RAPD analysis of *Colletotrichum* species causing chilli anthracnose disease in Thailand

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RAPD (Random Amplified Polymorphic DNA) analysis was performed on 18 isolates including 2 species, *Colletotrichum gloeosporioides* and *C. capsici*, isolated from three varieties of chilli, i.e. Chilli pepper (*Capsicum annuum*), Long cayenne pepper (*C. annuum var acuminatum*) and Bird's eye chilli (*C. frutescens*). The relationship among the species were analyzed based on the dendrogram of RAPD patterns using UPGMA (Unweighed Pair Group Method with Arithmetic Mean). RAPD analysis in our study showed a clear difference between *C. gloeosporioides* and *C. capsici*. Furthermore, *C. capsici* isolates were more closely related than *C. gloeosporioides* isolates.

Key words: Chilli, *Colletotrichum gloeosporioides*, *C. capsici*, RAPD

Introduction

Chilli (*Capsicum annuum* L.) is an important tropical and subtropical crop on the basis of its high consumption, nutritional and cash value to farmers and consumers both in developed and developing countries, particularly in Thailand. Anthracnose of chilli is one of the most destructive diseases of chilli-growing areas in Thailand (Oanh *et al.*, 2004; Taylor, 2007) and also in the tropical Asia (Sariah, 1989; Shin *et al*., 2000; Sharma *et al*., 2005). Anthracnose disease has been reported to be caused by several *Colletotrichum* species: *C. capsici, C. acutatum, C. gloeosporioides, C. coccodes* and *C. dematium* (Hong and Hwang, 1998; Gopinath *et al.*, 2006). Those species of *Colletotrichum*, *C. gloeosporioides* (Penz.) Penz. & Sacc. and *C. capsici* (Syd.) E. J. Butler & Bisby are the most frequently cited as causal agents of chilli anthracnose. Mannandhar *et al*. (1995) reported that *C. gloeosporioides* strains

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causing anthracnose disease on chilli fruits in Taiwan. Moreover, Gopinath *et al.* (2006) found that anthracnose of chilli caused by *C. capsici* which has been become a serious problem for chilli cultivation in India. The fungus is distributed throughout the tropics and very commonly occurs in chilli growing areas. *C. capsici* appeared to be the most severe being able to infect a range of *Capsicum* species and resistant genotypes (Taylor, 2007). The disease produces symptoms on leaves, stem and fruits. Decay of mature red fruit caused by anthracnose can result in substantial reduction in chilli yield. *Colletotrichum* species infecting diverse hosts have a high degree of pathogenic variability. It is common to find that a single botanical species of *Colletotrichum* infects multiple hosts. For example, *C. gloeosporioides* is found on a wide variety of fruits, including almond, avocado, apple, arabica coffee, guava, mango and strawberry (Agwanda *et al*., 1997; Freeman *et al*., 1998; Martínez-Culebras *et al*., 2000; Sanders and Korsten, 2003; Xiao *et al*., 2004; Amusa *et al*., 2005). The use of differential hosts is a viable option for the evaluation of pathogenic variability. Combined application of molecular diagnostic tools along with use of different isolates could be appropriated and reliable approach for studying pathological variability in *Colletotrichum* species. Molecular methods have been employed successfully to differentiate between populations of *Colletotrichum* from many hosts in general, according to the study of Agwanda *et al.* (1997), which the RAPD (random amplified polymorphic DNA) technique has been used to identify of RAPD markers for resistance to coffee berry disease, caused by *C. kahawae*, in arabica coffee. Backman *et al.* (1999) also used the RAPD technique to determine if *C. graminicola* isolates from annual bluegrass and creeping bentgrass are host specific. The comparative study of capsicum anthracnose pathogens, *C. gloeosporioides, C. coccodes, C. dematium, Glomerella cingulata* and *C. acutatum*, have been found on capsicum in Korea and China, that used RAPD-PCR technique to detect the variation among *Colletotrichum* species isolates (Shin *et al*., 2000). Weeds *et al.* (2003) reported that using molecular markers to compare the genetic diversity in *C. gloeosporioides* infecting species of the tropical forage legume *Stylosanthes* from Brazil, Australia, China and India had been clearly demonstrated. RAPD markers were used to determine genetic relationships among *C. gloeosporioides* isolates, causing crown rot of strawberry, recovered from noncultivated hosts and disease strawberry plants in Florida (Xiao *et al*., 2004). Furthermore, Sharma *et al.* (2005) studied on the genetic relationship between five morphological groups recognized within *C. capsici* by using RAPD analysis. Molecular polymorphism generated by RAPD confirmed the variation in virulence of *C. capsici*, the cause of fruit rot of chillies, and different isolates were grouped into five clusters (Sharma *et al*., 2005).

