Genetic and Breeding Studies on Functional Components of Galangal and Chili Pepper

(ナンキョウとトウガラシの機能性成分に関する遺伝育種学的研究)

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Chapter I

Introduction

Galangal and chili pepper are known as the popular spice in Thailand, especially as the typical cuisine of Thailand. For galangal, it is one of the important crops cultivated in tropical area. Its rhizome is used not only as a common spice for many dishes but also as a medicinal plant. The demands for such medical herbs are expected to increase. 1′-acetoxychavicol acetate (ACA) is one of principal ingredients in rhizome of galangal that has a lot of benefit such as anti-tumor (Kondo *et al.*, 1993) and anti-fungal (Itokawa *et al.*, 1987). It had power to resist cancer Sarcoma 180 ascites in mouse. It also showed the efficiency to control the anthracnose postharvest disease on the surface of mango's fruit (Jariyanusorn, 2002). Many studies on such ingredient of galangal had been carried out, however, there was little information about genetic relationship of galangal available for systematic breeding. Therefore, this study was described in the genetic relationship of galangals collected from different regions in Thailand by cluster analysis based on randomly amplified polymorphic DNA (RAPD) analysis and revealed the relation of ACA content, one of important characters of galangal, with the genetic relationship of galangal in Thailand from standpoint of breeding of high ACA content cultivar.

For chili pepper, it is one of the important spices in the world. Chili peppers and their various cultivars originated from the New World. Its domestication in the New World coincides with the development of human culture and civilization (Fari, 1986). By now, they are grown around the world and widely use as spices or vegetable in cuisine, and as

medicine. The nutritional content of chili peppers is relatively high. Chili pepper is good sources of vitamins, particularly vitamin C, and in the dried pungent types, vitamin A. It had many beneficial effects on health. In addition to its use as food or condiment and medicine, chili peppers still have a lot of value as industrial and ornamental plants (Armitage and Hamilton, 1987; Carmichael, 1991; Bosland *et al.*, 1994; Cichewicz and Thorpe, 1996; Bosland, 1997). In Thailand, chili pepper has also become a part of the Thai in the many cooking traditions, especially one of the chili pepper species *Capsicum frutescens*. This species is known as a pungent chili pepper species such as 'Phrikkhiinuu' in Thailand. However, 'S3212', one of *C. frutescens* accessions, showed very low capsaicinoid content, namely few-pungency in the fruits (Matsushima *et al.*, 2004; Konisho *et al.*, 2005a). This few-pungent trait is unique to 'S3212' because all other *C. frutescens* accessions had pungent fruits with high capsaicinoid content. Therefore, this study was analyzed in the inheritance of few-pungent trait in chili pepper 'S3212' (*C. frutescens*). The segregation of capsaicinoid contents in an interspecific hybrid of chili pepper (*C. frutescens* x *C. chinense*) was described. Isolocus test between the few-pungent locus and *C* locus controlling capsaicinoid production was carried out using a cleaved amplified polymorphic sequence (CAPS) marker SCY-800 linked to the *C* locus. In additions, confirmation of a single gene controlled few-pungent trait is different from *c* gene and investigation of some mechanism concerns with the few-pungent gene, the identification of markers linked to the gene controls few-pungent trait in these chili peppers was described by using the RAPD technique in combination with a bulked segregation analysis (BSA) (Michelmore *et al.*, 1991). Moreover, genetic analysis of molecular marker linked closely more to the *cf* locus for the few-pungency was observed using RAPD and CAPS marker. Furthermore, using of SSR marker concerning with SSR-based linkage map was also reported. Of all markers used in this study, molecular mapping of the *cf* locus for the few-

pungency in chili pepper (*C. frutescens* x *C. chinense*) was constructed. It will be useful starting points for marker development in breeding programs.

The information of galangal and chili pepper in the present study will be beneficial to genetic improvement through breeding for development of the new breeding lines in galangal and chili pepper further.

Chapter II

Genetic and Breeding Studies on Functional Components of Galangal

Experiment 1

Genetic Relationship of Galangal (*Alpinia galanga* Willd.) in Thailand by RAPD Analysis

1.1. Introduction

Zingiberaceae is a large family and includes many economically important species. Therefore, there are lots of studies on genetic diversity and phylogeny in Family Zingiberaceae (Sirirugsa, 1998; Kress *et al.*, 2002; Ngamriabsakul *et al.*, 2004) and Genus *Alpinia* (Kress *et al.*,1992; Rangsiruji *et al.*,2000; Saensouk *et al.*,2003) using molecular techniques due to the difficulty of phylogenetic study based on morphological characters (Sirirugsa, 1998).

Alpinia galanga Willd., one of the members in Zingiberaceae family, is commonly known as galanga, galangal or greater galangale and also known as Kha in Thailand. Galangal is an important aromatic plant and widely cultivated in Thailand as well as in East Bengal, South India and Southeast Asia (Rangsiruji *et al.*, 2000; Matsuda *et al.*, 2003b).

The aromatic rhizome of galangal is used as a condiment or spice for many dishes and also as an ingredient in traditional medicine (Dastur, 1962; Umaro *et al.*, 1983; Matsuda *et al.*, 2003b; Khattak *et al.*, 2005). The one of principal ingredients is 1′-acetoxychavicol acetate (ACA) in galangal rhizome. Some studies on galangal revealed the activities of ACA in anti-tumor (Qureshi *et al.*, 1992; Kondo *et al.*, 1993; Moffatt *et al.*, 2000; Murakami *et al.*, 2000; Zheng *et al.*, 2002), anti-oxidants (Kubota *et al.*, 2001;

Juntachote and Berghofer, 2005), anti-inflammatory (Nakamura *et al.*, 1998), anti-allergic (Matsuda *et al.*, 2003a), anti-gastric ulcer (Matsuda *et al.*, 2003b) and anti-fungal (Janssen and Scheffer, 1985; Itokawa *et al.*, 1987; Haraguchi *et al.*, 1996; Jariyanusorn, 2002; Ficker *et al.*, 2003; Khattak *et al.*, 2005).

Galangal is asexually propagated using rhizomes, as in the conventional cultivation. Farmers grow galangal landraces and select rhizomes with good yield and taste for next cultivation by themselves. Bad taste rhizomes are neglected in the home-garden or forest as wild. There is no developed cultivar in Thailand as well as in other countries.

As described above, studies on such ingredient of galangal had been reported, however, there was little information about genetic relationship of galangal available for systematic breeding. Therefore, the objective of the present study is to investigate genetic relationship of galangals collected from different regions in Thailand by cluster analysis based on RAPDs. In addition, ACA content, one of the important characters of galangal, was investigated in relation to genetic relationship among accessions in Thailand from standpoint of breeding of high ACA content cultivar.

1.2. Materials and Methods

1.2.1. Plant materials

Thirty-seven accessions of galangal were collected from Northern, Central, Eastern and Northeastern regions of Thailand (Table 1-1). The accessions were classified into the cultivated type 30 accessions and the wild type 7 accessions. For cultivated type, the galangal was grown as a crop for selling or eating by farmers. The wild ones were not used as spice because of their bad taste and were neglected in the home-garden or forest. Galangals were grown and examined growth characters in the Farm of Chiang Mai University, Chiang Mai, Thailand.

Table 1-1. Accessions of galangal collected from different regions in Thailand and their rhizome characters

Accession	Local name	District*[1]	Type*[2]	Rhizome		ACA*[4] (% w/dw)
				Skin color	Size*[3]	
1	KhaDang	N	Cult	Red	M	1.7
2	KhaDang	N	Cult	Red	M	0.0
3	KhaDang	N	Cult	Red	M	1.9
4	KhaDang	N	Cult	Red	M	3.8
5	KhaYuek	N	Cult	White	L	1.7
6	KhaYuek	N	Cult	White	L	1.5
7	KhaYuek	N	Cult	White	M	0.0
8	KhaYuek	N	Cult	White	M	2.5
9	KhaTadang	C	Cult	Red	L	0.0
10	Kha	C	Cult	Red	L	3.2
11	KhaDang	C	Cult	Red	M	4.2
12	KhaTadang	C	Cult	Red	M	5.4
13	Kha	C	Cult	Red	M	5.0
14	KhaKaw	C	Cult	Red	M	7.5
15	Kha	C	Cult	Red	M	4.7
16	KhaDang	C	Cult	Red	M	5.4
17	KhaTadang	C	Cult	Red	M	4.0
18	KhaNol	C	Cult	Red	M	4.6
19	KhaTadang	C	Cult	Red	M	3.0
20	KhaTadang	C	Cult	Red	M	5.2
21	KhaDang	C	Cult	Red	M	4.1
22	KhaDang	C	Cult	Red	M	4.4
23	KhaYuek	C	Cult	White	L	2.1
24	KhaYuek	C	Cult	White	L	2.1
25	KhaYai	C	Cult	White	L	0.0
26	KhaYai	C	Cult	White	L	2.9
27	KhaLuang	C	Cult	White	S	6.1
28	KhaNoldang	E	Cult	Red	L	4.0
29	Kha	E	Cult	Red	M	6.4
30	KhaDang	E	Cult	Red	M	2.6
W1	KhaPar	N	Wild	Red	M	0.0
W2	KhaSaku	N	Wild	Red	S	0.0
W3	KhaLeang	N	Wild	Yellow	M	0.0
W4	KhaLing	C	Wild	Red	M	1.5
W5	KhaLeang	C	Wild	Yellow	M	0.0
W6	KhaKut	E	Wild	White	L	5.1
W7	KhaLing	Ne	Wild	Red	M	6.2

*[1] Refer to Fig.1-2. N: Northern, C: Central, E: Eastern, Ne: Northeastern
*[2] Cult: Cultivated type, Wild: Wild type
*[3] Diameter of rhizome: $S < 2$ cm $\leq M < 4$ cm $\leq L$
*[4] ACA: 1′- acetoxychavicol acetate

1.2.2. DNA extraction and RAPD analysis

DNA was extracted from young leaves of each accession using the SDS extraction procedure (Kuntapanom and Ikeda, 1998). For RAPD analysis, 10-mer random primers (Operon Technologies) were used to detect polymorphisms among galangal accessions.

The total volume of each PCR was 20 μL consisting of 1x reaction buffer (QIAGEN), 2 mM $MgCl_2$, 150 μM dNTPs, 40 ng of each primer, 1 U *Taq* DNA polymerase (QIAGEN), and 30 ng template DNA. PCR was carried out in a thermal cycler: GeneAmp PCR system (MJ Research, PTC 100). The PCR condition was as follows. First step: 2 cycles of 1 min at 93°C, 1 min at 34°C, 2 min at 72°C and 2 cycles of 1 min at 93°C, 1 min at 36°C, 2 min at 72°C. Second step: 36 cycles of 1 min at 93°C, 1 min at 37°C, 2 min at 72°C. Final step: 5 min at 72°C and holding indefinitely at 4 °C. The PCR products were separated by 2% agarose gel with TAE buffer. Gels were stained with ethidium bromide. RAPD bands were manually scored 1 for presence or 0 for absence. The data matrices were analyzed by the similarity for qualitative data (SIMQUAL) program of NTSYS-pc Version 1.8, and similarities between accessions were estimated using the Jaccard's Coefficient. A dendrogram was constructed using the unweighted pair-group method with arithmetic mean (UPGMA) method. Correlations between the genetic and geographic matrices were assessed using Mantel's test (Mantel, 1967).

1.2.3. ACA quantitative analysis

Quantitative analysis of ACA was carried out by Thin Layer Chromatography (TLC) method (Jariyanusorn, 2002). The samples were extracted from rhizome of each galangal accessions harvested in the Farm of Chiang Mai University. The data of ACA content was subjected to analysis of variance with a level of significance of 0.01.

1.3. Results and Discussion

Out of 22 RAPD primers used in this study, eight primers (OPA20, OPB18, OPC09, OPD02, OPD11, OPG13, OPK12 and OPAX17) amplified 73 polymorphic RAPD bands. A dendrogram constructed by UPGMA method using RAPD data clustered the accessions into 5 major groups (Fig. 1-1). The largest cluster II consisted of 17 accessions. The second large cluster I consisted of 13 accessions. The other three clusters consisted of two or three accessions.

Although galangal is classified by color or size of rhizome, there was no relation between the cluster and morphological characters in color or size of rhizome (Table 1-1, Fig. 1-1). There was also no relation with the type of galangal. On the other hand, major cluster had relations with their collection sites. As for the dendrogram, large cluster I and II grouped accessions together by their collection site and ACA contents.

According to collection sites of galangal, two of five major clusters corresponded to three regions of Thailand with the exception of some accessions. Cluster II included 13 accessions collected from central, 1 accession from northern and 3 accessions from eastern district. By here, district is administrative division and collection sites of 3 eastern accessions are geographically near to the border of central and eastern district (Fig. 1-2). These accessions (28, 29 and w6) clustered with central accessions. Also, cluster I included 9 and 4 accessions from northern and central district, respectively. These 4 accessions were collected from the villages near to the northern border of central district. Hence, it is assumed that cluster I and II correspond with geographical distribution of accessions. This assumption is supported with correlation coefficient ($r = 0.25$, $p < 0.001$) between the RAPD and geographic distance matrices by Mantel's test (Mantel, 1967). Sugiyama *et al.* (2006) suggested that the presence of regional clustering based on AFLP

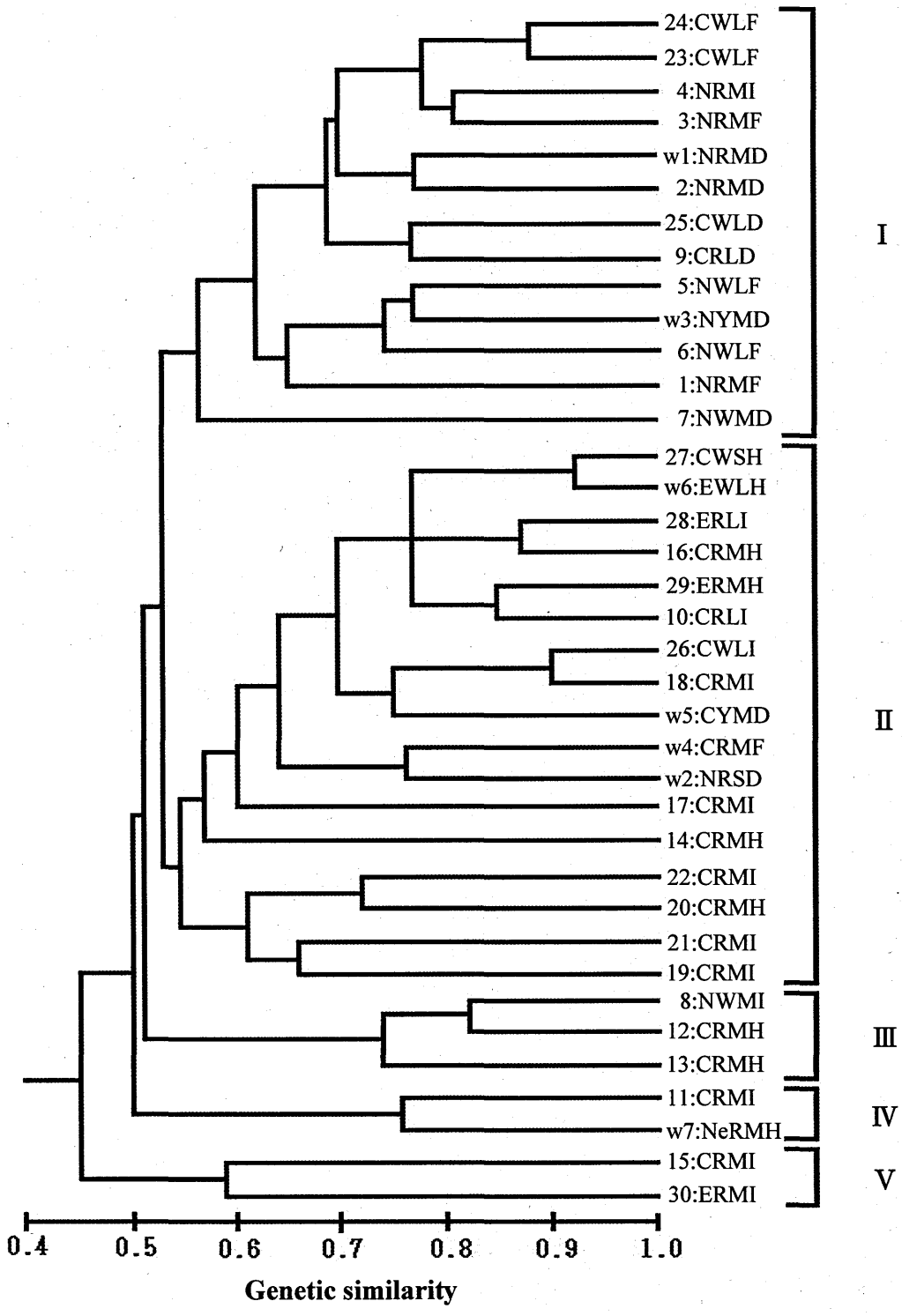

Fig. 1-1. UPGMA dendrogram of 37 galangal accessions from Thailand based on RAPD data. Abbreviations tagged on accession represent district (N: Northern, C: Central, E: Eastern, Ne: Northeastern), skin color (R: red, W: White, Y: Yellow), size of rhizome (S < 2 cm ≤ M < 4 cm ≤ L) and ACA content (D = 0 % < F < 2.5 % ≤ I < 5.0 % ≤ H).

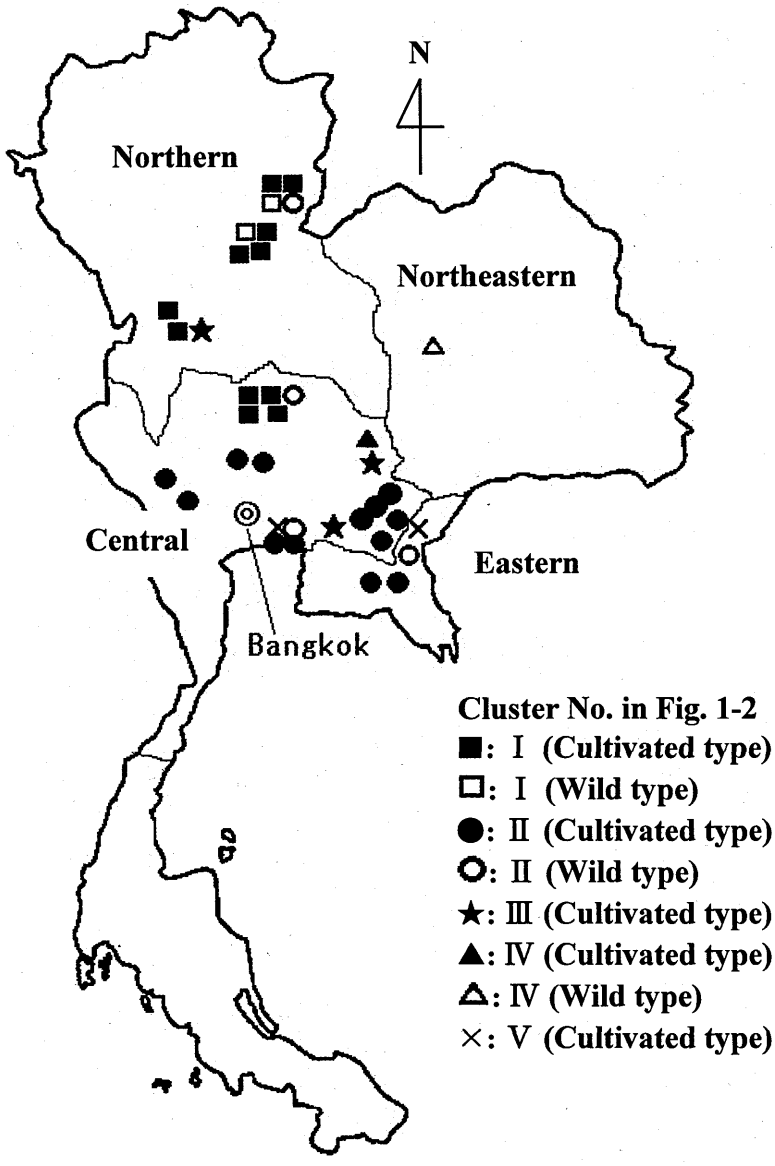

Fig. 1-2. The relationships between collection sites and clustering of galangal accessions in Thailand.

markers in elephant foot yam (*Amorphophallus paeoniifolius*) of Java, a vegetative propagation species same as galangal.

As for ACA content, 37 galangal accessions showed a wide variation ranging from the highest (7.5 %w/dw) for accession 14 to the lowest (0 %w/dw) for eight accessions (Table 1-1). Most of high ACA content accessions clustered together in cluster II such as accession 14, 16, 27 and 29. The accessions in cluster I had ACA contents significantly lower than ones in cluster II ($p < 0.01$). Furthermore, ACA non-detectable (0%w/dw) accessions, w1 and 2, 25 and 9 clustered together (Fig. 1-1). This indicates that polymorphic RAPD bands related with ACA content may exist in amplified 73 polymorphic RAPD bands used for dendrogram construction. In fact, some RAPD bands, probably linked to ACA content were observed as follows: the 450 bp (OPB18), 800 bp (OPC09), and 450 bp and 550 bp (OPAX17) fragments were specific in the high ACA accessions (Fig. 1-3). While, 800 bp (OPK12) fragment was present in the low ACA accessions. These fragments may be potential RAPD markers for screening ACA content and will be available in marker-assisted selection (MAS) of high ACA content galangal breeding.

Wild type accessions did not make an independent cluster. Four of seven wild accessions (w2, w4, w5 and w6) grouped together in cluster II, but the other wild accessions spread over cluster I and IV (Fig. 1-1). This result illustrates that cultivated type accessions had been selected by farmers from wild type accessions, and that wild accessions are useful source of genetic variation. For example, wild type accessions (w6 and w7) having high ACA content are available as breeding materials for high ACA content cultivar.

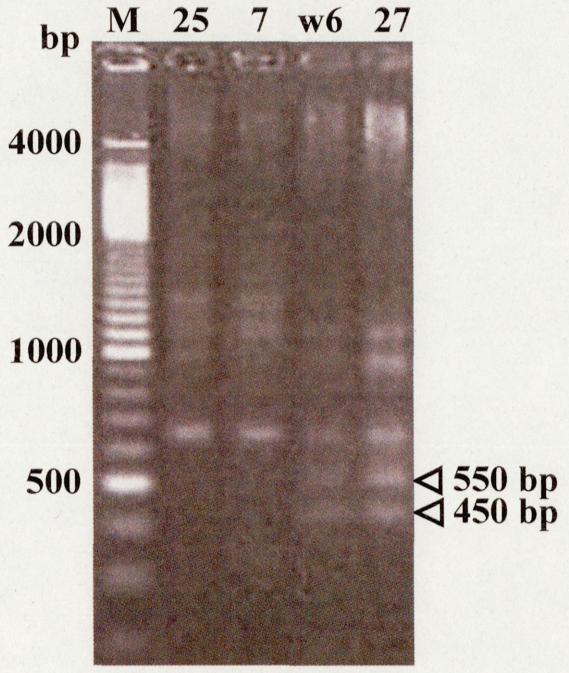

Fig. 1-3. Some specific band linked to ACA content in galangal using RAPD primer OPAX17. Number of accession refers to Table 1-1.

(M: 100 bp DNA Step Ladder size marker.)

Therefore, the genetic relationship of galangal collected in different areas of Thailand was observed. It is noteworthy that the clustering of galangal was related to the collection site in each galangal concerning with ACA content. RAPD markers were used to detect genetic relationships in galangal and provide a useful means of differential genetic structures within galangal. The highly informative primers identified in this study could be available for genetics and breeding in galangal further.

1.4. Abstract

The genetic relationship of galangal (*Alpinia galanga* Willd.) accessions collected from Northern, Central, Eastern, and Northeastern districts of Thailand was investigated by cluster analysis based on random amplified polymorphic DNAs (RAPD). In addition, RAPD marker linked to 1′- acetoxychavicol acetate (ACA) content, one of the most important characters of galangal, was explored with a view to breeding high ACA content cultivar. Eight 10-mer random primers amplified 73 polymorphic RAPD bands and a dendrogram constructed by UPGMA method clustered 30 cultivated and 7 wild accessions into 5 major clusters. Large cluster I and II corresponded with geographical distribution and ACA content. The cluster I and cluster II mainly consisted of accessions from Northern and Central district, respectively. Although a wide variation of ACA content (0-7.5 %w/dw) was observed, most of high ACA content accessions clustered together in cluster II and accessions in cluster I had significantly lower ACA contents than ones in cluster II. Four RAPD bands specific in the high ACA accessions were found: 450 bp (OPB18), 800 bp (OPC09), and 450 bp and 550 bp (OPAX17). These fragments are potential DNA markers for screening high ACA content accessions. Wild accessions did not make an independent cluster and spread over 3 clusters with a wide variation of ACA

content same as cultivated ones. Wild accessions having high ACA content are available as breeding materials for high ACA content cultivar.

Chapter Ⅲ

Genetic and Breeding Studies on Functional Components of Chili Pepper

Experiment 2

Inheritance of Few-pungent Trait in Chili Pepper 'S3212' (*Capsicum frutescens*)

2.1. Introduction

Capsaicinoids are strongly pungent alkaloids that accumulate in the placenta of chili pepper (*Capsicum* spp.) fruits (Fujiwake *et al.*, 1982; Suzuki *et al.*, 1980). In general, chili peppers have two types for pungency. One is pungent chili pepper that can produce capsaicinoids, and another is non-pungent chili pepper with a complete lack of pungency (Minamiyama *et al.*, 2005).

Some studies on non-pungent chili peppers have been carried out and revealed that capsaicinoid production is controlled by a single gene called *C* gene at the *C* locus. The dominant allele *C* at the *C* locus is essential for capsaicinoid production. The homozygous recessive condition, *cc*, results in a complete lack of ability to synthesize capsaicinoids (Blum *et al.*, 2002; Daskalov and Poulos, 1994) and this recessive *c* gene is found only in *C. annuum*. To date, the *C* locus has been mapped on chromosome 2 (Deshpande, 1935; Greenleaf, 1952; Ben-Chaim *et al.*, 2001; Blum *et al.*, 2002).

