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Doctoral Dissertation

Construction and Application of Linkage Maps for Azuki Bean (*Vigna angularis*)

アズキ連鎖地図の構築とその応用

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Contents

Chapter I. General introduction

Azuki bean (*Vigna angularis*) is one of the important legumina in East Asia (Yamaguchi 1992). Since red pigment from the seed coat has been traditionally used as the color of celebration, many native varieties exist throughout East Asia (Lumpkin and MacClay 1994). In Japan, based on their characteristic, cultivars of azuki beans including native varieties are usually divided into three groups, 'dainagon', 'shouzu' and 'shiroazuki'. 'Dainagon' having more than 16 gm of hundred-seed-weight (Narikawa 1976) is a commercially expensive group used for red 'an' (sweets ingredient) paste. 'Shiroazuki' with white seed coat is also an expensive group used for white 'an' paste. On the other hand, 'Shouzu' having less than 16 gm of hundred-seed-weight is sold at a lower price because of its large-scale production in Hokkaido Prefecture.

In Japan, azuki bean is the second most important pulse after soybean, and usually used as material for sweets. The annual production of azuki bean, however, is unstable because of the cold weather and pests. Therefore, it is necessary to develop high-yielding cultivars with cold tolerance and disease resistance. Little is known about the genetic diversity of azuki bean. Moreover, genetical research on agronomically important traits of azuki bean has lagged far behind those of other pulses, and only a few studies on morphological characters including seed and stem color (Matsuura 1933) and pod color and leaf shape (Kakizaki 1923) are available. At the present time, more genetic information is widely needed to support azuki bean breeding.

The number of genetic resources of *Vigna* species have been increased, and breeding programs have been initiated to introduce several desirable traits into azuki cultivars. For example, brown stem rot resistance has been introduced from one of the wild species, *V. nakashimae.* In Japan except Hokkaido area, bruchid, *Callosobruchus chinensis* L., often attacks stored azuki seeds and causes severe damages. The bruchid resistance was identified in rice bean, *V. umbellata* (Sawa and Tan 1976; Tomooka 1991). Although this bean is crossable with azuki bean and hybrids can be produced through embryo rescue, no resistant line has been developed yet. It has been pointed out that many undesirable traits might be transferred with desirable ones from wild species to the cultivar.

Molecular markers have been used successfully in phylogenetic studies, construction of linkage maps and gene tagging. A molecular linkage map provides a way to identify genes controlling quantitative traits and improved methods for selection in breeding programs. If molecular markers linked to the genes controlling desirable and undesirable traits are identified, it will be possible to monitor the useful genes and to eliminate the undesirable genes However, no genetic map for azuki bean have been constructed yet.

In the present investigation, the author studied the genetic diversity of azuki bean and its relatives using molecular makers, constructed linkage maps and tried to introduce desirable genes from related species into azuki cultivars.

The results from the following studies (chapter II-V) were reported.

In chapter II, genetic diversity in the subgenus *Ceratotropis* (genus *Vigna*) was investigated. RAPD analysis was carried out using 12 cultivars of azuki bean to reveal the intraspecific variation in *V. angularis*. RAPD and RFLP analyses were performed to elucidate the genetic diversity in the subgenus *Ceratotropis*. In chapter III, a genetic linkage map of azuki bean with molecular and morphological markers was constructed using interspecific population (*V. angularis* x *V. nakashimae*). In chapter IV, QTL analysis for some morphological and reproductive traits was performed using the molecular linkage map constructed in chapter III. In chapter V, another molecular linkage map was constructed using a interspecific population between *V. umbellata* and *V. angularis*, and the bruchid resistance and several morphological traits were characterized by QTL analysis based on this molecular linkage map.

Chapter II. Analysis of genetic diversity in the subgenus *Ceratotropis* (genus *Vigna*)

Introduction

The subgenus *Ceratotropis* of the genus *Vigna* includes five important Asian pulses; azuki bean, rice bean, mungbean, blackgram and mothbean. Rice bean (*V. umbellata*) is widely cultivated in Asia and the Pacific islands, and is often used as a substitute for azuki bean. Mungbean (*V. radiata*) and blackgram (*V. mungo*) have been major beans from ancient times in Asia. At present, mungbean cultivation spread worldwide because it is digested easier than blackgram (Smartt 1990). Mothbean (*V. aconitifolia*), which has drought tolerance, is cultivated in arid and semi-arid areas of western Asia (Jain and Mehra 1980). Wild relatives of these beans except mothbean were reported as follows: *V. angularis* var. *nipponensis* is known as the wild form of *V. angularis*, and *V. umbellata* var. *gracilis* is of *V. umbellata*. *V. radiata* var. *sublobata* and *V. mungo* var. *silvestris* are wild forms of *V. radiata* and *V. mungo,* respectively (Arora et al., 1973; Miyazaki, 1982; Chandel, 1984).

The wild *Ceratotropis* species in Japan include *V. angularis* var. *nipponensis*, *V. nakashimae*, *V. riukiuensis* and *V. reflexo-pilosa*. However, Tomooka et al. (1991) proposed to classify *V. nakashimae* and *V. riukiuensis* into a single species, *V. minima*. The *V. reflexo-pilosa* is an unique wild form having allotetraploid genome in this subgenus (Egawa et al. 1996), and has been thought to be a wild ancestral form of *V. grabrescens,* which is cultivated as a forage crop in Mauritius and

West Bengal (Tomooka et al. 1991). On the basis of isozyme analysis and crossability experiments, *V. reflexo-pilosa* was suggested to occur by interspecific hybridization between *V. trinervia* and *V. minima,* followed by natural chromosome doubling (Tomooka et al. 1991).

The subgenus *Ceratotropis* is one of seven subgenera in the genus *Vigna* (Verdcourt 1970) and has some characteristics in common with the species of the related genus, *Phaseolus.* The taxonomic treatment of *Ceratotropis* species has been confusing, and it has been called *Phaseolous- Vigna* complex. The seedling characteristics are useful to classify *Ceratotropis* species; *V. radiata* and *V. mungo* show epigeal germination and have primary leaves with sessile petioles, while *V. angularis* and *V. umbellata* show hypogeal germination and have primary leaves with long petioles. Previously, Maekawa (1955) treated the former group as the genus *Rudua* and the latter group as the genus *Azukia.* This classification were followed by other researchers, and they called them as 'Azuki group' and 'mungbean group', respectively (Tomooka 1991). The strong reproductive isolation barrier was observed between these two groups (Chen et al. 1983; Egawa 1988).

One of the *Vigna* species, V. *aconitifolia* shows epigeal germination and has primary leaves with petiole length intermediate between those two groups. The phylogenetic relationship of this species remains unknown, although Dana (1980) classified it into the group together with *V. radiata* and *V. mungo.*

Knowledge of genetic diversity is useful in making a population for mapping experiments because it facilitates efficient sampling and utilization of germ plasm resources. The researcher can use genetic similarity information to make decision regarding the choice of

genotypes to cross for development of population. Restriction fragment length polymorphism (RFLP) analysis has been shown to be one of powerful tools for estimation of genetic similarity. Fatokun et al. (1993) analyzed 18 *Vigna* species including five of the subgenus *Ceratotropis* by RFLP analysis to determine the relationships between the subgenus *Ceratotropis* and the other subgenera. Their results are consistent with expectation based on seed protein (D'Urzo et al. 1990; Rao et al. 1992) and isozyme (Jaaska and Jaaska 1990) analyses. However, still little is known about the genetic similarity and the genetic relationships within the subgenus *Ceratotropis* species.

Recently, another molecular analysis called random amplified polymorphic DNA (RAPD) analysis was reported to be useful for evaluating intraspecific variation (dos Santos et al. 1994; Thormann et al. 1994). RAPD markers are generated by the polymerase chain reaction (PCR) with single short oligonucleotides of arbitrary sequences and provide genetic information at a DNA level with relative ease (Williams et al. 1990). Since the genetic difference is reflected as the presence or absence of RAPDs and a large number of RAPDs can be obtained easily, RAPD analysis has been successfully used to reveal interspecific relationships in many plants, such as *Brassica* (Demeke et al. 1992), *Azolla* (Van Coppenolle et al. 1993), *Stylosanthes* (Kazan et al., 1993), *Lotus* (Campos et al. 1994) and *Panicum* (M'Ribu and Hilu 1994).

In this chapter, results of the following two studies were reported. (1) RAPD analysis using 12 cultivars of azuki bean to reveal the intraspecific variation of *V. angularis*. (2) RAPD and RFLP analyses to elucidate the genetic diversity in the subgenus *Ceratotropis*. The

correlation between RAPD and RFLP analyses was discussed.

Materials and methods

Plant materials

Two groups of *Vigna* cultivars and wild accessions belonging to the subgenus *Ceratotropis* were used.

Group 1 included 12 cultivars of azuki bean (*V. angularis*), one of common bean (*Phaseolus vulgaris*) and one of cowpea (*V. unguiculata*). This group was used for RAPD analysis to clarify the relationships among cultivars of azuki bean and its related genera (Table II-1).

