	Pink white
24	Cg24	Faizabad	75%	*C. gloeosporioides*	+++	(Rounded) yellow white
25	Cg25	Darjeeling	100%	*C. gloeosporioides*	+++	Yellowish white
26	Cg26	Gorakhpur	10-30%	*C. gloeosporioides*	+++	Yellowish white
27	Cg27	(Other leaf)	100%	*C. gloeosporioides*	+	White
28	Cg28	Badlapur, (U.P.)	100%	*C. gloeosporioides*	++	White
29	Cg29	Mujaffarnagar	100%	*C. gloeosporioides*	+++	(Rounded) white black
30	Cg30	Bihar	100%	*C. gloeosporioides*	+++	White

Table 1: Details of Colletotrichum gloeosporioides isolates collected from different agro-climatic regions of India causing mango anthracnose disease.

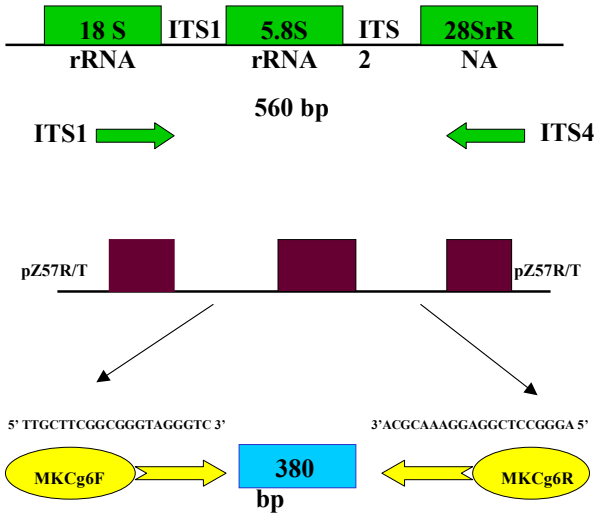

Figure 1: Internal Transcribed spacer (ITS) region primers ITS1 and ITS 4 [8] used for Colletotrichum species-specific primer designing.

Primer	Sequence	Product Size (bp)	Origin
ITS1	TCCGTAGGTGAACCTGCGG	**560**	White et al. [8]
ITS 4	TCCTCCGCTTATTGATATGC		
MKCgF	TTGCTTCGGCGGGTAGGGTC	**380**	Species-specific primer
MKCgR	ACGCAAAGGAGGCTCCGGGA		

Table 2: Details of primer sequences used for ITS-PCR.

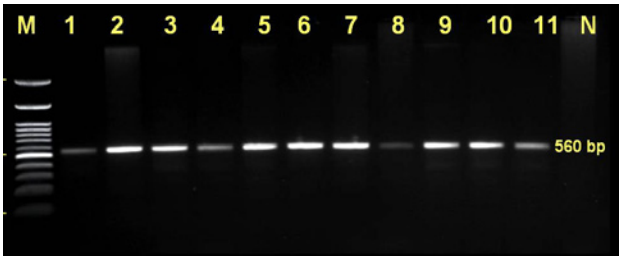

Figure 2: PCR amplification of *Colletotrichum gloeosporioides* using Fungus specific universal ITS1 and ITS4 primers which amplifies 560 bp uniform amplicon among all the 11 isolates collected from different geographical regions and LaneM-100 bp DNA Ladder (Biochem), Lane N is negative.

require the presence of viable organisms and can work even when there is a limited amount of sample [23]. In the present investigation, a sensitive PCR-based diagnostic assay were developed with the aim to detect *Colletotrichum gloeosporioides* mango anthracnose pathogen in infected plant and fruit tissue using species-specific designed primer-pair. The specificity of this sensitive PCR-based assay was also verified by the absence of reactivity with DNA from uninfected (healthy) tissues and other *Colletotrichum* species. The 10 pg to 10 mg of genomic DNA of *Colletotrichum* spp. was found to be sufficient for a detectable PCR amplification. This is for the first time a PCR based diagnostic assay for early detection of mango anthracnose pathogen *C. gloeosporioides* was carried out using species-specific designed primer which amplifies a uniform band of 380 bp among all the isolates (Figure 3) and clearly discriminated other species having no amplification with the designed primer as specific.

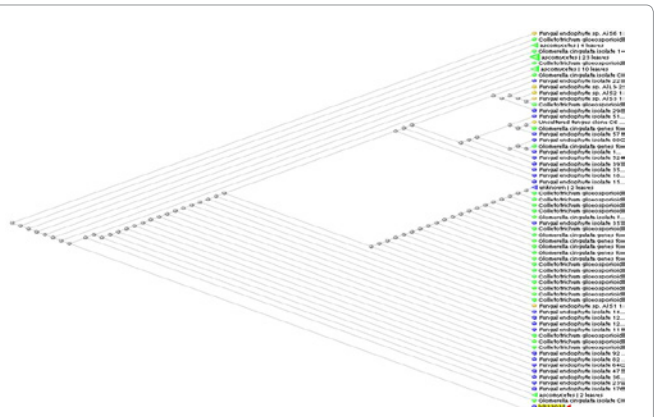

Figure 3: Taxonomy report of 11 *Colletotrichum* isolates showing maximum similarity about 99-100% with *C. gloeosporioides* with already available sequences at the NCBI web.