Thus, the objective of this study was to investigate the variability in *C. gloeosporioides* and *C. capsici* populations infecting chillies in Thailand by using molecular approaches.

Materials and methods

Isolation and identification of the pathogens from infected chilli

The pathogens were isolated from the symptoms on three varieties of chilli as follows: Chilli pepper (*Capsicum annuum*), Long cayenne pepper (*C. annuum var acuminatum*) and Bird's eye chilli (*C. frutescens*). The disease samples of leaves and fruits were collected in the fields in Ratchaburi province, Thailand, then kept in moisten chamber and brought to laboratory. Isolation of causing agent was done by using tissue transplanting technique. The disease plant parts were cut at the advanced margin of lesions in to small pieces (5 mm × 5 mm) and then surface were disinfected with 10% Clorox for 1 min, followed by washing in sterile distilled water, and transferred onto isolating medium (water agar, WA). The mycelia growing out of the plant tissue were sub-cultured to potato dextrose agar (PDA) medium, and incubated at room temperature for 7-10 days (approximately 28-30°C). Single spore isolation was also done to be pure culture. The isolate was identified into species by observation of morphology under compound microscope.

Genomic DNA isolation and PCR amplification of DNA

The total genomic DNA of *Colletotrichum* sp. was isolated from mycelia. Isolates were incubated at 28^{o}C for 4 days in tubes containing 20 ml of potato dextrose broth, agitated at 180 rpm. Mycelia were harvested by filtration through filter paper, dried between two layers of filter paper and stored at -80^{o}C for further use. Dried mycelium was ground to fine powder with pestle and mortar using liquid nitrogen and transferred to 1.5 ml Eppendorf tube. 600 µl Cetyltrimethylammonium bromide (CTAB) was added and incubated at 65^{o}C for 30 min, tubes were vortexed every 10 min. After cooling at room temperature equal volume (600 µl) of chloroform:isoamyl alcohol (24:1, v/v) was added in fume hood cabinet, gently mixed for 20-30 min and centrifuged at 7000 rpm for 5 min at 4^{o}C. The aqueous phase was transferred to new tubes and repeat CIA extraction. After the second CIA wash, the DNA was precipitated by adding 300 µl isopropanol, tubes were gently mixed and incubated at room temperature for 30 min. Tubes were centrifuged at 12000

rpm for 10 min and supernatant was decanted. The DNA pellet was dissolved in 50 µl of ddH_2O.

Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) procedure described by Williams *et al*. (1990) was used by the following of a reaction mixture of 25 µl volume which consisting of 2.5µl of 10X PCR buffer, 2.5µl of 25 mM $MgCl_2$, 0.5 µl dNTPs mix (10 mM each of dATP, dCTP, dGTP, dTTP), 0.4 µl Taq DNA polymerase, 2.0 µl of primer, 1.0 µl of genomic DNA and 16.1 µl of sterilized double-distilled water. The reaction mixture was vortexed and centrifuged at 12000 rpm for 2 min. Amplification was carried out in a thermal cycle by using three temperature profiles, programmed for initial DNA denaturation at 94^{o}C for 3 min, followed by 35 cycles consisting of DNA denaturation for 30 sec at 94^{o}C, primer annealing at 35^{o}C for 30 sec and polymerization for 1 min at 72^{o}C with a final extension period of 10 min at 72^{o}C. Amplification products were separated on 1.5% agarose gel in 1X TAE buffer at 110 V for about 3 h 1 kb DNA ladder (Fermentas) (0.5 µg/µl) was run for weight size comparison. Gels were stained with ethidium bromide for 30 min, then were visualized with UV light and photographed.

Analysis of RAPD profiles

Differences in fingerprinting patterns between isolates were assessed visually. Polymorphisms including faint bands that could be scored unequivocally were included in the analyses. Presumed homologous bands were scored as present (1) or absent (0) to create a binary matrix. A similarity matrix was generated from the binary data using DICE similarities coefficient in SIMQUAL program of NTSYS-PC Package (Rohlf, 2000). Cluster analysis was done with the Unweighed Pair Group Method with Arithetic Mean (UPGMA) in the SAHN program of NTSYS-PC Package, and constructed dendrogram based on genetic distances.

