รายงานวิจัยฉบับสมบูรณ์

โครงการ การสร้างโมเลกุลเครื่องหมายสำหรับลักษณะความต้านทานโรคแอนแทรคโนสของพริกลูกผสมข้ามชนิดระหว่าง *Capsicum annuum* L. และ *C. chinense* Jacquin

The development of molecular markers for resistance to anthracnose in the interspecific hybrid chilli between *Capsicum annuum* L. and *C. chinense* Jacquin

โดย อรรัตน์ มงคลพร

กันยายน 2546

สัญญาเลขที่ PDF/44/2544

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วิทยาเขตกำแพงแสน นครปฐม

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

ขอขอบคุณสำนักงานกองทุนสนับสนุนการวิจัยที่ให้การสนับสนุนงานวิจัยชิ้นนี้ และขอขอบคุณ Dr Paul Taylor ในฐานะนักวิจัยพี่เลี้ยงที่ให้คำปรึกษาและคำแนะนำที่เป็นประโยชน์ต่อโครงการ

ขอขอบคุณ Department of Education, Science and Training ประเทศออสเตรเลีย ที่ให้ทุนสนับสนุนงานวิจัยเรื่อง Prospecting for anthracnose disease resistance genes in chili ซึ่งจากผลการวิจัยทำให้มีการสร้าง expressed sequenced tag (EST) marker และได้นำมาใช้ประโยชน์ต่อในโครงการนี้ และขอขอบคุณ Dr Paul Taylor (BioMarka, School of Agriculture and Food Systems, Institute of Land and Food Resources, The University of Melbourne) และ Dr Eddie Pang (Department of Biotechnology and Environmental Biology, RMIT University) ในฐานะผู้ร่วมโครงการ Prospecting for anthracnose disease resistance genes in chili

ขอขอบคุณศูนย์เทคโนโลยีชีวภาพเกษตร มหาวิทยาลัยเกษตรศาสตร์ ที่ให้ทุนสนับสนุนค่าวัสดุบางส่วนสำหรับงานวิจัยนี้

ขอขอบคุณสภาวิจัยแห่งชาติ ที่ให้การสนับสนุนงานวิจัยในส่วนการทดสอบลูกผสมด้วยเทคนิคไมเครุดเครื่องหมาย ซึ่งจำเป็นต่อการสร้างประชากรพริกที่ใช้ในโครงการนี้

ขอขอบคุณ รองศาสตราจารย์ ดร. สมศิริ แสงโชติ ภาควิชาโรคพืช มหาวิทยาลัยเกษตรศาสตร์ ที่ให้ความอนุเคราะห์เชื้อ *Colletotrichum capsici* ขอขอบคุณศูนย์วิจัยพืชผักเขตร้อนและ Asian Regional Center of the Asian Vegetable Research and Development Center (ARC-AVRDC) มหาวิทยาลัยเกษตรศาสตร์ ที่ให้ความอนุเคราะห์สถานที่ทำการทดลองในแปลง โรงเรือน และ ห้องปฏิบัติการโรคพืช รวมทั้งสายพันธุ์พริกที่ใช้สร้างประชากรสำหรับการศึกษาครั้งนี้

ธรรัตน์ มงคลพร

29 ธันวาคม 2546

บทคัดย่อ

รหัสโครงการ : PDF/44/2544

ชื่อโครงการ : การสร้างไมเลกุลเครื่องหมายสำหรับลักษณะความต้านทานโรคแอนแทรคโนสของพริกลูกผสมข้ามชนิดระหว่าง *Capsicum annuum* L. และ *C. chinense* Jacquin

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โรคแอนแทรคโนสเป็นปัญหาหนึ่งที่สำคัญของพริกในประเทศไทย สร้างความเสียหายแก่ผลผลิตพริกมากกว่า 50% พบความต้านทานโรคระดับสูง (immune) ในพริก *Capsicum chinense* Jacquin 'CM021' จึงทำการผสมข้ามชนิดระหว่างพริกพันธุ์ต้านทานนี้และพริก *C. annuum* L. พันธุ์บางช้าง เพื่อถ่ายทอดลักษณะต้านทานสู่พันธุ์บางช้างซึ่งเป็นพริกที่มีความสำคัญทางอุตสาหกรรมของไทย แต่อ่อนแอต่อโรค จากการศึกษาการกระจายตัวของลักษณะต้านทานโรคในประชากรพริกรุ่นที่ 2 (F_2) และประชากรผสมกลับรุ่นที่ 1 (BC_1) พบว่าลักษณะต้านทานโรคเป็นลักษณะด้อย และถูกควบคุมด้วยยีน 1 ตำแหน่ง ทำการค้นหาไมเลกุลเครื่องหมายของลักษณะต้านทาน ด้วยเทคนิค bulked segregant analysis โดยใช้ประชากร BC_1 พบเครื่องหมายชนิด RAPD 7 ตำแหน่ง และ EST 2 ตำแหน่ง ที่มีความสัมพันธ์กับลักษณะต้านทานโรค โดยมีเครื่องหมาย 5 ตำแหน่งสัมพันธ์กับ recessive allele และอีก 4 ตำแหน่งสัมพันธ์กับ dominant allele จากการทดสอบ linkage พบว่าเครื่องหมาย RAPD $SAI02_{800}$ แสดงค่า recombination ประมาณ 7%