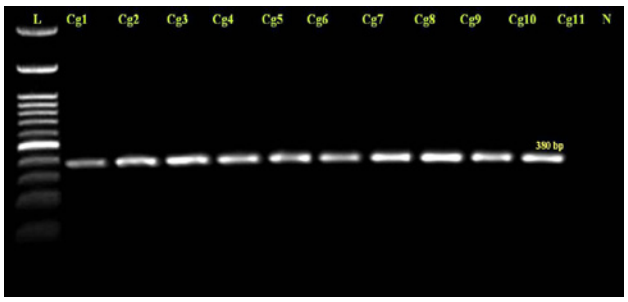

Figure 4: PCR amplification of *C. gloeosporioides* isolates using species-specific designed primer amplifies a uniform band of 380 bp as desired among all the 11 isolates (Lane1-11) (Lane-N) negative control and Lane L-100 bp DNA Ladder (Biochem).

Sequencing and data analysis

The sequence information obtained for the twenty *C. gloeosporioides* isolates were analyzed by local alignment tool using BLASTn. Taxonomic correlation of the isolates upon NCBI web proved that the isolates were more related to C. gloeosporioides. All annotations were based on BLAST searches with a score threshold of e" 200 for BLASTn and e-values 10^{-5} with a minimum of 99-100% identity over at least 80% of the length of the nucleotide sequence which are the commonly used thresholds for reliable sequence annotation. Sequences were confirmed for identification using repeated blast with already available sequences for the identification and taxonomy of the *C. gloeosporioides* using the NCBI-BLAST search for sequence identification of BLAST 2.0 (http://www.ncbi.nlm.nih.gov/BLAST) Figure 3.

PCR amplification of species-specific designed primer

DNA fragment of approximately 380 bp were amplified for *C. gloeosporioides* using primer-pair MKCgF and MKCgR (Figure 4). No cross-amplification in other *Colletotrichum* sp., viz., *C. falcatum, C. capsici* (Figure 5). Thus, the diagnostic PCR approach yielded highly consistent and reproducible results. The first amplification with fungus specific primers preferentially increased the population of fungal ITS molecules, thereby increasing the ratio of fungus by host ITS molecules. Then, the PCR amplification with species-specific designed primer amplifies a PCR product of 380 bp as specific for *Colletotrichum gloeosporioides*. Similarly, the existence of species-specific primers

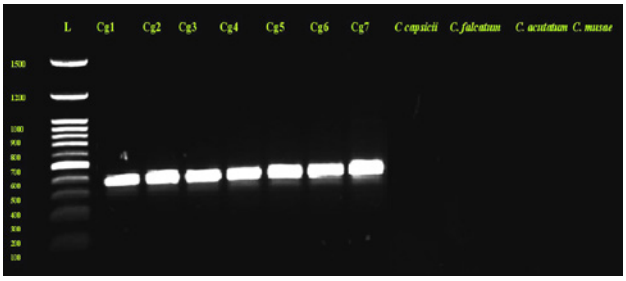

Figure 5: PCR amplification of *C. gloeosporioides* species-specific primer amplified a uniform band of 380 bp as desired among all the 6 isolates (Lane2-7) Lane-L-100 bp DNA Ladder (Biochem) and no amplification in other host Colletotrichum species viz., *C. falcatum, C. acutatum, C. musae* and *C. capsici.*

based on nucleotides sequences of the ITS1 rDNA region have made the Polymerase Chain Reaction (PCR) a powerful tool for the identification of *Colletotrichum* species [23-27].

Validation of species-specific primer with other *Colletotrichum* species

PCR amplification of species-specific designed primer MKCgF and MKCgR gives amplicon size of 380 bp uniformly amplifies in all the isolates of *Colletotrichum gloeosporioides* but, no amplification in other with other *Colletotrichum* species viz. *C. accutatum, C. falcatum and C. capsici.* In context to our findings, the designed primer-pair does not amplified in other *Colletotrichum* species which; clearly discriminates *Colletotrichum gloeosporioides* the pathogen of mango anthracnose (Figure 5) on the basis of species-specificity.

Identification of the causal agent and prevalence of a disease is very essential for adequate and timely management of disease, which in turns depends on accurate diagnosis and early detection of the pathogen. Often, it may be desirable to examine the planting material for prevalence of any potential pathogen even before the crop is sown. So, in the present investigation the species-specific designed primer-pair MKCgF and MKCgR for *Colletotrichum gloeosporioides* specifically for mango was designed and the primer has been validated with other *Colletotrichum* species. Thus, the designed primer proved to be an efficient marker for species-specific discrimination which would be useful in developing a rapid and sensitive diagnostic PCR based assay for early detection and timely management of *Colletotrichum gloeosporioides* causing mango anthracnose disease.

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