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DETECTION OF *COLLETOTRICHUM CAPSICI* CAUSING CHILI ANTHRACNOSE THROUGH MORPHOLOGICAL AND SPECIES-SPECIFIC MARKER AND ITS GENETIC DIVERSITY

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Abstract

Anthracnose of chili is caused by different *Colletotrichum* species. Precise detection of the species responsible is essential in strategizing disease management and control. We collected ten *Colletotrichum* isolates causing chili anthracnose to identify the organism by species-specific SSR marker, characterized them morphologically and determined genetic diversity by RAPD-PCR technique. The fungal pathogens were taken from anthracnose lesions without visible spores. Identification and characterization based on the morphology of the fungal colony, and the dimension and shape of the conidia formed were done. Two pathogen species-specific microsatellite loci were used for molecular detection. As was expected, the markers amplified DNA bands of 394 and 447bp, which confirmed the pathogen as *Colletotrichum capsici*. Five RAPD primers generated 64 distinct bands, of which 84.38% showed polymorphism. UPGMA Dendrogram segregated ten isolates into two distinct clades. Wide variability was detected among the isolates indicating the existence of different pathotypes of anthracnose causing *C. capsici* in Bangladesh. This paper identifies *Colletotrichum capsici* as the causal agent of chilli anthracnose in Bangladesh by molecular and conventional methods and detects molecular diversity among the *C. capsici* isolates. The information obtained has potential application in the detection and effective control of chill anthracnose.

Key words: Fruit rot, Diseases management, Molecular divergence, Polymorphism, RAPD, SSR.

Introduction

Colletotrichum is a destructive pathogen that is responsible for anthracnose disease of various vegetables, legumes, cereals, and also in some perennial crops and fruit trees causing considerable economic loss. In addition, it is considered as a model organism in knowing many aspects of infection, disease developmental processes, signal transduction, host resistance, and biology of the interactions of host and pathogen. Different species of *Colletotrichum* can cause anthracnose in a single host plant or different hosts. However, the interactions and number of the *Colletotrichum* species causing anthracnose of chili are not yet clearly understood.

Chili (*Capsicum frutescens*) is an important vegetable cum spice crop with considerable economic value. Chili anthracnose is a serious disease that renders up to 50% yield loss (Than *et al*., 2008b; Dev *et al*., 2012; Yadav & Singh, 2016; Yadav *et al*., 2017; Kiran *et al*., 2020). At least four species of *Colletotrichum* were found responsible for the fruit rot disease of chili in different countries. *C. capsici* together with *C. gloeosporioides* was reported in Indonesia, India, Thailand, and Korea; *C. acutatum* was detected to cause anthracnose of chili in Indonesia as well as Australia; while *C. coccodes* was reported from New Zealand (Than *et al*., 2008a). For resistance breeding and effective management and control of disease, accurate identification of the causal agent is essential (Freeman *et al*., 1998). The disease-causing pathotype isolates of *C. capsici* show variations in the size of lesions, necrotic tissue, and the appearance and disappearance of acervulus on Capsicum fruits (Cai *et al*., 2009). Therefore, proper detection of the species concerned and adequate information on pathogen populations capable of causing epidemics are necessary for proper control and management of fruit rot. Traditional morphology-based detection has many imitations, hence, DNA sequence-based molecular approach is found efficient to know, analyze and characterize the complex taxonomic characters of *Colletotrichum*. DNA characters are independent of environmental influences and hence DNA analysis is a reliable and accurate identification and classification technique for *Colletotrichum* (Than *et al*., 2008b; Sangdee *et al*., 2011).

Nayaka *et al*., (2009) and Torres-Calzada *et al*., (2011) described the most reliable method for quick detection of *C. capsici* based on PCR using species-specific SSR primers. The RAPD technique is efficient to differentiate among the *Colletotrichum* species from various host plants as was reported for *C. acutatum, C. coccodes, C. dematium*, and *C. gloeosporioides* isolate collected from capsicum grown in China and Korea (Hyunjoo *et al*., 2000). RAPD analysis was applied for diversity assessment of *Colletotrichum capsici* and classification and study of genetic relatedness existing between various morphologically distinct groups of *C. capsici* at the molecular level (Sharma *et al*., 2005). A combination of traditional morphological characterization and molecular diagnosis is suggested as a reliable approach for studying *Colletotrichum* species complexes causing anthracnose and managing the disease and pest effectively. However, none of the above-mentioned approaches were used for the identification and characterization of *Colletotrichum* species causing chili fruit rot in Bangladesh. This research work aimed at precise characterization of chili fruit rot causing *Colletotrichum* in Bangladesh by morphological markers, identify the species by species-specific SSR marker and determine genetic diversity among 10 *Colletotrichum* capsicum isolates collected from different places using RAPD markers.