In pungent chili peppers with the *C* gene, considerable variation of pungency exists due to their capsaicinoid contents. Since some chili pepper cultivars such as 'Manganji' or 'Shishitou' with the *C* gene show very low capsaicinoid contents, they are treated as vegetables, while high pungent cultivars such as 'Takanotsume' are used as spices.

Hereby, chili peppers that have dominant *C* gene had the wide range of capsaicinoid variability from the chili pepper treated as vegetable for low capsaicinoid contents to spice for high capsaicinoid contents.

As described above, capsaicinoid production is controlled by a single dominant *C* gene, but capsaicinoid content is a quantitatively inherited trait (Zewdie and Bosland, 2000a, 2000b) controlled by polygene in quantitative trait locus (QTL).

Several studies have focused on this pungent trait. Blum *et al.* (2003) found a major QTL for capsaicinoid content, termed *cap*, on chromosome 7. Ben-Chaim *et al.* (2006) also reported that six QTLs on three chromosomes controlled capsaicinoid content in *Capsicum*.

Chili pepper with low or no capsaicinoid content have been observed only in *C. annuum*, while in *C. frutescens*, only highly pungent cultivars are known such as 'Phrikkhiinuu' in Thailand or 'Shima-tougarashi' in Japan. There is no report on non-pungent chili pepper in this species; however, 'S3212', an accession belonging to *C. frutescens*, showed very low capsaicinoid contents, namely few-pungency in the fruits (Matsushima *et al.*, 2004; Konisho *et al.*, 2005a). This few-pungent trait is novel and unique to 'S3212' in *C. frutescens*.

Therefore, the objective of this study is to analyze the inheritance of the few-pungent trait in *C. frutescens*. At first, segregation of capsaicinoid contents was observed in populations derived from an interspecific hybrid between 'S3212' and 'S3010', a pungent accession of *C. chinense*. Then, an isolocus test between the few-pungent gene locus with the *C* locus, a single major gene locus controlling capsaicinoid production, was carried out using a cleaved amplified polymorphic sequence (CAPS) marker. In addition, random amplified polymorphic DNA (RAPD) marker linked to the few-pungent trait in these chili peppers was explored by bulked segregant analysis (Michelmore *et al.*, 1991).

2.2. Materials and Methods

2.2.1. Plant materials

The materials used in this study were a very high pungent accession of *C. chinense* 'S3010' (P_1), a few-pungent accession of *C. frutescens* 'S3212' (P_2), F_1 ($P_2 \times P_1$), F_2 ($P_2 \times P_1$) and BC_1s (BCP_1 and BCP_2) (Table 1). Both parents were diploid species. All seeds produced by hand pollination with bagging were sown and seedlings were grown under field conditions in 2005 in an experimental field of the Faculty of Agriculture, Shinshu University (Minamiminowa, Nagano, Japan).

In addition, 6 chili pepper lines; 'Ace' (*C. annuum*), 'Wonderbell' (*C. annuum*), 'Takanotsume' (*C. annuum*), 'T-1' Phrikkhiinuu (*C. frutescens*), 'White habanero' (*C. chinense*), and 'Rocoto' (*C. pubescens*) were examined for PCR polymorphism of CAPS marker comparatively with previous study (Minamiyama *et al.*, 2005).

2.2.2. Capsaicinoid content measurement

For quantification of capsaicinoid content, 5 to 22 fruits from each individual plant were harvested when half of each fruit had turned to red from September 19 to October 6, 2005. Capsaicinoid content was measured as the total amount of capsaicin, dihydrocapsaicin, and nordihydrocapsaicin using high performance liquid chromatography (HPLC) according to Minami *et al.* (1998). The measuring conditions are indicated in Table 2-1.

2.2.3. DNA extraction

Total DNA was extracted from young leaves using an Automatic Nucleic Acids Extractor (CompacBio MX-16, COMPACBIO SCIENCES, Japan).

Table 2-1. HPLC analysis (Minami *et al.*, 1998).

Analyzed system	Shimadzu class VP
Column	YMC-Pack ODS-A, S-5 μm, 12 nm, 75x4.6mm
Guard column	YMC-Guardpack ODS-A, S-5 μm, 12 nm, 10x4.0mm
Mobile phase	Methanol : H_2O = 6 : 4 (v : v)
Sample solution	5 μl
Flow rate	$ml \cdot min^{-1}$
Temperature	40 °c
UV detector	280 nm

2.2.4. CAPS analysis

CAPS analyses were carried out according to Minamiyama *et al.* (2005). Two specific primers, a forward primer of 24 bp SCY-800FW, 5′-CCATGGATT GTTGCTCGGGCCTCC-3′, and a reverse primer of 23 bp SCY-800RV, 5′-CCGTA CCGCCCCATTGCGATTCC-3′, were used in parent lines, F_2 population and 6 chili pepper lines. Amplifications were performed using a standard PCR reaction. The PCR reaction mixture consisted of 12 ng genomic DNA, 0.21 μM of each primer, 1x PCR buffer (Takara Bio, Japan), 200 μM each of dNTPs and 0.5 units of *Ex Taq*™ DNA polymerase (Takara Bio, Japan) in a total reaction volume of 12 μL. PCR amplifications were performed in a BIO-RAD i Cycler (BIO-RAD, Japan) with the following conditions: initial 3 min pre-denaturation at 94°C, 30 cycles of 1 min denaturation at 94°C, 2 min annealing at 65°C and 2 min extension at 72°C, and a final extension for 5 min at 72°C. The amplified fragments were digested in a final volume of 14 μL that contained 12 μL of the PCR reaction and 1 unit of restriction endonuclease (*Mbo*I). The digests were resolved by electrophoresis in 13% acrylamide gel and visualized on a UV transilluminator after staining with ethidium bromide.

2.2.5. RAPD analysis

DNA samples from seven pungent and seven few-pungent F_2 individuals were pooled respectively and used as a template for detecting RAPD in bulked segregant analysis (Michelmore *et al.*, 1991).

PCR was carried out using a 12 μL volume containing 0.5 units of *Taq* DNA polymerase (Takara Bio, Japan), 1x buffer (Takara Bio, Japan), 200 μM each of dNTPs, 0.42 μM arbitrary primer, and 12 ng genomic DNA. Two hundred and eighty-eight 10-mer arbitrary primers (Operon Technologies, USA) were used to screen the amplified products.

DNA amplifications were performed using a BIO-RAD i Cycler (BIO-RAD, Japan) with the following conditions: one step of 3 min at 94 °C, then 40 cycles of 1 min at 94 °C, 2 min at 40 °C, 2 min at 72 °C, and a final step of 5 min at 72 °C. The amplification products were resolved by electrophoresis in 1.7% agarose gel and visualized on a UV transilluminator after staining with ethidium bromide.

2.3. Results and Discussion

2.3.1. Capsaicinoid contents and segregation of pungency

C. chinense 'S3010' (P_1) which was a pungent chili pepper had the range of capsaicinoid contents from the highest 52,992 $\mu g \cdot g^{-1}$DW to the lowest 33,001 $\mu g \cdot g^{-1}$DW. The average was 44,581 $\mu g \cdot g^{-1}$DW. *C. frutescens* 'S3212' (P_2) showed their range of capsaicinoid contents from the highest to lowest in 14 to 0 $\mu g \cdot g^{-1}$DW. The average was 5 $\mu g \cdot g^{-1}$DW. There are no plants that were complete lack of capsaicinoid, except for one individual.

In the F_1 population, the highest capsaicinoid content was 5,863 $\mu g \cdot g^{-1}$DW and the lowest was 4,220 $\mu g \cdot g^{-1}$DW. The average was 4,967 $\mu g \cdot g^{-1}$DW that they had been closely to few-pungent 'S3212' (P_2).

In the F_2 population, 134 plants were measured for capsaicinoid contents. For capsaicinoid contents, F_2 population distributed closer to few-pungent parent 'S3212' (P_2) than to pungent parent 'S3010' (P_1) with a wide range from the highest 20,547 $\mu g \cdot g^{-1}$DW to the lowest 5 $\mu g \cdot g^{-1}$DW (Fig. 2-1A). And there was no plant that had complete non-pungency. This F_2 population separated into two groups by the capsaicinoid contents. One was the few-pungent group (capsaicinoid content is less than 100 $\mu g \cdot g^{-1}$DW) and another was the pungent group (capsaicinoid content is more than 100 $\mu g \cdot g^{-1}$DW). The range of the pungent group was 1,963 to 20,547 $\mu g \cdot g^{-1}$DW, while the few-pungent group was 5 to 77

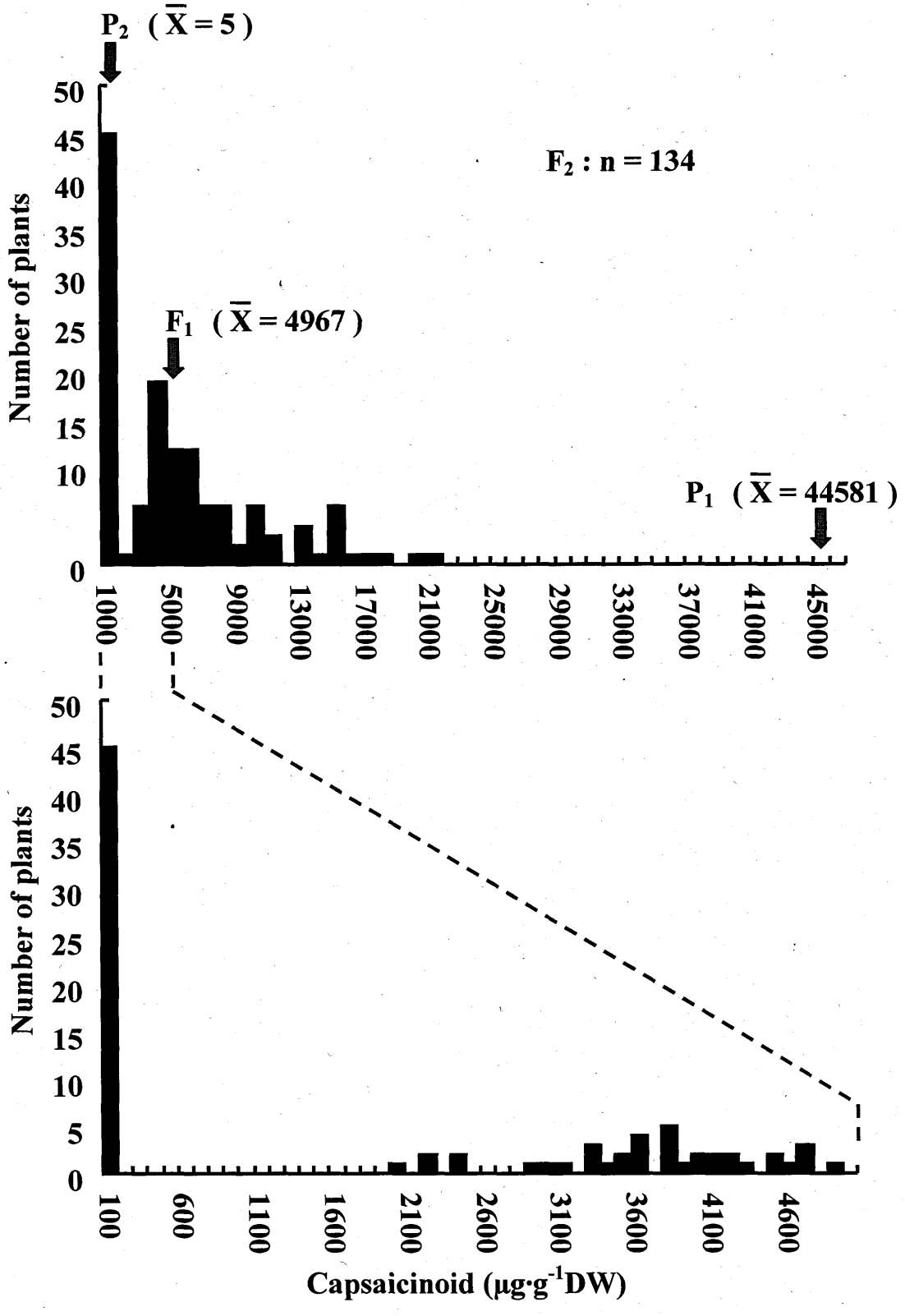

Fig. 2-1. Frequency distribution of capsaicinoid contents in F_2 population of *C. frutescens* 'S3212' (P_2) × *C. chinense* 'S3010' (P_1) (A: above) and focus of frequency distribution in the scale of capsaicinoid contents from 0 to 5,000 $\mu g \cdot g^{-1}$DW(B: below).

$\mu g \cdot g^{-1}$DW (Fig. 2-1B). It showed the blank between the pungent and few-pungent group clearly.

In backcross populations, all BCP_1 plants were pungent, ranging from 4,211 to 36,526 $\mu g \cdot g^{-1}$DW (Fig. 2-2A), while the BCP_2 population separated into 2 clear groups: a pungent group (1,009 to 2,974 $\mu g \cdot g^{-1}$DW), and a few-pungent group (13 to 45 $\mu g \cdot g^{-1}$DW). It showed the blank between the pungent and few-pungent group clearly (Fig. 2-2B).

According to chi-square tests, segregation ratio of two groups in the BCP_2 population fitted significantly to the expected 1 : 1 ratio ($\chi^2 = 2.778$, $0.10 > P > 0.05$, Table 2-2) while, the F_2 population did not conform to the expected 3 : 1 ratio ($\chi^2 = 5.264$, $0.05 > P > 0.01$, Table 2-2). However, the results indicate that few-pungency is controlled by a single recessive gene. The deviation from the expected segregation ratio 3:1 at $P = 0.05$ in the F_2 population might be caused by environmental effects (Harvell and Bosland, 1997; Zewdie and Bosland, 2000b) or the effect of minor genes (Ben-Chaim *et al.*, 2006; Blum *et al.*, 2003). In additions, with respect to the result of F_1 or F_2 populations that they had been closely to the few-pungent 'S3212' (P_2), it maybe caused from the other minor genes which have regulated to control the high contents of pungency from 'S3010' (P_1) were inhibited or no expression in these populations.

Owing to there was no individual that had complete lack of capsaicinoid, the gene controls few-pungent trait is different from *c* gene that controls complete lack of pungency in *C. annuum*. Therefore, we designated the recessive gene controlling few- pungency as the *cf* gene in this study.

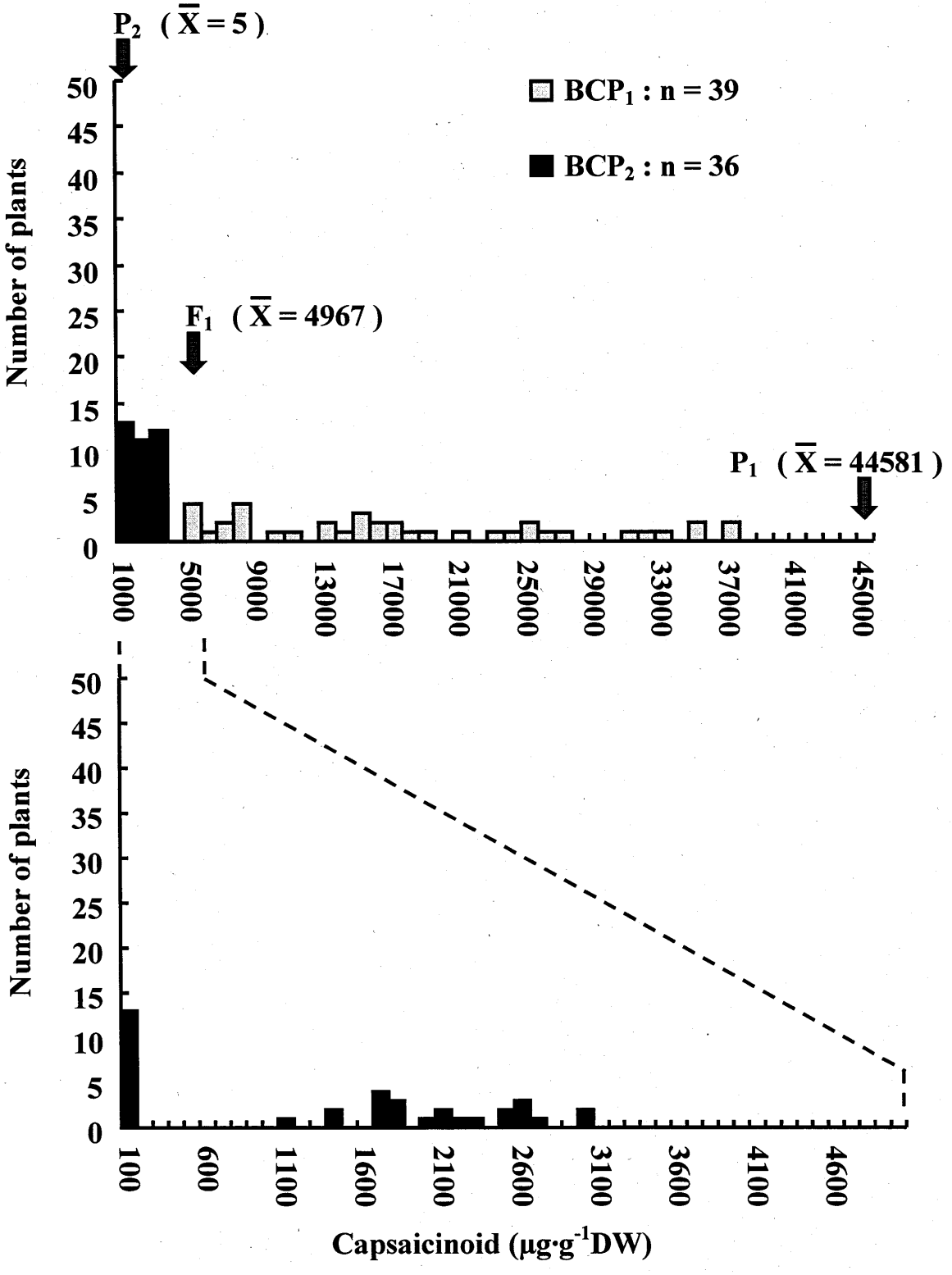

Fig. 2-2. Frequency distribution of capsaicinoid contents in BCP_s population of *C. frutescens* 'S3212' (P_2) × *C. chinense* 'S3010' (P_1) (A: above) and frequency distribution of capsaicinoid contents in BCP_2 population (B: below).

Table 2-2. The segregation of capsaicinoid contents in the populations of *C. frutescens* 'S3212' (P_2) x *C. chinense* 'S3010' (P_1).

Population	Number of plants		Goodness of fit		
	<100 $\mu g \cdot g^{-1}DW$	>100 $\mu g \cdot g^{-1}DW$	Ratio	χ^2	*P*
P_1	0	13			
P_2	16	0			
F_1	0	37			
F_2	45	89	1 : 3	5.26	0.05-0.01
BCP_1	0	39			
BCP_2	13	23	1 : 1	2.78	0.10-0.05

2.3.2. DNA extraction

An example of extracted DNA using Automatic Nucleic Acids Extractor (CompacBio MX-16, COMPACBIO SCIENCES, Japan) was presented in Fig. 2-3.

2.3.3. Isolocus test between *cf* locus and *C* locus using CAPS marker

The *C* locus on chromosome 2 is known as the only locus qualitatively controlling capsaicinoid production and a recessive *c* gene has been reported only in *C. annuum*. Therefore, in the present study, an isolocus test between *cf* locus and *C* locus was carried out using CAPS marker SCY-800 linked to the *C* locus (Minamiyama *et al.*, 2005).

2.3.3.1. Evaluation of CAPS pattern in parent lines of an interspecific hybrid (*C. frutescens* x *C. chinense*)

To evaluate whether the CAPS patterns are different in each parent lines, a CAPS marker was examined. The CAPS patterns have to be different between P_1 (*C. chinense* 'S3030') and P_2 (*C. frutescens* 'S3212) that would be available for segregation analysis in F_2 population. In addition, 6 chili pepper lines also were examined for pattern of CAPS marker comparatively with previous study (Minamiyama *et al.*, 2005).

Using this CAPS marker, a common single fragment of 800 bp, designated SCY-800 was amplified from genomic DNA of each line and polymorphic restriction sites within the amplified fragment were identified by digestion with *Mbo*I.

As a result, the digestion of fragment SCY-800 from 6 chili pepper lines by *Mbo*I showed the different pattern in each species (Table 2-3, Fig. 2-4). Three lines of *C. annuum* showed in 4 fragments, 135, 170, 230, and 240 bp, as in CAPS pattern 'A'. *C. frutescens* showed three fragments, 175, 240, and 350 bp, as in CAPS pattern 'E', also *C. chinense* gave three fragments, 155, 230, and 300 bp, as in CAPS pattern 'D'.

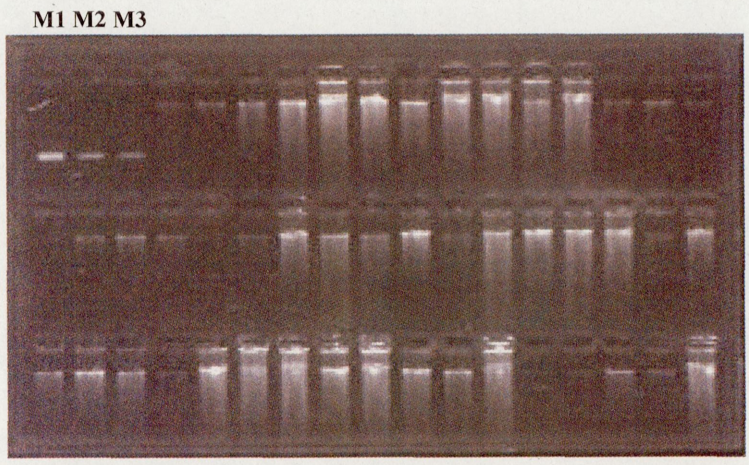

Fig. 2-3. An example of extracted DNA of F_2 population using Automatic Nucleic Acids Extractor (CompacBio MX-16, COMPACBIO SCIENCES, Japan).

M: DNA Quantitative Standard marker (M1; M2; M3: DNA concentration 160 ng; 80 ng; 40 ng, respectively).

Table 2-3. Pungency traits and CAPS patterns among 8 chili pepper lines of *Capsicum* spp.

Lines	Species	Pungency	CAPS patterns[Z] (SCY-800 + *Mbo*I)
Ace	*C. annuum*	Non-pungent	A
Wonderbell	*C. annuum*	Non-pungent	A
Takanotsume	*C. annuum*	Pungent	A
S3212	*C. frutescens*	Few-pungent	E
T-1 Phrikkhiinuu	*C. frutescens*	Pungent	E
S3010	*C. chinense*	Pungent	D
White habanero	*C. chinense*	Pungent	D
Rocoto	*C. pubescens*	Pungent	-

[Z] The same letter shows that the CAPS pattern is the same; - is not amplified.

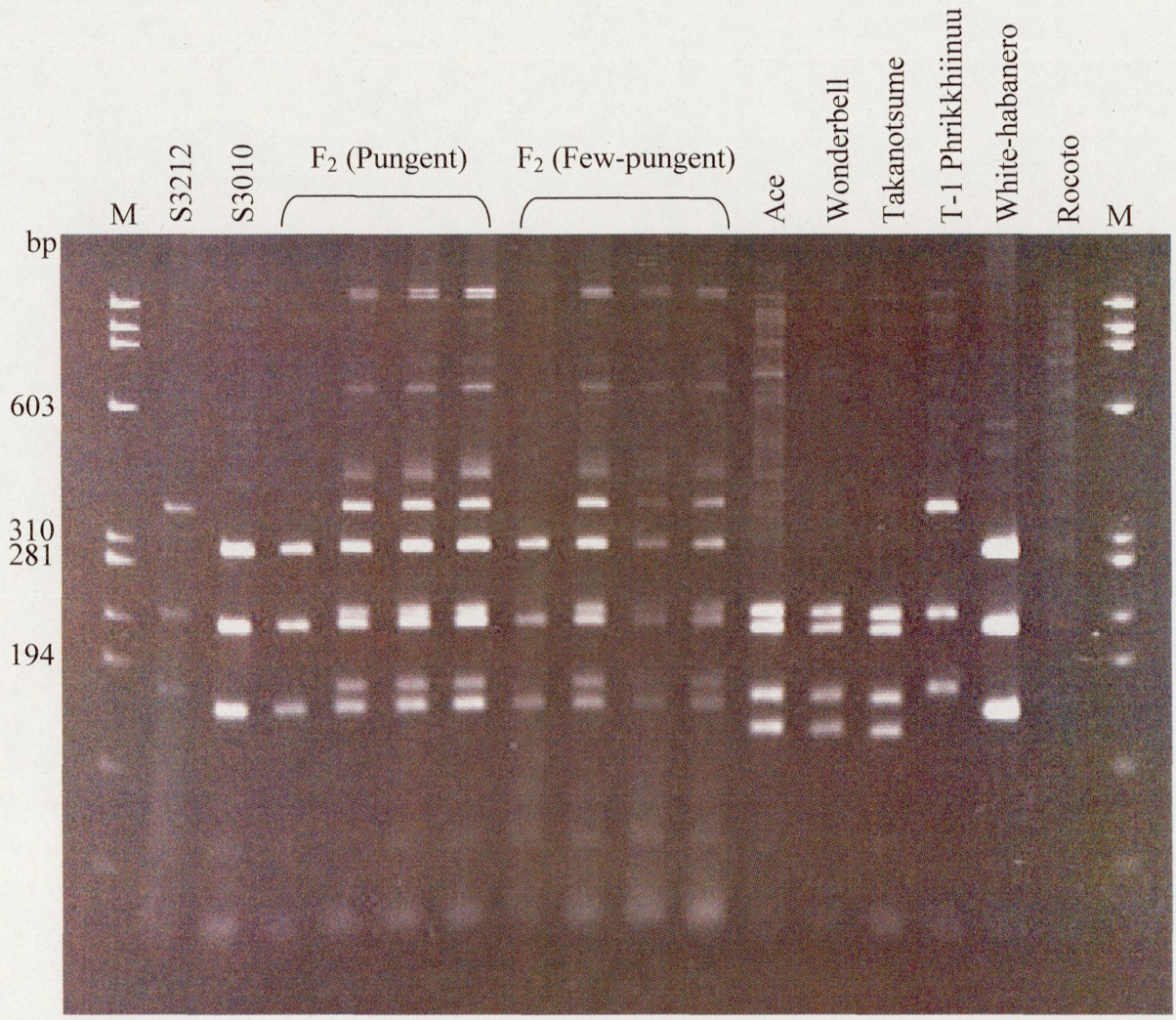

Fig. 2-4. Segregation of codominant CAPS marker linked to the *C* locus. PCR with specific primers was followed by restriction with *Mbo*I.