คำหลัก : พริก, ความต้านทานโรคแอนแทรคโนส, ไมเลกุลเครื่องหมาย

Abstract

Project Code: PDF/44/2544

Project Title : The development of molecular markers for resistance to anthracnose in the interspecific hybrid chilli between *Capsicum annuum* L. and *C. chinense* Jacquin

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Project Period : 1 July 2001 – 30 September 2003

Anthracnose is a major disease of chili in Thailand, causing over 50% yield loss. High resistance (immune) to anthracnose has been identified in *Capsicum chinense* Jacquin 'CM021'. The resistance was transferred to a Thai elite cultivar but very susceptible to anthracnose, *C. annuum* L. 'Bangchang'. Segregation of the resistance in the F_2 and BC_1 populations of a cross between 'CM021' and 'Bangchang' indicated that the resistance was conferred by a recessive gene. Bulked segregant analysis (BSA) technique was applied to the BC_1 populations to identify molecular markers linked to the resistance. Seven RAPD and two EST markers were identified through BSA. Of the nine markers, five associated with the recessive allele (repulsion phase) and four associated with the dominant allele (coupling phase). Linkage analysis of the markers and the trait performed in the BC_1s indicated that the RAPD $SAI02_{650}$ marker was the closest with 7% recombination.

Keywords : chili, *Capsicum*, resistance to anthracnose, molecular marker

Introduction

Chili (*Capsicum annuum* L.) is an economically important crop of Thailand. Anthracnose (*Colletotrichum* spp). is one of the most severe diseases of chili in the tropics. Under conditions favorable to the disease development, significant pre- and post-harvested fruit losses of over 50% have been reported. Two significant causal pathogens found in Thailand are *C. capsici* (Syd.) Butler & Bisby and *C. gloeosporiodes* Penz. (Sangchote S., personal comm). Up to date no high resistance has been found in *C. annuum* which is the only species widely grown in Thailand and worldwide.

High resistance to anthracnose has been identified in an accession of *C. chinense* (AVRDC Report 1997, unpublished data). Our preliminary study have exhibited this accession was resistant to both species of *Colletotrichum* found in Thailand (unpublished data). Characterisation of the resistance genes in this *C. chinense* genotype is important as it may be a source of novel resistance, which may be transferred and incorporated to Thai elite chili varieties to develop anthracnose resistant genotypes.

Molecular marker has proven a great tool to assist selecting some difficult traits such as disease resistance in several crops. Random amplified polymorphic DNA (RAPD, (Williams et al., 1990) is the simplest marker technique using an arbitrary decamer oligonucleotides as a primer to generate polymorphisms between DNA samples. RAPDs have proven a useful and fast tool to identify markers for some important traits such as resistance to siliqua shattering in *Brassica* ((Mongkolporn et al., 2003), ascochyta blight resistance in lentil (Ford et al., 1999), anthracnose resistance in common bean (Young et al., 1998), and in sorghum (Boora et al., 1998).

Expressed sequenced tags (EST) is one of the latest marker technology developed from genes that express in a certain condition. In this study, ESTs were developed from genes that were found expressed in *C. chinense* 'CM021' the anthracnose resistant chili after being inoculated with *C. capsici*, compared to *C. annuum* 'Bangchang' (anthracnose susceptible chili) using microarray analysis (Mongkolporn O., unpublished data).

This paper reports genetic study of anthracnose resistance that expressed in the interspecific cross of Thai susceptible *C. annuum* cv. 'Bangchang' and an anthracnose resistant *C. chinense* 'CM021', and the identification of molecular markers that are linked to the resistance.

Materials and Methods

1. Genetic analysis of resistance to anthracnose

1.1 Production of interspecific populations segregating for anthracnose resistance

Reciprocal crosses by hand pollination were performed between an anthracnose resistant *Capsicum chinense* Jacq. 'CM021' and a susceptible Thai elite cultivar *C. annuum* L. 'Bangchang'. Emasculation of a flower from a female parent was carried out in the early morning one day before flowering by using a pair of very fine tip forceps to completely remove anthers and then the emasculated flower was covered with a cotton ball to protect cross contamination from other pollen until pollination was performed on the following day. Pollen was collected from newly opened flowers of a male parent on the day of pollination. The pollination was executed in the morning during 7.00-9.00 am. The pollinated flowers were covered with a cotton ball to protect contamination from undesired pollen.

Advanced generations were produced by self-pollinating one F_1 hybrid to generate F_2 and backcrossing the F_1 to each parent to generate BC_1s. Two BC_1 populations were BC_1R which was derived from F_1 x 'CM021' and BC_1S from F_1 x 'Bangchang'. The F_1 genetic purity was approved using molecular analysis (Mongkolporn et al., 2004) (data not shown).

Total seven chili populations including: 10 plants each of the parental varieties 'CM021' and 'Bangchang', 10 plants each of F_1 ('CM021' x 'Bangchang') and F_1 ('Bangchang' x 'CM021'), 150 F_2, 31 BC_1R , and 85 BC_1S, were grown in a field at the Tropical Vegetable Research Center (TVRC), Kasetsart University, Kamphaengsean Campus (KU-KPS), Nakhon Pathom, from January to May 2002. Plant spacing was 50 x 100 cm^2 on a single rowed bed.

Another F_2 population of 98 individuals were regrown in a shade house at the TVRC from July to November 2003 to confirm the genetic analysis of the resistance to anthracnose obtained from the previous crop. Each plant was planted in a 30-cm plastic pot. Both parents and F_1, ten plants each, were also included.