Group 2 included three cultivars and two ancestral wild forms of *V. angularis*, two cultivars and two ancestral wild forms of *V. umbellata*, three cultivars and three ancestral wild forms of *V. radiata*, two cultivars of *V. aconitifolia,* two cultivars and four ancestral wild forms of *V. mungo*, one wild accession of *V. nakashimae,* one of *V. riukiuensis* and one of *V. reflexo-pilosa.* In order to reveal phylogenetic relationships in *Ceratotropis* species, 25 and 23 accessions from Group 2 were used for RAPD and RFLP analyses, respectively (Table II-2).

These species are self-pollinating and have same number of chromosome (2n=22) except for *V. reflexo-pilosa* (2n=44). The accessions 5-2 and 6-2 of *V. umbellata* var. *gracilis* have been proposed to be the most probable ancestral wild form of *V. umbellata* (Tomooka 1991). The registered azuki bean cultivars and native varieties of Group 1 were obtained from the National Institute of Hokkaido Prefectural Agricultural Experimental Station and Hontakasagoya

Table II-1. Plant materials used for RAPD analysis of azuki bean (*V. angularis*), common bean (*Phaseolus vulgaris*) and cowpea (*V. unguiculata*) cultivars.

Code	Cultivar	Origin
	Azuki bean	
1	Iwaidainagon	Native variety in Hokkaido
2	Akanedainagon	Breeding line in Hokkaido
3	Notodainagon	Native variety in Ishikawa
4	Hokkaidainagon	Native variety in Hokkaido
5	Hokkaishouzu	Native variety in Hokkaido
6	Benidainagon	Breeding line in Hokkaido
7	Hyoukei 1	Breeding line in Hyogo
8	Hyoukei 2	Breeding line in Hyogo
9	Sasayamanatu	Native variety in Kyoto
10	Erimoshouzu	Breeding line in Hokkaido
11	Otofukeshouzu	Native variety in Hokkaido
12	Kyotodainagon	Breeding line in Kyoto
	Common bean	
13	Satsukimidori 2	Commercial cultivar
	Cowpea	
14	Sanjakusasage	Commercial cultivar

Table II-2. Plant materials used for RAPD and RFLP analyses of Ceratotropis species.

Code	Species	Origin	Accession
1	*V. angularis*	Japan	Hyoukei 2
2	*V. angularis*	Japan	Hokkaisyouzu
3	*V. angularis*	Japan	Tokushima
4	*V. angularis* var. *nipponensis**	Japan	Sendai
5	*V. angularis* var. *nipponensis**	Japan	VA0001
6	*V. umbellata*	Japan	Kagoshima
7	*V. umbellata*	Nepal	EN337
8	*V. umbellata* var. *gracilis**	Thailand	5-2
9	*V. umbellata* var. *gracilis**	Thailand	6-2
10	*V. radiata* [1]	Indonesia	R51
11	*V. radiata*	Afghanistan	R107
12	*V. radiata*	China	Kitsurin
13	*V. radiata* var. *sublobata**	Madagascar	TC1966
14	*V. radiata* var. *sublobata**	India	TC2207
15	*V. radiata* var. *sublobata**	Australia	TC1965
16	*V. aconitifolia*	Pakistan	2725-3
17	*V. aconitifolia*	India	Nilgiri
18	*V. mungo*	Thailand	U-Tong 2
19	*V. mungo*	Thailand	NT9172
20	*V. mungo* var. *silvestris**	India	TC2208
21	*V. mungo* var. *silvestris**	India	TC2209
22	*V. mungo* var. *silvestris**	India	TC2210
23	*V. mungo* var. *silvestris**	India	TC2211
24	*V. nakashimae** [2]	Korea	Korea
25	*V. riukiuensis** [2]	Japan	90Y28
26	*V. reflexo-pilosa** [2]	Japan	90Y6

* : wild form.

1 : Materials used only for RAPD analysis.

2 : Materials used only for RFLP analysis.

Corporation, respectively. The seeds of Group 2 were obtained from the National Institute of Agrobiological Resources.

Total DNA extraction for RAPD analysis

Total DNA was isolated by the method of Doyle and Doyle (1987) with slight modifications: approximately 200 mg of primary leaves were collected from five one-week-old seedlings, ground in liquid nitrogen, and suspended in 1 ml of 2X CTAB isolation buffer [100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 0.2 % 2-mercaptoethanol, 2% hexadecyltrimethylammonium bromide] containing 1% sodium metabisulfite (Fritsch et al. 1993). The suspension were incubated at 60 °C for 30 min with optional gentle shaking. Protein of the mixture was removed by chloroform-isoamyl alcohol (24:1). Total DNA was precipitated with 2/3 vol of isopropanol. The recovered total DNA was centrifuged for 5 min at 15000 rpm in refrigerated centrifuge at 4 °C. The pellet was washed with 75 % ethanol containing 10 mM ammonium acetate and subsequently with 75 % ethanol. The dried pellet was suspended in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 1 μg RNase A, and incubated 30 min at 37 °C. Using the mini-gel method (Sambrook et al. 1989) the appropriate DNA amount for PCR amplification was determined by visual comparison with lambda DNA of known concentration.

Total DNA extraction for RFLP analysis

Total DNA was isolated by the method of Draper and Scott (1988) with some modifications: approximately 3.5 g of leaves were collected from three two-week-old seedlings, ground in liquid nitrogen, and

suspended in 15 ml of extraction buffer [100 mM Tris-HCl (pH 8.0), 500 mM NaCl, 50 mM EDTA (pH 8.0), 10 mM 2-mercaptoethanol]. When a fine suspension had been achieved, 2 ml of 10% SDS was add to this suspension. The mixture was shaken thoroughly and incubated at 65 °C for 20 min with optional gentle shaking. Protein of the mixture was removed by the protein/SDS precipitation; the sample was mixed with 5 ml of 5M potassium acetate and incubated at 0 °C for 30 min with optional gentle shaking. The mixture was centrifuged for 30 min at 12000 rpm in refrigerated centrifuge at 4 °C. The supernatant was filtered through Miracloth. Total DNA was precipitated with 2/3 vol. of isopropanol. The recovered total DNA was spooled out with a pipette tip and transferred to the tube containing 10 ml of 70% ethanol. The mixture was centrifuged for 10 min at 8000 rpm and the pellet was washed with 70 % ethanol. The dried pellet was suspended in 10 ml of TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)] containing 1 μg RNase A, and incubated 1 hr at 37 °C. Polysaccharides of the mixture was removed by the ethanol precipitation (Scott et al. 1994). The suspension was mixed with 500 μl of 5M potassium chloride by vigorous shaking, then mixed with 0.35 vol of absolute ethanol by immediate vigorous shaking and incubated at 0 °C for 20 min without shaking. The mixture was centrifuged for 5 min at 12000 rpm in refrigerated centrifuge at 4 °C. Total DNA was precipitated with 2/3 vol of isopropanol. The recovered total DNA was spooled out with a pipette tip and transferred to 10 ml 70 % ethanol. The mixture was centrifuged for 10 min. at 12000 rpm. The pellet was washed again with 70 % ethanol. The dried pellet was suspended in 500 μl of TE buffer. Using the mini-gel method (Sambrook et al. 1989) the

appropriate DNA amount for restriction enzyme digestion was determined by visual comparison with lambda DNA of known concentration.

RAPD analysis

PCR was performed in a 0.5 ml tube in volume of 10 μl consisting of 0.2 ng of genomic DNA, 0.2 μM primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 0.001 % gelatin, 0.4 mM each of dATP, dTTP, dCTP and dGTP, and 0.2 unit *Taq* DNA polymerase (TOYOBO, Osaka, Japan). Decamer oligonucleotide primers were purchased either from TOYOBO, Japan or Operon Technologies, USA. The reaction mixture was overlaid with a drop of mineral oil. Amplifications were carried out in BioOven (BioTherm, USA) programmed for 45 cycles of 30 sec at 93 °C, 1 min at 36 °C and 2 min at 72 °C, and ending with 1 min at 72 °C. Amplified products were electrophoresed on 2% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and stained with ethidium bromide.

Hybridization with RAPD probes recovered from agarose gel

Some of the RAPD fragments were excised from the agarose gel, purified by GENECLEAN 2 (BIO 101 Inc.) and labeled with digoxigenin under the manufacture's instructions (DIG DNA labeling Kit, Boehringer Mannheim). The amplified DNA products were separated on 1.2% agarose gel and transferred by southern-blotting onto a Hybond N+ membrane (Amersham). Hybridization was carried out as follows. The membrane was hybridized overnight with the probe in the Hybridization Solution (5x SSC, 0.5% Blocking reagent, 0.1% N-lauroylsarcosine Na-salt, 0.02% SDS) at 65 °C. The membrane was

washed twice in 2X SSC and 0.1% SDS solution at room temperature for 5 min, and twice in 0.1X SSC and 0.1% SDS solution at 65 °C for 15 min. Chemiluminescent detection was carried out with 15 min exposure to X-ray film according to the supplier's instruction (Lumi-Phos 530, Boehringer Mannheim).