M: molecular-weight marker (∅X-174 digested with *Hae*III).

They showed completely different pattern in each species. All of chili pepper lines in this study showed the same result with the previous study as described by Minamiyama *et al.* (2005). Although, the SCY-800 fragment was not amplified in a line of *C. pubescens* that this result may be also similar to *C. chacoense* in that previous study, it confirmed the SCY-800 CAPS pattern was clear and available for this study. This CAPS marker was linked to the *C* locus for pungency in *Capsicum*.

For the parent lines in this study, S3212 (*C. frutescens*) and S3010 (*C. chinense*) also showed the CAPS pattern of each species in 'E' and 'D', respectively (Table 2-3, Fig. 2-4). These parent lines showed different CAPS patterns clearly. So, this SCY-800 CAPS marker was available to investigate segregation of the CAPS pattern linked to the *C* gene in F_2 population.

2.3.3.2. Analysis of the *C* gene linked to pungency using CAPS marker

To investigate whether the segregated inheritance of *C* gene is appropriate in F_2 population, a CAPS marker was examined. The specific primer pair amplified a common fragment of 800 bp in both parents. A polymorphic restriction site within the amplified region was identified by digestion with *Mbo*I. The digestion of the SCY-800 fragment from 'S3212' by *Mbo*I showed in CAPS pattern 'E', and 'S3010', showed in CAPS pattern 'D'. In F_2 population, it showed CAPS patterns of either the 'S3212' type 'E' or 'S3010' type 'D'. And some plants were heterozygous that they were showed in both types 'D+E' (Fig. 2-4).

Segregation of the CAPS pattern fitted well to the expected D : D+E : E = 1 : 2 : 1 ratio (35 : 67 : 32, $\chi^2 = 0.13$, $P = 0.94$) because *C. frutescens* and *C. chinense* had close relation (Konisho *et al.*, 2005b) even if they belong to different species. So, this segregated inheritance of *C* gene is appropriate in F_2 population.

Analysis of relation between few-pungency linked to *cf* locus and CAPS pattern linked to *C* locus in F_2 individuals, it showed that the CAPS patterns were not related with the few-pungency in these chili peppers. If *cf* locus and *C* locus are isolocus, F_2 individuals with CAPS pattern 'E' are few-pungent and individuals with pattern 'D' or 'D+E' are pungent, but there was no relationship between few-pungency and CAPS pattern in the F_2 population (Fig. 2-5). It showed the recombination frequency in 42.0% (Table 2-4). This result illustrates that *cf* locus is different from *C* locus.

2.3.4. Identification of RAPD markers linked to *cf* locus in an interspecific hybrid (*C. frutescens x C. chinense*)

To find RAPD markers linked to pungency, trait as the allele of few-pungency for *cf* locus, bulked segregant analysis was conducted in the F_2 population using 288 RAPD primers. As a result, three bands were specifically amplified in pungent parent 'S3010' and the pungent bulk. The first band was a 350 bp fragment amplified by the OPD03 (5′-GTCGCCGTCA-3′) primer. The second band was a 400 bp fragment amplified by the OPD11 (5′-AGCGCCATTG-3′) primer. The third band was an 1100 bp fragment amplified by the OPQ09 (5′-GGCTAACCGA-3′) primer. These three bands were designated OPD03-350, OPD11-400 and OPQ09-1100 respectively.

Segregation of the three bands was examined in 134 individuals of the F_2 population. According to chi-square tests, the segregation of pungency and three RAPD markers did not deviate from the expected 3:1 ratio, but co-segregation of pungency and RAPD markers differed significantly from the expected 9:3:3:1 ratio of independent inheritance (Fig. 2-6A, B, C; Table 2-5). This indicates that these three RAPD markers link to *cf* locus and may be available as DNA markers for screening for few-pungency in these chili peppers. However, they showed recombination frequency of 22.6%, 10.3%, and

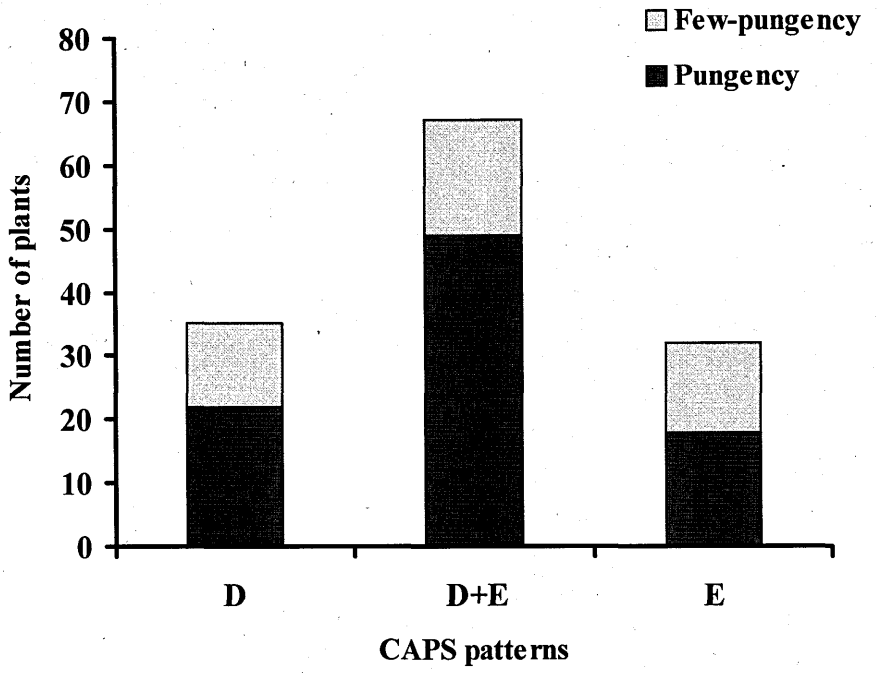

Fig. 2-5. Segregation of CAPS patterns (SCY-800) and few-pungency in F_2 population of *C. frutescens* 'S3212' (P_2) × *C. chinense* 'S3010' (P_1).

Table 2-4. Segregation of pungency and CAPS marker linked to *C* locus, and chi-square tests for fitting to 3:1 (χ^2A; χ^2B) and to 9:3:3:1 (χ^2AB) expected in F_2 population of *C. frutescens* 'S3212' (P_2) × *C. chinense* 'S3010'(P_1).

Locus		Number of plants observed [Z]				Chi-square values			Recombination frequency (%)
A	B	AB	Ab	aB	ab	χ^2A	χ^2B	χ^2AB	
Pungency	SCY-800	71	18	31	14	5.26**[Y]	0.09	7.43*	42.0

[Z] A, a: Pungency or few-pungency; B, b: Presence of specific band in co-dominant marker.

[Y] * and ** indicate significance at $P \leq 0.1$ and $P \leq 0.05$, respectively, by chi-square test.

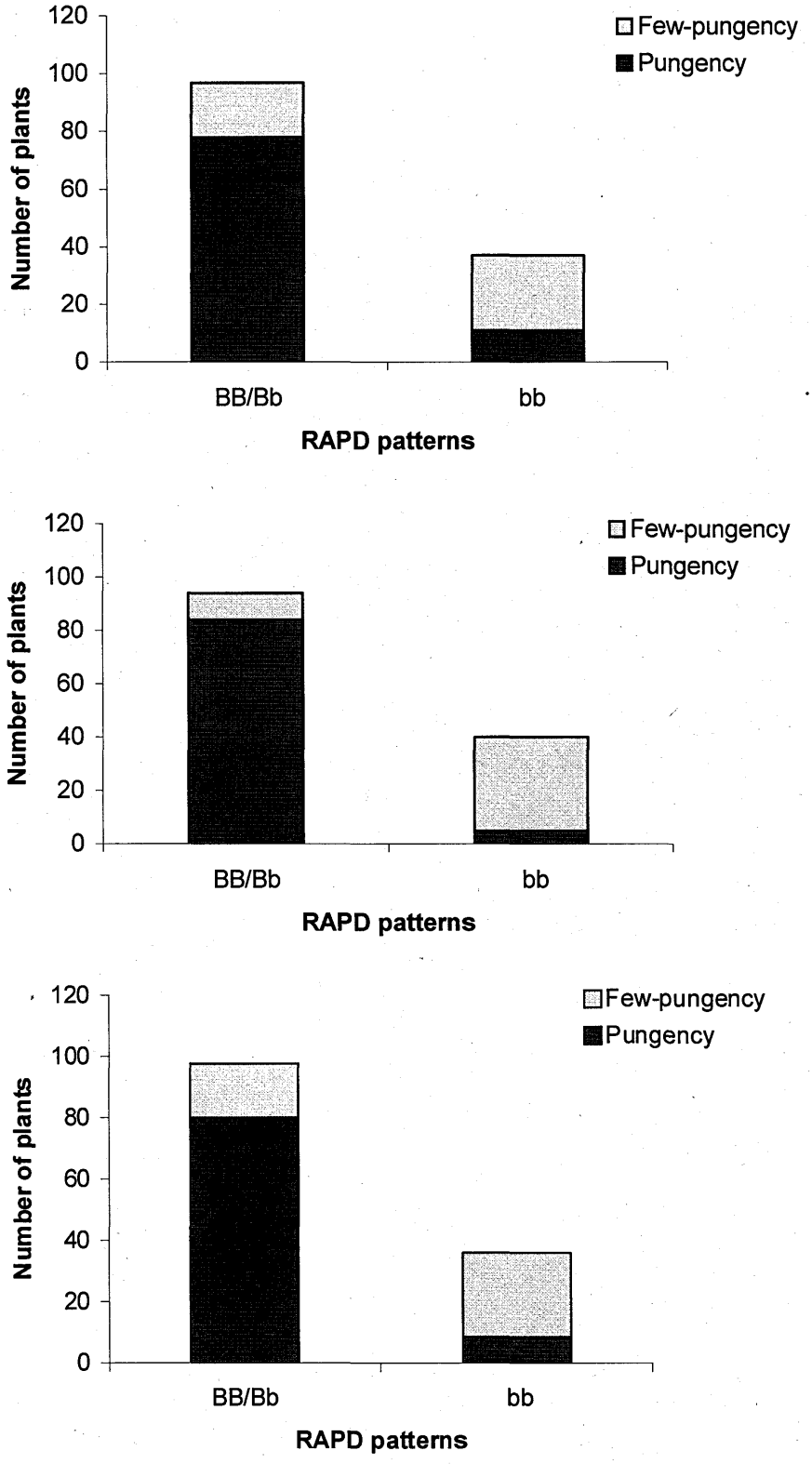

Fig. 2-6. Segregation of RAPD patterns and few-pungency in F_2 population of *C. frutescens* 'S3212' (P_2) × *C. chinense* 'S3010' (P_1) using RAPD marker; OPD03 (A: above), OPD11 (B: middle) and OPQ09 (C: below), respectively.

Table 2-5. Segregation of pungency and RAPD marker, and chi-square tests for fitting to 3:1 (χ^2A; χ^2B) and to 9:3:3:1 (χ^2AB) expected in F_2 population of *C. frutescens* 'S3212' (P_2) × *C. chinense* 'S3010' (P_1).

Locus		Number of plants observed [Z]				Chi-square values			Recombination frequency (%)
A	B	AB	Ab	aB	ab	χ^2A	χ^2B	χ^2AB	
Pungency	OPD03-350	78	11	19	26	5.26**[Y]	0.49	46.62***	22.6
	OPD11-400	84	5	10	35	5.26**	1.68	110.86***	10.3
	OPQ09-1100	80	9	18	27	5.26**	0.25	54.07***	19.8

[Z] A, a: Pungency or few-pungency; B, b: Presence or absence of RAPD marker.

[Y] ** and *** indicate significance at $P \leq 0.05$ and $P \leq 0.01$, respectively, by chi-square test.

19.8% in OPD03-350, OPD11-400, and OPQ09-1100 with few-pungency, respectively, indicating that these three RAPD markers had some linkage distance from *cf* locus in these chili peppers (Table 2-5); therefore, DNA markers more closely linked to *cf* locus have to be discovered.

Regarding to the segregation of pungency by HPLC data in F_2 population showed the ratio that was not fit to the expected ratio 3:1, that result might be caused to the recombination. On the other hand, F_2 population in this study was separated into the pungent group and few-pungent group at the range of 100-1,000 $\mu g \cdot g^{-1}$DW in capsaicinoid contents, because there were no individuals in this range. However, it has possibility that the lowest range of pungent group was not 1,000 $\mu g \cdot g^{-1}$DW but was 0 $\mu g \cdot g^{-1}$DW. In fact, in *C. annuum*, some of sweet varieties like 'Shishitou' and 'Manganji' are classified to pungent pepper genetically, in spite of little of pungency. In such as case, the few-pungent group in the F_2 population was including some individuals that did not have genotype of recessive *cf* gene, but have genotype of dominant pungent gene even if they were not so pungent. This may be also one of the reasons that the segregation of pungency by HPLC data in F_2 population was not fit with 3:1.

Therefore, these three RAPD markers confirmed the linkage to the *cf* locus in these chili peppers. It reinforced that the few-pungency in these chili peppers was controlled mainly by a single recessive gene, namely *cf* gene.

Knowledge of the inheritance of the few-pungent trait in few-pungent chili pepper 'S3212' (*C. frutescens*) in this study will facilitate further genetic analysis of the pungency trait in *Capsicum*, giving breeders more information about the development of new chili pepper cultivars such as the few-pungent chili pepper in *C. frutescens* or *C. chinense*. Quantitative characters such as the level of pungency controlled by polygenes are susceptible to the environmental effect. In *C. annuum*, it has been reported that fruits with

strong pungency are sometimes found in few-pungent vegetable-type chili pepper cultivars grown under stressed conditions such as low temperature or water deficiency (Estrada *et al.*, 1999; Harvell and Bosland, 1997). A single *cf* gene controlling few-pungency found in this study is expected to use also in *C. annuum* to develop new stable few-pungent cultivars. There will be good product as vegetable for consuming.

Based on the present study, more detailed mapping around the *cf* locus and investigation of some mechanism concerns with the few-pungent gene, molecular approach will be used further. The information will be useful to develop the co-dominant marker for marker-assisted selection (MAS) in breeding program.

2.4. Abstract

Capsicum frutescens is known as a highly pungent chili pepper species, but an accession of *C. frutescens* showed very low capsaicinoid content. This few-pungent trait is novel and unique to 'S3212' in *C. frutescens*. To investigate the inheritance of the few-pungent trait, segregation of capsaicinoid content in F_2 and backcross populations (BC_1) crossed with high pungent *C. chinense* 'S3010' were observed. The segregation ratio indicated that few-pungency was controlled by a single recessive gene designated as the *cf* gene. Isolocus test between *cf* locus and *C* locus controlling capsaicinoid production was carried out using cleaved amplified polymorphic sequence (CAPS) marker SCY-800 linked to the *C* locus. CAPS pattern and few-pungency were not related, so *cf* locus is different from *C* locus. Moreover, RAPD markers confirmed the linkage to the *cf* locus in these chili peppers. It reinforced that the few-pungency in these chili peppers was controlled mainly by a single recessive gene. The single *cf* gene controlling few-pungency found in this study is expected to be used to develop new cultivars stable in few-pungency.

Experiment 3

Genetic Analysis of Molecular Marker Linked to Few-pungent Gene in Chili Pepper (*Capsicum frutescens*)

3.1. Introduction

Of five domesticated chili pepper species, *Capsicum frutescens* has remarkably high pungency (IBPGR, 1983). However, 'S3212' showed very low capsaicinoid contents, namely few-pungency in the fruits (Matsushima *et al.*, 2004; Konisho *et al.*, 2005a). The few-pungent trait is unique to chili pepper 'S3212' (*Capsicum frutescens*) because all other *C. frutescens* accessions had pungent fruits with high capsaicinoid contents. This few-pungency was controlled by a single recessive gene (Saritnum *et al.*, 2006). The *c* gene locates in the *C* locus is controlled complete lack of pungency in *C. annuum*. However, the gene controls few-pungent trait, namely *cf*, is located differently from the *C* locus (Saritnum *et al.*, 2007).

Therefore, we have analyzed to the few-pungency gene, *cf* gene, in chili pepper 'S3212' (*C. frutescens*). The identification of markers more linked closely to the *cf* locus in these chili peppers was described using the RAPD marker in combination with bulked segregant analysis (BSA). However, RAPD marker was the dominant marker. To develop the PCR-based molecular marker linked closely more to the *cf* locus, one of the RAPD marker was converted to the co-dominant marker (CAPS marker) linked to few-pungent gene, the gene controls few-pungent trait, *cf* gene, at the *cf* locus by cloning and sequencing. This CAPS marker will be close enough to be useful starting points for marker-assisted selection (MAS) in breeding programs further.

3.2. Materials and Methods

3.2.1. Plant materials (refer to Experiment 2)

An F_2 population of 134 plants from a crossing of *C. frutescens* 'S3212' (a few-pungent chili pepper) and *C. chinense* 'S3010' (a pungent chili pepper) was used for genetic analysis.

3.2.2. DNA extraction (refer to Experiment 2)

DNA was extracted from young leaves using Automatic Nucleic Acids Extractor (CompacBio MX-16, COMPACBIO SCIENCES, Japan).

3.2.3. Bulked segregant analysis using RAPD marker

Bulked segregant analysis (BSA) was conducted using the 480 RAPD primers. DNA samples from seven pungent and seven few-pungent F_2 individuals were pooled respectively and used as a template for detecting RAPD in bulked segregant analysis (Michelmore *et al.*, 1991). The RAPD method was performed as described by Minamiyama *et al.* (2005).

3.2.4. Cloning and sequencing of the RAPD product

The specific RAPD band linked to the pungency was amplified using DNA from F_2 plants, which was excised from the gel, and then purified using a QIAquick Gel Extraction Kit (Qiagen, CA, USA). The fragment was ligated into a pCR-TOPO vector using TOPO® TA Cloning Kit (Invitrogen), and the *Escherichia coli* DH5α strain was transformed with the ligated plasmids. The identity of the cloned DNA fragment was examined by hybridization to Southern blots of the RAPD of individuals that segregated with this marker. Sequencing was carried out using an Applied Biosystems 310 sequencer.

3.2.5. Primer design and CAPS analysis

The sequence information of the cloned RAPD fragment OPQ09-1100 was used to design two specific primers, a forward primer of 20 bp SCQ-600FW, 5′-CAATACAAATTGGCCCCTCT-3′, and a reverse primer of 20 bp SCQ-600RV, 5′-TAAAGACGGCTCCTGGAATG-3′. Sequences of the arbitrary primer were not included. Amplifications were carried out using a standard PCR reaction with 0.21 μM of each primer, and the PCR conditions were performed as follows: one step of 94°C for 3 min; 35 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min; a final step of 72°C for 5 min. The amplified fragment was digested in a final volume of 14 μL that contained 12 μL of the PCR reaction and 1 unit of the chosen restriction endonuclease (*Alu*I). The digests were resolved by electrophoresis in 3% agarose gel or 13% acrylamide gel and visualized on a UV transilluminator after staining with ethidium bromide.

3.3. Results and Discussion

3.3.1. Identification of RAPD markers more linked closely to the *cf* locus

To identify the RAPD markers more linked closely to pungency, trait as the allele of few-pungency, bulked segregant analysis was conducted in the F_2 population using the 480 RAPD primers. In the result, total of seven bands were specifically amplified in 'S3010' highly pungent chili pepper and in the pungent bulk. To inquire that band is linked to the pungent trait, some of F_2 individual also was used to the second screening of the specific bands. It was showed in the same result of specific bands in pungency. The first band was a 350 bp fragment amplified by the OPB01 (5′-GTTTCGCTCC-3′) primer. The second band was a 350 bp fragment amplified by the OPD03 (5′-GTCGCCGTCA-3′) primer. The third band was a 400 bp fragment amplified by the OPD11 (5′-AGCGCCATTG-3′) primer. The fourth band was an 1100 bp fragment amplified by the

OPQ09 (5′-GGCTAACCGA-3′) primer. The fifth band was a 400 bp fragment amplified by the OPAD05 (5′-ACCGCATGGG-3′) primer. The sixth band was an 1000 bp fragment amplified by the OPAI03 (5′-GGGTCCAAAG-3′) primer. The seventh band was a 550 bp fragment amplified by the OPAN17 (5′-TCAGCACAGG-3′) primer. The seven specific bands were designated OPB01-350, OPD03-350, OPD11-400, OPQ09-1100, OPAD05-400, OPAI03-1000, and OPAN17-550, respectively (Fig. 3-1.1; 3-1.2; 3-1.3; 3-1.4; 3-1.5; 3-1.6; 3-1.7, respectively). Of these seven specific bands, OPQ09-1100 was clear and reproducible band, the other bands were rather faint or poorly reproducible.

Segregation of these seven bands was examined in 134 individuals of the F_2 population. According to chi-square tests, the segregation of pungency and OPQ09-1100 did not deviate significantly from the expected 3 : 1 ratio (98:36, $\chi^2 = 0.25$, $P = 0.63$). OPB01-350, OPD03-350, OPD11-400, OPAD05-400, OPAI03-1000, and OPAN17-550 also conformed to this ratio ((97:37, $\chi^2 = 0.49$ $P = 0.50$), (97:37, $\chi^2 = 0.49$, $P = 0.50$), (94:40, $\chi^2 = 1.68$, $P = 0.22$), (101:33, $\chi^2 = 0.01$, $P = 0.92$), (87:40, $\chi^2 = 2.86$, $P = 0.12$), and (92:42, $\chi^2 = 2.88$, $P = 0.11$), respectively). By this result, segregation of OPAI03-1000 could not detect in all 134 individuals because of poor reproducibility of the band. RAPD markers were found more to link with the few-pungent gene, *cf* gene, at the *cf* locus in these chili peppers.

Nevertheless, they showed the recombination frequency with few-pungency in 13.9%, 22.6%, 10.3%, 19.8%, 22.2%, 19.6%, and 28.7% in OPB01-350, OPD03-350, OPD11-400, OPQ09-1100, OPAD05-400, OPAI03-1000, and OPAN17-550, respectively (Fig 3-2.1A, B; 3-2.2A, B; 3-2.3A, B; 3-2.4A, B; 3-2.5A, B; 3-2.6A, B; 3-2.7A, B; Table 3-1). By this result, it assumed that these seven markers had some linkage distance from the *cf* locus in these chili peppers. On the other hand, it also assumed that the few-pungent trait in these chili peppers was controlled mainly by single gene with the other minor genes

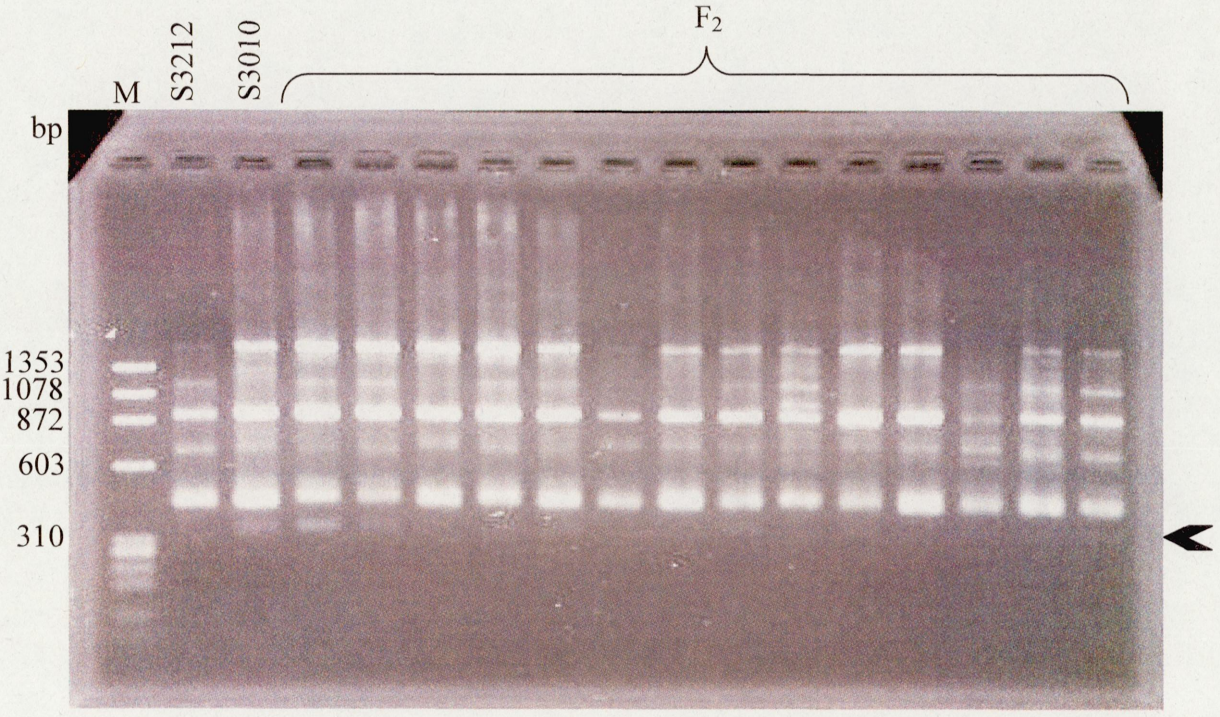

Fig. 3-1.1. The specific band in parent line and some F_2 population using RAPD primer OPB01. M: molecular-weight marker (∅X-174 digested with *Hae*III).