1.2 Assessment of anthracnose resistance

1.2.1 Preparation of chili fruit for inoculation: Assessment of anthracnose resistance was performed at the Pathology Laboratory of the Asian Regional Center-Asian Vegetable Research and Development Center (ARC-AVRDC), KU-KPS. Five fruits at

mature green stage (35 days after flowering) were harvested from each plant. Calyces were removed from the harvested fruits. The fruits were surface sterilised by soaking in 1% (v/v) sodium hypochlorite solution for 1 min, washed twice with distilled water and dried with paper towels. The fruits were subsequently placed on a stainless steel rack (16x18x4 cm^3) in a plastic box (20x20x10 cm^3), which was filled with 500 ml distilled water.

1.2.2 *Preparation of inoculum*: A Thai highly aggressive single-spore isolate of *Colletotrichum capsici* (Syd.) E.J. Butler & Bisby strain number 158*ci*. Conidia from seven-day-old cultures were harvested by adding 5-10 ml of sterilised distilled water onto the culture grown on potato dextrose agar (PDA) at 27°C under continuous fluorescence, which was then gently swirled to dislodge the conidia. The conidia suspension was filtered through two layers of muslin cloth. Number of conidia was counted under a microscopy using a haemocytometer. The concentration of conidia suspension was finally adjusted to 10^6 conidia/ml.

1.2.3 *Inoculation and evaluation of anthracnose*: The prepared fruits were inoculated with *C. capsici* using an injection method modified from Lin, 2002 #293 and AVRDC Report 1997 (unpublished data). One µl of the adjusted conidial suspension was injected into each individual fruit at 1 mm depth using an injector Micro syringe™ model 1705 TLL with dispenser PB600-1 (Hamilton, Switzerland). The inoculated fruits were incubated in the dark for the first 24 hrs, and then in 12/12 hrs light/dark cycle at 25°C with 100% relative humidity. Lesion area at the inoculated site was measured at 9 days after inoculation (DAI).

1.3 Genetic analysis of anthracnose resistance

Frequency distribution of mean values for lesion area/fruit area (LFA) in all chili populations was investigated. Separation of phenotypic classes in the F_2 was based on difference between the two parents. Segregation of the resistant and susceptible phenotypes in the F_2, BC_1R and BC_1S were determined to fit a Mendelian ratio using Chi-squared goodness-of-fit test.

$$\chi^2 = \sum \frac{[(O-E)-0.5]^2}{E}$$

whereby, O and E are observed and expected frequency respectively.

2. Identification of molecular markers linked to genes for anthracnose resistance

2.1 Plant materials and genomic DNA isolation

Two backcross populations BC_1R and BC_1S containing 31 and 85 plant individuals respectively were used to generate DNA for marker identification study. These plants were grown in a field at the TVRC, KU-KPS from November 2002 to May 2003. Eight young and healthy leaves (approximately 200 mg) were detached from each individual 3-month-old plant. The leaves were wrapped in an aluminum foil and placed on ice. Shortly the leaves were then snap frozen in liquid nitrogen and kept at –80°C for subsequent DNA extraction. Total genomic DNA was extracted from the frozen leaves using a modified CTAB (cetyltrimethylammonium bromide) microprep method ((Mongkolporn et al., 2004)). DNA was stored in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0) at -20°C.

2.2 Assessment of DNA quality and quantity

DNA quantity and quality was assessed using a spectrophotometric method by measuring the absorbance of DNA solution at 260 and 280 nm with a UV spectrophotometer GeneQuant Pro® (Amersham Bioscience, UK). The DNA solution was 100 fold diluted from DNA stock solution for 100 µl. The DNA quantity was calculated as follows,

$$\text{DNA concentration (ng/µl)} = (OD_{260} \times 50) \times \text{dilution factor}$$

OD_{260} and OD_{280} ratio indicates DNA purity, which should range 1.8-2.0 for relatively good DNA purity (Sambrook and Russell, 2001).

2.3 DNA amplification

2.3.1 *RAPD assays*: An optimised PCR condition was obtained from (Mongkolporn et al., 2004), which contained 50 ng of genomic DNA, 0.75 unit of DyNAzyme™ DNA polymerase (Finnzyme, Finland), 0.24 mM each of dATP, dCTP, dTTP and dGTP (Finnzyme, Finland), 0.4 µM of primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 3 mM of $MgCl_2$ in a final volume of 25 µl. Two hundred and twenty four random decamer primers (Operon Technologies, USA) were used to amplify chili DNA.

The PCR reaction was performed in a thermal cycler 'Cooled-Palm' (Corbett Research, Australia) which was programmed as follows: initial denaturation at 94°C for 1 min followed by 35 cycles of 94°C for 30 sec, 37°C for 30 sec and 72°C for 1 min with

final extension at 72°C for 5 min. Amplified products were separated by electrophoresis on a 1.5% agarose gel in TAE buffer, stained with ethidium bromide, visualised with UV illumination, and photographed with a gel documentation system TCX-20M (Vilber Lourmat, France)

2.3.2 *EST assays*. Sixteen EST primer pairs, which were developed from gene expression analysis of *Capsicum* anthracnose resistance using microarray approach (Mongkolporn O., unpublished data), were used to amplify chili DNA. A PCR reaction contained 50 ng of genomic DNA, 0.75 unit of DyNAzyme™ DNA polymerase, 0.24 mM each of dATP, dCTP, dTTP and dGTP (Finnzyme, Finland), 0.2 µM each a primer in a pair, 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 2.5 mM of $MgCl_2$ in a final volume of 25 µl.