Materials and Methods

Colletotrichum samples of chili fruits with fruit rot lesions were collected from Jamalpur (S, S1, S3, S6, S9, S10, and S14), Mymensingh (S5), and Chittagong (S11 and S15) districts in Bangladesh. Isolated samples were collected from diseased chili fruits that did not have any visible sporulation (Photita *et al*., 2005). Three pieces of tissues measuring 5x5 mm^2 were excised from the margins of infected fruits. Earlier, the surface of the fruits was sterilized for 3-5 min dipped in 1% sodium hypochlorite and a subsequent rinsing at least thrice with sterilized water. The fruit tissues were then placed on agar solidified medium for incubation at ambient room temperature that ranged from 28 to 30°C. Hyphal mycelium developed from the disease tissue discs. The growing edges of the hyphal mycelia were transferred under an aseptic condition to the previously prepared potato dextrose agar (PDA) media. Single-spores were isolated from diseased fruits with sporulation. The fungal pathogens were first identified by visual observation of their sporulation. A single-germinated spore with conidia from 7 days old cultures was picked up by sterilized needles and was then cultured on PDA to get pure cultures. The cultures were stored for future use at 4°C.

Prepared cultures were placed onto PDA at the ambient temperature of the room (~25°C). Sterilized 4 mm plugs from areas near the growing and actively sporulating edge of each isolate were punched after 25 days of culture. This was then incubated with a similar condition to the previous for about 10 days. Then the size (cm) and color of the colony were recorded individually on the 5th, 7th, and 10th days. Conidia length and width were also calculated through an occulometer (Mesa-Arango *et al*., 2002). Length at three separate places of conidia was calculated and the average was recorded. Similarly, the width was also calculated. Confirmation of setae was done by visualization, and by counting conidia numbers within a µm3 space, the percentage of conidia was calculated.

Genomic DNA from each isolate was extracted from mycelium following Sangdee *et al*., (2011) with little modification. The mycelia of the pathogen were carefully mixed with 200 g of sterilized white quartz sand and preheated 600 µl 2X CTAB buffer. Initially, 400 µl of CTAB buffer was added first and then crushed with a homogenizer for 5-10 min, and then another 200 µl CTAB buffer was added. Then the mixture was incubated in a water bath at 60°C for 20 min and vortexed for 20 sec and re-incubated for another 20 min in the same water bath. Only the aqueous part was taken to a new centrifuge tube and into each tube, 600 µl mixture (25:4:1) of phenol: chloroform: isoamyl alcohol was carefully mixed. It was then centrifuged at a speed of 13000 rpm for a period of 30 min, and the aqueous extract was once again transferred to another Eppendorf tube. Subsequently, 100% cold ethanol was added to it @ two times (2X) the volume of the aqueous extract. About 40 µl 5M sodium acetate was then added and the tubes were inverted gently. To precipitate DNA, the samples were stored overnight at -20°C. Then it was subjected to centrifugation at a speed of 13000 rpm for 30 min followed by the addition and gentle mixing of 1000 µl 70% ethanol. Pellets were washed with 1000 µl of 70% ethanol and spun at 13000 rpm for 10 min. The DNA samples were air-dried for 3 hours after removal of ethanol. The DNA pellets were re-suspended adding 100µl 1x TE buffer, kept at -20°C temperature until used.