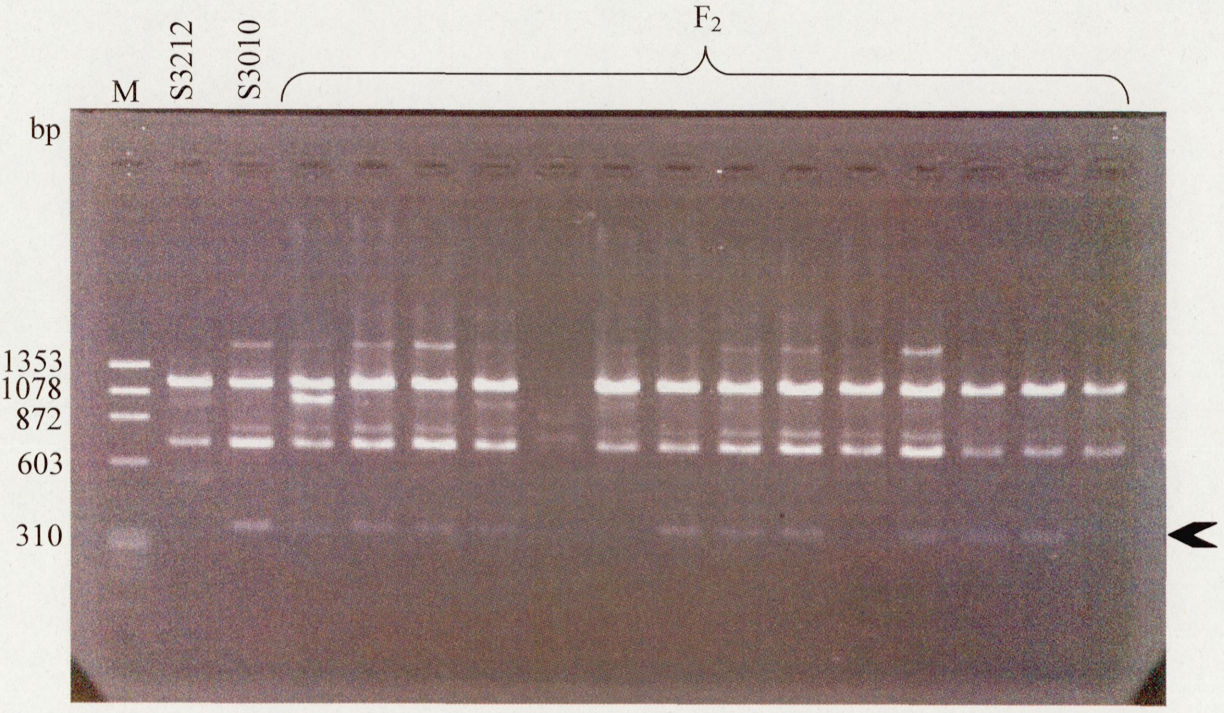

Fig. 3-1.2. The specific band in parent line and some F_2 population using RAPD primer OPD03. M: molecular-weight marker (∅X-174 digested with *Hae*III).

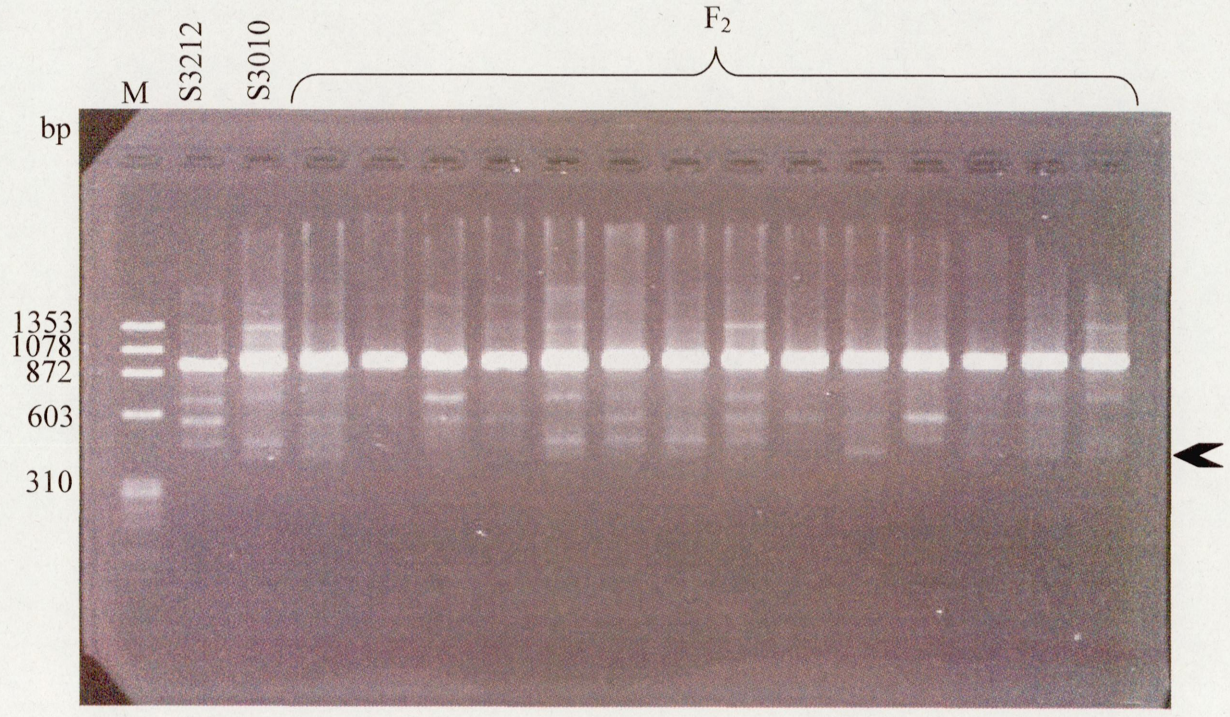

Fig. 3-1.3. The specific band in parent line and some F_2 population using RAPD primer OPD11. M: molecular-weight marker (∅X-174 digested with *Hae*III).

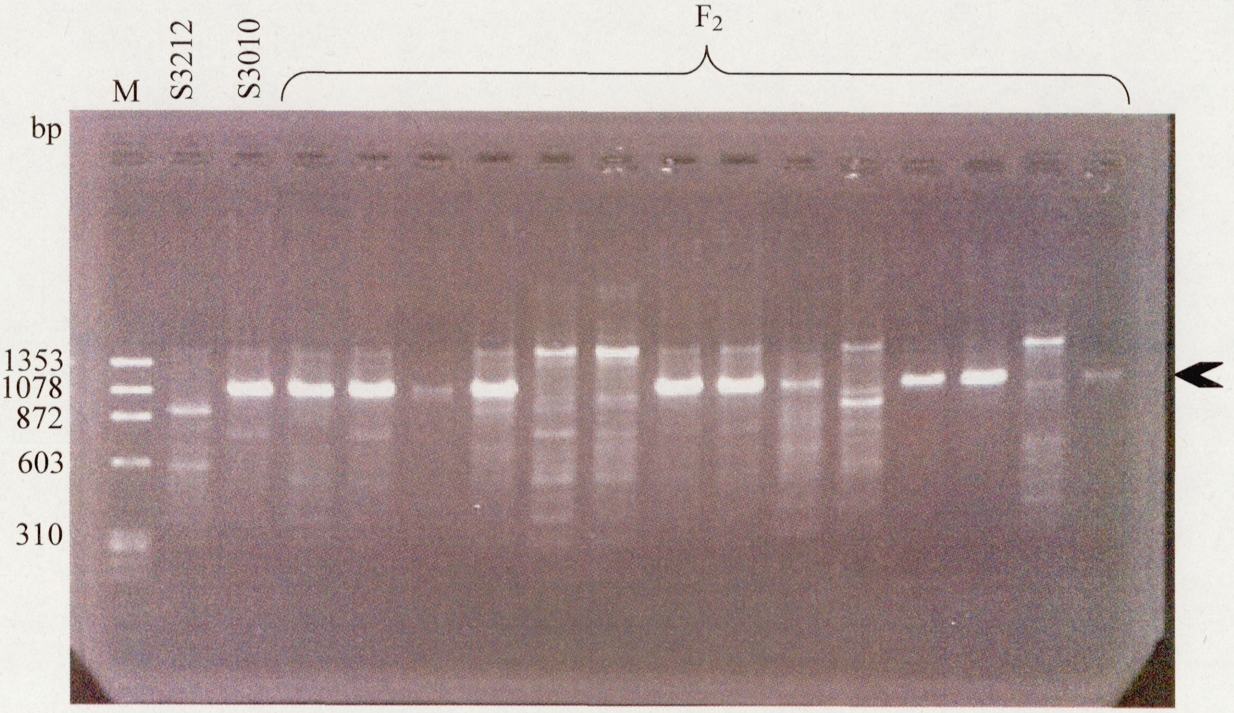

Fig. 3-1.4. The specific band in parent line and some F_2 population using RAPD primer OPQ09. M: molecular-weight marker (∅X-174 digested with *Hae*III).

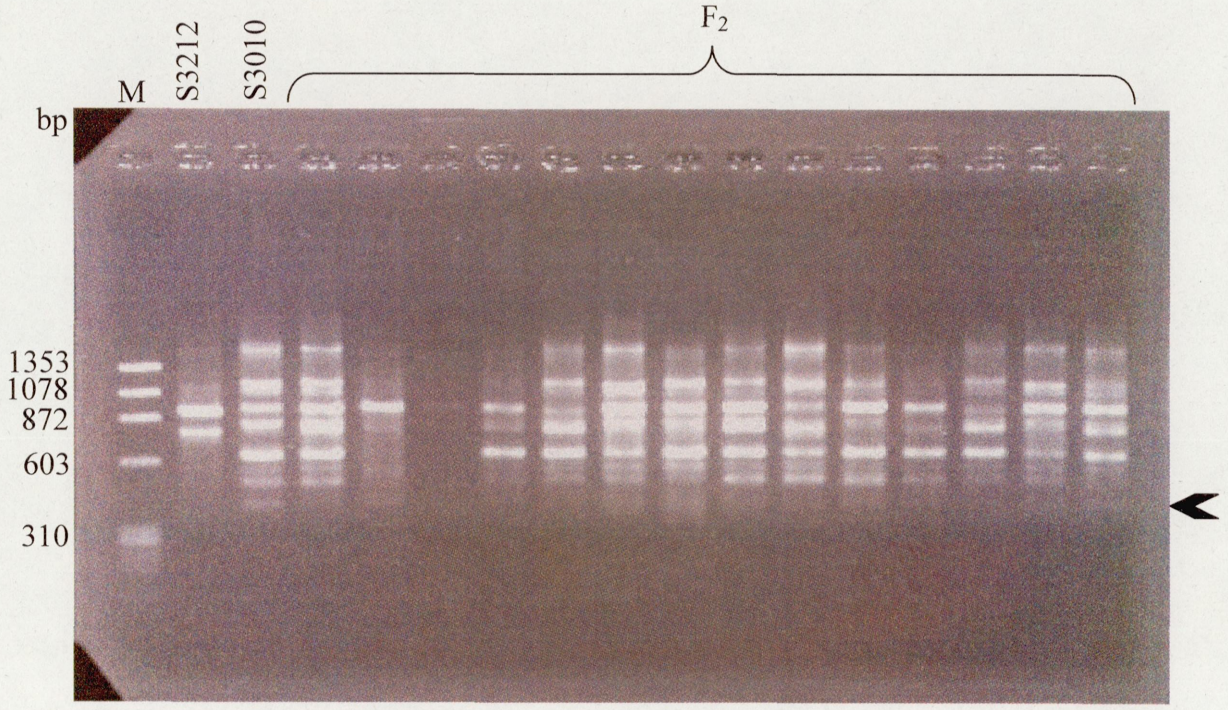

Fig. 3-1.5. The specific band in parent line and some F_2 population using RAPD primer OPAD05. M: molecular-weight marker (∅X-174 digested with *Hae*III).

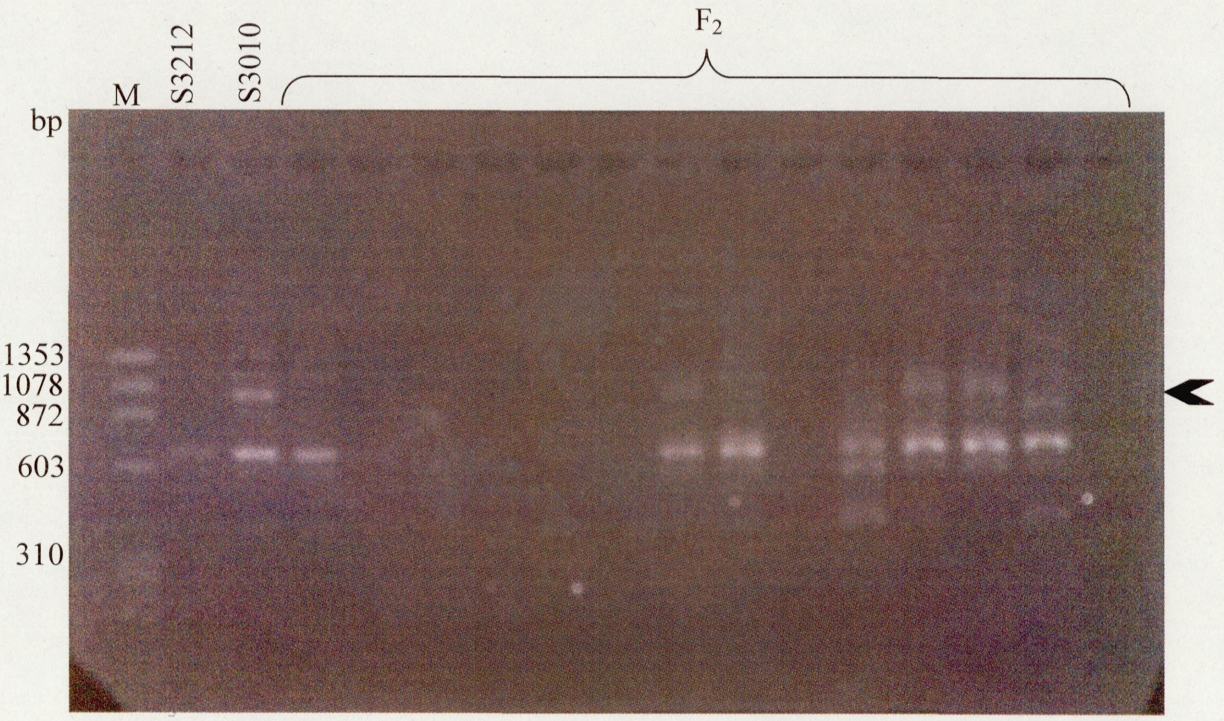

Fig. 3-1.6. The specific band in parent line and some F_2 population using RAPD primer OPAI03. M: molecular-weight marker (∅X-174 digested with *Hae*III).

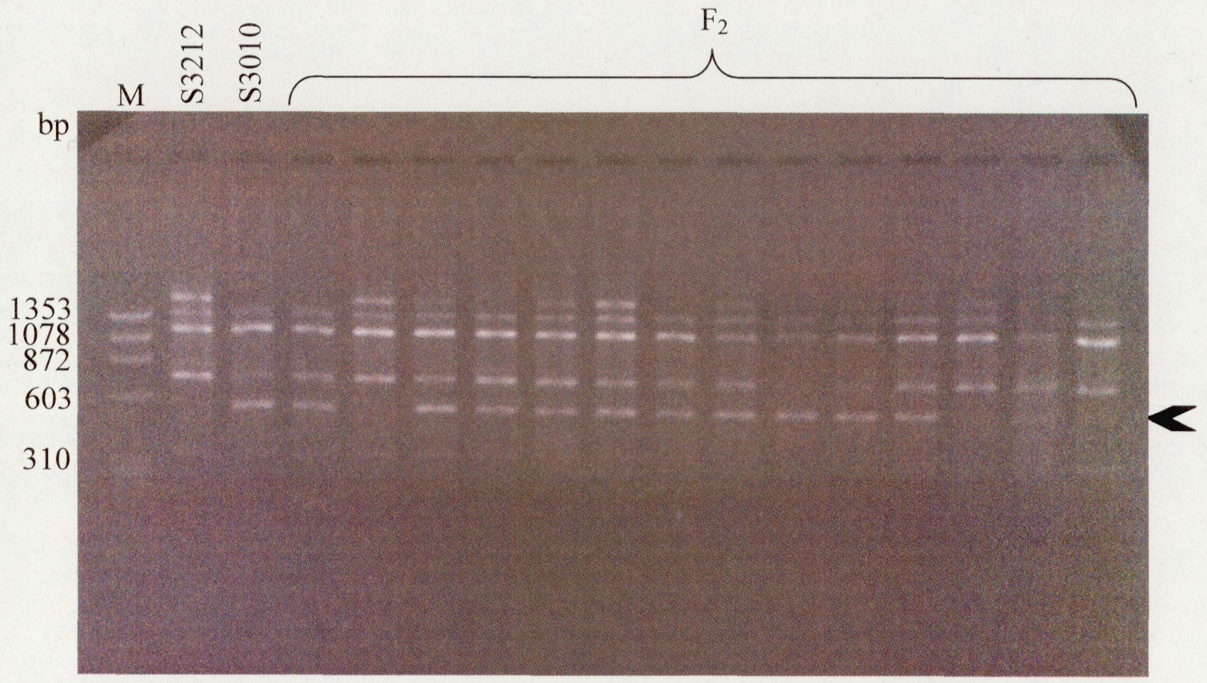

Fig. 3-1.7. The specific band in parent line and some F_2 population using RAPD primer OPAN17. M: molecular-weight marker (∅X-174 digested with *Hae*III).

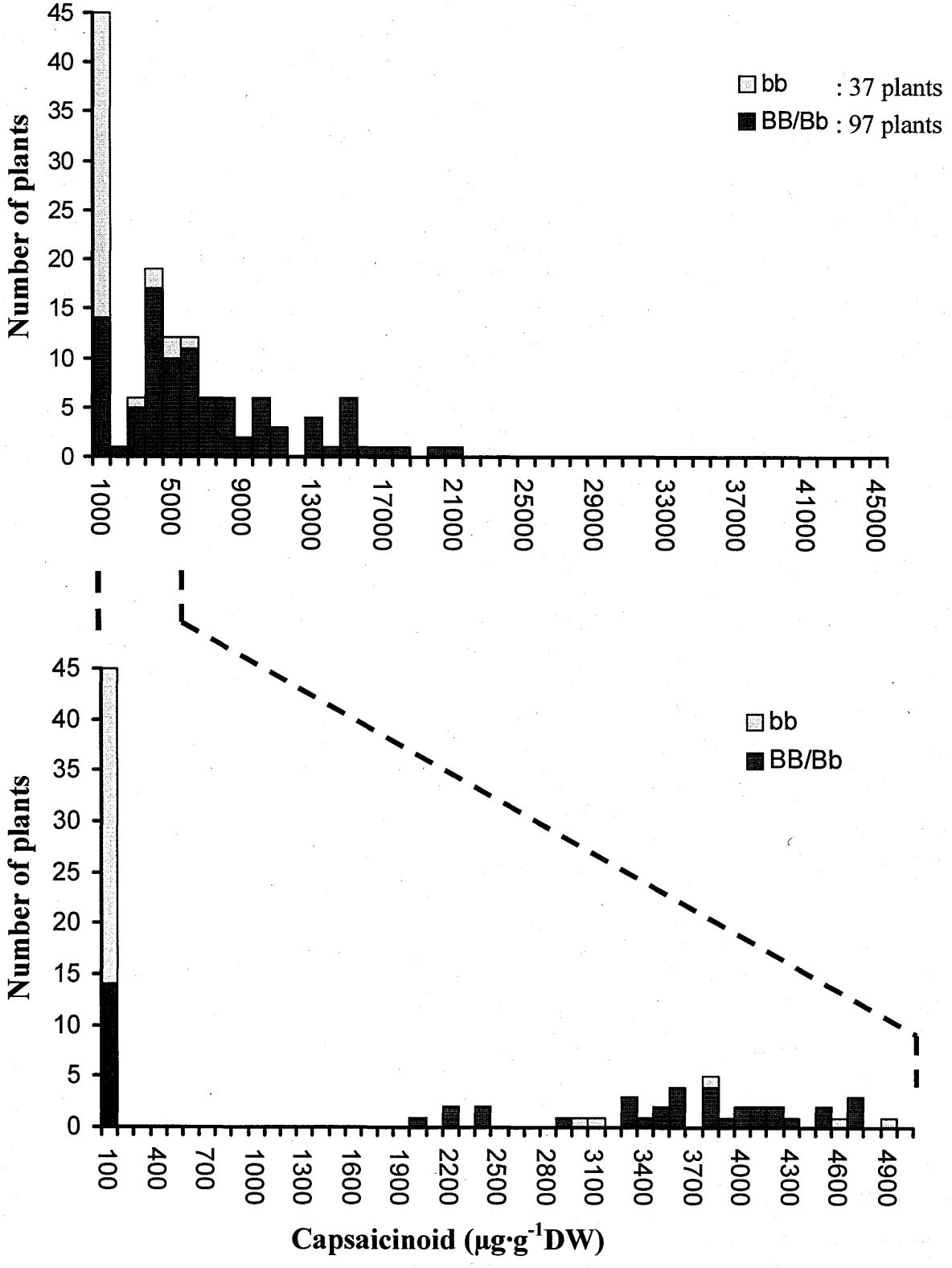

Fig. 3-2.1. Frequency distribution of capsaicinoid contents related with RAPD marker OPB01 in F_2 population of an interspecific hybrid (*C. frutescens* x *C. chinense*) (A: above) and focus of frequency distribution in the scale of capsaicinoid contents from 0 to 5,000 $\mu g \cdot g^{-1}$DW (B: below)

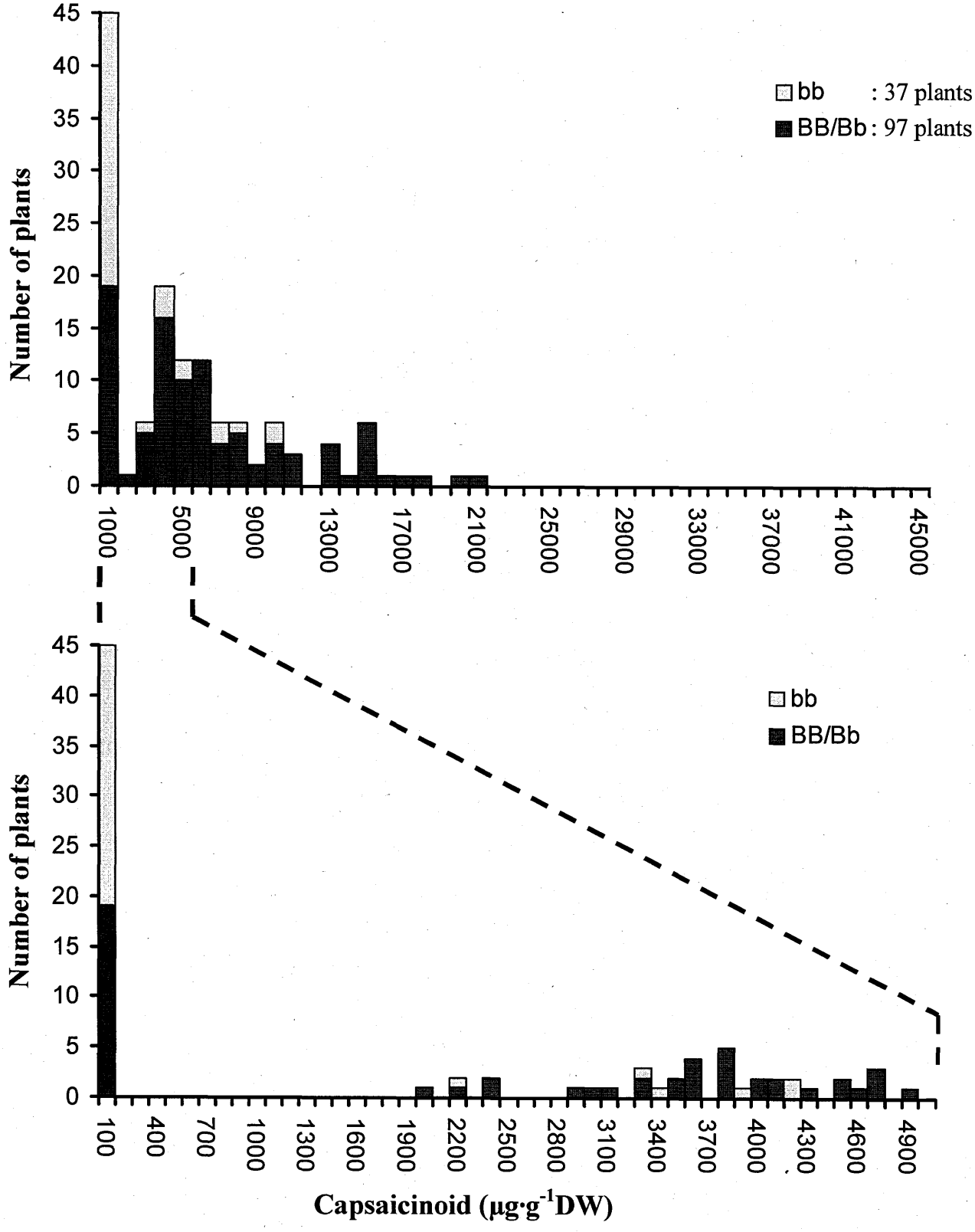

Fig. 3-2.2. Frequency distribution of capsaicinoid contents related with RAPD marker OPD03 in F_2 population of an interspecific hybrid (*C. frutescens* x *C. chinense*) (A: above) and focus of frequency distribution in the scale of capsaicinoid contents from 0 to 5,000 $\mu g \cdot g^{-1}$DW (B: below)