The PCR reaction was performed in a thermal cycler programmed as follows: 35 cycles of 94°C for 1 min, 50-60°C for 1 min and 72°C for 2 min with final extension at 72°C for 7 min. Separation and visualisation of the PCR amplified products were carried out as previously described.

2.4 Bulked segregant analysis (BSA)

Ten individuals that were extremely resistant were selected from the BC_1R, and another ten that were extremely susceptible were selected from the BC_1S populations. The same amount of DNA from each individual within the same phenotypic class was pooled together, and thus two DNA bulks 'R' and 'S' were generated. Polymorphisms were identified both between the two parental DNA and between the two DNA bulks using 224 RAPD and 16 EST primers.

2.5 Linkage analysis

The markers that were identified in the 'R' or 'S' DNA bulks were tested with all individual members of the bulked DNA. Putative markers were investigated further with the entire BC_1s population to determine linkage of the markers and the resistance trait. Segregation distortion of the markers was tested whether the segregation performed in a Mendelian fashion for dominant marker using the Chi-squared goodness-of-fit test.

Results and Discussion

1. Genetic analysis of resistance to anthracnose

1.1 Anthracnose development in the parents and F_1

Development of anthracnose symptom was earliest visually detected at 3 DAI in 'Bangchang', while 'CM021' remained no lesion throughout the study. Mean lesion area of 'Bangchang' was 6.83 cm^2 at 9 DAI. The evidence of no symptom in 'CM021' indicated a high level of resistance to *C. capsici* strain 158*ci*. 'CM021' has been proven to be a high resistant variety to *C. gleosporioides* isolate *Cg*-153 (AVRDC, 1998). In the F_1 progeny, mean lesion areas at 9 DAI were 5.17 cm^2 and 5.83 cm^2 for both F_1 ('CM021' x 'Bangchang') and F_1 ('Bangchang' x 'CM021') respectively.

1.2 Inheritance of resistance to anthracnose

Mean lesion area at 9 DAI from each individual plant of all seven chili populations was recorded. Frequency distribution of mean lesion area/fruit area (LFA) from all populations was observed. The LFA values of 'CM021' and 'Bangchang' were significantly different (0 and 0.24-0.80) without overlapping values (Fig 1). All F_1s from both crosses resembled 'Bangchang' in anthracnose susceptibility with the LFA ranging from 0.11-0.36. The appearance of anthracnose symptom in both F_1s indicated the resistance as measured by LFA was a recessive trait and was inherited through nuclear genome.

The distribution of LFA ranged from 0 to 0.42 in the BC_1R, and from 0.12 to 0.99 in the BC_1S populations (Fig 1). The F_2 data were not collectable in this crop, thus an F_2 population was repeated and regrown in 2003. The LFA of the second F_2 ranged from 0 to 1.00 (Fig 2). High resistance (no symptom) was able to transfer from 'CM021' to the F_2 progeny, and was found in 24 of 98 plants in total.

Based on the difference of the LFA values between the parents, two phenotypes were considered. The LFA values resembling 'CM021' the resistant parent, which was 0, were classified as resistant, while the LFAs greater than 0 were classified as susceptible. Frequency distribution of resistance and susceptibility in the F_2 and BC_1 populations was investigated. Segregation ratio of resistance: susceptibility in the F_2 and BC_1R well fitted a 1: 3 and 1: 1 Mendelian fashion (Table 1). The segregation ratio was approved by Chi-squared test and suggested a recessive gene conferring anthracnose resistance.