Fifteen RAPD primers were collected from Japan and were screened on randomly chosen samples to know how many of them can amplify fungal DNA for accurate scoring. By displaying praiseworthy banding patterns, 5 primers (OPAB02, OPAB06, OPA02, S1155, S1234) were selected for the analysis of all 10 isolates. The PCR reactions were performed in 10 µL mixture that consisted of 2 µL of genomic DNA, 1µL of 10X AmpliTaq polymerase buffer, 0.6 µL of MgCl2, 1 µLdNTPs, 2 µL primer, 0.2 µLTaq polymerase, and 3.2 µL sterilized deionized water. Then the PCR reactions were programmed for 40 cycles. The sample was denatured initially at 94°C for 3 min and subsequently at 94°C for another 1 min, annealed at 35°C for 1 min with a subsequent elongation at 72ºC for 2 min. Finally, elongation was performed following the completion of cycles for 7 min at 72°C. The DNA products amplified by PCR were then separated electrophoretically on a gel of 1.4% agarose. Alongside the PCR products, 1 kb and 100 bp DNA ladders were electrophoresed to determine the molecular weight of the loci. The gel, after electrophoresis, was stained in Ethidium Bromide (Et-Br), solution for a duration of 20 minutes placed on the Gel documentation system (UV-transilluminator) for visualization of DNA bands, and a photograph was taken.

C. capsici was detected by PCR using CcapF/CcapR and CC1F1/CC2R2, two species-specific primers. These primers amplified an amplicon size of 394 and 447 bp as expected (Table 1). The reaction mixture for PCR consisted of 10x PCR buffer, 10 mMdNTPs, 1 U Taq DNA polymerase, 0.2 µM each of the forward primer and 0.2 µM reverse primer. All the amplifications were performed using a Thermal Cycler (Eppendorf, Germany). The amplification was done by: initially denatured for 5 min at 95°C, denaturation was performed for 1 min at 94°C, annealing for 1 min at 56°C was done, and then allowed for the extension at 72°C for a period of 2 min with a final extension for 7 min at the same temperature. All the PCR amplified DNA were electrophoretically separated on 1.4% agarose gel, then were stained with Et-Br, and finally photographed by a Gel Documentation System.

A single matrix constructed based on the pooled data of the scores of all primers helped in estimating polymorphic loci, genetic distance, gene diversity (Nei, 1972; Nei, 1973), and construction of a UPGMA dendrogram among populations using version 1.31 of POPGENE computer program. The size of the amplified product was measured in base pairing AlphaEaseFc 4.0 software (Protein Simple, 2011).

Table 1. Sequence of species-specific primers used for detection of *Colletotrichum capsici.*

Primer name		Sequence	Product size (bp)
CC1F1/CC2R2	Reverse	5' AAATTTGGGGGTTTTACGGC 3'	447
	Forward	5'ACCTAACTGTTGCTTCGGCG3'	
CcapF/CcapR	Reverse	5'CCCAATGCGAGACGAAATG 3'	394
	Forward	5'GTAGGCGTCCCCTAAAAAGG 3'	

Table 2. Summary of morphological data of colony growth and color for *Colletotrichum* isolates.

Isolate number	Colony character (after 10 days) and conidia	Growth rate (cm)		
		5 day	7 day	10 day
S	Cottony white and a little grey-Falcate conidia	5.0	6.0	7.5
S1	Cottony white with slight grey- Falcate conidia	5.0	7.0	8.2
S3	Cottony white to darken grey outside- Falcate conidia	5.0	7.0	8.0
S5	Greenish white to pale grey, Around slight orange at first- Falcate conidia	5.0	6.0	8.0
S6	Slight to dark grey and green, Salmon white- Falcate conidia	5.0	8.0	9.0
S9	Cottony white to slight grey, lastly green and ash color- Falcate conidia	5.5	7.5	8.0
S10	Cotton white, ash to dark grey- Falcate conidia	5.3	8	8.5
S11	Cottony white to light grey, lastly green- Falcate conidia. Pale orange colony	3.8	5.6	8.0
S14	Cottony white to slight grey, lastly green- Falcate conidia	4.5	8.0	8.5
S15	Cottony white to slight grey, lastly green- Falcate conidia	4.7	6.0	6.7

Results and Discussion

Identification and morphological characterization: Differences in culture morphological characters and colony characters of 10 isolates gave rise to identifiable morphological groups similar to the *Colletotrichum* species. Fungal colonies grown in PDA media showed differences after 10 days of pure culture (Fig. 1). S, S1, S3, S10 expressed as Cottony white with little grey-falcate conidia whereas, S5, S6, S9, S11, S14, S15 showed Cottony white to slight grey, lastly green and ash color with same falcate conidia and aerial mycelium. Isolates of sample S11 formed pale orange colonies that had little white mycelial growth (Table 2). The growth rates of the different colonies of the isolates differed considerably as measured on the 5th, 7th, and 10th days. There were many setae in three isolates (S, S5, S10) and the percentage of conidia was significantly highest in S5 and S10 (100%). Conidial length and width were measured highest in S5 (16.472 µm and 3.093 µm, respectively) (data not shown). The morphological characters revealed the isolates to be *Colletotrichum capsici.*