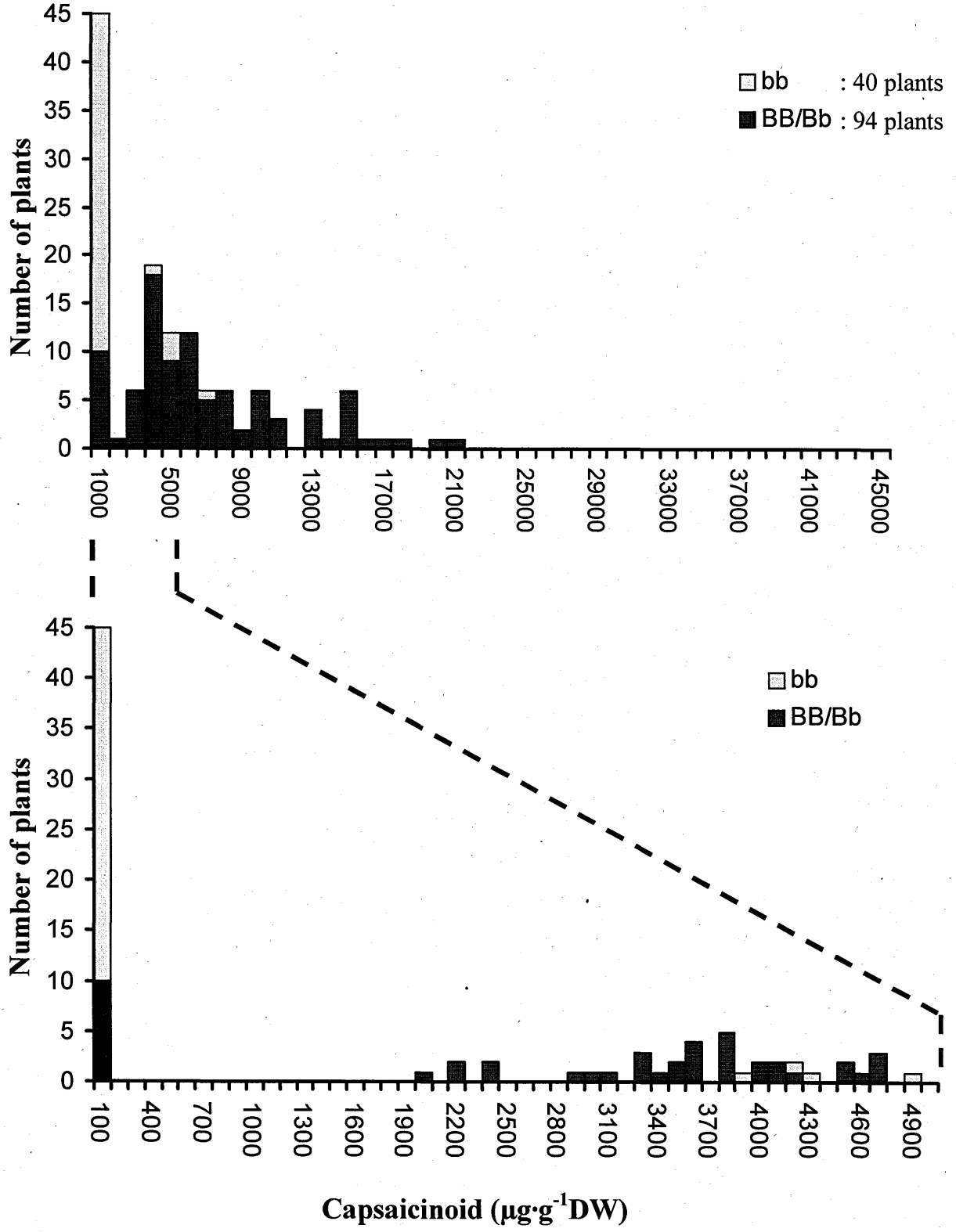

Fig. 3-2.3. Frequency distribution of capsaicinoid contents related with RAPD marker OPD11 in F_2 population of an interspecific hybrid (*C. frutescens* x *C. chinense*) (A: above) and focus of frequency distribution in the scale of capsaicinoid contents from 0 to 5,000 $\mu g \cdot g^{-1}$DW (B: below)

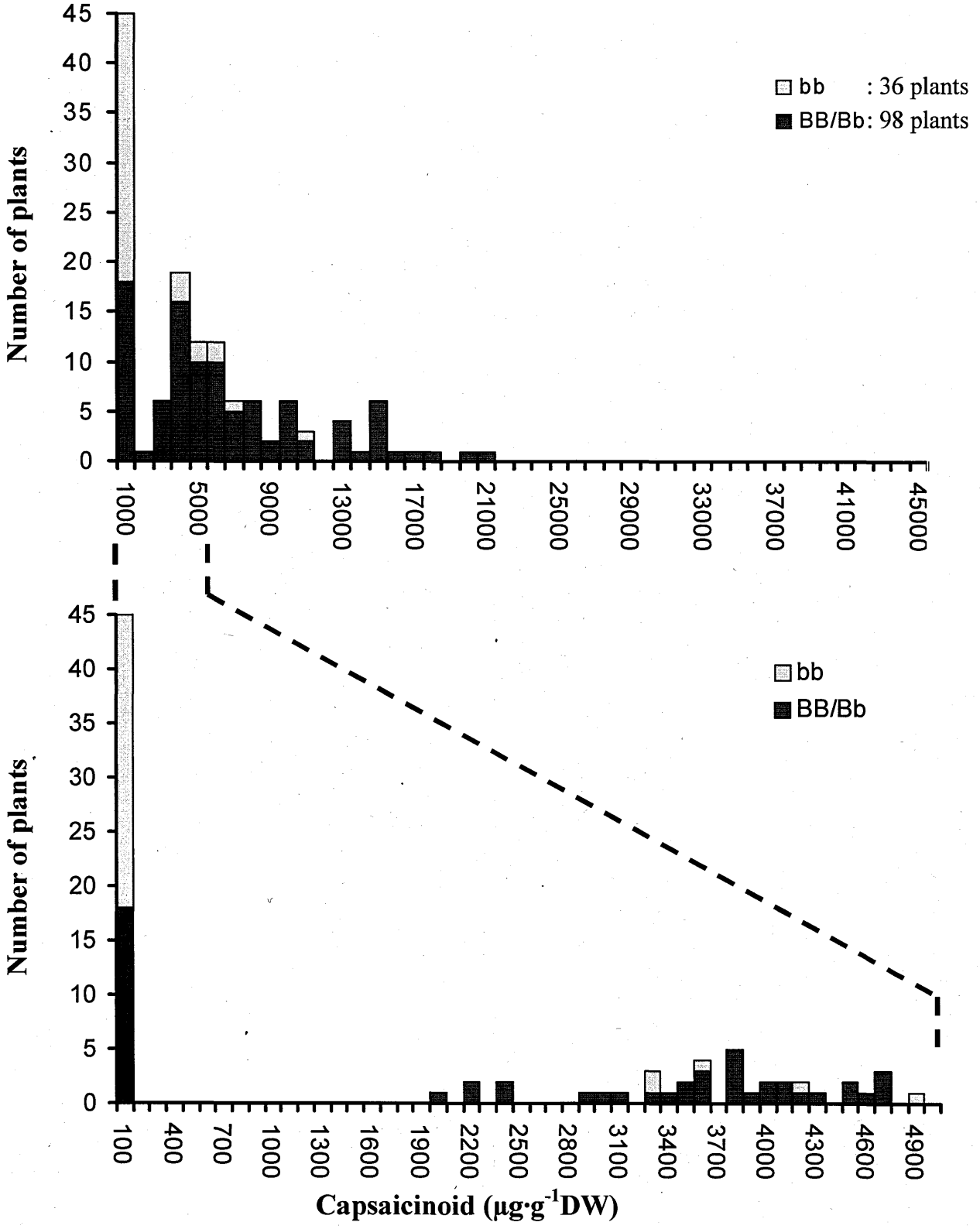

Fig. 3-2.4. Frequency distribution of capsaicinoid contents related with RAPD marker OPQ09 in F_2 population of an interspecific hybrid (*C. frutescens* x *C. chinense*) (A: above) and focus of frequency distribution in the scale of capsaicinoid contents from 0 to 5,000 $\mu g \cdot g^{-1}$DW (B: below)

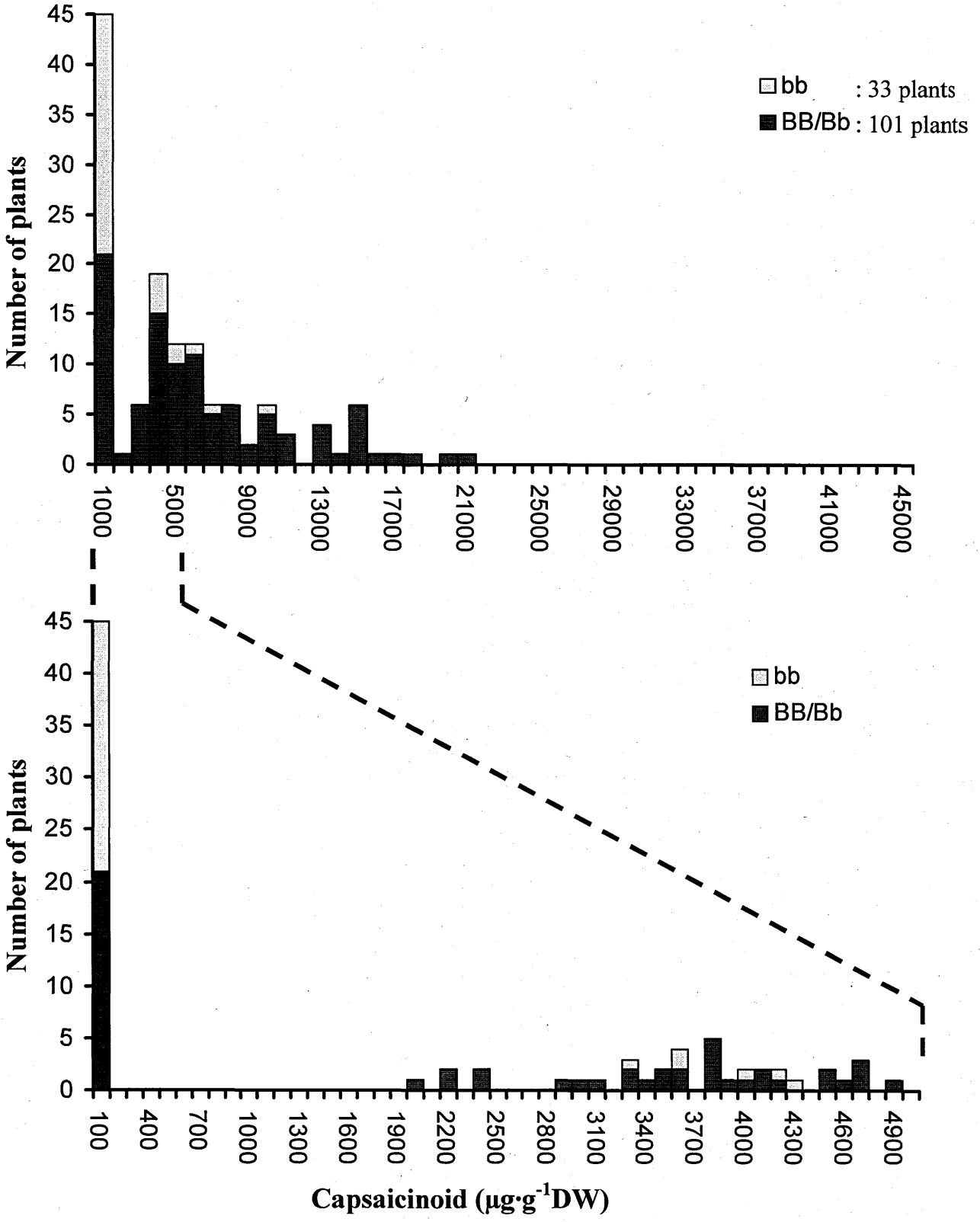

Fig. 3-2.5. Frequency distribution of capsaicinoid contents related with RAPD marker OPAD05 in F_2 population of an interspecific hybrid (*C. frutescens* x *C. chinense*) (A: above) and focus of frequency distribution in the scale of capsaicinoid contents from 0 to 5,000 $\mu g \cdot g^{-1}$DW (B: below)

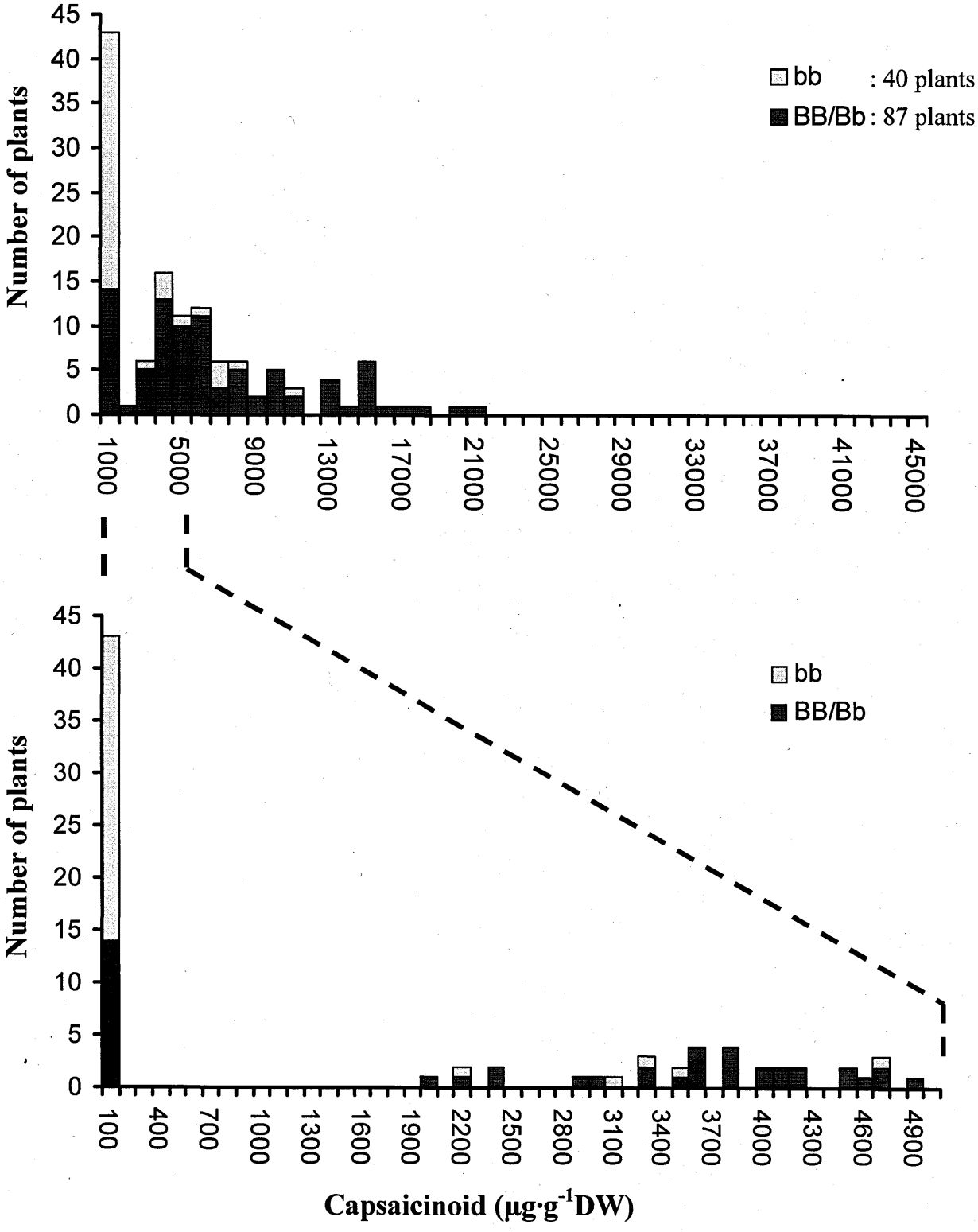

Fig. 3-2.6. Frequency distribution of capsaicinoid contents related with RAPD marker OPAI03 in F_2 population of an interspecific hybrid (*C. frutescens* x *C. chinense*) (A: above) and focus of frequency distribution in the scale of capsaicinoid contents from 0 to 5,000 $\mu g \cdot g^{-1}$DW (B: below)

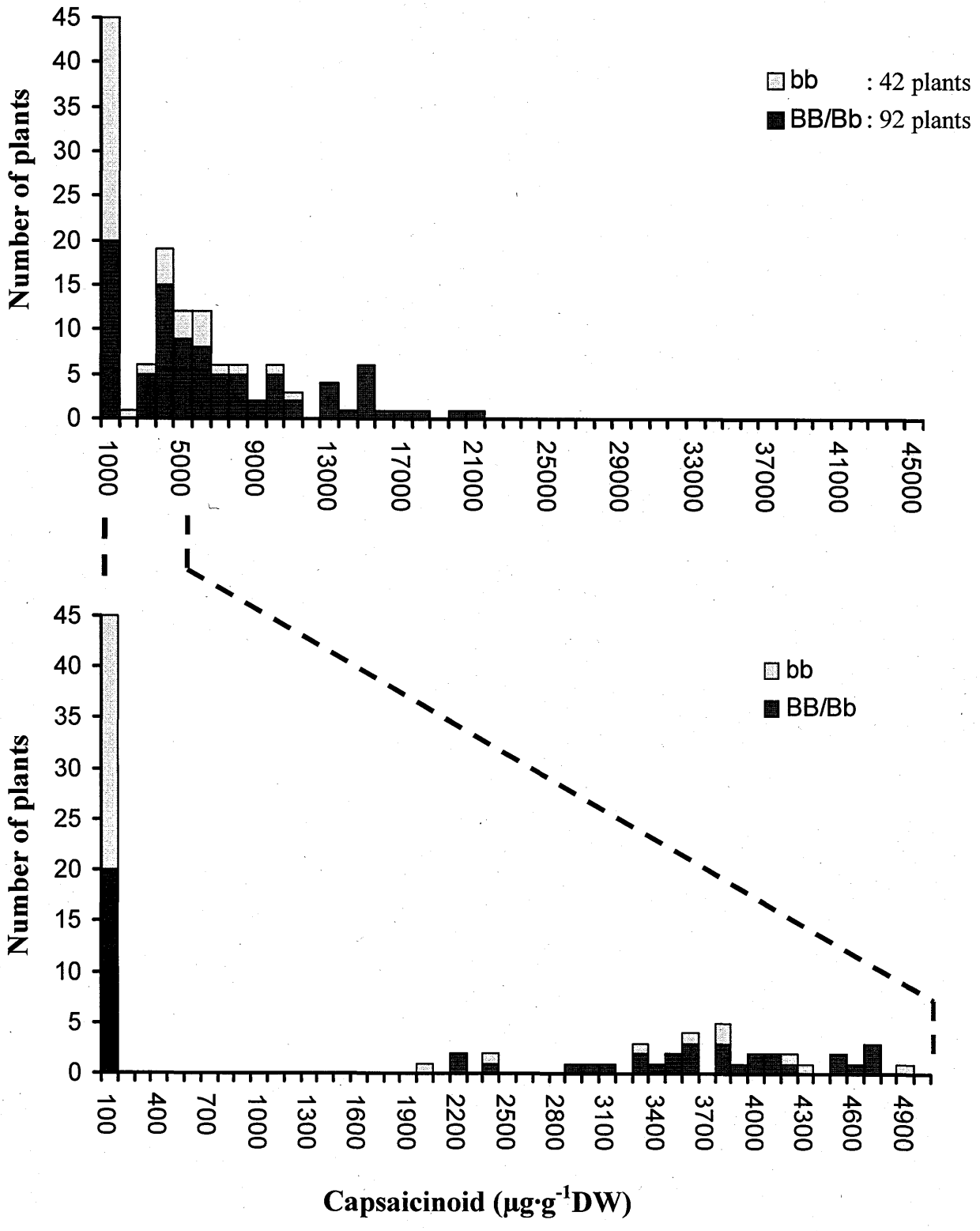

Fig. 3-2.7. Frequency distribution of capsaicinoid contents related with RAPD marker OPAN17 in F_2 population of an interspecific hybrid (*C. frutescens* x *C. chinense*) (A: above) and focus of frequency distribution in the scale of capsaicinoid contents from 0 to 5,000 $\mu g \cdot g^{-1}$DW (B: below)

Table 3-1. Segregation of pungency and molecular marker, and chi-square tests for fitting to 3:1 (χ^2A; χ^2B) and to 9:3:3:1 (χ^2AB) expected in F_2 population of *C. frutescens* 'S3212' (P_2) × *C. chinense* 'S3010' (P_1).

Locus		Number of plants observed [Z]				Chi-square values			Recombination frequency (%)
A	B	AB	Ab	aB	ab	χ^2A	χ^2B	χ^2AB	
Pungency	OPB01-350	83	6	14	31	5.26**[Y]	0.49	81.38***	13.9
	OPD03-350	78	11	19	26	5.26**	0.49	46.62***	22.6
	OPD11-400	84	5	10	35	5.26**	1.68	110.86***	10.3
	OPQ09-1100	80	9	18	27	5.26**	0.25	54.07***	19.8
	OPAD05-400	80	9	21	24	5.26**	0.01	40.46***	22.2
	OPAI03-1000	73	11	14	29	5.31**	2.86*	66.86***	19.6
	OPAN17-550	72	17	20	25	5.26**	2.88*	36.83***	28.7
	SCQ-600	85	3	15	29	4.89**	0.04	76.70***	10.7

[Z] A, a: Pungency or few-pungency; B, b: Presence or absence of dominant marker/ presence of specific band in co-dominant marker.

[Y] *, ** and *** indicate significance at $P \leq 0.1$, $P \leq 0.05$ and $P \leq 0.01$, respectively, by chi-square test.

participated to control the high content of pungency in QTL. However, these seven RAPD markers confirmed the linkage to the *cf* locus in these chili peppers.

3.3.2. Development of a cleaved amplified polymorphic sequence (CAPS) marker linked to the *cf* locus

To develop the PCR-based molecular marker linked closely more to the *cf* locus, one of the RAPD marker, OPQ09-1100 had a clear and reproducible band, was converted to the co-dominant CAPS marker linked to the *cf* locus by cloning and sequencing. A specific primer pair designed from the sequence data amplified a single band of 600 bp using genomic DNA from parents and some F_2 plants. The band was designated SCQ-600. As suggested by the sequence, digestion of the SCQ-600 fragment from 'S3010' by *Alu*I resulted in two major fragments, 305 and 240 bp, also that of 'S3212' resulted in two major fragments, 300 and 260 bp (Fig. 3-3).

Segregation of this polymorphism in F_2 population fitted to the expected 1 : 2 : 1 ratio (24 : 76 : 32, $\chi^2 = 4.00$, $P = 0.14$) (Fig. 3-4). By this result, the segregation of this CAPS marker for the *cf* locus was also somewhat different from that of the original RAPD marker OPQ09-1100, the difference might be caused by the unstable amplification of the band. However, the CAPS marker SCQ-600 showed in the 10.7 percentage of recombination frequency that was less than the RAPD marker OPQ09-1100 from which SCQ-600 was converted to the *cf* locus (Fig. 3-5, Table 3-1). So, this SCQ-600 marker was found to be closely more linked to the *cf* locus in these chili peppers.

Based on the present markers, more detailed mapping around the *cf* locus and DNA markers more closely linked to *cf* locus have to be discovered further.

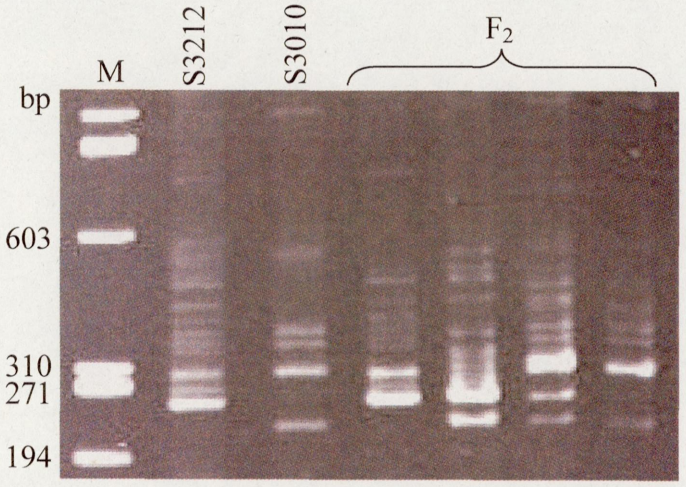

Fig. 3-3. Segregation of the co-dominant CAPS marker linked to the *cf* locus in few-pungent trait of chili pepper (*C. frutescens* x *C. chinense*). PCR with specific primers was followed by restriction with *Alu*I. M: molecular-weight marker (∅X-174 digested with *Hae*III).

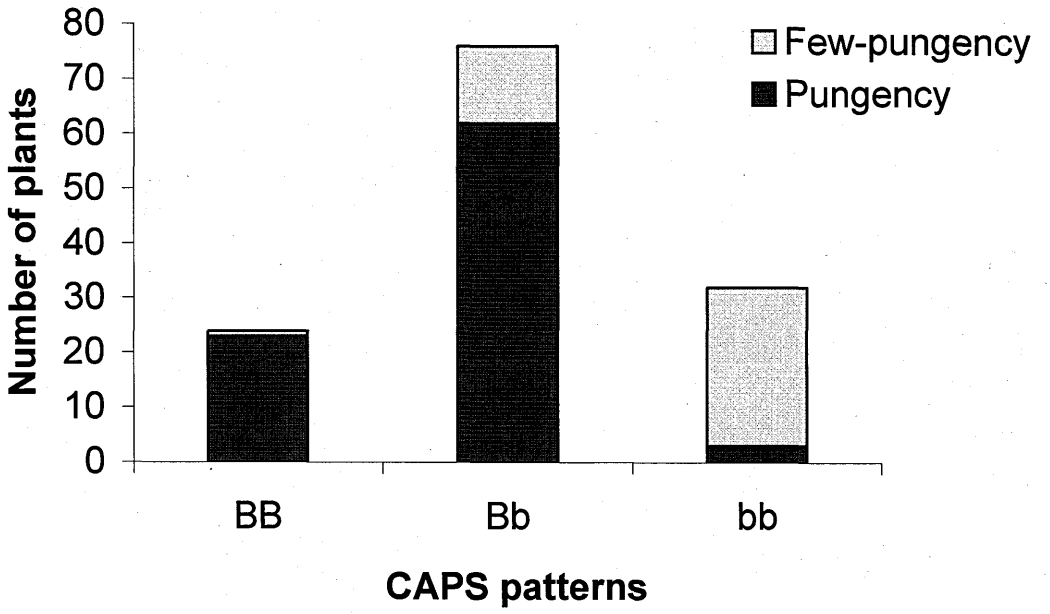

Fig. 3-4. Segregation of CAPS patterns (SCQ-600) and few-pungency in F_2 population of *C. frutescens* 'S3212' (P_2) × *C. chinense* 'S3010' (P_1).