RFLP analysis

Total DNA was digested with *Dra*I, *Eco*RI, *Eco*RV and *Hin*dIII, electrophoresed (5-10 μg/lane) using 0.8% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA), and then transferred by Southern blotting onto Hybond N+ membrane (Amersham). Mungbean and cowpea random genomic DNA probes were kindly provided by Dr. N. D. Young, University of Minnesota, USA. They were constructed using *Pst*I site of pUC18. Probe labeling and band detection were carried out using ECL direct nucleic acid labeling and detection systems (Amersham) according to the manufacture's instruction.

Data analysis for RAPD and RFLP data

RAPD and RFLP data were recorded based on the presence or the absence of the fragments. Each polymorphic fragment was treated as a unit character and compared between each pair of accessions. In the investigation of *Ceratotropis* species, the genetic dissimilarity in each pair of accessions was given by the number of different fragments / the number of total fragments detected between the two accessions. Basic statistics and cluster analysis were conducted using STATISTICA ver. 4.1J (StatSoft, USA). The cluster analysis was performed, based on the percentage of different fragments, using unweighted-pair-group

method (Sneath and Sokal, 1973).

Results

RAPD analysis of azuki bean and its related genera

Thirty-seven decamer primers (Table II-3) were used in evaluating of the 12 cultivars of azuki bean, one of common bean and one of cowpea. In total, 184 fragments were visually scored among azuki bean cultivars (Fig. II-1). Of these, 174 (95 %) were common in all cultivars, and 10 (5 %) were polymorphic and shared by at least two. On the other hand, many polymorphic fragments were detected between azuki bean, common bean and cowpea cultivars. Since small variation was observed among azuki bean cultivars, 'Notodainagon' was used as a representative to compare with common bean and cowpea. Using the same primers, 180, 170 and 168 fragments were observed in the banding patterns of azuki bean (Notodainagon), cowpea and common bean, respectively. Of these fragments, 36 fragments (11.4 %) were common between azuki bean and cowpea, which belong to the same genus *Vigna*. However, 37 (11.9 %) fragments were detected between azuki bean and common bean, which are classified as different genera.

Genetic diversity among azuki cultivars was estimated based on the number of different fragments between the two compared. Table II-4 shows the numbers of different fragments in all possible combinations of 12 azuki cultivars. Using these values, the dendrogram showing genetic relationships among azuki cultivars was drawn (Fig. II-2). They were mainly divided into three groups; Group A ('Iwaidainagon', 'Hokkaishouzu' and 'Erimoshouzu'), Group B ('Hyoukei 2',

Table II-3. Decamer primers used in this experiment.

Sequence (5' to 3')	Sequence (5' to 3')
TGGTCACTGA	TGGTCACAGA
TGGTGACTGA	TGGTCACCGA
TGGTCTCTGA	TGGTCACGGA
TGGTCCCTGA	TGGTCACTCA
TGGTCGCTGA	TGGTCACTAA
TGGTAACTGA	TGGTCACTTA
TGGTTACTGA	TGGTCACTGT
TGGACACTGA	TGGTCACTGC
TGGCCACTGA	TGGTCACTGG
TGGGCACTGA	AGGTCACTGA
TGCTCACTGA	CGGTCACTGA
TGATCACTGA	GGGTCACTGA
TGTTCACTGA	TGGTGACTGA
TCGTCACTGA	TGGTGAGTGA
TAGTCACTGA	TGGTGATTGA
TTGTCACTGA	TGGTCAATGA
TGGTCAGTGA	TGGTCACCGA
TGGTCAATGA	TGGTGACAGA
TGGTCATTGA	

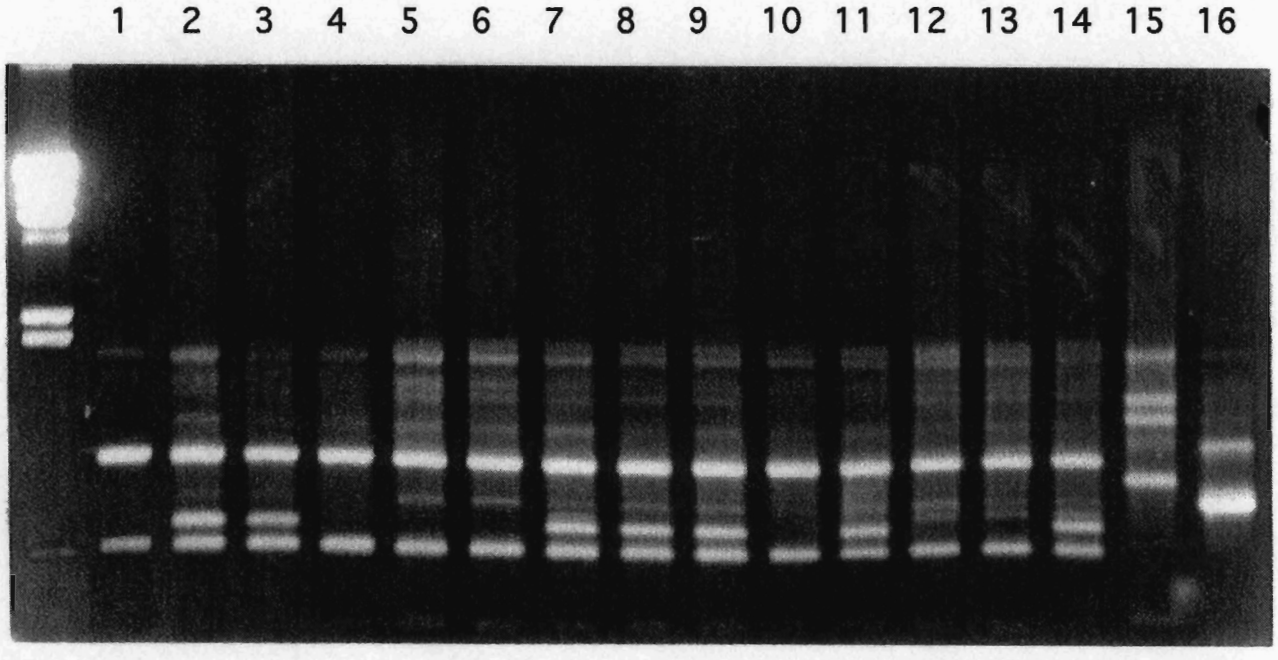

Fig. II-1. DNA polymorphisms detected by amplification of genomic DNA via PCR, using 5'-TGGACACTGA-3'. The left most lane contains *Hind* III digested lambda DNA. Codes of the accessions are listed in Table II-1.

Table II-4. The numbers of different fragments detected between 12 azuki cultivars. Codes of the accessions are listed in Table II-1.

Code	2	3	4	5	6	7	8	9	10	11	12
1	7	5	8	0	6	5	4	9	0	9	5
2		2	3	7	5	4	3	2	7	2	4
3			5	5	3	2	1	4	5	4	2
4				8	2	7	6	1	8	1	7
5					6	5	4	9	0	9	5
6						5	4	3	6	3	5
7							1	6	5	6	0
8								5	4	5	1
9									9	0	6
10										9	5
11											6

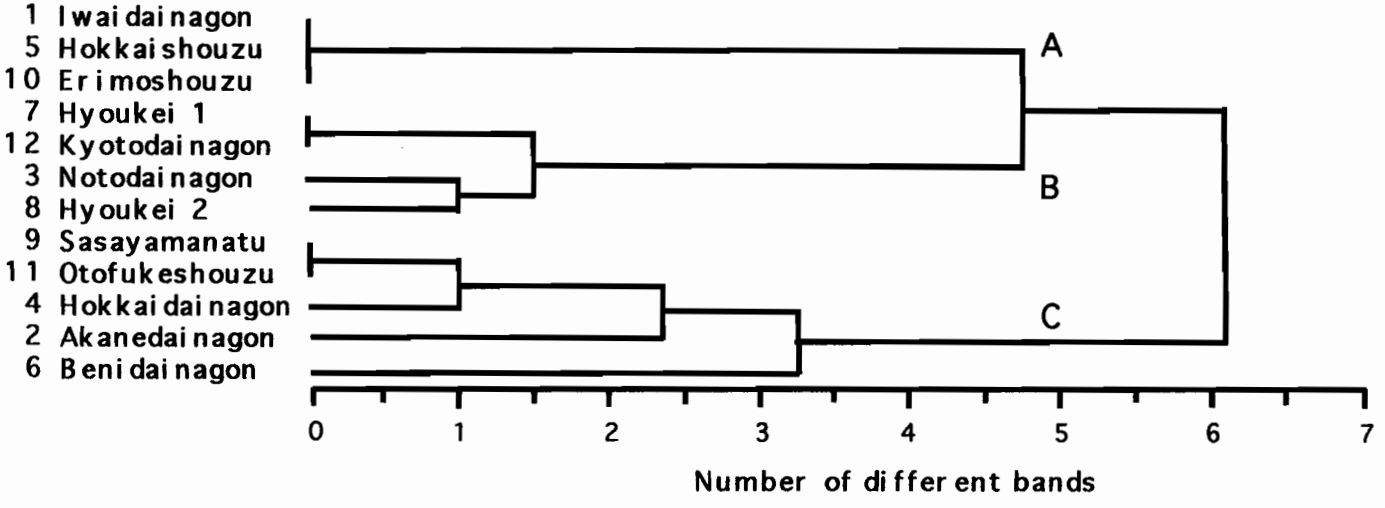

Fig. II-2. A dendrogram showing relationships among 12 azuki cultivars based on the RAPD analysis. Code number of the accessions are listed in Table II-1.