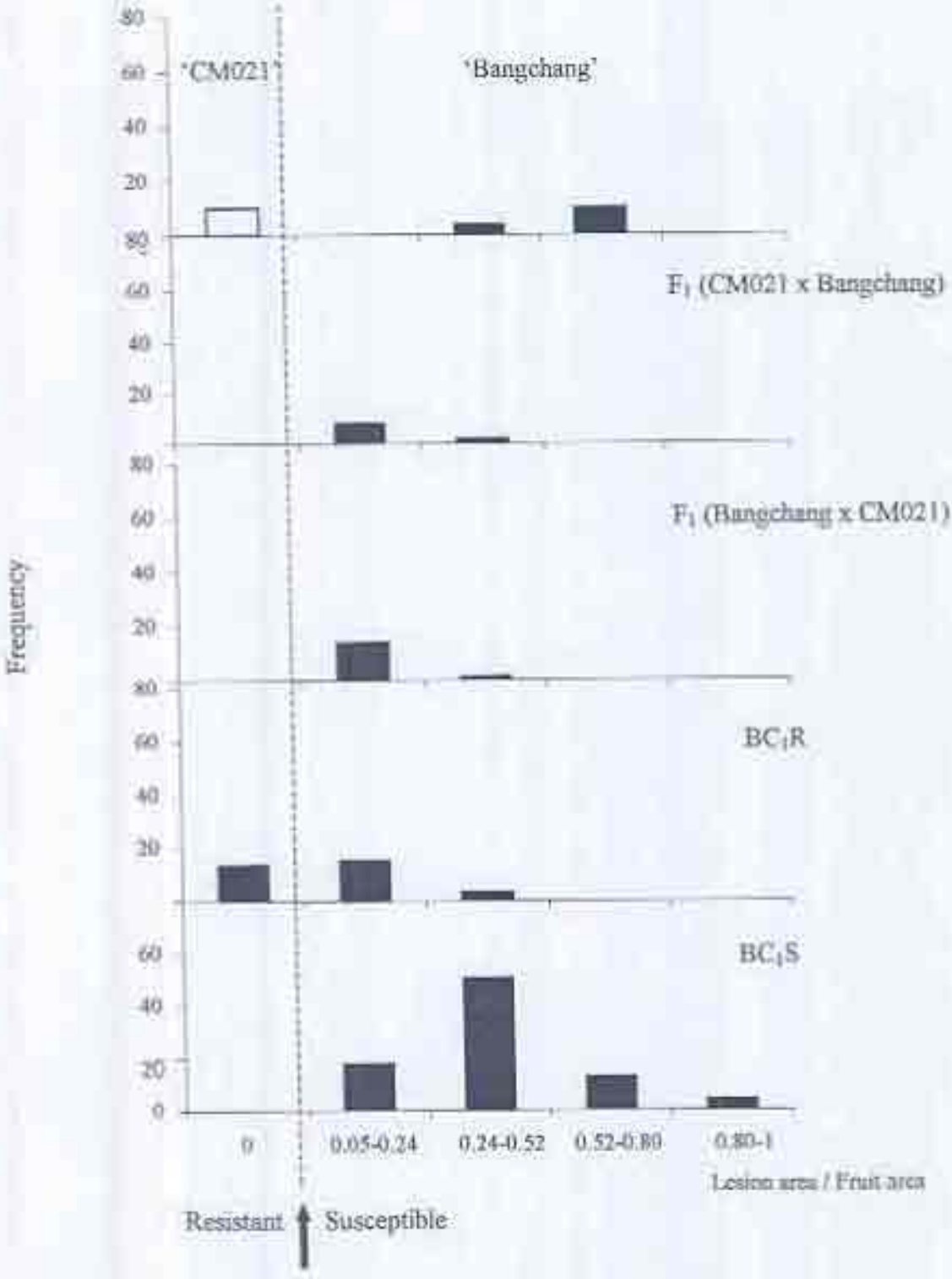

Figure 1 Frequency distribution of lesion area/fruit area in the chili populations grown in 2002-3: 'CM021', Bangchang', and their progeny, F_1s and BC_1s populations at 9 days after inoculation

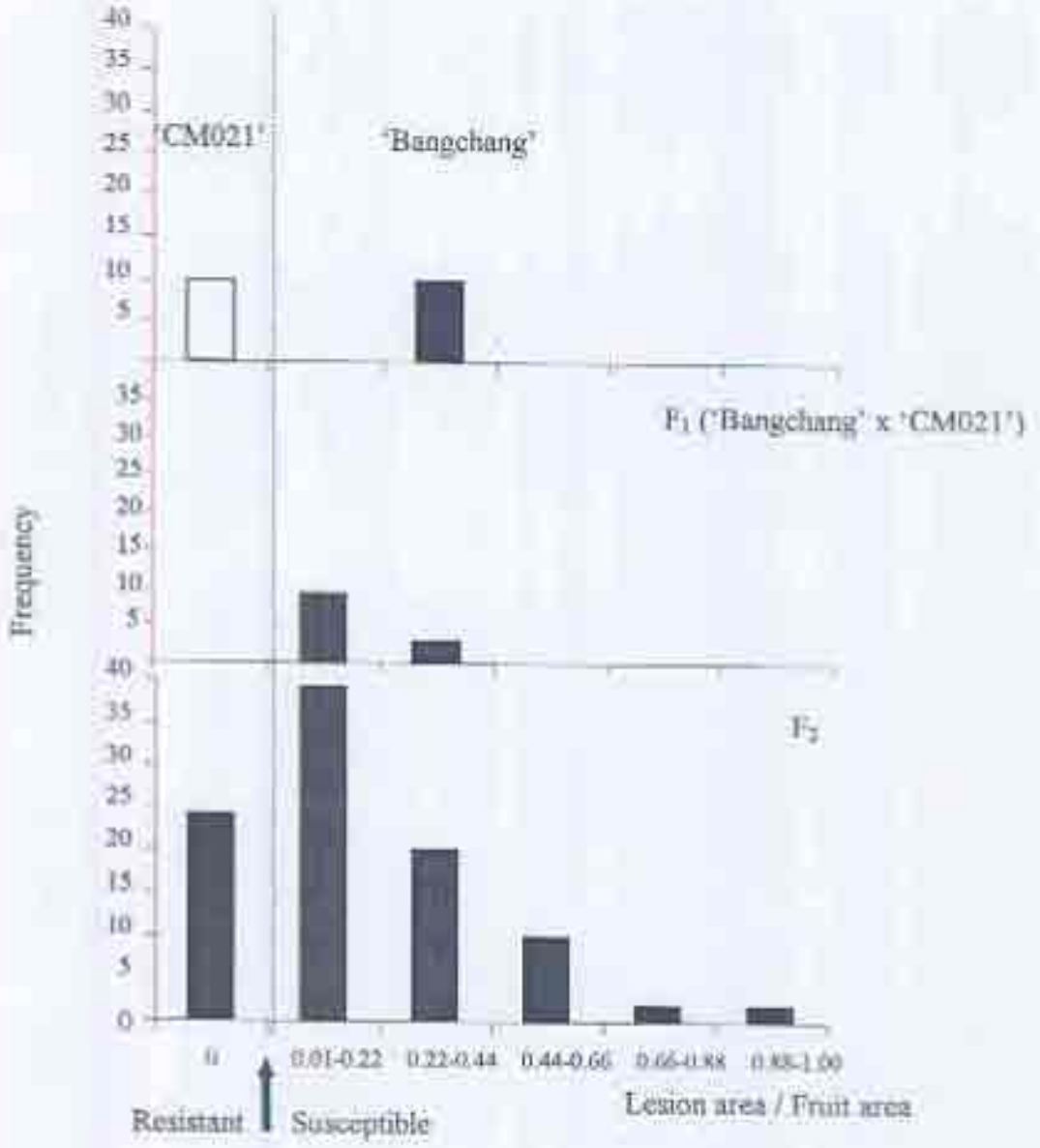

Figure 2 Frequency distribution of lesion area/fruit area in the chili populations grown in 2003: 'CM021', Bangchang', and the F_2 at 9 days after inoculation