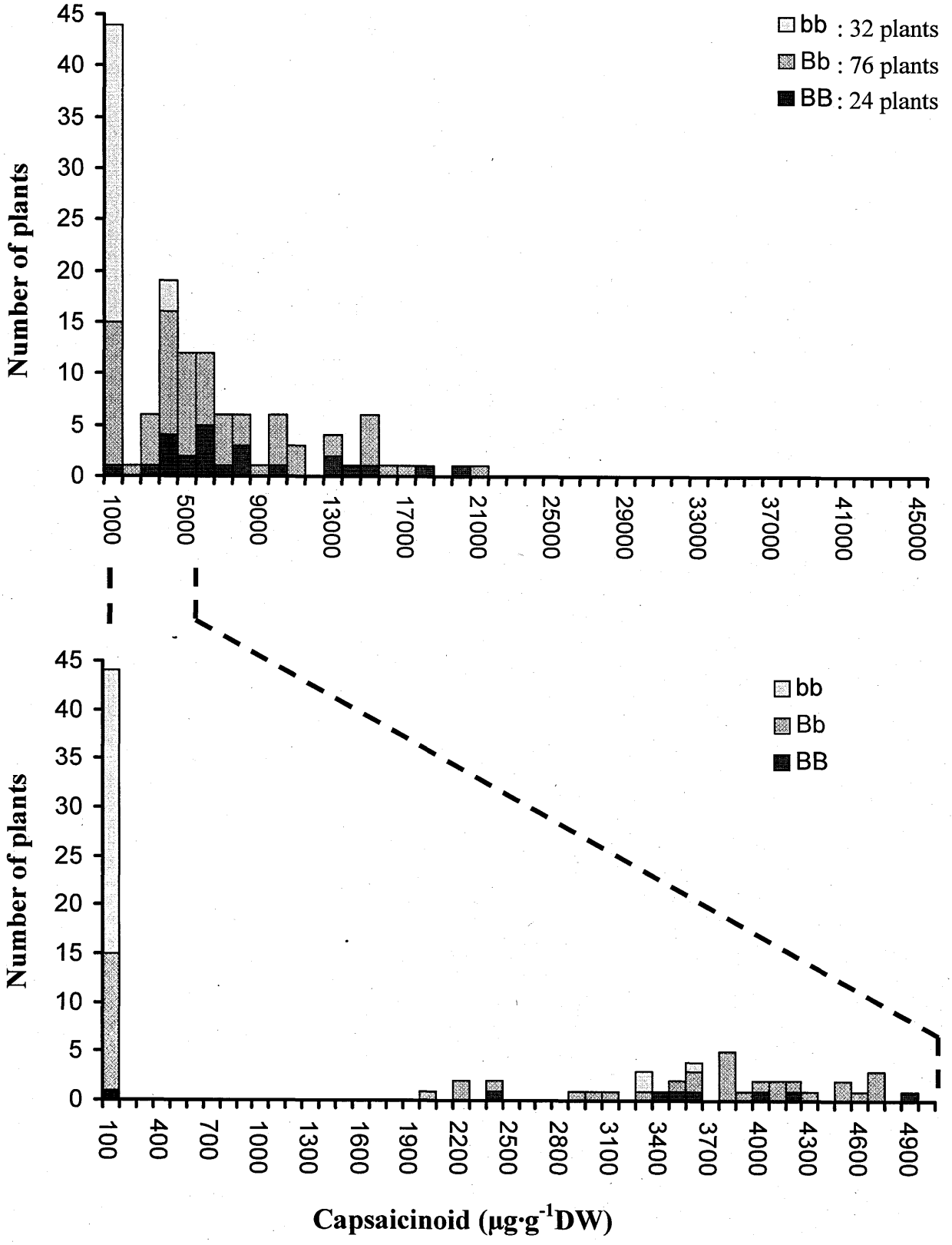

Fig. 3-5. Frequency distribution of capsaicinoid contents related with CAPS marker SCQ-600 in F_2 population of an interspecific hybrid (*C. frutescens* x *C. chinense*) (A: above) and focus of frequency distribution in the scale of capsaicinoid contents from 0 to 5,000 $\mu g \cdot g^{-1}$DW (B: below).

3.4. Abstract

To find molecular markers linked to *cf* locus, bulked segregant analysis was conducted in the F_2 population using 480 RAPD primers. Seven bands designated OPB01-350, OPD03-350, OPD11-400, OPQ09-1100, OPAD05-400, OPAI03-1000, OPAN17-550 were amplified in 'S3010' pungent chili pepper and in the pungent bulk. The segregation of pungency and RAPD markers did not deviate from the expected 3 : 1 ratio, but co-segregation of pungency and RAPD markers differed significantly from the expected 9 : 3 : 3 : 1 ratio of independent inheritance. This indicates that these seven RAPD markers link to *cf* locus and may be available as DNA markers for screening for few-pungency. However, they showed 10.3-28.7% of recombination with *cf* locus indicating that they had some linkage distance from *cf* locus. To develop more closely linked marker, a clear and reproducible RAPD marker OPQ09-1100 was converted into a co-dominant CAPS marker, SCQ-600. This CAPS marker was found to link closely to *cf* locus, however, it had 10.7% of recombination indicating some linkage distance from *cf* locus.

Experiment 4

Analysis of SSR-based Linkage Map in Chili Pepper

4.1. Introduction

Simple sequence repeats (SSRs) or microsatellite markers are PCR-based marker that have been developed in many plant species, including most major crops, such as rice, maize, sorghum, wheat, and barley, as well as vegetable crops (Mba *et al.*, 2001; Suwabe *et al.*, 2002; Chiba *et al.*, 2003). SSR markers have the advantage of being multiallelic, highly polymorphic, and co-dominant, and have been developed and used for the genetic mapping of solanaceous vegetables, including tomato, potato, and eggplant (Broun and Tanksley, 1996; Smulders *et al.*, 1997; Milbourne *et al.*, 1998; Nunome *et al.*, 2003). In this study, the interest of few-pungent trait linked to *cf* locus in an interspecific hybrid of *C. frutescens* x *C. chinense* was observed. We have analyzed a linkage map using SSR markers linked to pungency in *C. frutescens* x *C. chinense*.

4.2. Materials and Methods

4.2.1. Plant materials (refer to Experiment 2)

The F_2 population of 134 plants derived from a cross between *C. frutescens* 'S3212' (a few-pungent chili pepper) and *C. chinense* 'S3010' (a pungent chili pepper) was used for genetic analysis.

4.2.2. DNA extraction (refer to Experiment 2)

DNA was extracted from young leaves using Automatic Nucleic Acids Extractor (CompacBio MX-16, COMPACBIO SCIENCES, Japan).

4.2.3. SSR analysis

For SSR analysis, amplifications were performed using a standard PCR reaction. The PCR reaction mixture consisted of 20 ng genomic DNA, 0.25 μM of each primer, 1x PCR buffer (Takara Bio, Japan), 200 μM each of dNTPs and 0.25 units of *Taq* DNA polymerase (Takara Bio, Japan) in a total reaction volume of 10 μL. PCR amplifications were performed in a BIO-RAD i Cycler (Japan). The mixture was initially denatured at 94°C for 3 min, followed by 30 cycles of PCR amplification with denaturation at 94°C for 30 sec, primer annealing at 50°C for 1 min, primer extension at 72°C for 1 min, and finally incubated at 72°C for 5 min. The PCR products were separated by electrophoresis in 13% acrylamide gel and visualized on a UV transilluminator after staining with ethidium bromide.

4.3. Results and discussion

4.3.1. Identification of SSR markers linked to the *cf* locus in chili pepper (*C. frutescens* x *C. chinense*)

To identify markers linked to the *cf* locus, the parent lines were screened with 259 SSR primer pairs. Ninety-five primer pairs showed the PCR amplification. Out of them, seventeen primer pairs showed the polymorphic bands between the parent lines (Table 4-1). Then, using these 17 SSR primer pairs in 134 individual plants of F_2 population, it found that 100 individual plants were approximate to be amplified, however, only 10 primer pairs (CAMS090; CAMS191; CAMS340; CAMS396; CAMS424; CAMS451; CAMS460; CAMS630; CAMS679; CAMS855) showed the differential PCR amplification in all of homozygous and heterozygous patterns (Fig. 4-1.1; 4-1.2; 4-1.3; 4-1.4; 4-1.5; 4-1.6; 4-1.7; 4-1.8; 4-1.9; 4-1.10, respectively). The other 7 primer pairs showed the differential PCR

Table 4-1. List of 17 SSR markers showed the polymorphic bands between the parent lines.

SSR marker	Repeat motif
CAMS065	(ac)12
CAMS090	(ca)3a(ac)15
CAMS142	(ta)3…(ac)7…(ac)12a(ta)8
CAMS191	(ac)10a(ta)4
CAMS207-2	(ac)4at(ac)8(at)3
CAMS336	(tc)16
CAMS340	(ta)3…(ag)13
CAMS396	(ag)12
CAMS424	(ag)16
CAMS451-1	(tc)21t(ac)3
CAMS460	(tc)20
CAMS630-2	(tct)5…(ctt)3
CAMS679	(tat)16
CAMS855	(agt)14a(gaa)9
CAMS861	(aga)11
CAMS865	(gaa)7
CAMS891	(gaa)8

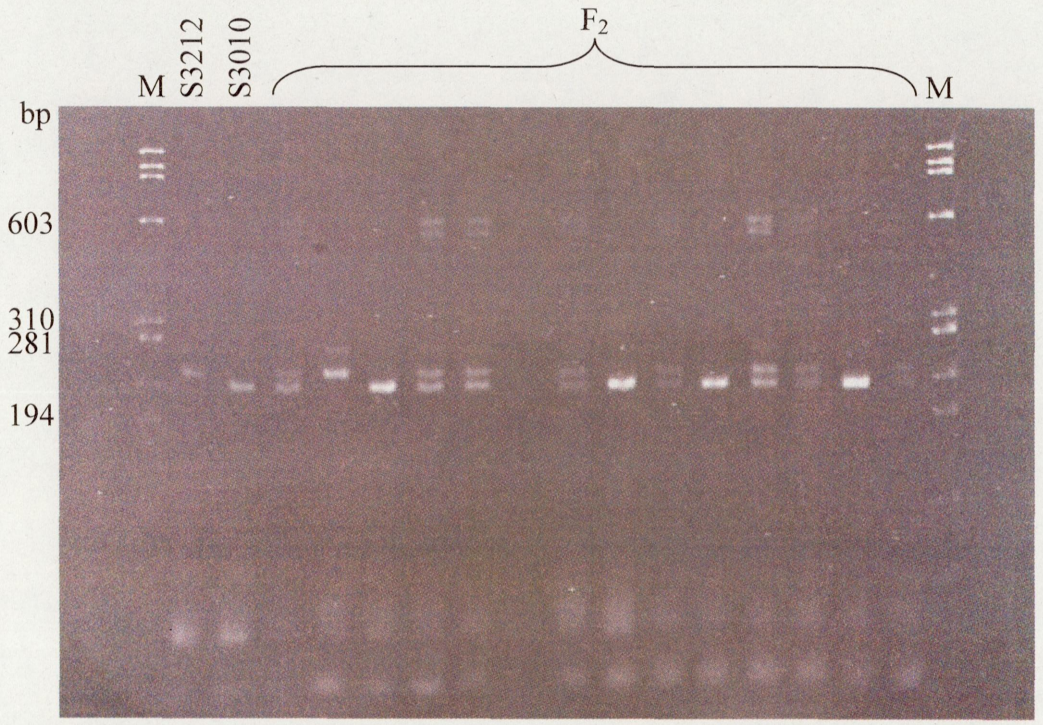

Fig. 4-1.1. The differential PCR amplification in parent lines and some F_2 population using CAMS090. M: molecular-weight marker (∅X-174 digested with *Hae*III).

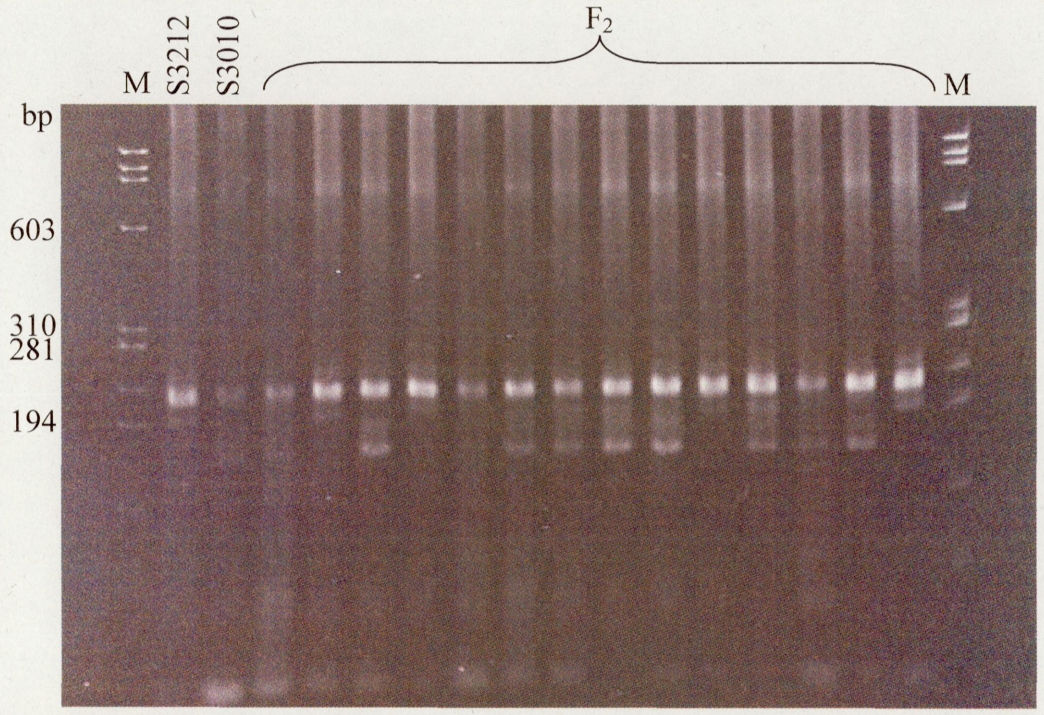

Fig. 4-1.2. The differential PCR amplification in parent lines and some F_2 population using CAMS191. M: molecular-weight marker (∅X-174 digested with *Hae*III).

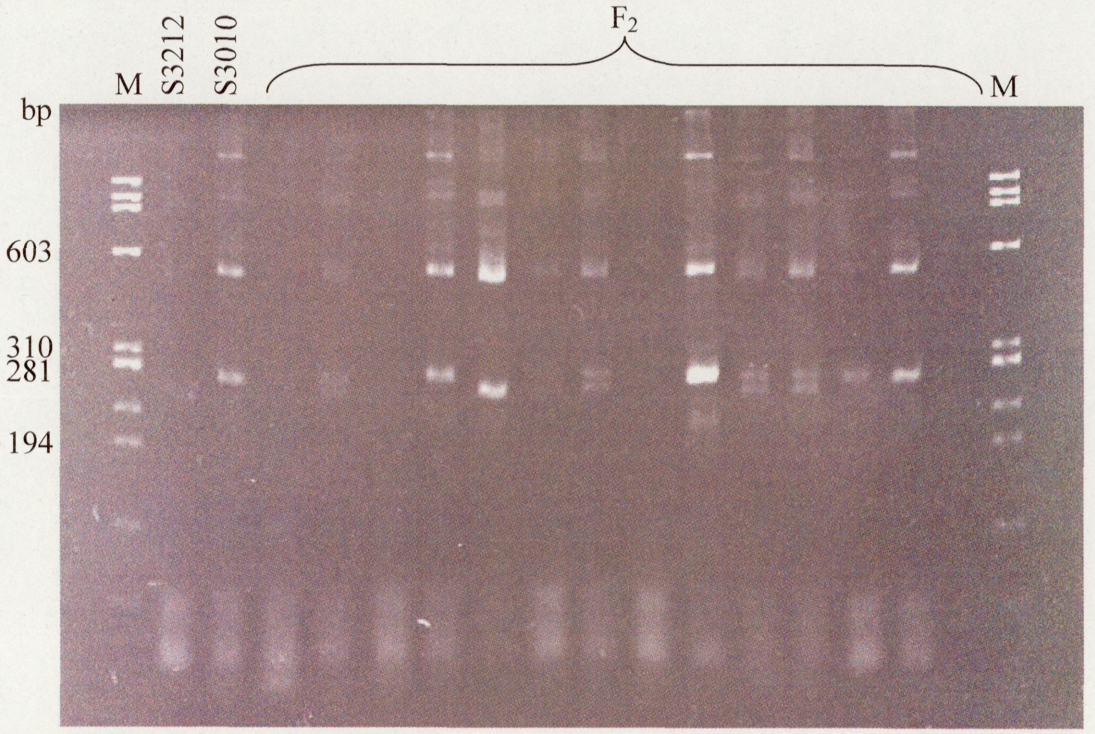

Fig. 4-1.3. The differential PCR amplification in parent lines and some F_2 population using CAMS340. M: molecular-weight marker (∅X-174 digested with *Hae*III).

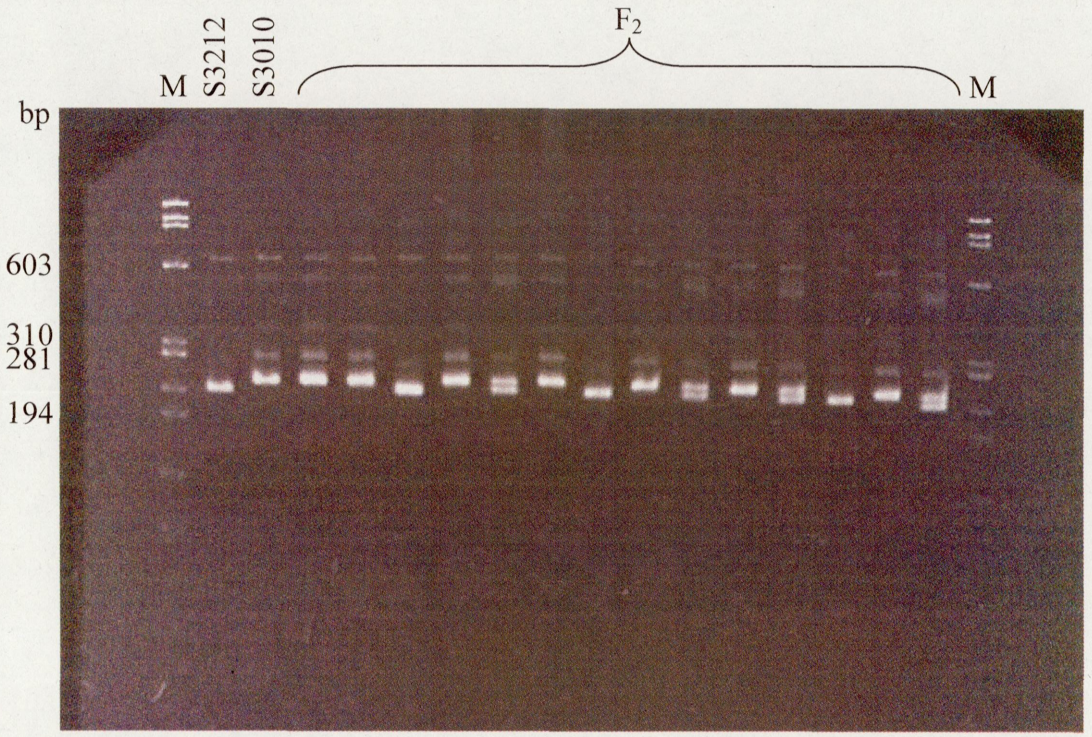

Fig. 4-1.4. The differential PCR amplification in parent lines and some F_2 population using CAMS396. M: molecular-weight marker (∅X-174 digested with *Hae*III).

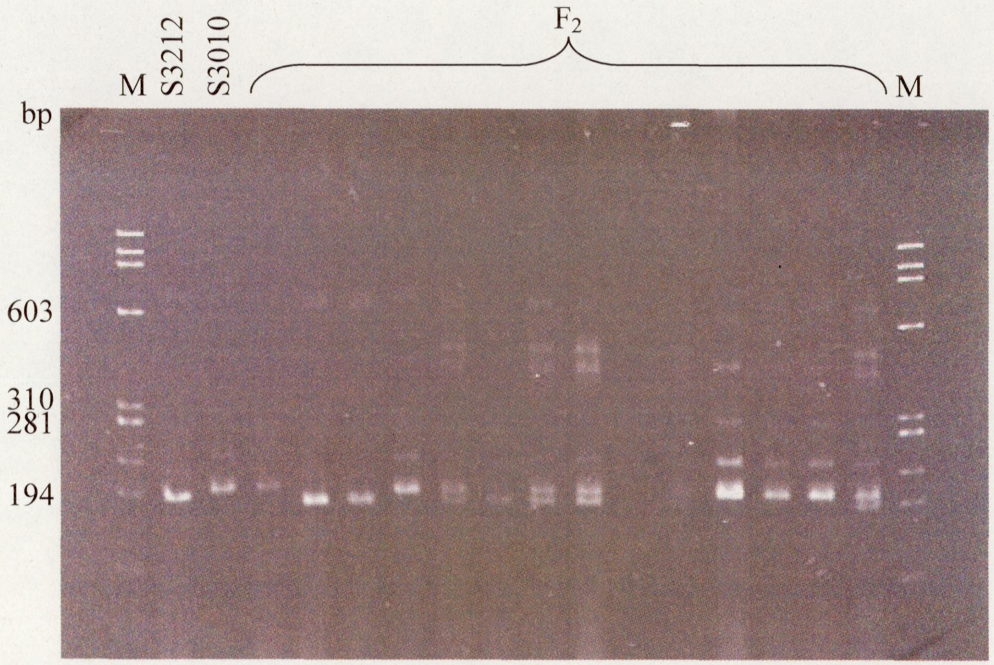

Fig. 4-1.5. The differential PCR amplification in parent lines and some F_2 population using CAMS424. M: molecular-weight marker (∅X-174 digested with *Hae*III).

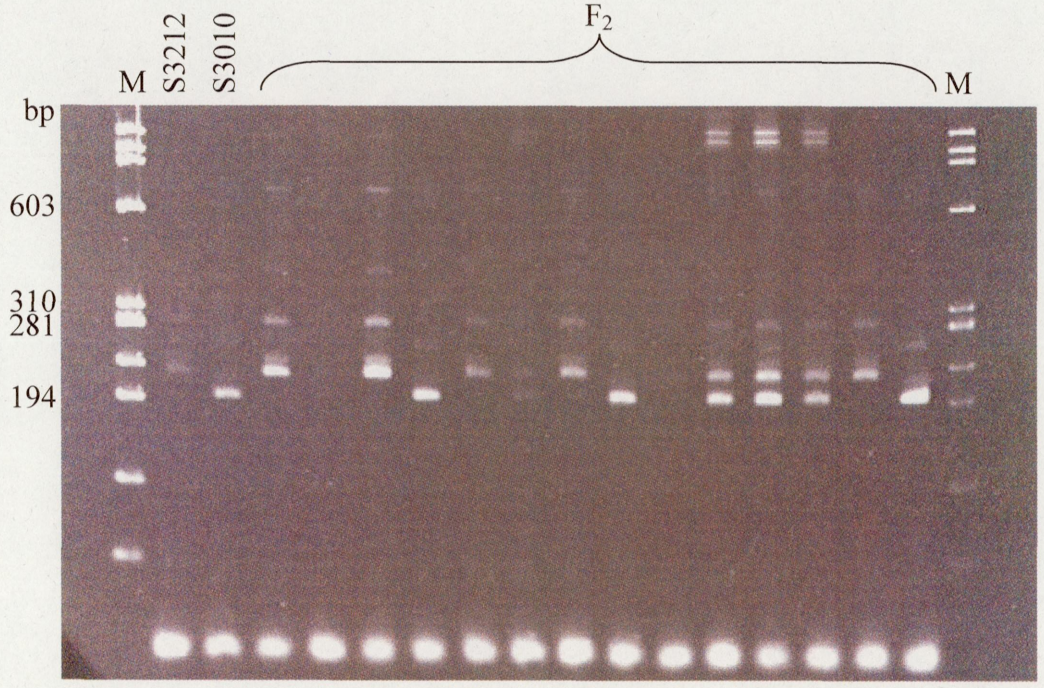

Fig. 4-1.6. The differential PCR amplification in parent lines and some F_2 population using CAMS451. M: molecular-weight marker (∅X-174 digested with *Hae*III).

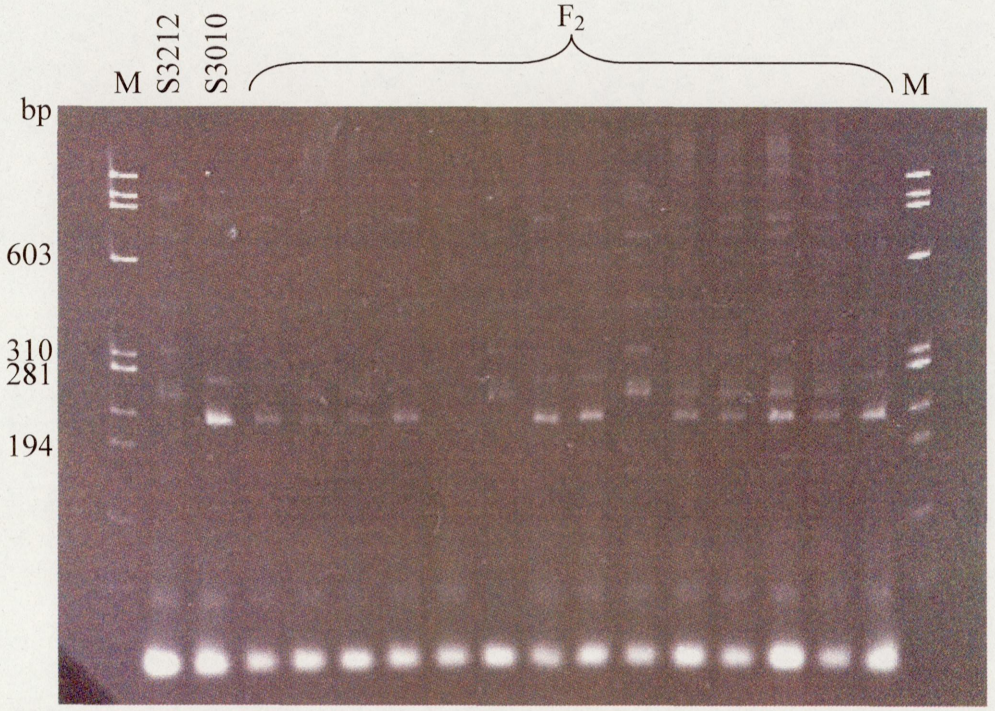

Fig. 4-1.7. The differential PCR amplification in parent lines and some F_2 population using CAMS460. M: molecular-weight marker (∅X-174 digested with *Hae*III).