'Kyotodainagon', 'Notodainagon' and 'Hyoukei 1') and Group C ('Sasayamanatu', 'Otofukeshouzu', 'Hokkaidainagon', 'Akanedainagon' and 'Benidainagon').

RAPD analysis of Ceratotropis *species*

Initially, 80 primers were surveyed using two accessions from each species. Distinct fragment patterns were obtained with 38 primers in all accessions. The banding patterns with 10 primers were either too complicated to score or without distinct fragments. With the remaining 32 primers, amplifications were failed or ambiguous fragment patterns were observed in some of the accessions.

After primary screening, 24 primers were selected from the 38 potentially usable primers and applied for evaluation of all other accessions (Fig. II-3). A total of 404 fragments were visually scored among 23 accessions (Table II-5). Of these fragments, 12 (3 %) were common in all accessions, 47 (12 %) were unique to only one accession, and 345 (85 %) were polymorphic and shared by at least two accessions.

Relationships among 23 accessions were analyzed by cluster analysis using the genetic dissimilarities of different fragments in all possible combinations as a distance matrix (Table II-6). Two main groups, A (*V. angularis* and *V. umbellata*) and B (*V. radiata*, *V. aconitifolia* and *V. mungo*), were recognized at 70 % average difference (Fig. II-4). Each of them was further divided into two or three subgroups which were in complete agreement with taxonomic species. Genetic dissimilarities between subgroups (or species) in Group B were 56 % between *V. radiata* and *V. aconitifolia*, 61 % between *V. radiata* and *V. mungo* and 60 % between *V. aconitifolia* and *V. mungo*. The

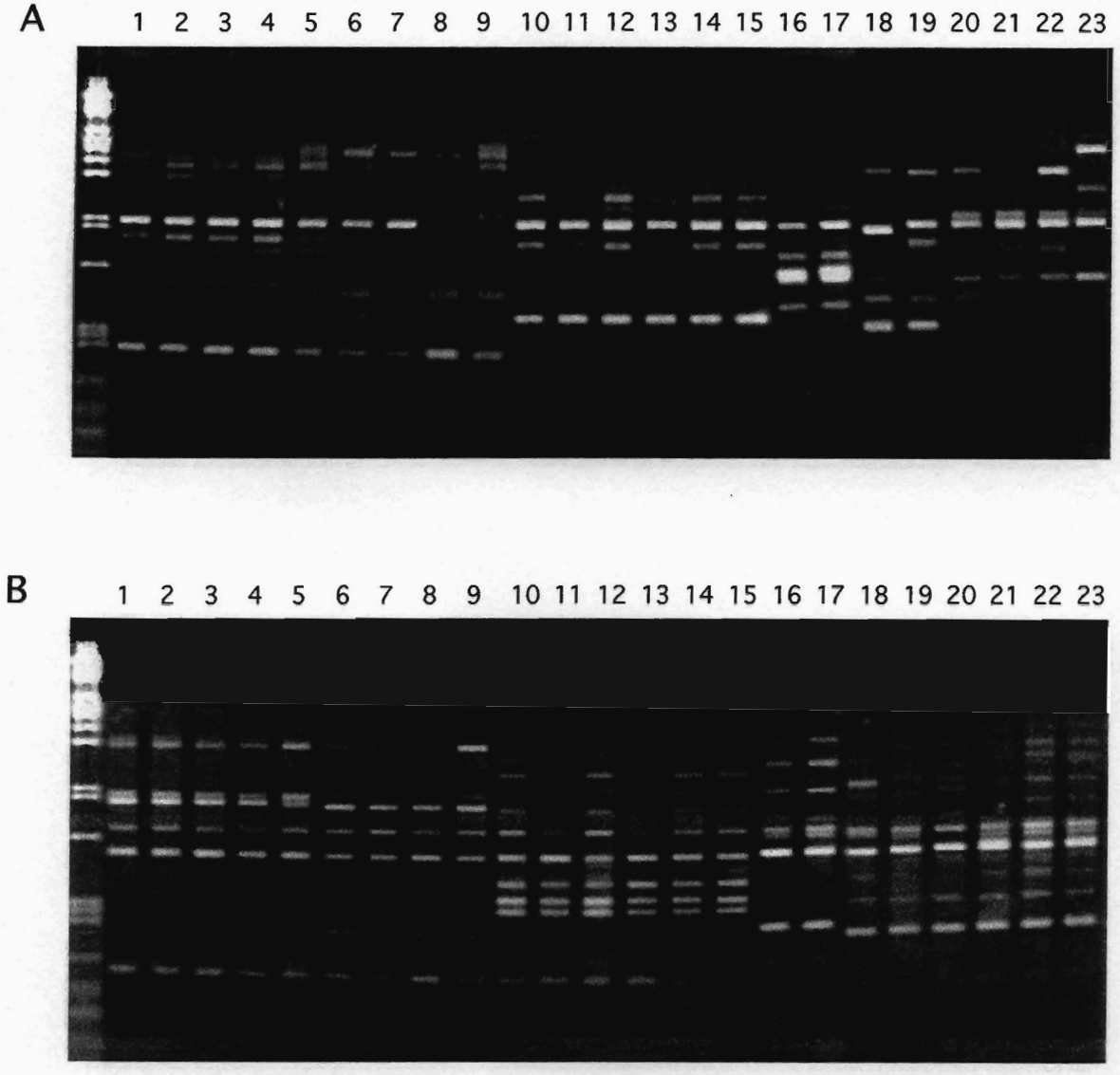

Fig. II-3. DNA polymorphisms detected by amplification of genomic DNA via PCR , using 5'-CCCAAGGTCC-3' (A) and 5'-TGGCCACTGA-3'(B). The left most lane contains *Hind* III digested lambda DNA. Codes of the accession are listed in Table II-2.

Table II-5. Decamer primers used in this experiment and the number of amplified fragments detected among 23 accessions.

Sequence (5' to 3')	Number of fragments		
	Polymorphic bands	Monomorphic bands	Total
TGGTCACTGA	23	0	23
TGGTGACTGA	21	0	21
TGGTCGCTGA	23	1	24
TGGACACTGA	22	0	22
TGGCCACTGA	14	1	15
TGGGCACTGA	11	0	11
TGCTCACTGA	14	0	14
TGGTCACCGA	20	0	20
TGGTCACGGA	24	0	24
CCGCATCTAC	26	0	26
GATGACCGCC	13	2	15
AAAGCTGCGG	26	1	27
GTCGCCGTCA	15	0	15
TTGGCACGGG	9	1	10
AGCGCCATTG	24	1	25
GGGGTGACGA	8	0	8
CATCCGTGCT	11	1	12
CCCAAGGTCC	11	0	11
GGTGCGGGAA	12	0	12
AAGACCCCTC	14	0	14
GAGTCTCAGG	10	1	11
TGCGGCTGAG	14	1	15
ACGCACAACC	22	0	22
GTGATCGCAG	5	2	7
Total	392	12	404

Table II-6. Genetic dissimilarities between 23 accessions in subgenus *Ceratotropis* based on the RAPD analysis. Codes of the accessions are listed in Table II-2.