Identification and characterization of the *Colletotrichum* species on the basis of their morphology related characters including conidial size and shape, the presence of setae or existence of a teleomorph, and some special features of the culture like growth rate, and color, and texture of the colony have been reported by Ratanacherdchai *et al*., (2010) and subsequently by Torres-Calzada *et al*., (2011). Differences of the pathogen in colony colors and type of *Colletotrichum* populations are not uncommon and were observed previously (Baxter *et al*., 1983; Lardner *et al*., 1999; Rajapakse & Ranasinghe, 2002; Sharma *et al*., 2005; Than *et al*., 2008a). In the present study, differences in colony characters of the isolates were used for the differentiation of *Colletotrichum* isolates. Some of the collected isolates were characterized by their fluffy, cottony, or suppressed colonies on PDA that had different colors including grayish-black to grayish-white, grey, or even green on their surface. However, all isolates had falcate conidia. Similar morphological differences were reported in earlier studies (Rajapakshe & Ranasinghe, 2002; Sharma *et al*., 2005; Akhtar & Singh, 2007).

Detection and identification of *Colletotrichum*: The pathogens isolates of the infected chili collected from different districts were detected by PCR amplification of fungal DNA by CcapF/CcapR and CC1F1/CC2R2, a pair of species-specific primers. The PCR detection by the species-specific SSR primer pairs revealed that all the isolates tested generated a positive band of a specific size (Fig. 2). Amplification with the two pairs of primers yielded bands of approximately 394 and 447 bp, respectively for all the 10 isolates. This investigation thus confirmed based on two species-specific primers for the first time that *C. capsici*is the species responsible for the fruit rot of chili in Bangladesh.

This study aimed to identify and detect *Colletotrichum capsici* by PCR amplification of fungal DNA. Using two SSR primers CcapF/CcapR and CC1F1/CC2R2, the *Colletotrichum capsici* DNA amplification of all isolates under study generated 394 and 447 bp, products. Torres-Calzada *et al*., (2011) confirmed that the use of the same CcapF/CcapR and CC1F1/CC2R2 primers identified *C. capsici* from among other similar pathogens and thus were able to facilitate the diagnosis and observation of the pathogens accurately and precisely irrespective of hosts and locations. Nayaka *et al*., (2009) also reported CC1F1/CC2R2 as a specific primer for the detection of *C. capsici*. Earlier, a previous study conducted in India confirmed 5 isolates, out of 10 (50%), as *Colletotrichum capsici* (Sharma *et al*., 2005). This report confirmed all the isolates to be *C. capsici* causing chilli anthracnose in Bangladesh for the first time.

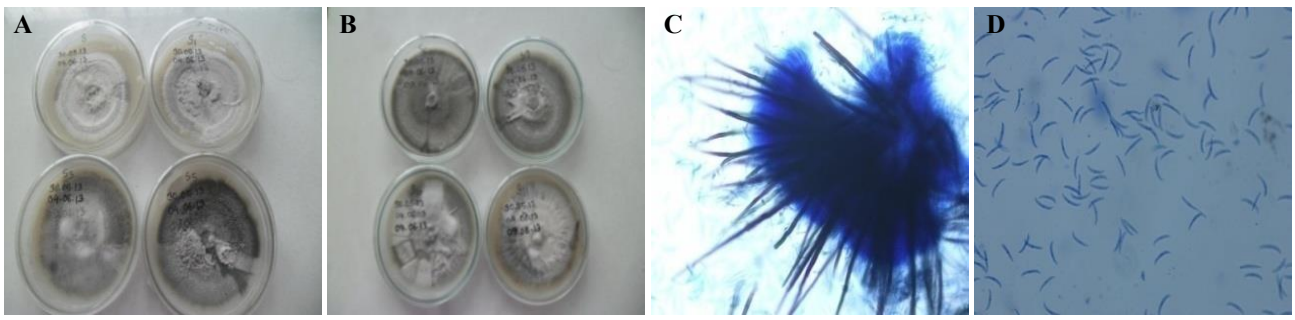

Fig. 1. Colony growth on 10th day showing isolates S, S1, S3, S5 (A), isolates S6, S9, S10, S11 (B), Acervuli with setae (C) and close view of conidia (D) of *Colletotrichum.*