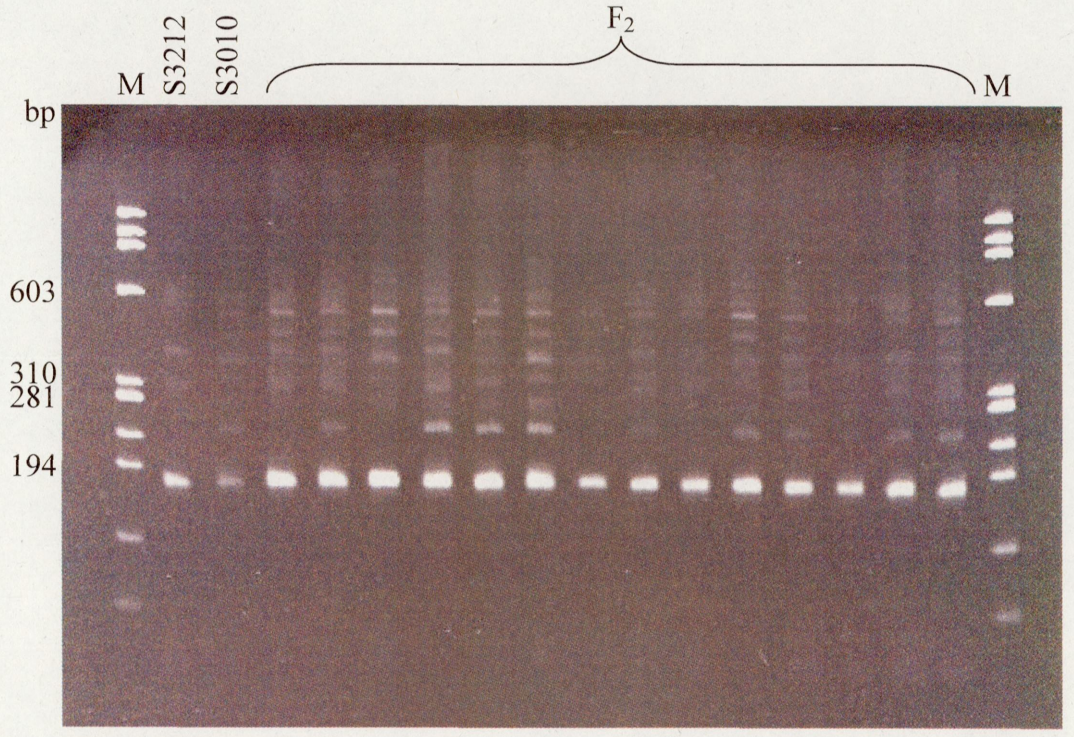

Fig. 4-1.8. The differential PCR amplification in parent lines and some F_2 population using CAMS630. M: molecular-weight marker (∅X-174 digested with *Hae*III).

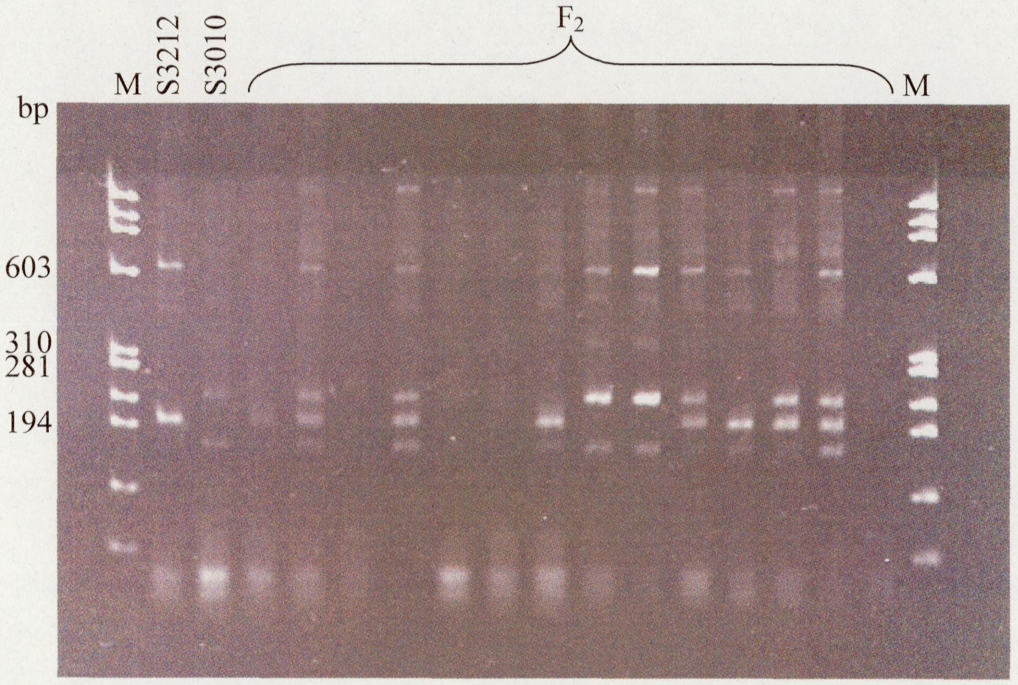

Fig. 4-1.9. The differential PCR amplification in parent lines and some F_2 population using CAMS679. M: molecular-weight marker (∅X-174 digested with *Hae*III).

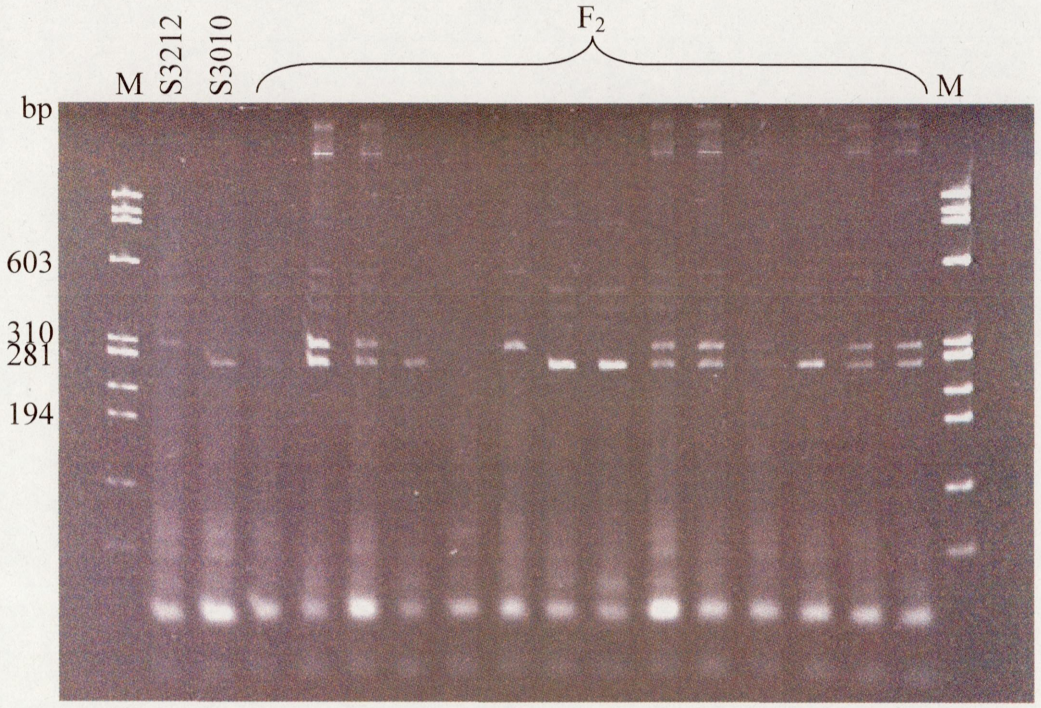

Fig. 4-1.10. The differential PCR amplification in parent lines and some F_2 population using CAMS855. M: molecular-weight marker (∅X-174 digested with *Hae*III).

amplification only in the homozygous patterns. They could not distinguish the differential PCR amplification between the homozygous and heterozygous patterns.

As the results, some DNA individuals were not be amplified, it might be caused by the quality of DNA that extracted by machine. Some DNA fragments might be broken during process of extraction. Also, the PCR conditions might be not suitable for amplification. In additions, the amplified bands from seven primer pairs that could not distinguish the differences in the gel level by electrophoresis might be had the nucleotide polymorphism differently in 1-2 base pair. So, it should be used the other methods such as sequencing to detect the different bands.

4.3.2. Analysis of SSR markers linked to pungency of *C. frutescens* x *C. chinense* on a linkage map of *C. annuum*

SSR markers in this study were used to analyze the relation with SSR markers in the linkage map of *C. annuum* (Minamiyama *et al.*, 2006). In 13 linkage groups (LGs) of *C. annuum* linkage map, it found that CAMS630 and CAMS679 were mapped on LG1. CAMS090 and CAMS855 were mapped on LG8. Also, CAMS191, CAMS424, CAMS460, CAMS340, and CAMS451 were mapped on LG3, LG6, LG7, LG10, and LG11, respectively.

In additions, the relation between each SSR markers in this study was examined. The percentage of relation was showed in Table 4-2. Each pair of marker had the percentage of relation about 30-50%. However, CAMS090 and CAMS855 had the percentage of relation in 97.1%. Also, CAMS630 and CAMS679 had the percentage of relation in 58.9%. These results were conformed to the result on the linkage map of *C. annuum* that CAMS090 and CAMS855 were mapped on the same LG8; or CAMS630 and CAMS679 were mapped on the same LG1. It has possibility that CAMS090 and

Table 4-2. Percentage of relation between each 10 SSR markers

Marker	CAMS090	CAMS191	CAMS340	CAMS396	CAMS424	CAMS451	CAMS460	CAMS630	CAMS679	CAMS855
CAMS090										
CAMS191	38.8									
CAMS340	42.5	47.7								
CAMS396	40.0	29.1	32.3							
CAMS424	44.6	28.9	36.7	35.9						
CAMS451	32.6	34.0	36.7	33.6	37.7					
CAMS460	54.7	38.1	36.0	40.0	38.7	30.4				
CAMS630	43.2	38.3	34.1	38.5	41.7	41.6	36.6			
CAMS679	35.3	28.0	37.7	42.1	37.0	36.1	35.3	58.9		
CAMS855	97.1	42.9	40.6	39.3	43.9	33.8	50.7	42.2	34.2	

CAMS855; or CAMS630 and CAMS679 are mapped on the same in each LG of *C. frutescens* and *C. chinense* combination.

4.3.3. Analysis of the relation between SSR marker and the few-pungency linked to the *cf* locus in *C. frutescens* x *C. chinense*

To find the SSR marker linked to the *cf* locus in *C. frutescens* x *C. chinense*, the parent lines and F_2 population was used. For SSR marker, the very pungent chili pepper 'S3010' (*C. chinense*) (P_1) and the few-pungent chili pepper 'S3212' (*C.frutescens*) (P_2) showed the differential PCR amplification in SSR pattern '1' and '2', respectively. In F_2 population, the homozygous individuals showed the SSR patterns of either the P_1 'S3010' SSR pattern '1' or the P_2 'S3212' SSR pattern '2'. Some individuals were heterozygous that they showed in SSR pattern '1+2'. For pungency, the result was referred to the previous experiment (Experiment 2) that the F_2 population separated into two groups by capsaicinoid contents. One was the few-pungent group (capsaicinoid content is less than 100 $\mu g \cdot g^{-1}DW$) and another was the pungent group (capsaicinoid content is more than 100 $\mu g \cdot g^{-1}DW$).

Analysis of relation between SSR pattern and pungency in F_2 individuals, it showed that the SSR patterns were not related with the few-pungent trait in these chili peppers. If the SSR patterns were related with the few-pungent trait, the F_2 individuals showed the SSR pattern '2' that was inherited from few-pungent parent (P_2) should be the few-pungent pepper in all but some individuals were pungent. Also, F_2 individuals in pattern '1' or '1+2' that they should be pungent in all but some individuals were few-pungent (Table 4-3). Therefore, it revealed that the 10 SSR markers that were found in this study were not so linked to the few-pungent gene, *cf* gene, at the *cf* locus in these chili peppers.

Table 4-3. The relation between SSR marker and pungency in an interspecific hybrid of *C. frutescens* x *C. chinense*

Marker	1		1+2		2	
	Pungency	Few-pungency	Pungency	Few-pungency	Pungency	Few-pungency
CAMS090	19	12	35	15	11	8
CAMS191	24	7	25	26	24	7
CAMS340	32	12	24	20	6	2
CAMS396	30	12	36	22	18	10
CAMS424	25	2	46	11	8	28
CAMS451	22	9	29	18	28	11
CAMS460	23	4	37	11	11	12
CAMS630	16	4	38	23	30	16
CAMS679	12	7	44	18	19	11
CAMS855	17	9	32	11	13	5

1: SSR pattern 1; homozygous individual that had pattern same as parent line (P_1)
2: SSR pattern 2; homozygous individual that had pattern same as parent line (P_2)
1+2: SSR pattern 1+2; heterozygous individual that had pattern in both parent

4.3.4. Analysis of the relation between SSR marker and immature fruit color in *C. frutescens* x *C. chinense*

To investigate the SSR marker linked to immature fruit color in *C. frutescens* x *C. chinense*, the parent lines and F_2 population was used. For SSR marker, *C. chinense* (P_1) and *C. frutescens* (P_2) showed the differential PCR amplification in SSR pattern '1' and '2', respectively. In F_2 population, the homozygous individuals showed the SSR patterns of either the P_1 SSR pattern '1' or the P_2 SSR pattern '2'. Some individuals were heterozygous that they showed in SSR pattern '1+2'. For immature fruit color, the result was referred to Baba *et al.* (2006) that the F_2 population showed the immature fruit color into three groups. Group 1 was deep green color, group 2 was medium green color, and group 3 was white green color.

Analysis of relation between SSR pattern and immature fruit color in F_2 individuals, it showed that the SSR patterns were not related with the immature fruit color trait in these chili peppers (Table 4-4). Therefore, it revealed that the 10 SSR markers that were found in this study were not linked to the immature fruit color trait in these chili peppers.

4.3.5. Analysis of the relation between SSR marker and CAPS marker SCY-800 linked to the *C* locus

To investigate the SSR marker related to the CAPS marker SCY-800 linked to the *C* locus in *C. frutescens* x *C. chinense*, the parent lines and F_2 population was used. For SSR marker, the 'S3010' (*C. chinense*) (P_1) and the 'S3212' (*C. frutescens*) (P_2) showed the differential PCR amplification in SSR pattern '1' and '2', respectively. In F_2 population, the homozygous individuals showed the SSR patterns of either the P_1 'S3010' SSR pattern '1' or the P_2 'S3212' SSR pattern '2'. Some individuals were heterozygous that they showed in SSR pattern '1+2'. For CAPS marker SCY-800 linked to the *C* locus, the result

Table 4-4. The relation between SSR marker and immature fruit color in an interspecific hybrid of *C. frutescens* x *C. chinense*

Marker	1			1+2			2		
	Deep green	Medium green	White green	Deep green	Medium green	White green	Deep green	Medium green	White green
CAMS090	30	0	1	47	2	1	7	7	4
CAMS191	26	1	4	44	3	2	26	3	2
CAMS340	34	6	3	35	7	2	7	0	1
CAMS396	35	3	4	47	6	4	24	3	0
CAMS424	23	1	3	51	4	2	26	6	2
CAMS451	25	5	1	37	5	4	36	3	0
CAMS460	23	2	2	41	5	1	18	3	2
CAMS630	16	3	1	54	5	1	37	4	4
CAMS679	16	1	2	51	9	2	26	1	3
CAMS855	24	1	1	41	2	0	8	6	4

1: SSR pattern 1; homozygous individual that had pattern same as parent line (P_1)
2: SSR pattern 2; homozygous individual that had pattern same as parent line (P_2)
1+2: SSR pattern 1+2; heterozygous individual that had pattern in both parent

was referred to the previous experiment (Experiment 2) that the F_2 population showed CAPS patterns of either the 'S3212' type 'E' or 'S3010' type 'D'. And some plants were heterozygous that they were showed in both types 'D+E'.

Analysis of relation between SSR pattern and CAPS pattern in F_2 individuals, it showed that the SSR patterns were not related with the CAPS pattern in these chili peppers. If the SSR patterns were related with the CAPS pattern, the F_2 individuals showed the SSR pattern '2' that was inherited from parent (P_2) should be showed in CAPS pattern 'E' in all but some individuals were showed in CAPS patterns 'D' or 'D+E'. Also, F_2 individuals in pattern '1' or '1+2' that they should be showed in CAPS patterns 'D' or 'D+E' in all but some individuals were showed in CAPS pattern 'E' (Table 4-5). Therefore, it revealed that 10 SSR markers were found in this study were not linked to the *C* locus in these chili peppers (*C. frutescens* x *C. chinense*). This result might be conformed to Minamiyama *et al.* (2006) in the linkage map of *C. annuum* that reported the CAPS marker (SCY-800) linked to *C* locus was mapped on the LG13 where was no these 10 SSR markers mapped together. So, in the present study, it has possibility that the CAPS marker (SCY-800) is mapped on the LG where is no these 10 SSR markers mapped together in the linkage map of *C. frutescens* and *C. chinense* combination.

Regarding to this study, the information of these 10 SSR markers concerning with the linkage map of *C. annuum* will be useful for genetic analysis of chili pepper further. However, the approach of the marker linked more closely with the few-pungent gene (*cf* gene) at the *cf* locus in these chili peppers, more information of molecular marker combining with sequencing is needed to clarify the discrepancies. In additions, further study shall be focus on the other marker or method such as AFLP marker or adding the number of primer for construction of linkage map of *C. frutescens* x *C. chinense*. The knowledge will be useful to detect and develop the co-dominant marker for marker-

Table 4-5. The relation between SSR marker and CAPS marker SCY-800 linked to the *C* locus in an interspecific hybrid of *C. frutescens* x *C. chinense*

Marker	1			1+2			2		
	D	D+E	E	D	D+E	E	D	D+E	E
CAMS090	11	8	12	12	30	8	3	11	5
CAMS191	10	17	4	10	26	15	11	12	8
CAMS340	13	22	9	10	22	12	1	4	3
CAMS396	8	21	13	17	27	14	9	15	4
CAMS424	7	13	7	16	28	13	7	19	10
CAMS451	8	16	7	15	24	8	9	17	13
CAMS460	6	14	7	12	27	9	7	9	7
CAMS630	6	12	2	15	31	15	14	18	14
CAMS679	6	9	4	15	31	16	10	11	9
CAMS855	10	7	9	12	23	8	3	11	4

1: SSR pattern 1; homozygous individual that had pattern same as parent line (P_1)
2: SSR pattern 2; homozygous individual that had pattern same as parent line (P_2)
1+2: SSR pattern 1+2; heterozygous individual that had pattern in both parent
D: CAPS pattern D; homozygous individual that had pattern same as parent line (P_1)
E: CAPS pattern E; homozygous individual that had pattern same as parent line (P_2)
D+E: CAPS pattern D+E; heterozygous individual that had pattern in both parent

assisted selection (MAS) to breeding the new variety of chili pepper which is stable in few-pungency.

4.4. Abstract

To find more markers linked to *cf* locus, 259 SSR primer pairs were examined in F_2 population. Ten SSR markers were found to be distinguishable high pungent and few-pungent parents, however, close relationship with few-pungency was not observed. Some pairs of SSR markers, linkages were observed with high percentage same as reported in *C. annuum* by Minamiyama *et al.* (2006). This indicates that linkage map of *C. frutescens* x *C. chinense* in this experiment may be unified with that of *C. annuum.*

Experiment 5

Molecular Mapping of the *cf* locus for Few-pungency in Chili Pepper (*Capsicum frutescens* x *Capsicum chinense*)

5.1. Introduction

Capsicum spp. is a plant giving rise to very large phenotypic diversity depending on the country where it is cultivated and its intended use, such as fresh or cooked vegetable and spice. Pungency is one of the most important characteristics in chili peppers. Capsaicinoids, which are produced in the placenta of the fruit, are the origin of this pungency (Blum *et al.*, 2003; Suzuki *et al.*, 1980). Chili pepper cultivars differ in their level of pungency because of quantitative variation in capsaicinoid content.

Due to the simple selection based on phenotype is not always effective for the quantitative trait such as capsaicinoid content. More recently, the selection of this trait using molecular linkage markers – marker-assisted selection (MAS) – has been recognized as a powerful tool for breeding. Information on linkage map is indispensable for elucidating quantitative trait loci and obtaining linkage markers.

In this study, the few-pungency which was novel and unique to 'S3212' in *C. frutescens* found to be controlled by a single recessive gene, namely *cf* gene at the *cf* locus (Saritnum *et al.*, 2008). We report the identification of several markers linked to the *cf* locus in chili pepper (*C. frutescens* x *C. chinense*). To demonstrate the potential of these linked markers for the *cf* locus, we tried to construct the linkage map of these chili peppers (*C. frutescens* x *C. chinense*) using RAPD, CAPS, and SSR marker. It will be useful to starting points of marker developing for breeding programs.

5.2. Materials and Methods

5.2.1. Plant materials (refer to Experiment 2)

The F_2 population of 134 plants derived from a cross between *C. frutescens* 'S3212' (a few-pungent chili pepper) and *C. chinense* 'S3010' (a pungent chili pepper) was used for genetic analysis.

5.2.2. Data analysis and map construction

Linkage analysis using the molecular markers and *cf* locus of few-pungent trait in chili pepper (*C. frutescens* x *C. chinense*) was performed with JoinMap 4 software (Van Ooijen, 2006) under the conditions of threshold values of a minimum LOD score above 2.0. Genetic linkage map was drawn using MapChart software (Voorrips, 2002).

5.3. Results and discussion

5.3.1. Construction of the linkage map of chili pepper (*C. frutescens* x *C. chinense*)

Linkage analysis was done using a total of 20 molecular markers that consisted of 7 RAPD, 2 CAPS and 11 SSR markers. Out of 20 molecular markers analyzed, 13 were placed on 3 linkage groups by the use of a minimum LOD score of above 2.0 and a maximum recombination value of 0.25. The groups ranged from 1.5 to 86.4 cM in length and carried 2 to 9 markers (Fig. 5-1).

5.3.2. Analysis of molecular marker linked to the *cf* locus for the few-pungency in the linkage map of chili pepper (*C. frutescens* x *C. chinense*)

The genetic distances and order of the *cf* locus and molecular markers were shown in Fig. 5-1. Nine markers were mapped in the same group with *cf* locus. However, the side of *cf* locus indicated in this study might be not only locus of *cf* gene controls the few-

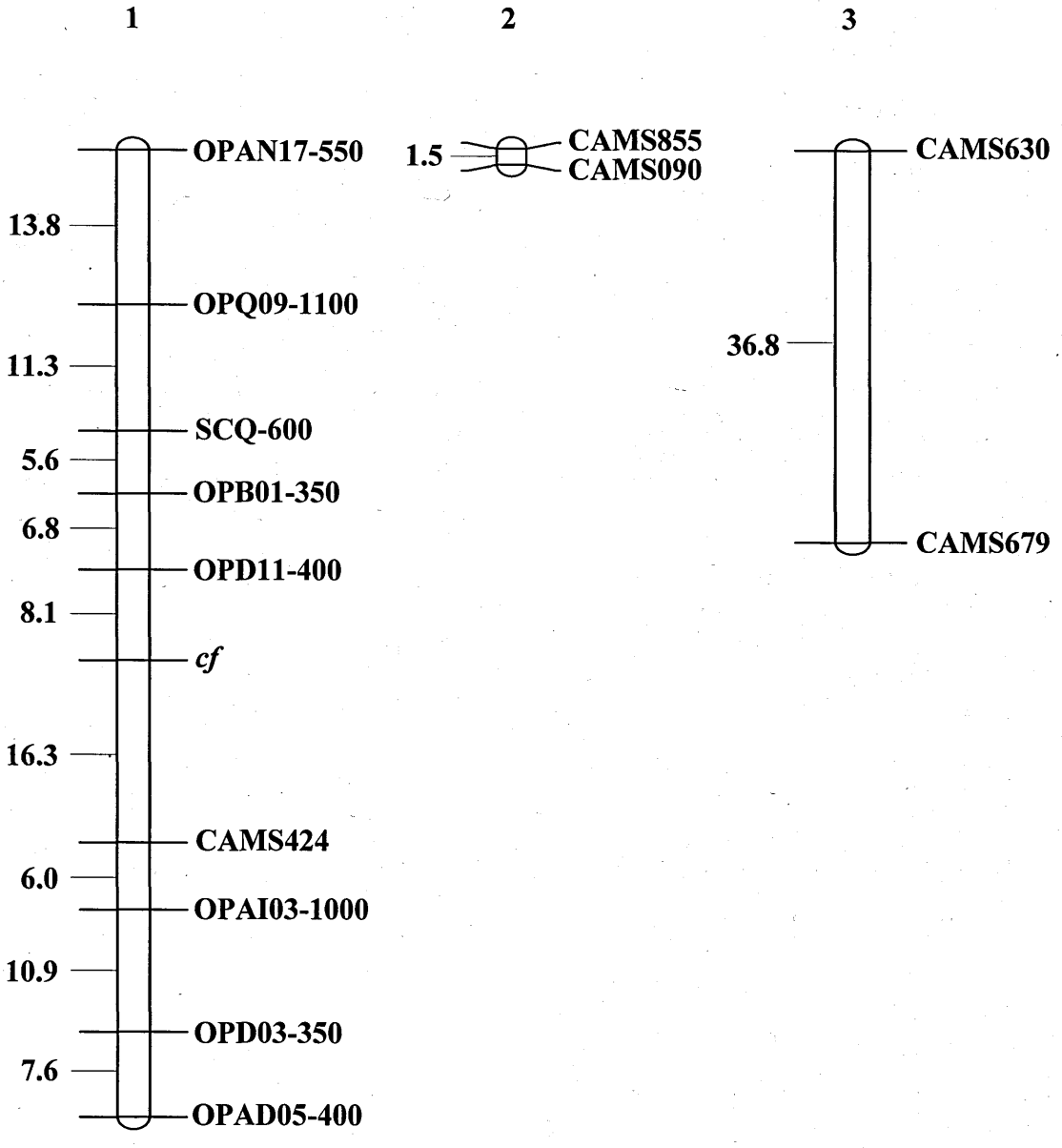

Fig. 5-1. Linkage map of the *cf* locus and molecular markers in F_2 population of chili pepper (*C. frutescens* x *C. chinense*). Genetic distances were calculated using Joinmap 4. Map distances are in centiMorgans.

pungent trait but also included the other minor genes participated to control the pungency from 0 $\mu g \cdot g^{-1}$DW in QTL. By here, it is possible to have affected to the distance of marker linked to *cf* locus for few-pungency of these chili peppers.