Code	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	0.014	0.004	0.009	0.042	0.348	0.357	0.417	0.400	0.730	0.722	0.718	0.717	0.761	0.708	0.744	0.751	0.640	0.648	0.639	0.645	0.639	0.632
2		0.009	0.014	0.038	0.357	0.376	0.427	0.410	0.727	0.718	0.714	0.722	0.758	0.704	0.741	0.748	0.644	0.652	0.634	0.640	0.634	0.627
3			0.014	0.037	0.354	0.363	0.423	0.406	0.729	0.721	0.717	0.716	0.760	0.706	0.743	0.750	0.648	0.655	0.637	0.643	0.637	0.630
4				0.033	0.351	0.360	0.421	0.404	0.728	0.719	0.716	0.714	0.759	0.705	0.742	0.749	0.637	0.644	0.636	0.642	0.636	0.628
5					0.358	0.367	0.420	0.403	0.729	0.719	0.716	0.705	0.751	0.695	0.734	0.741	0.635	0.642	0.624	0.631	0.624	0.616
6						0.080	0.120	0.100	0.730	0.730	0.726	0.717	0.726	0.689	0.727	0.724	0.684	0.683	0.700	0.688	0.683	0.693
7							0.167	0.136	0.722	0.730	0.726	0.689	0.718	0.671	0.736	0.733	0.675	0.674	0.700	0.688	0.683	0.693
8								0.113	0.739	0.739	0.735	0.725	0.743	0.697	0.744	0.742	0.664	0.662	0.689	0.677	0.671	0.682
9									0.726	0.717	0.704	0.693	0.722	0.647	0.731	0.729	0.670	0.668	0.695	0.683	0.677	0.688
10										0.059	0.083	0.333	0.275	0.351	0.554	0.558	0.590	0.588	0.614	0.586	0.605	0.573
11											0.058	0.342	0.300	0.351	0.545	0.550	0.590	0.588	0.605	0.578	0.597	0.564
12												0.354	0.279	0.345	0.561	0.574	0.622	0.620	0.620	0.610	0.620	0.588
13													0.389	0.383	0.577	0.582	0.614	0.613	0.640	0.619	0.631	0.596
14														0.345	0.578	0.591	0.613	0.612	0.629	0.610	0.629	0.605
15															0.532	0.545	0.623	0.622	0.658	0.628	0.658	0.623
16																0.053	0.602	0.591	0.617	0.590	0.617	0.576
17																	0.598	0.588	0.605	0.586	0.605	0.572
18																		0.030	0.143	0.064	0.126	0.095
19																			0.157	0.060	0.130	0.091
20																				0.111	0.113	0.134
21																					0.077	0.072
22																						0.091

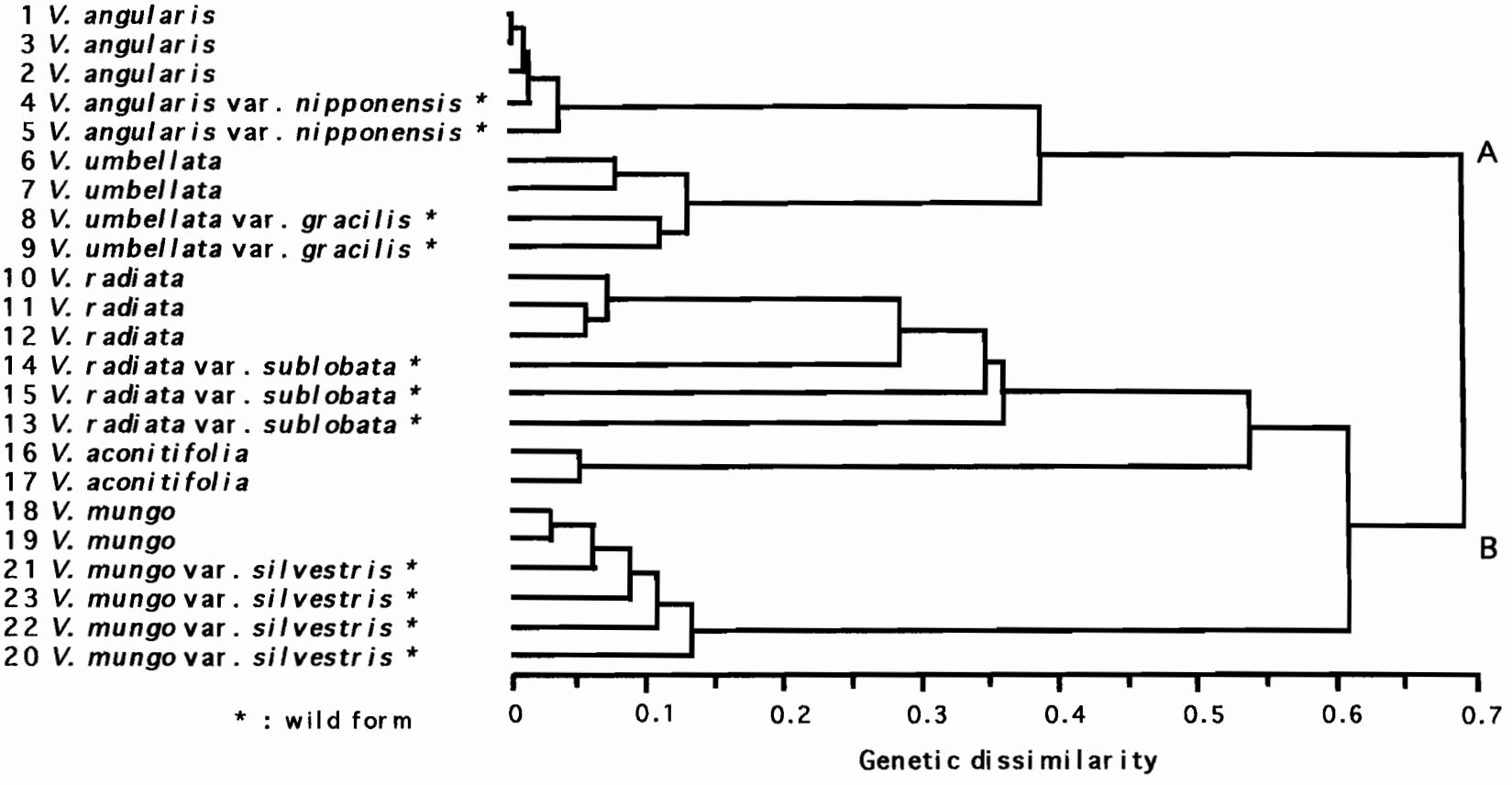

Fig. II-4. A dendrogram showing genetic relationships among 23 accessions in subgenus *Ceratotropis* based on the RAPD analysis. Codes of the accessions are listed in Table II-2.

average interspecific dissimilarity was 59 %. On the other hand, the dissimilarity between *V. angularis* and *V. umbellata* in Group A was 39%, which was apparently lower than the interspecific differences in Group B. In each subgroup or species except *V. aconitifolia*, there are both cultivated and its wild forms. The greatest intraspecific dissimilarity was found in *V. radiata* ; 37 % within wild forms, 7 % within cultivated forms, and 33 % between cultivated and wild forms. The differences within cultivated forms were always smaller than those within wild forms.

RFLP analysis of Ceratotropis *species*

In order to examine the genetic diversity among 25 accessions in *Ceratotropis* species, RFLP analysis was carried out with 15 DNA probes. Since they are mungbean and cowpea single-copy genomic probes (Fatokun et al., 1993; Menancio-Hautea et al., 1993), small numbers of the fragments were detected in the hybridization patterns of mungbean (Fig. II-5). However, relatively higher numbers of bands were observed in other species. In total, 510 fragments were scored with 55 combinations of hybridization patterns using 15 probes and four enzymes (Table II-7). Of these, five (1 %) were observed in all accessions, 112 (22 %) were unique to only one accession, and 393 (77 %) were polymorphic and shared by at least two accessions.

Genetic dissimilarities between all the combinations of 25 accessions were calculated in Table II-8. Based on these data, a dendrogram showing genetic relationships in *Ceratotropis* species were constructed (Fig. II-6). Twenty-five accessions were mainly divided into three groups, Group I (*V. angularis* ,*V. umbellata* , *V. reflexo-pilosa*,