Table 1 Segregation of resistance (R) and susceptibility (S) to anthracnose caused by *Colletotrichum capsici* '158*ci*' in the chili F_2 and backcross populations derived from a cross between 'CM021' and 'Bangchang', as evaluated by contingency Chi-square values for goodness-of-fit

Population	Mendelian ratio (S : R)	Observed frequency		χ^2	Probability
		S	R		
Crop 1 (2002-3)					
CM021	-	0	10	-	-
Bangchang	-	10	0	-	-
F_1 (CM021 x Bangchang)	-	10	0	-	-
F_1 (Bangchang x CM021)	-	10	0	-	-
F_2	*	*	*	*	*
BC_1R (F_1 x CM021)	1 : 1	18	13	0.8	0.39
BC_1S (F_1 x Bangchang)	1 : 0	85	0	not available	not available
Crop 2 (2003)					
CM021	-	0	10	-	-
Bangchang	-	10	0	-	-
F_1 (Bangchang x CM021)	-	10	0	-	-
F_2	3 : 1	74	24	0.034	0.89

* No data were available

Previous genetic study in Thailand has reported that the resistance to anthracnose was a dominant trait controlled by a single gene ((Lin et al., 2002). However, the chili parental materials used in that study were different from this current report. In addition, the resistance derived from '83-168' variety was only moderately resistant. Approximately 30-90 % anthracnose incidences (from 3-7 DAI) were detected on the inoculated fruits of the '83-168'. In contrast, the 'CM021' remained no visual symptoms throughout the experiment (9 DAI).

2. Identification of molecular markers linked to genes for anthracnose resistance

2.1 Bulked segregant analysis

2.1.1 *RAPD analysis:* Of total 224 RAPD primers screened, 157 produced polymorphisms between the parental DNA yielding 327 and 326 specific markers for 'Bangchang' and 'CM021' respectively. Up to date, only two primers AI02 and R12 detected polymorphisms between the two DNA bulks (Table 2). AI02 generated two markers, each of which associated with the resistance and susceptibility. While R12 generated five markers, of which three associated with the resistance and two associated with the susceptibility.

2.1.2 EST analysis: Of total 16 EST primer pairs used, ten successfully amplified the chili parental DNA. Five produced polymorphisms between the parents, and only two including OM14 and OM11 generated markers between the two DNA bulks, which associated with the resistance (Table 2).

The markers identified from BSA had the prefix either R or S, which associated to the resistance or susceptibility respectively, followed by the primer name. Each marker was followed by lower-case number indicating molecular weight size (base pairs) of the marker. Therefore, five markers including $RAI02_{300}$, $RR12_{500}$, $RR12_{900}$, $ROM11_{700}$ and $ROM14_{700}$ were associated with the resistance, in the other words they were linked in repulsion phase to the dominant allele of the trait. Oppositely, the rest four markers *i.e.* $SAI02_{800}$, $SR12_{500}$, $SR12_{700}$ and $SR12_{950}$ were associated with the susceptibility, thus linked in coupling to the dominant allele.

2.2 Linkage analysis

Only eight candidate markers, excluding $ROM11_{700}$ as identified from BSA were investigated their linkage with the resistance trait. All the markers used in this study were dominant markers. The markers in coupling theoretically segregate 1: 1 for presence: absence of the marker in the BC_1R and do not segregate (1: 0) in the BC_1S populations. While the markers in repulsion theoretically segregate 1: 1 for presence: absence of the marker in the BC_1S and do not segregate in the BC_1R populations. All the eight markers tested fitted well with the expected segregation ratio (Table 3).

Considering the resistance trait being conferred by a recessive gene on one hand and the identified markers being dominant markers on the other, only markers linked in

coupling to the dominant allele are informative. Therefore among the eight markers only four S- markers were able to be estimated their % recombination with the trait. $SAI02_{800}$ appeared to be the closest marker to the trait with 7.41 % recombination (Table 3).

Further marker linkage analysis is being investigated in the F_2 population. Up to date, the investigation has been done with the two EST markers $ROM11_{200}$ and $ROM14_{700}$, which showed similar % recombination of 11.46 and 10.42 respectively (Table 4). However, the segregation of these markers deviated from the theoretical 3: 1 ratio. The segregation distortion of the markers arose from some resistant individuals did not present the markers. The PCR amplification of these markers was repeated three times and produced the same results. A significant level of marker segregation distortion is generally found in an interspecific population, where 27-39% distortion was reported in an interspecific *Cicer* ((Collard et al., 2003; Winter et al., 1999; Winter et al., 2000), and approximately 50% in *Capsicum* (*C. annuum* and *C. chinense*; Livingstone et al., 1999).