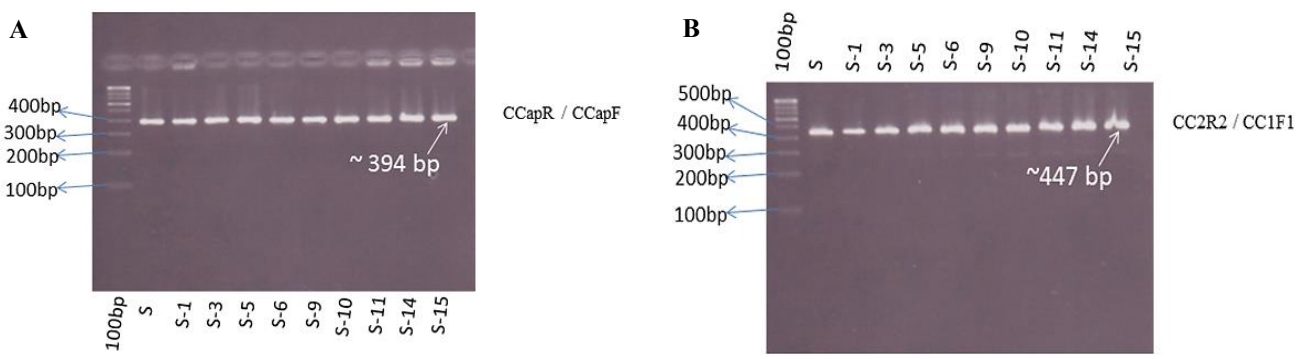

Fig. 2. Detection of *Colletotrichum capsici* by PCR amplification with two pairs of species-specific primers (A) CcapR/CcapF giving a band of ~394 bp and (B) CC2R2/CC1F1 giving a band of ~447 bp.