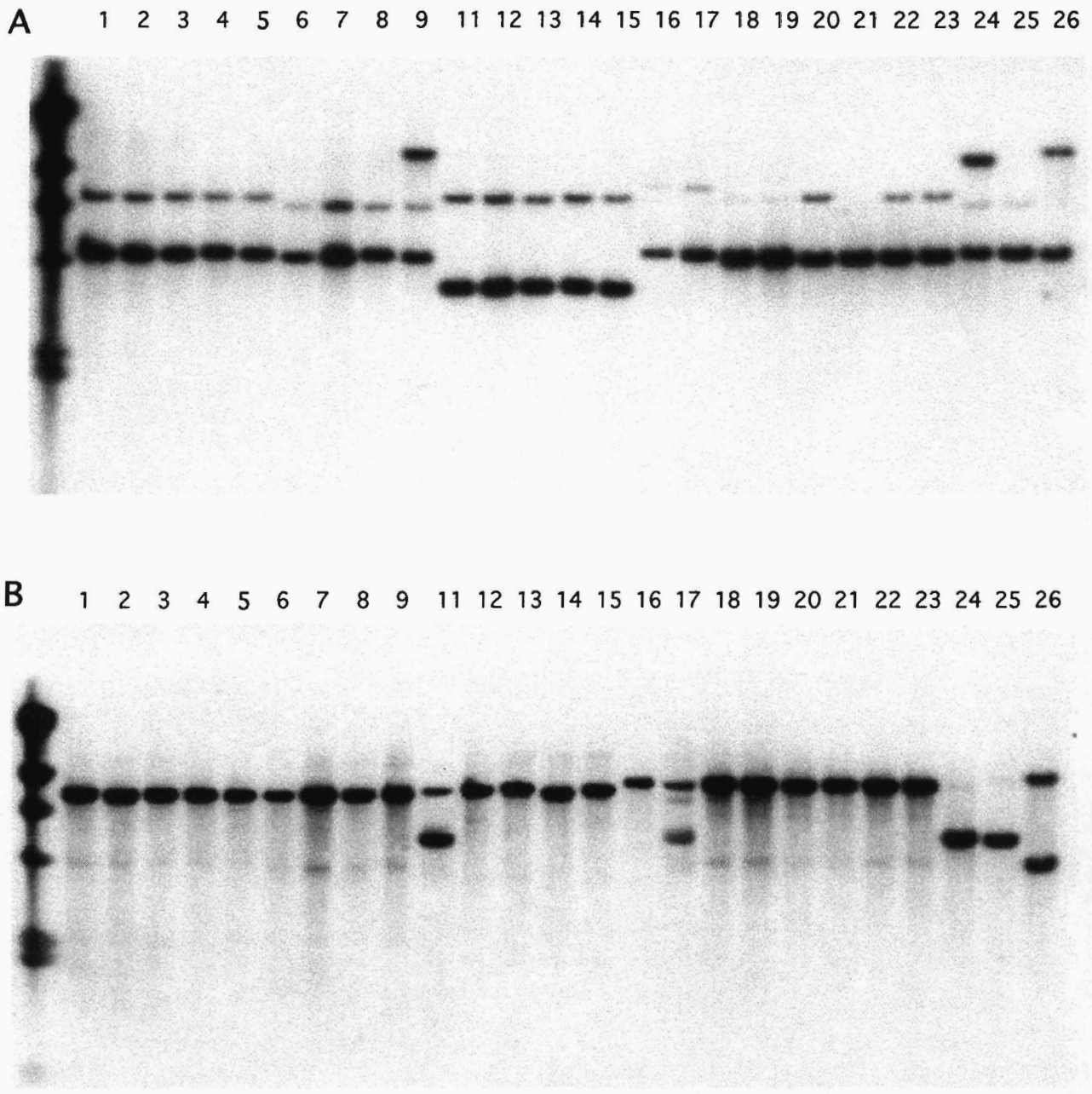

Fig. II-5. DNA polymorphisms detected by Sourthern hybridization with a probe cM4 to *Eco* RV (A) and pP170 to *Hind* III digested total DNA(B), respectively. The left most lane contains *Hind* III digested lambda DNA. Codes of the accession are listed in Table II-2.

Table II-7. Genomic DNA probes used in this experiment and the numbers of the fragments detected among 25 accessions in *Ceratotropis* species using four restriction enzymes.

Probe	Number of fragments				
	Dra I	*Eco*RI	*Eco*RV	*Hin*dIII	Total
cM3	15	21	11	14	61
cM4	7	8	7	15	37
cM11	4	-	-	-	4
cM14	5	9	7	11	32
cM93	-	16	7	7	30
pM100	3	11	8	16	38
pM186	14	10	12	18	54
pM392	8	9	7	6	30
pO95	6	-	11	8	24
pO109	8	8	6	9	31
pO111	4	1	1	9	20
pP170	5	2	8	6	21
pQ22	7	8	11	8	34
pR2	1	4	5	9	18
pR34	10	10	6	8	34
pR67	9	15	14	4	42
Total					510

- : not determined.

Table II-8. Genetic dissimilarities between 25 accessions in subgenus *Ceratotropis* based on the RFLP analysis. Codes of the accessions are listed in Table II-2.

	2	3	4	5	6	7	8	9	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1	0.01	0.06	0.05	0.05	0.48	0.49	0.47	0.51	0.79	0.77	0.80	0.80	0.81	0.82	0.83	0.74	0.74	0.74	0.71	0.72	0.77	0.59	0.60	0.57
2		0.06	0.06	0.06	0.47	0.48	0.46	0.50	0.78	0.76	0.79	0.79	0.80	0.82	0.83	0.73	0.73	0.73	0.72	0.73	0.76	0.60	0.61	0.56
3			0.05	0.05	0.48	0.48	0.46	0.49	0.80	0.78	0.80	0.79	0.81	0.83	0.83	0.72	0.72	0.72	0.70	0.71	0.75	0.61	0.62	0.57
4				0.01	0.47	0.48	0.46	0.48	0.79	0.79	0.80	0.80	0.82	0.82	0.83	0.74	0.74	0.73	0.71	0.72	0.76	0.59	0.61	0.58
5					0.46	0.48	0.46	0.49	0.79	0.79	0.80	0.80	0.82	0.82	0.83	0.74	0.74	0.73	0.71	0.72	0.76	0.60	0.60	0.58
6						0.09	0.11	0.12	0.82	0.81	0.80	0.81	0.81	0.82	0.81	0.76	0.76	0.76	0.74	0.76	0.79	0.62	0.62	0.59
7							0.09	0.17	0.84	0.84	0.82	0.83	0.83	0.82	0.81	0.76	0.76	0.76	0.74	0.76	0.79	0.64	0.63	0.61
8								0.20	0.84	0.83	0.81	0.82	0.83	0.81	0.80	0.77	0.76	0.77	0.74	0.77	0.80	0.62	0.64	0.58
9									0.82	0.81	0.84	0.81	0.82	0.81	0.82	0.77	0.76	0.75	0.72	0.76	0.79	0.63	0.64	0.58
11										0.15	0.43	0.38	0.45	0.82	0.80	0.73	0.72	0.76	0.74	0.75	0.74	0.79	0.79	0.75
12											0.40	0.36	0.40	0.83	0.84	0.75	0.74	0.78	0.74	0.76	0.74	0.81	0.79	0.73
13												0.43	0.44	0.86	0.86	0.72	0.71	0.74	0.71	0.71	0.72	0.85	0.84	0.74
14													0.46	0.85	0.85	0.74	0.74	0.72	0.75	0.74	0.75	0.82	0.84	0.74
15														0.84	0.85	0.74	0.74	0.72	0.72	0.71	0.72	0.85	0.82	0.75
16															0.07	0.81	0.82	0.82	0.77	0.76	0.82	0.83	0.84	0.79
17																0.81	0.82	0.82	0.77	0.76	0.82	0.83	0.83	0.79
18																	0.02	0.22	0.18	0.26	0.30	0.83	0.81	0.77
19																		0.22	0.17	0.25	0.29	0.83	0.81	0.78
20																			0.16	0.15	0.22	0.82	0.80	0.78
21																				0.11	0.25	0.79	0.79	0.76
22																					0.21	0.79	0.78	0.76
23																						0.83	0.81	0.79
24																							0.30	0.65
25																								0.63