Future directions

To achieve more accurate linkage analysis of these identified markers, the markers (nine in total) are to be tested further in the F_2 population with 98 individuals. The F_2 population is more informative for recombination analysis than the BC_1s. More markers (approximately 100-200) will be generated using RAPD, ISSR, EST and STMS to construct a linkage map from this F_2 population. The benefits of the map to be constructed are to locate the gene conferring the anthracnose resistance, to identify quantitative trait loci (QTL) for the resistance, and to identify closely linked markers to the trait and its QTL.

Table 2 RAPD and EST markers identified in 'R' and 'S' bulks generated from BC_1 population derived from a cross 'Bangchang' x 'CM021'

Primer	Markers identified in 'R' bulk*	Markers identified in 'S' bulk*
AI02	$RAI02_{380}$	$SAI02_{800}$
R12	$RR12_{500}$ $RR12_{900}$	$SR12_{600}$ $SR12_{700}$ $SR12_{850}$
OM11	$ROM11_{700}$	
OM14	$ROM14_{700}$	

*Subscripted number indicates size of the DNA fragment in base pairs

Table 3 Segregation and linkage analysis of the eight candidate markers identified from bulked segregant analysis, performing in the BC_1 populations grown in 2002-3

Marker	BC_1R				BC_1S				%R***
	P:A*	E**	χ^2	Prob	P:A*	E**	χ^2	Prob	
$RAI02_{380}$	27: 0	1: 0	na	-	34: 45	1: 1	1.86	0.17	na
$RR12_{500}$	28: 0	1: 0	na	-	39: 43	1: 1	0.19	0.66	na
$RR12_{900}$	28: 0	1: 0	na	-	38: 44	1: 1	0.44	0.51	na
$ROM14_{700}$	30: 0	1: 0	na	-	36: 49	1: 1	1.99	0.16	na
$SAI02_{800}$	15: 12	1: 1	0.33	0.56	78: 0	1: 0	na	-	7.41
$SR12_{600}$	15: 14	1: 1	0.03	0.86	84: 0	1: 0	na	-	31.03
$SR12_{700}$	16: 12	1: 1	0.57	0.45	82: 0	1: 0	na	-	22.22
$SR12_{850}$	13: 15	1: 1	0.14	0.71	83: 0	1: 0	na	-	28.57

* number of presence: absence of the marker
** expected segregation ratio
*** % recombination between the marker and the phenotype in the BC_1s
na = not available

Table 4 Segregation and linkage analysis of the two EST markers identified from bulked segregant analysis, performing in the F_2 population grown in 2003, where the segregation of the markers was expected to be 3: 1 for presence: absence

EST marker	P:A*	χ^2	Prob	%R**
ROM11$_{708}$	60: 36	8.00	0.0046	11.46
ROM14$_{710}$	59: 37	9.39	0.0021	10.42

* number of presence: absence of the marker
** % recombination between the marker and the phenotype in the F_2

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Project Output

1. International publication

Mongkolporn O., Dokmaihom Y., Kanchana-udomkan C., and Pakdeevaraporn P. 2004. Genetic purity test of F_1 hybrid *Capsicum* using molecular analysis. Journal of Horticultural Science and Biotechnology (*in press*).

- Manuscript is attached, see Appendix

Kanchana-udomkan C., Taylor P.W.J., and **Mongkolporn O.** 2003. Development of a bioassay to study anthracnose infection of chili fruit caused by *Colletotrichum capsici*. Australasian Plant Pathology (*submitted*).

- Manuscript is attached, see Appendix

Pakdeevaraporn P., Taylor P.W.J., Wasee S. and **Mongkolporn O.** 2004. Inheritance of resistance to anthracnose from *Capsicum chinense* Jaq. (*in preparation*)

2. การนำผลงานวิจัยไปใช้ประโยชน์

ผลงานวิจัยมีประโยชน์เชิงวิชาการ

- พัฒนางานวิจัยและสร้างองค์ความรู้ใหม่เกี่ยวกับพันธุกรรมที่ควบคุมความต้านทานโรคแอนแทรคโนสในพริก และการพัฒนาเทคโนโลยีที่ใช้ในการศึกษาจีโนมพริก
- พัฒนาสายพันธุ์พริกให้ต้านทานโรคแอนแทรคโนส
- พัฒนาการเรียนการสอน ความรู้ที่ได้จากงานวิจัยนี้เกี่ยวข้องกับการพัฒนาโมเลกุลเครื่องหมายที่มีความสัมพันธ์กับลักษณะต่อย สามารถนำไปเป็นตัวอย่างสอนในหัวข้อการศึกษาจีโนมพืช การใช้โมเลกุลเครื่องหมายเพื่อช่วยคัดเลือกลักษณะ เป็นต้น
- สร้างนักวิจัยใหม่ เนื่องงานวิจัยมีการใช้เทคนิคด้านการปรับปรุงพันธุ์พืช และพัฒนาเทคโนโลยีด้านชีวโมเลกุล ซึ่งเป็นการฝึกฝนวิทยาการแก่นิสิตและผู้ช่วยวิจัยที่มีส่วนร่วมในโครงการ
- พัฒนาโมเลกุลเครื่องหมายเพื่อใช้ช่วยคัดเลือกลักษณะความต้านทานในโครงการปรับปรุงพันธุ์พริก

3. อื่นๆ

Kanchana-udomkan C., Watcharawetsaringkharn S., and **Mongkolporn O.** 2002. A laboratory evaluation for resistance to antracnose in chili, p.186. Proceedings of the First International Conference on Tropical and Subtropical Plant Diseases, Chiangmai, Thailand.