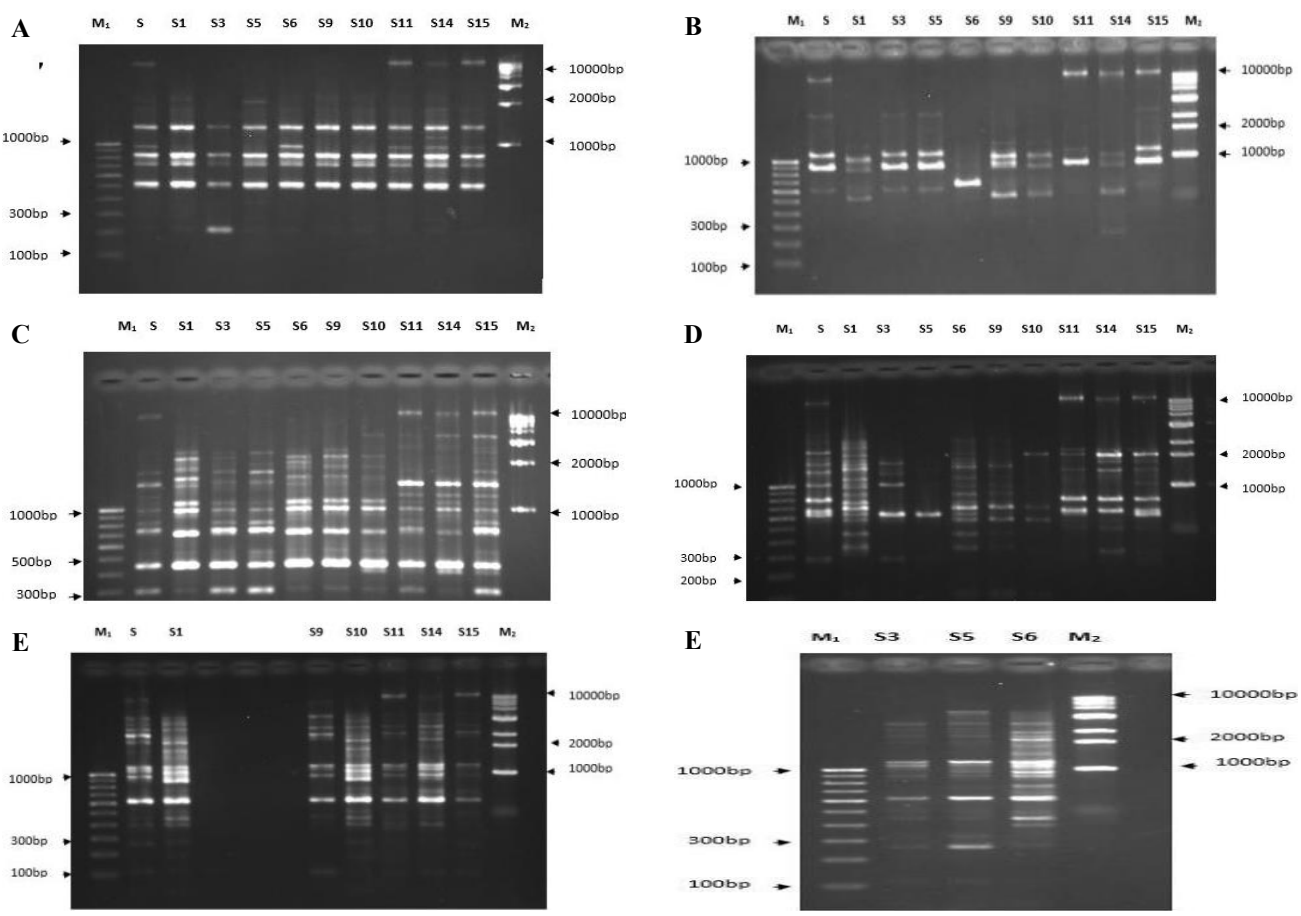

Fig. 3. RAPD profile of 10 isolates of *C. capsici* using primer OPAB06 (A), S1234 (B), S1155(C), OPA02(D). Lane M_1 (100bp ladder, Lane M_2 (1Kb ladder) Lane 1-10, *C. capsici.* isolates namely S, S1, S3, S5, S6, S9, S10, S11, S14, S15, respectively. Figure 3E (isolates S, S1, S9, S10, S11, S14, S15) and 3F (isolates S3, S5, S6) together consist of RAPD profile of 10 isolates with primer OPAB 02.

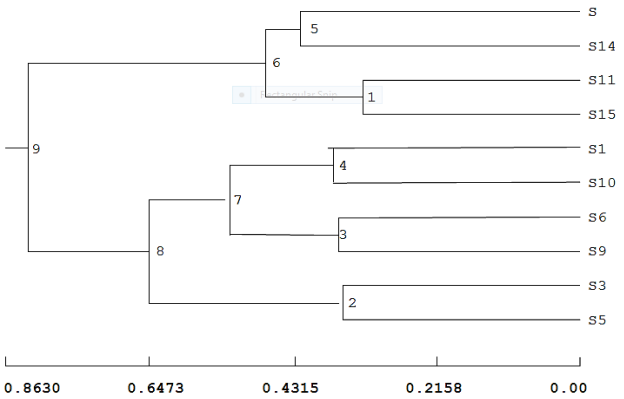

Fig. 4. UPGMA dendrogram was constructed based on Nei's genetic distance depicting diversity among the isolates.

Molecular characterization by RAPD analysis: Out of 15 primers, 5 were found suitable for use in RAPD analysis of these 10 isolates with clear banding zone in each and minimum smearing. RAPD data information of these isolates was analyzed with all selected primers. The fingerprints obtained were scored for mainly particular positioned banding patterns on the gel (Figs. 3A-F). Polymorphism was evaluated, and the bands of 104-4740 bp were detected among the studied genotypes. These versatile banding patterns of the primers were considered for estimating genetic distance among pathogen samples.

Isolates that have a higher Inter population similarity index with a lower polymorphic loci frequency possibly have lower heterozygosity in comparison to the isolates having a lower Inter isolate similarity index. It means that a lower similarity among the samples is indicative of their less homozygosity. The higher inter isolate similarity index (Si) was found between S11 Vs S15 (84.4%), and the lowest was found between the two isolates S Vs S10 (53%) of the studied samples. The isolates collected from the same or closer locations will likely to have higher similarities than those collected from distant places. Band sharing-based inter genotype similarity indices (Si) were higher (Average 82.67%). Among the 5 primers, OPAB06 showed the highest (82.67%) inter isolate similarity indices. However, the primer S1234 produced the lowest (50.89%) inter isolate similarity indices (data not shown).