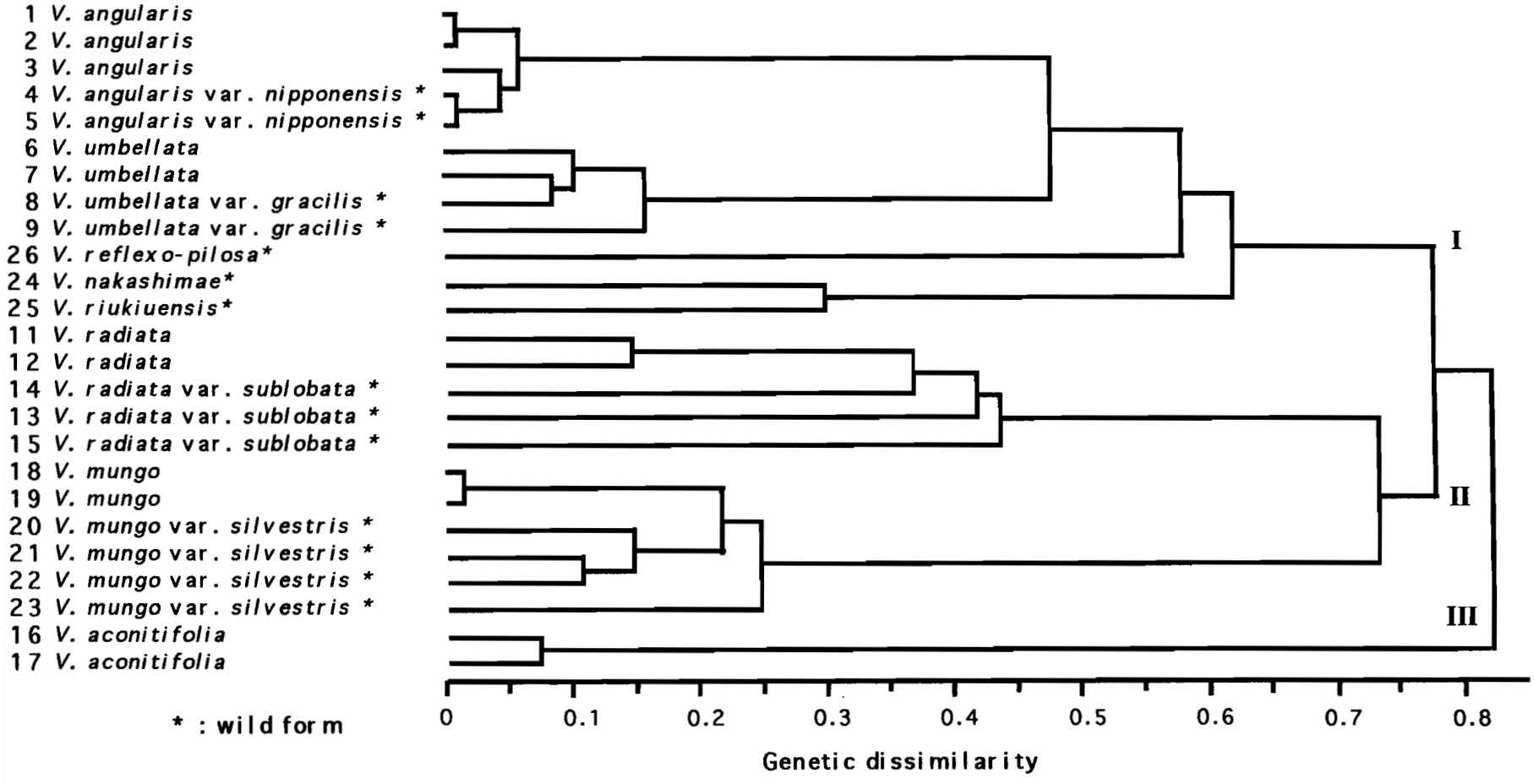

Fig. II-6. A dendrogram showing genetic relationships among 25 accessions in subgenus *Ceratotropis* based on the RFLP analysis. Codes of the accessions are listed in Table II-2.

V. nakashimae and *V. riukiuensis*), Group II (*V. radiata* and *V. mungo*) and Group III (*V. aconitifolia*). Groups I and II were further separated into four and two subgroups, respectively. These subgroups were found to be in good agreement with taxonomic species. The wild species, *V. nakashimae, V. riukiuensis* and *V. reflexo-pilosa*, displayed high level of polymorphism and did not make small clusters with other cultivated accessions.

Identify of RAPD fragments

In the above study, RAPD fragments amplified from different accessions were compared based on the molecular weight, and the fragments with the same molecular weight were treated as the common fragments. In order to examine whether these fragments are homologous in their sequences or not, Southern hybridization was carried out as follows: Three different RAPD fragments, 'a', 'b' and 'c', amplified from 'Hyoukei 2' (*V. angularis*) using primer 5'-AAAGCTGCGG-3' (Fig. II-7), were excised from the agarose gel. After labeling, they were probed to the amplification products from other species. The fragment 'a', appeared in *V. angularis* and *V. umbellata*, hybridized with only the similar-sized fragments in these two species. The common fragment 'b' in all accessions hybridized with the similar-sizes fragments in all the accessions (Fig. II-7). However, the fragment 'c' hybridized with both similar-sized fragments and smaller fragments in all accessions. Thus, the three amplification products of 'Hyoukei 2' were apparently homologous to those with similar sizes in the other species, although some other homologous fragments with different

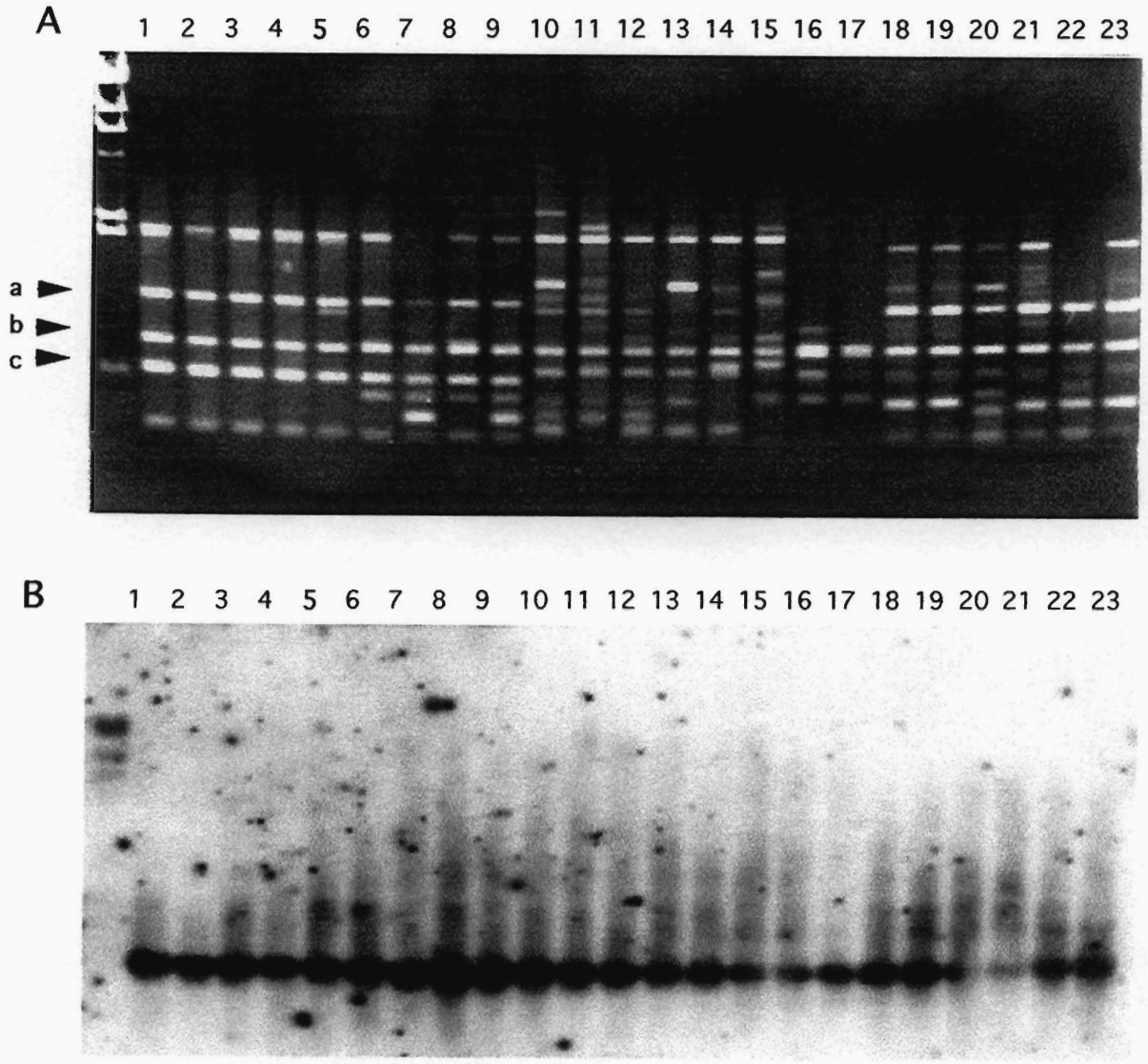

Fig. II-7. A, RAPDs among 23 accessions amplified by a primer 5'-AAAGCTGCGG-3'. Codes of the accession are listed in Table II-2. The arrowed fragments 'a', 'b' and 'c' in lane 1 were used as probes for Southen hybrdization. B, the fragment 'b' Southern hybridized with the similar sized fragments in all the accessions. The left most lane contains *Hind* III digested lambda DNA.

sizes were also detected in the other species.

Comparison of genetic dissimilarities between RAPD and RFLP analyses

In order to compare RAPD and RFLP analyses for estimating genetic relationships among *Ceratotropis* species, the pairs of genetic dissimilarities from RAPD and RFLP data sets (Tables II-7 and 9) were compared for all possible combinations. The strong correlation was observed between these genetic dissimilarities ($r=0.97$, $p<0.01$), and a trimodal distribution was appeared (Fig. II-8). High and low dissimilarity values were from interspecific and intraspecific comparisons, respectively. The middle group was mixture of lower interspecific values, e.g., between *V. angularis* and *V. umbellata*, and higher intraspecific values, e.g., among wild *V. radiata*. Since this strong correlation was due to the arrangement of three groups, the correlation was separately reexamined for the intra- and interspecific values. As a result, significant correlation were again observed for intraspecific ($r=0.68$, $n=33$, $p<0.001$) and interspecific comparison ($r=0.34$, $n=169$, $p<0.001$), indicating that there was no significant difference between the results from RAPD and RFLP analyses.