- Document is attached, see Appendix

Pakdeevaraporn P., Dokmaihom Y., Kanchana-udomkan C., and **Mongkolporn O.** 2002. A novel source of resistance to anthracnose in chili, p.187. Proceedings of the First International Conference on Tropical and Subtropical Plant Diseases, Chiangmai, Thailand.

- Document is attached, see Appendix

ภาคผนวก

เอกสารประกอบ 4 เรื่อง

1. Manuscript titled 'Genetic purity test of F_1 hybrid *Capsicum* using molecular analysis' with the acceptance letter from the Journal Editor.
2. Manuscript titled 'Development of a bioassay to study anthracnose infection of chili fruit caused by *Colletotrichum capsici*'.
3. Proceedings document: A laboratory evaluation for resistance to antracnose in chili.
4. Proceedings document: A novel source of resistance to anthracnose in chili.

Genetic purity test of F_1 hybrid *Capsicum* using molecular analysis

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SUMMARY

A fast and accurate genetic purity test of F_1 hybrid plants is essential for seed production and accelerating advanced breeding generations in breeding programs. DNA technology has great potential for enhancing purity assessment of hybrids. Genetic purity of three F_1 chili hybrids was determined using two molecular marker techniques RAPD and ISSR. RAPD analysis successfully detected all three F_1 hybridity, while ISSR only detected two. This was due to the RAPD marker system producing greater number of markers than the ISSR system.

Genetic purity is a vital component of hybrid seed production for both commercial and breeding purposes. Morphological characteristics of hybrid seed are normally similar to one of the parents therefore, genetic purity of the hybrid seed is difficult to visually assess. Genetic purity test is conventionally conducted by field trials to observe the morphological characters of the hybrid progeny, which takes a few months to evaluate. However, morphological variations are frequently limited for varietal identification.

The time requirement to have purity field tests has created a great disadvantage in seed production business. Contracted farmers are not paid until the purity test is executed, which normally takes several months to prove F_1 hybrid seed is genetically true. In addition, in crop breeding procedures, fast determination of true hybrids are also essential, so advanced generations can be produced further.

Some molecular techniques have proven to be excellent tools for identification purposes. PCR-based markers such as random amplified polymorphic DNA (Williams *et al.*, 1990) and inter simple sequence repeat; (Gupta *et al.*, 1994; Zietkiewicz *et al.*, 1994) are relatively simple and inexpensive compared to hybridisation-based markers such as restriction fragment length polymorphisms (RFLP). RAPD analysis has been shown to be effective in varietal identification of several crops including potato (Ford and Taylor, 1997), fruits (Koller *et al.*, 1993; Lu *et al.*, 1996; Elisiario *et al.*, 1999; Luo *et al.*, 2002), flowers (Takatsu *et al.*, 2001; Yamagishi *et al.*, 2002) and *Capsicum* (Ilbi, 2003).

ISSR markers have been efficiently used for cultivar identification in crop plants (Li and Ge, 2001; Yamagishi *et al.*, 2002; Ge *et al.*, 2003). Furthermore, ISSR has successfully identified somatic hybrids of citrus (Scarano *et al.*, 2002) and potato (Matthews *et al.*, 1999).

Based on the above information, the two marker techniques exhibited a great potential to determine genetic purity of F_1 hybrid. This study aimed to compare the effectiveness of RAPD and ISSR marker techniques to determine genetic purity of three *Capsicum* hybrids.

MATERIALS AND METHODS

Plant materials and genomic DNA isolation

Plant materials included three F_1 *Capsicum* hybrids and their six respective parents (Table 1). Total genomic DNA was extracted from approximately 150 mg of the youngest and healthy leaves of 1-month-old *Capsicum* seedlings using a modified CTAB method (Taylor *et al.*, 1995). The quantity and quality of DNA was determined using a spectrophotometer GeneQuant Pro® (Amersham Biosciences, Cambridge, UK) by measuring the absorbance at 260 and 280 nm (Sambrook and Russell, 2001)

DNA amplification

RAPD assays: Twenty-five decamer primers from Operon Technologies (USA) were used to amplify the *Capsicum* DNA. A PCR reaction of 25 µl final volume consisted of 50 ng genomic DNA template, 1 unit of DyNAzyme™ DNA polymerase (Finnzymme, Finland), 0.24 mM each of dATP, dCTP, dGTP and dTTP, 0.4 µM of RAPD primer, and PCR buffer with a final concentration of 0.01 M Tris-HCl, 2.5 mM of $MgCl_2$, 0.05 M KCl, 0.1 mg ml^{-1} gelatin, pH 8.3. The reaction was subsequently performed in a 'Cooled-Palm' thermal cycler (Corbett Research, Australia), which was programmed as follows: initial denaturation at 94°C for 1min followed by 35 cycles of 94°C for 30 s, 40°C for 30 s and 72°C for 1min with a final extension at 72°C for 5 min. Amplified products were separated by electrophoresis on a 1.4% agarose gel in TAE buffer at 100 V, stained with 1 mg ml^{-1} ethidium bromide and visualised with UV illumination. The gel was photographed with gel documentation system TCX-20M (Vilber Lourmat, France). Amplified products were compared with Lamda DNA digested with *Eco*RI and *Hind*III restriction enzymes (Promega, USA), and their accurate sizes were determined using PhotoCaptMW® computer software (Vilber Lourmat, France).