Gene diversity among the varieties for the identified loci in this study was 0.3566. We detected higher (above 0.5) gene diversity (0.50) together with Shannon's information index (0.6931) in OPAB06-5, S1234-2, S1234-8, S1155-3, S1155-4, S1155-6, S1155-11, OPA02-7, OPA02-7, OPA02-9, OPAB02-11, OPAB02-12, and OPAB02-13 loci. While the lowest gene diversity together with the lowest Shannon's information indices was also generated in this study by the primers OPAB06-4, OPAB06-6, OPAB06-7, OPAB06-8, S1155-10, S1155-12, S1155-13, OPA02-14, OPAB02-4, and OPAB02-7. The primers generated 64 distinct loci among which 52 were polymorphic that gave a higher polymorphism of 81.25%. Among the 5 primers, OPA02 alone produced 17 polymorphic loci that indicated its high efficiency to find polymorphism. The least polymorphic loci (4) were generated by the primer OPAB06. On average, the 5 primers generated 13 total and 10 polymorphic RAPD bands per primer (data not shown). Both strong, as well as weak bands, are common in the RAPD based genetic analysis (Wahab *et al*., 2020). The weak bands have resulted when a low homology exists in between the primers with their complementary sites available in the DNA template.

The pair-wise Nei's genetic distance among 10 *Colletotrichum* isolates calculated from combined data generated from 5 primers varied between 0.2469 and 0.8630. The isolates S1 and S11 were found to have the highest genetic distance (0.8630). On the contrary, the lowest extent of genetic distance (0.2469) was found between isolates S11 and S15. The highest variability within varieties expresses the richness of the genetic material of a species. A dendrogram was constructed using Nei's (1972) genetic distance on the basis Unweighted Pair Group Method with Arithmetic Means (UPGMA). The dendrogram segregated the 10 isolates into two main clusters (6, 8). Four isolates S, S14, S11, and S15 formed cluster 6, where there were two sub-clusters (5, 1). Sub-cluster 5 formed with S, S14 isolates, and Sub-cluster 1 formed with S11 and S15 isolates (Fig. 4).

Different molecular markers are used to estimate the variability among the populations of pathogens. Only limited research has been conducted to assess the diversity among the *Colletotrichum* species. This study identified genetic relationships and diversities among 10 isolates of *Colletotrichum* using RAPD markers. RAPD markers efficiently measured the variability among chili plants (Ratanacherdchai *et al*., 2010; Sangdee *et al*., 2011). RAPD markers have been used for the intra-specific characterization of many fungal pathogens. A high degree of variability at the molecular level among the *Colletotrichum* species has been identified. These studies have proved RAPD-DNA fingerprinting as the most preferred technique of estimating molecular variability among *C. capsici*. The technique we used in detecting the variability of the *Colletotrichum* species isolates generated useful information on the variations among the populations in Bangladesh.

In this study, we measured the amplified product size that ranged from 0.150 kb to 4.500 kb, although the majority was below 1.5 kb. This result showed similarity with molecular characterization of 37 *C. capsici* isolates using 10 oligonucleotide RAPD primers exhibiting higher consistent polymorphism and producing an average number of 8-12 bands with the size of 0.2-3.5 kb (Sharma *et al*., 2005). The dendrogram constructed in this study separated 10 *C. capsici* isolates of Bangladesh into two major clusters. A similar pattern of clustering was earlier reported by Sangdee *et al*., (2011). However, the clustering could not be correlated to cultural morphology data. Our results suggest that the establishment of a correlation between molecular and morphological data is not always possible. Previous studies showed that RAPD and ISSR-PCR analyses were not always correlated with the rates of growth during the culture period, geographical location of collection, color, pathological or average IVCM of the isolates (Sharma *et al*., 2005; Silveira *et al*., 2019). However, it is evident that the RAPD approach identifies *Colletotrichum* sp. efficiently.

Conclusion

The study aimed to identify specifically and detect precisely the species of *Colletotrichum* by morphological and molecular markers and measure the extent of variability among the isolates. This study identified, characterized the *Colletotrichum capsici* and estimated the diversity of *Colletotrichum* at the molecular level. The detection by CcapF/CcapR and CC1F1/CC2R2 primers and RAPD based molecular fingerprinting could serve as a sound basis in the identification of *Colletotrichum capsici*. We report for the first time that *Colletotrichum capsici* is responsible for anthracnose of chili in Bangladesh.

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