Results and discussion

Isolation and identification of the pathogens from infected chilli

Three isolates of *Colletotrichum gloeosporioides* were isolated form Chilli pepper (*Capsicum annuum*). In addition, four isolates of *C. capsici* were isolated from Long cayenne pepper (*C. annuum var acuminatum*). Moreover, three isolates of *C. Gloeosporioides* were isolated from Bird's eye chilli (*C.*

frutescens) and eight isolates of *C. capsici* were isolated from Bird's eye chilli (*C. frutescens*) were also used in this study (Table 1).

Table 1. *Colletotrichum gloeosporioides* and *Colletotrichum capsici* isolates used in this study.

Hosts	Isolates	
	Colletotrichum gloeosporioides	***Colletotrichum capsici***
Chilli pepper (*Capsicum annuum*)	C101, C105, C106	-
Total	3	
Long cayenne pepper (*Capsicum annuum var acuminatum*)	-	C201, C202, C203, C204
Total	4	
Bird's eye chilli (*Capsicum frutescens*)	C301-1, C301-2, C304	C307, C308-1, C308-2, C310, C311, C312, C313, C314
Total	11	

RAPD analysis

Initially, 90 random 10-mer primers (Shanghai Sangon Biological Engineering Technology & Service Co., Ltd.) were screened to select primers exhibiting maximum polymorphism. Of these 13 primers (Table 2) which produced easily scorable and consistent banding patterns were used for RAPD analysis of test isolates. A total of 429 bands were produced by 13 primers (Fig. 1), and the dendrogram (Fig 2) drawn from the RAPD patterns using UPGMA (Unweighed Pair Group Method with Arithmetic Mean).

Table 2. Nucleotide sequence of primers generating amplification products.

Primer code	Base sequence (5'-3')
S1027	ACGAGCATGG
S1063	GGTCCTACCA
S1089	CAGCGAGTAG
S1136	GTGTCGAGTC
S1155	GAAGGCTCCC
S1184	GACGGCTATC
S1189	AGTCCCCCTC
S1234	TCGCAGCGTT
S1239	TGACAGCCCC
S1265	GAGCTACCGT
S1313	CTACGATGCC
S1320	TGTCCTAGCC
S1358	ACCCCAACCA

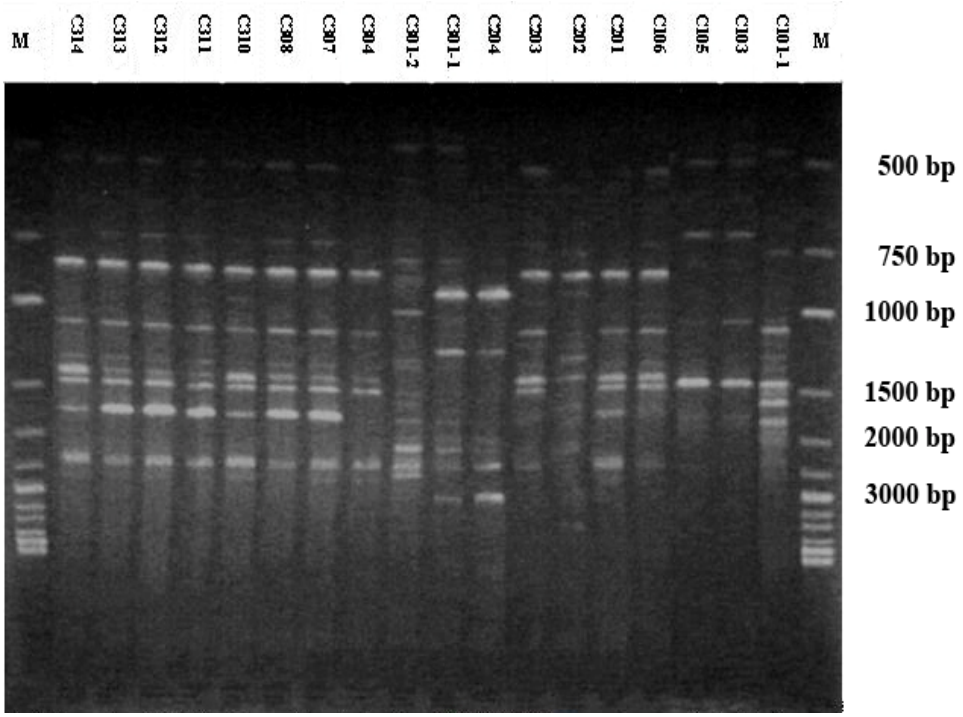

Fig. 1. Examples of RAPD polymorphism in isolates of *Colletotrichum* species generated with primers S1136. Molecular weight markers (in base pairs) are indicated on the left and right side (1 kb ladder, Fermentas).