Of this study, the RAPD marker, OPD11-400, was found to be the closest marker linked to the *cf* locus. However, it had a distance of 8.1 cM with the *cf* locus (Fig. 5-1). This result was concerned to the recombination frequency that this marker also showed in the lowest recombination frequency of 10.3% with the few-pungency (Table 5-1).

For the co-dominant marker such as CAPS marker, SCQ-600 had a distance of 20.5 cM with the *cf* locus (Fig. 5-1). However, this CAPS marker was mapped closer to the *cf* locus than RAPD marker OPQ09-1100 converted to this CAPS marker, it confirmed that this CAPS marker was linked closely more to the *cf* locus. This result also concerned to the recombination frequency that CAPS marker SCQ-600 had the recombination frequency lower than RAPD marker OPQ09-1100 (Table 5-1). For SSR marker, CAMS424 was mapped in the same linkage group of *cf* locus. It showed a distance of 16.3 cM with the *cf* locus and a recombination frequency of 17.0% with the few-pungency (Fig. 5-1, Table 5-1). By this way, the distance and recombination frequency of this SSR marker might be caused by the small size of analyzed data. Hence, this marker was found to have some link to the *cf* locus with some distance. A partial linkage map around the *cf* locus was shown in Fig. 5-1.

Table 5-1. Segregation of pungency and molecular marker, and chi-square tests for fitting to 3:1 (χ^2A; χ^2B) and to 9:3:3:1 (χ^2AB) expected in F_2 population of *C. frutescens* 'S3212' (P_2) × *C. chinense* 'S3010' (P_1).

Locus		Number of plants observed [Z]				Chi-square values			Recombination frequency (%)
A	B	AB	Ab	aB	ab	χ^2A	χ^2B	χ^2AB	
Pungency	OPB01-350	83	6	14	31	5.26**[Y]	0.49	81.38***	13.9
	OPD03-350	78	11	19	26	5.26**	0.49	46.62***	22.6
	OPD11-400	84	5	10	35	5.26**	1.68	110.86***	10.3
	OPQ09-1100	80	9	18	27	5.26**	0.25	54.07***	19.8
	OPAD05-400	80	9	21	24	5.26**	0.01	40.46***	22.2
	OPAI03-1000	73	11	14	29	5.31**	2.86*	66.86***	19.6
	OPAN17-550	72	17	20	25	5.26**	2.88*	36.83***	28.7
	SCQ-600	85	3	15	29	4.89**	0.04	76.70***	10.7
	CAMS424	71	8	13	28	5.38**	1.60	69.57***	17.0

[Z] A, a: Pungency or few-pungency; B, b: Presence or absence of dominant marker/ presence of specific band in co-dominant marker.

[Y] *, ** and *** indicate significance at $P \leq 0.1$, $P \leq 0.05$ and $P \leq 0.01$, respectively, by chi-square test.

In the present study, the other 2 groups also showed in the linkage map of *C. frutescens* x *C. chinense*, resulting in some SSR markers mapped on the same in each linkage group of the linkage map of *C. annuum* (Minamiyama *et al.*, 2006). As the results of the linkage map of *C. frutescens* x *C. chinense*, one group was included with CAMS090 mapped at a distance of 1.5 cM with CAMS855. Another one group was included with CAMS679 mapped at a distance of 36.8 cM with CAMS630 (Fig. 5-1). These results also concerned to the previous result of the relation in each marker showing in high percentage of each pair related marker.

Therefore, based on the present study, the information of molecular markers linked to the *cf* locus might be useful as a source of PCR-based markers for developing of marker linked tightly more to the *cf* locus of *cf* gene controls the few-pungent trait in chili pepper. Fine mapping of the *cf* locus and development of more closely linked markers are expected to facilitate marker-assisted selection (MAS) in chili pepper breeding.

5.4. Abstract

A linkage map of chili pepper (*C. frutescens* x *C. chinense*) was constructed. Thirteen markers were mapped on 3 linkage groups. RAPD marker OPD11-400 located closest to *cf* locus with a distance of 8.1 cM. As for the co-dominant marker, CAPS marker SCQ-600 was mapped closer to *cf* locus than RAPD marker OPQ09-1100 from which SCQ-600 was converted. SSR marker, CAMS424 was linked to *cf* locus with some distance. As for the other 2 linkage groups, one included CAMS090 and CAMS855 and another included CAMS630 and CAMS679. These two linkage groups accorded with the linkage map of *C. annuum* (Minamiyama *et al.*, 2006). By developing the fine map around *cf* locus, more closely linked markers to *cf* locus will be constructed and be used for marker-assisted selection (MAS) in stable few-pungent chili pepper breeding.

Chapter IV

General discussion

The information of plant genetic improvement through breeding is essential for development of the new breeding lines. Major breeding efforts on plants focus on fruit traits, productivity, disease resistance, and the regulation of components. However, many of these traits are quantitative, and simple selection based on phenotype is not always effective. More recently, the selection of these traits using molecular linkage markers – marker-assisted selection (MAS) – has been recognized as a powerful tool for breeding. For the important spice of Thailand such as galangal and chili pepper, this information is still insufficiently studied. So, the present study had focused on the genetic and breeding concerning with the functional components of galangal and chili pepper that will be utilized for plant improvement and breeding further.

For galangal, the basic information on genetic variation related with geographical distribution was observed using cluster analysis based on RAPDs. ACA content, an important character of galangal, was also investigated in relation to genetic relationship for standpoint of breeding of high ACA content cultivar.

On the basis of randomly amplified polymorphic DNA (RAPD), this technique has been increasingly used for determination of genetic variability in various taxa. RAPD is particularly useful for rapid detection of divergence and for identification of DNA markers between investigated taxa (Hadrys *et al.*, 1992). As Swoboda and Bhalla (1997) used this technique to determine inter- and intraspecific variation of wild and cultivated forms of fan flower, *Scaevola* spp., large genetic differences among these species were found from RAPD analysis suggesting the possibility to apply this approach for breeding programs of these taxa at both intra- and interspecific levels.

In this experiment, randomly amplified polymorphic DNA (RAPD) technique had been able to cluster 37 accessions of galangal into 5 major groups using 8 RAPD primers. As for the type of galangal, they did not make an independent cluster. This suggests that the wild type was developed to the cultivated type. Most of cultivated accessions might be improved from local varieties by conscious breeding. The wild accessions might be useful for source of genetic variation. As for cultivated accessions, they were classified into five clusters without any relation with their morphological characters such as color or size of rhizome. Nevertheless, the clustering of galangal was related to the collection site in each galangal concerning with the ACA content. These regional clustering results were corresponded to the other vegetative propagating plants such as elephant foot yams (Sugiyama *et al.*, 2006), suggesting the presence of regional clustering based on AFLP markers.

Regarding to the genetic information and the genetic similarity in this study, it was found that some RAPD markers were probably linked to the high potential ACA content from these galangals. It may be useful as RAPD markers for plant improvement and breeding. Some reports revealed that RAPD markers appear to use for assessing genetic relationships routinely by plant breeders to identify genetic variation (Keil and Griffin, 1994; Lashermes *et al.*, 1996; Perron *et al.*, 1995; Fico *et al.*, 2003), locate regions of the genome linked to agronomically important genes (Reiter *et al.*, 1992; Martin *et al.*, 1991; Michelmore *et al.*, 1991; Pillay and Kenny, 1996; Ochiai *et al.*, 2001), and facilitate introgression of desirable genes into commercial accessions (Stuber, 1992; Lavi *et al.*, 1994).

Therefore, in the present study, the information of genetic variation and relationship of galangal related with geographical distribution in Thailand will be beneficial to galangal breeding further.

For chili pepper, *C. frutescens* has remarkably high pungency (IBPGR, 1983). There was not reported about non-pungent chili pepper in this species. However, 'S3212' was found to be unique to few-pungency in *C. frutescens*. So, this study reported about the inheritance of few-pungent trait in chili pepper 'S3212' (*C. frutescens*). The segregation of capsaicinoid contents in an interspecific hybrid of chili pepper (*C. frutescens* x *C. chinense*) revealed that the few-pungency in these chili peppers is controlled by a single recessive gene mainly. Even the F_2 population did not conform to the expected 3 : 1 ratio, it estimates that some of minor genes also participate to control the pungency trait in QTL. Owing to there was no individual that had complete lack of capsaicinoid, the gene controls few-pungent trait, *cf* gene, is different from *c* gene that controls complete lack of pungency in *C. annuum* (Deshpande, 1935; Daskalov and Poulos, 1994).

For the isolocus test between *cf* locus and *C* locus, CAPS marker SCY-800 linked to the *C* locus was used (Minamiyama *et al.*, 2005). Analysis in the relation between pungency trait and CAPS pattern linked to *C* locus in F_2 individuals, it showed that the CAPS patterns were not related with the pungency in these chili peppers. It confirmed that the gene controls few-pungency, namely *cf* gene located differently from the *C* locus. It also illustrates that the *cf* locus is different from the *C* locus.

Furthermore, three RAPD markers were found to link with the few-pungency in these chili peppers. It was clear that the few-pungent trait in these chili peppers was controlled mainly by single recessive gene, namely *cf* gene. However, there is no evidence of that single gene locates on the *cf* locus. Because these three RAPD markers showed the recombination frequency concerning to the linkage distance with the *cf* locus linked to few-pungency in these chili peppers. On the contrary, the results also assumed that the few-pungent trait in these chili peppers was controlled mainly by single recessive gene with the other minor genes participated to control the high contents of pungency in QTL with the

wide range of capsaicinoid from the lowest to highest content. By this way, some few-pungent individuals that were controlled by polygene in QTL were included in the few-pungent group. So, the reason of pungency segregation ratio in F_2 population that it was not fit to the expected ratio 3:1 might be caused from the effect of minor genes (Ben-Chaim *et al.*, 2006; Blum *et al.*, 2003) or environmental effects (Harvell and Bosland, 1997; Zewdie and Bosland, 2000b). However, these three RAPD markers confirmed the linkage to the few-pungency in these chili peppers. The confirmation of a single gene (*cf* gene) controlled few-pungent trait is different from *c* gene and some mechanism concerns with the *cf* gene were described. It reinforced that the few-pungency in these chili peppers was controlled mainly by a single recessive gene, namely *cf* gene.

In additions, the identification of molecular marker more linked closely to the *cf* gene at the *cf* locus for few-pungency, RAPD, and CAPS marker were analyzed with the parents and F_2 population. Many markers were used, however, they also showed the recombination frequency with the few-pungency. Even the CAPS marker SCQ-600 converted from RAPD marker OPQ09-1100 was developed to link closely more to the *cf* locus, it also showed some recombination frequency with the few-pungency, indicating that the marker had some linkage distance from the *cf* locus.

Regarding to SSR marker, this markers are PCR-based marker that have been developed in many plant species, including most major crops, such as rice, maize, sorghum, wheat, and barley, as well as vegetable crops (Mba *et al.*, 2001; Suwabe *et al.*, 2002; Chiba *et al.*, 2003). SSR markers have the advantage of a high reproducibility with high variability and potentially increasing both reliability and resolution (Wim *et al.*,2008). It can been showed in multiallelic, highly polymorphic, and co-dominant, and have been developed and used for the genetic mapping of solanaceous vegetables, including tomato,

potato, and eggplant (Broun and Tanksley, 1996; Smulders *et al.*, 1997; Milbourne *et al.*, 1998; Nunome *et al.*, 2003).

In this study, the SSR markers also were used to analyze in the SSR-based linkage map in chili pepper. Using these 17 SSR primer pairs in 134 individual plants of F_2 population, it found that 100 individual plants were approximate to be amplified, however, only 10 primer pairs showed the differential PCR amplification in all of homozygous and heterozygous patterns. It suggested that some DNA fragment might be broken during process of DNA extraction by machine or the condition of PCR might be not suitable for amplification. Moreover, some polymorphic bands might be had the nucleotide polymorphism differently in 1-2 base pair which could not be separated well in the gel by electrophoresis. It suggests to the other method such as sequencing for detecting the differences.

Furthermore, some reports also revealed that the low level of DNA polymorphism in crop species is an obstacle to applying molecular marker technology in breeding programs. Self-incompatible *Brassica* crops tend to show high levels of polymorphism among breeding lines and cultivars, whereas other crops, such as eggplant, soybean, and cucumber, tend to show low levels of polymorphic loci within each species (Maughan *et al.*, 1996; Bradeen *et al.*, 2001; Nunome *et al.*, 2001). The breeding lines of sweet and hot *C. annuum* also show low levels of polymorphism (Minamiyama *et al.*, 2006). Therefore, interspecific crosses are sometimes used for mapping populations in these low polymorphic species. In the present study, although an interspecific crossing was used, it showed the low level of polymorphism. Lee *et al.* (2004) described that the low number of primer pairs used reflects the low polymorphism. Other problems might be related to technical aspects that some of the SSR primer pairs amplified poor or non-reproducible bands, and others sometime amplified more complex bands but were not suitable for

scoring alleles, as described by Roder *et al.*, (1998). Therefore, in the present study, it suggests to use more primer numbers to detect the high polymorphism linked closely more to the few-pungency in these chili peppers (*C. frutescens* x *C. chinense*).

In this study, within 10 SSR markers, it suggested some relation of SSR marker in the linkage map of *C. annuum* (Minamiyama *et al.*, 2006) such as CAMS090 and CAMS855; CAMS630 and CAMS679. These markers have possibility to map on the same in each LG of *C. frutescens* and *C. chinense* combination. However, these 10 SSR primers were not so relation with pungency trait, immature fruit color trait, and CAPS marker linked to *C* gene. Approach of the SSR marker linked with the few-pungent gene (*cf* gene) in these chili peppers, the combining data with sequencing is needed to clarify the discrepancies.

Owing to information on linkage map is indispensable for elucidating quantitative trait loci and obtaining linkage markers, molecular mapping of chili pepper (*C. frutescens* x *C. chinense*) was constructed using RAPD, CAPS and SSR marker. Among 20 markers mapped, 9 markers were mapped in the same linkage group with the *cf* locus linked to *cf* gene for few-pungency; 4 markers were mapped into the other two linkage groups; and 7 markers remained no linkage in the mapping. It might be caused to no enough data for linkage analysis.

In this study, RAPD marker, OPD11-400 was the closest marker mapped with the *cf* locus. However, RAPD marker is dominant marker that could not be used to detect in heterozygous. So, RAPD marker OPQ09-1100 showed the clear and reproducible band was converted to CAPS marker SCQ-600. The CAPS marker, SCQ-600, was mapped closer to the *cf* locus than RAPD marker OPQ09-1100. In here, although this CAPS marker showed the low recombination frequency likely to the closest marker of this study, it was not the closest marker seemly mapped with the *cf* locus. Mokrani *et al.* (2002) described in

many factor as to the nature of the population studied, the number of individuals and the number of markers that might change the recombination frequency and in consequence the distance between two loci.

Overcoming in low polymorphism is a key to marker technology, AFLP is a powerful technique with which to score a number of polymorphic loci in a single experiment (Vos *et al.*, 1995). So, in this study, it suggests to using the other marker such as AFLP or adding the number of primer, more information will be available and useful for detecting of molecular marker linked closely more to the *cf* locus for few-pungency in these chili peppers. Fine mapping of *cf* locus and development of more closely linked markers are expected to facilitate marker-assisted selection (MAS) in chili pepper breeding.

Beyond the Genetic and Breeding Studies on Functional Components of Galangal and Chili Pepper, the information of galangal and chili pepper in the present study will be beneficial to plant improvement and breeding further.

Chapter V

Summary

Galangal and chili pepper are the highly potential plants that have a lot of benefit. Studies on such these two plants had been reported, however, the information of genetic improvement through breeding in galangal and chili pepper (*Capsicum frutescens* x *Capsicum chinense*) is still insufficiently studied. This information is essential for development of the new breeding lines. So, the present study was undertaken to analyze in genetic of galangal and chili pepper based on molecular marker for plant improvement and breeding.

Galangal (*Alpinia galanga* Willd.), its rhizome is used not only as a common spice for flavor soups and many other dishes but also as a medicinal and aromatic plant. 1′-acetoxychavicol acetate (ACA) is one of principal ingredients in rhizome of galangal that has a lot of benefit such as anti-tumor (Kondo *et al.*, 1993) and anti-fungal (Itokawa *et al.*, 1987). Many studies on such ingredient of galangal had been carried out, however, there was little information about genetic relationship of galangal available for systematic breeding.

In experiment 1 "Genetic Relationship of Galangal (*Alpinia galanga* Willd.) in Thailand by RAPD Analysis", 37 galangal accessions were used. Out of 22 RAPD primers, eight primers produced a total of 73 polymorphic bands. A dendrogram constructed by UPGMA method using RAPD data clustered the accessions into 5 major groups. There was no relation between major cluster and type or morphological characters such as size or color of rhizome. But major cluster had some relation with their collection sites. Two of five major clusters corresponded to three regions of Thailand with the exception of some accessions. These results were also concerned with the quantity of ACA contents. The

largest cluster (No. II) included the accessions collected from central and eastern region. Some accessions of this cluster had high quantity of ACA contents. Another cluster (No. I) included the accessions collected from northern region and most of accessions had low quantity of ACA contents. The specific RAPD bands probably linked to the quantity of ACA contents were presented. These bands may be potential RAPD markers for screening ACA content and will be used in marker-assisted selection (MAS) of high ACA content galangal breeding.

Chili pepper, *Capsicum frutescens* is known as a pungent chili pepper species. However, a line, 'S3212' (*C. frutescens*) showed very low capsaicinoid contents, namely few-pungency in the fruits (Matsushima *et al.*, 2004; Konisho *et al.*, 2005a). This few-pungency is unique to 'S3212' because all other *C. frutescens* accessions had pungent fruits with high capsaicinoid contents.

In experiment 2 "Inheritance of Few-pungent Trait in Chili Pepper 'S3212' (*Capsicum frutescens*)", the pungent accessions of *C. chinense* 'S3010' (P_1, 44,581 $\mu g \cdot g^{-1}DW$), few-pungent accessions of *C. frutescens* 'S3212' (P_2, 5 $\mu g \cdot g^{-1}DW$), F_1 ($P_2 \times P_1$, 4,967 $\mu g \cdot g^{-1}DW$), F_2 and backcross populations (BCPs) were used. Capsaicinoid contents were measured by using HPLC method. For capsaicinoid contents, 134 F_2 plants distributed closer to few-pungent parent 'S3212' (P_2) than to pungent parent 'S3010' (P_1) with range from the highest 20,547 $\mu g \cdot g^{-1}DW$ to the lowest 5 $\mu g \cdot g^{-1}DW$. And there was no plant that had complete non-pungency (0 $\mu g \cdot g^{-1}DW$). The F_2 and BCP_2 populations separated into two groups by capsaicinoid contents, respectively. One was the few-pungent group (capsaicinoid content is less than 100 $\mu g \cdot g^{-1}DW$) and another was the pungent group (>1,000 $\mu g \cdot g^{-1}DW$). The segregation of capsaicinoid contents in HPLC data indicates that few-pungency in these chili peppers is controlled by a single recessive gene. There was no individual that had complete lack of capsaicinoid, the gene controls few-pungent trait

called temperately as *cf* gene in this study is different from *c* gene that controls complete lack of pungency in *C. annuum*. Then, isolocus test between *cf* locus and *C* locus controlling capsaicinoid production was carried out using cleaved amplified polymorphic sequence (CAPS) marker SCY-800 linked to the *C* locus. The segregation of CAPS patterns was appropriate. CAPS pattern and few-pungency were not related, so the *cf* locus is different from the *C* locus. Furthermore, using a bulk segregation analysis, three RAPD markers linked to the pungency trait, trait as the allele of few-pungency, in these chili peppers were found. These three markers confirmed the linkage to the few-pungency in these chili peppers. It reinforced that the few-pungency in these chili peppers was controlled by a single recessive gene.

In experiment 3 "Genetic Analysis of Molecular Marker Linked to Few-pungent Gene in Chili Pepper (*Capsicum frutescens*)", RAPD and CAPS marker was examined in 134 plants of F_2 population (*C. frutescens* x *C. chinense*). A bulked segregation analysis was conducted using the 480 RAPD primers. Seven bands designated OPB01-350, OPD03-350, OPD11-400, OPQ09-1100, OPAD05-400, OPAI03-1000 and OPAN17-550 were amplified in 'S3010' pungent chili pepper and in the pungent bulk. According to chi-square tests, the segregation of pungency and RAPD marker did not deviate significantly from the expected 3:1 ratio. RAPD markers were found more to link with the *cf* locus. Nevertheless, they showed the recombination frequency in 10.3-28.7%, indicating that they had some linkage distance with the *cf* locus.

Development of the more closely linked marker, RAPD marker OPQ09-1100 had a clear and reproducible band was converted into a co-dominant CAPS marker, SCQ-600. This CAPS marker was found to link closely more to the *cf* locus, however, it showed the recombination frequency 10.7% indicating some linkage distance with the *cf* locus.

In experiment 4 "Analysis of SSR-based Linkage Map in Chili Pepper", seventeen SSR primer pairs from 259 primer pairs were used in 134 F_2 plants (*C. frutescens x C. chinense*). It found that 100 individual plants were approximate to be amplified. Ten SSR markers were found to be distinguishable high-pungent and few-pungent patterns, however, close relationship with few-pungency was not determined. Some pairs of SSR markers, the linkage was observed with high percentage same as reported in *C. annuum* by Minamiyama *et al.* (2006). This indicates that linkage map of *C. frutescens* x *C. chinense* in this experiment may be unified with that of *C. annuum.*

In experiment 5 "Molecular Mapping of the *cf* locus for Few-pungency in Chili Pepper (*Capsicum frutescens* x *Capsicum chinense*)", based on the above results, a linkage map of chili pepper (*C. frutescens* x *C. chinense*) was constructed. Thirteen markers were mapped on 3 linkage groups. RAPD marker, OPD11-400, was found to be the closest marker linked to the *cf* locus. It had a distance of 8.1 cM with the *cf* locus. For the co-dominant marker, CAPS marker SCQ-600 mapped closer to the *cf* locus than RAPD marker OPQ09-1100 converted to this CAPS marker, it confirmed that this CAPS marker was linked closely more to the *cf* locus. However, it had a distance of 20.5 cM with the *cf* locus. SSR marker, CAMS424 also mapped in the same linkage group of the *cf* locus with some distance. For the other 2 linkage groups in this linkage map (*C. frutescens* x *C. chinense*), one included CAMS090 and CAMS855 and another included CAMS630 and CAMS679. These two linkage groups accorded with the linkage map of *C. annuum* (Minamiyama *et al.*, 2006).

Based on the present study, the information of molecular markers linked to the *cf* locus might be useful as a source of PCR-based markers for developing of marker linked tightly more to the *cf* locus of the *cf* gene controls few-pungency in chili pepper. Fine

mapping of the *cf* locus and development of more closely linked markers are expected to facilitate marker-assisted selection (MAS) in stable few-pungent chili pepper breeding.

Therefore, the information based on "Genetic and Breeding Studies on Functional Components of Galangal and Chili Pepper" from the present study will be useful for the improvement and breeding of galangal and chili pepper further.

Chapter VI

Acknowledgements

The dissertation would not have been completed without a good gesture from many persons. I would like to express my heartfelt gratitude, sincere appreciation and immense indebtedness to my supervisor, Dr. Mineo Minami, Professor, Advanced Course of Sciences of Functional Foods (Integrated Department), Graduate School of Agriculture, Shinshu University, for his constant supervision and proper guidance throughout the period of this research works and preparing this manuscript.

I wish to express my sincere gratitude and appreciation to Dr. Ken-ichi Matsushima, Associate Professor, and Kazuhiro Nemoto, Assistant Professor, Advanced Course of Sciences of Functional Foods (Integrated Department), Graduate School of Agriculture, Shinshu University, for their helpful guidance, valuable advice, encouragement and care.

I am grateful to Dr. Pittaya Sruamsiri, Associate Professor, Faculty of Agriculture, Chiang Mai University, for his constant cooperation and invaluable suggestions.

Together with this, I am very grateful to Professor Masashi Hirai, Faculty of Agriculture, Kyoto Prefectural University, and Dr. Yasuhiro Minamiyama, Kyoto Prefectural Institute of Agricultural Biotechnology, for kind cooperation, training, suggestion and comments.

My profound gratitude and grateful thanks to the members of advisory committee, Professor Yoshihiko Furuta, Gifu University, Professor Mitsuo Omura, Shizuoka University, Professor Shuichi Miyagawa, Gifu University, and Associate Professor Yasunori Hamauzu, Shinshu University for their great encouragement and keen interest in the present studies.

Thanks and gratitude are due to all members of the Laboratory of Plant Genetics and Breeding, both past and present, for their friendship, encouragement and help in different ways.

Acknowledgement goes to a Japanese Government (Monbukagakusyo) Scholarship, The United Graduate School of Agricultural Science, Gifu University, and Faculty of Agriculture, Shinshu University for providing all sorts of facilities and kind cooperation throughout this research period.

Finally, I would like to express my deepest gratitude to my parents, sister, relatives and friends for their constant inspiration, unlimited love, care, understanding and encouragement extended throughout my study.

Chapter Ⅶ

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