According to the dedrogram of *Colletotrichum* spp. that can be divided into three main groups (Fig. 2). The first group includes three isolates, most of them were *C. gloeosporioides*, which isolated from Chilli pepper (*Capsicum annuum*). The second group contains the other 3 isolates of *C. gloeosporioides*, most of them were isolated from Bird's eye chilli (*C. frutescens*). The last group includes the 11 isolates of *C. capsici*, which isolated from both of Long cayenne pepper (*C. annuum var acuminatum*) and Bird's eye chilli (*C. frutescens*). RAPD analysis conducted in this study showing less variation in *C. gloeosporioides* isolates than *C. capsici* isolates. Result from the dedrogram showed a clear distinction between Chilli pepper (*Capsicum annuum*) and Bird's eye chilli (*C. frutescens*) isolates of *C. gloeosporioides*, thereby supporting the study was made by Sharma *et al.* (2005) which reported that the phylogenetic grouping of *C. capsici* causing fruit rot/die back or anthracnose of chillies in the north-western region of India based on RAPD data did not appear to be congruent with morphological and virulence pattern. RAPD-based

DNA fingerprinting could be one of the methods of studying genetic diversity in *C. capsici* in the absence of a definite differential set. Xiao *et al.* (2004) reported that RAPD markers were used to determine genetic relationships among isolates that recovered from noncultivated hosts and diseased strawberry plants. Phylogenetic analysis using RAPD marker data divided isolates of *C. gloeosporioides* from noncultivated hosts into two separate clusters. Isolates from strawberry were interspersed within the cluster containing the isolates that were recovered from noncultivated hosts. However, Backman *et al.* (1999) studied on anthracnose basal rot caused by *C. graminicola* (Ces.) Wils., which is a destructive disease of annual bluegrass (*Poa annua* L.) and creeping bentgrass (*Agrostis palustris* Huds.) golf course putting greens in North America and Europe. Cluster analyses of RAPD markers showed that isolates from bluegrass and creeping bentgrass separated into two distinct groups. Furthermore, Alahakoon *et al.* (1994) reported that when inoculated into non-host, *C. gloeosporioides* could genetically adapt to a new host, and resultant isolates could not be distinguished morphologically from the wild type. Thus requires further investigation for the cross-infection of different hosts by *Colletotrichum* species.

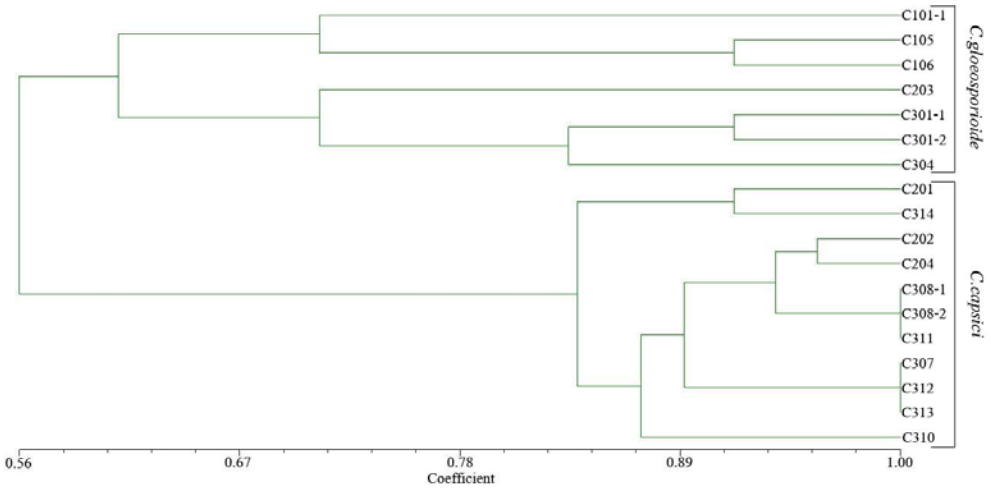

Fig. 2. Generated dendrogram showing the clustering of strain of *Colletotrichum gloeosporioides* and *Colletotrichum capsici* species by the combination of all the RAPD data for all thirteen selected 10-mer random primers used. Scale at the bottom depicts the similarity values.

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