Discussion

RAPD and RFLP analyses for evaluating species relationships

We used both RAPD and RFLP analyses as tools for determine the relationships in the subgenus *Ceratotropis*. These analyses revealed various degree of differentiation. According to Gower (1985), the number of bands to obtain stable classification is required for 10 % of

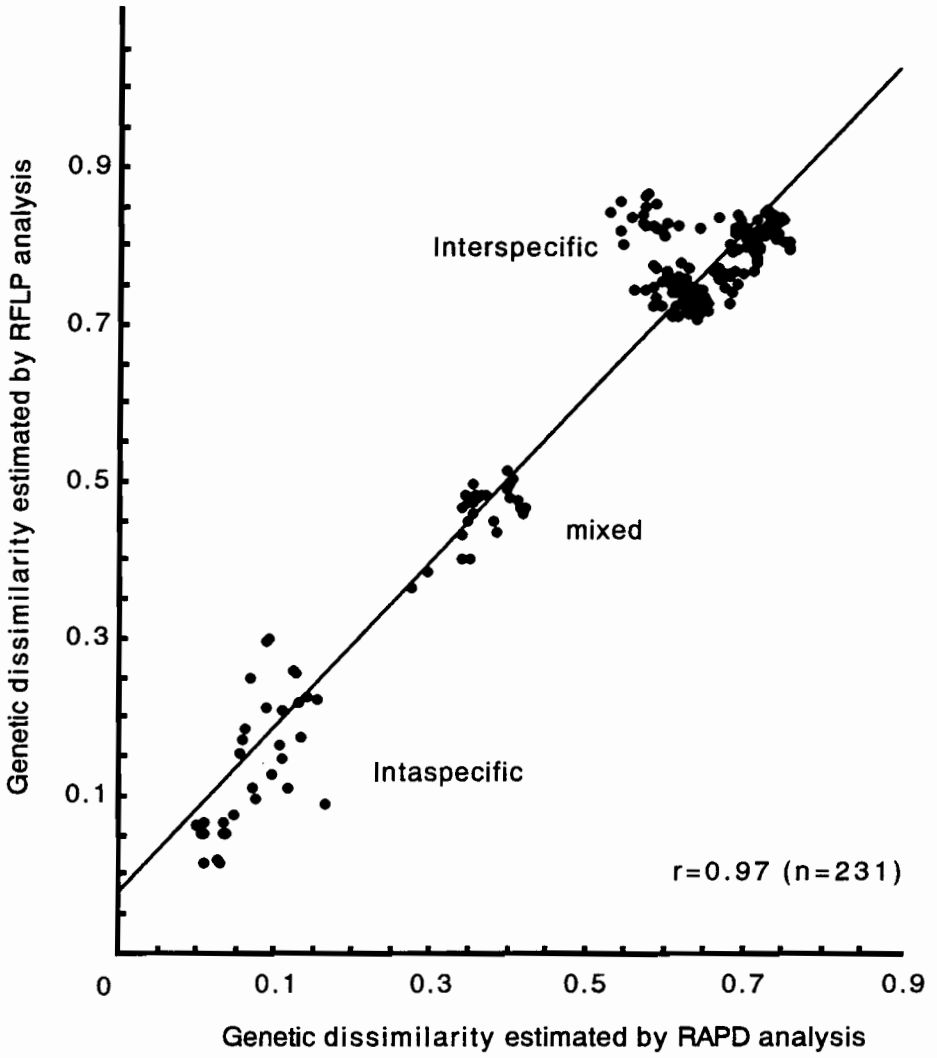

Fig. II-8 Comparison of genetic dissimilarities between RAPD and RFLP analysis.

mean CV. The classifications with CV as low as 10% were reported using 300 RAPDs for comparison of maize and bean (Skroch et al. 1992) and using 327 RAPDs and 294 RFLP fragments for cruciferous species (Thormann et al. 1994). In the present study, the sampling errors that might influence the comparison were thought to be relatively small in both RAPD and RFLP analyses because larger number of fragments than above studies were examined.

Williams et al. (1993) pointed out the possibility that similar-sized fragments with no sequence homology might bias the data obtained. Thormann et al. (1994) showed such biased data obtained from RAPD analysis by comparing with RFLP analysis. However, in this study, the high correlation of genetic dissimilarities was observed between RAPD and RFLP analyses. Southern hybridization with three amplification products gave signals to those of similar sizes in the other species. These results support that treatment of common bands did not significantly biased the data obtained in this study.

The nature of polymorphisms detected by RFLP analysis is different from those by RAPD analysis. The majority of amplified fragments by RAPD analysis were thought to arise from repeated sequences (Devos and Gale 1992; Williams et al. 1993; Paran and Michelmore 1993), and the polymorphisms are mainly caused by the point mutations. On the other hand, RFLP analysis usually detects the mutations occurring in single-copy sequences, such as deletion, insertion, base substitution, genome rearrangements. Although those two analyses therefore examine different parts of the genome, the similar results were obtained in the subgenus *Ceratotropis*, indicating that the differentiation in the subgenus is well reflected by both

polymorphisms.

Genetic relationships among azuki bean cultivars

Based on the RAPD analysis, 12 cultivars of azuki bean were mainly divided into 3 main groups by cluster analysis (Fig. II-2). Group B consists of four 'dainagon' varieties, but the other groups were the mixtures of 'dainagon' and 'shouzu' varieties. Since these nomenclature are only based on the size of the beans, their hundred-seed-weights were compared in Table II-9. The seed weights of Group A were much less than 16 gm, corresponding to 'shouzu'. On the other hand, the seed weights of Group B were much more than 16 gm, and those of Group C were relatively close to 16 gm, both corresponding to 'dainagon'. There are some discrepancies; 'Iwaidainagon' for less than 10 gm of hundred-seed-weight variety, and 'Otofukeshouzu' for 13.8 gm. However, in this study azuki cultivars were divided into three groups agreed with the classification based on the hundred-seed-weight.

The cultivars of Group B were from the western part of Japan, and those of Group C were mainly from the northern part of Japan (Hokkaido Prefecture). They share the same name 'dainagon', but their ancestral varieties are different. Cultivars in the western Japan were originated from traditional Japanese varieties in 'Edo' period, whereas most of the cultivars in Hokkaido Prefecture were from the varieties introduced from north-east China in 1904-1905. In this study, these different genetic backgrounds of Japanese 'dainagon' were confirmed by RAPD analysis.

Table II-9. Correspondence between the groups classified by RAPD analysis and their hundred-seed-weights using 12 azuki cultivars.

Group	Code	Cultivar	Origin	100 seed weight (g)
A	1	Iwaidainagon	Hokkaido	9.9
	5	Hokkaishouzu	Hokkaido	11.4
	10	Erimoshouzu	Hokkaido	12.3 (11.7-13)
B	7	Hyoukei 1	Hyogo	23.0
	12	Kyotodainagon	Kyoto	21.1
	3	Notodainagon	Ishikawa	19.6
	8	Hyoukei 2	Hyogo	27.9
C	9	Sasayamanatu	Kyoto	16.8
	11	Otofukeshouzu	Hokkaido	13.8
	4	Hokkaidainagon	Hokkaido	14.0
	2	Akanedainagon	Hokkaido	13.6 (15.2-19)
	6	Benidainagon	Hokkaido	18.0 (16.8)

Parentheses in seed-weight column indicate the weight from the registered reference (Lumpkin and McClary, 1994)

Species relationships in the subgenus Ceratotropis

Based on the RAPD and RFLP analyses, *Ceratotropis* species were divided into two (A and B) and three (I, II and III) main groups, respectively (Figs. II-4 and 6). In both analyses, *V. angularis* - *V. umbellata* and *V. radiata* - *V. mungo* made clusters in the main groups. They correspond to the genus *Azukia* and *Rudua* proposed by Maekawa (1955). The cross-incompatibility between them were often reported (Ahn and Hartmann 1978; Miyazaki 1982; Chen et al. 1983; Egawa 1988). Numerical taxonomy based on ecological, morphological, biochemical and physiological characteristics (Maréchal et al. 1978) and RFLP analysis (Fatokun et al. 1993) also provided clear differentiation between them. All these analyses suggested that these two groups identified by RAPD and RFLP analyses are highly differentiated.

Previously, Fatokun et al. (1993) studied variation among *V. aconitifolia*, *V. radiata* and *V. mungo* by RFLP analysis. In this study, they were clearly separated according to the taxonomic groups. These results agreed with each other. Jaaska and Jaaska (1990) reported that *V. aconitifolia* differentiated widely from the other species based on the 20 isozyme characters. In ancient India, these species were described as different beans (Jain and Merha 1980).

V. angularis and *V. umbellata* were relatively close compared with other species, *V. aconitifolia*, *V. radiata*, and *V. mungo* (Figs. II-4 and 6). *V. angularis* is crossable with *V. umbellata* to produce hybrids through embryo rescue. Their hybrids show regular meiosis with 11 bivalents and high pollen fertility (Ahn and Hartmann 1978; Chen et al. 1983). In contrast, the hybrids between *V. radiata* and *V. mungo* exhibit irregular meiotic configuration with a high frequency of

univalent formation (Chen et al. 1983; Egawa 1988) and low pollen fertility (Miyazaki 1982). Therefore, genetic differences revealed at DNA levels are in agreement with cytological observations.

In the present study, the ancestral wild forms were always grouped with their most closely related cultivated forms (Figs. II-4 and 6). The degree of similarity between cultivated and presumed ancestral wild forms varied in each species. Wild forms of *V. radiata* having high variation showed the most wide differentiation from the cultivated forms. The degree of similarity between them were almost equivalent to that between *V. angularis* and *V. umbellata*. Miyazaki (1982) reported that in *V. radiata* the pollen fertility of the hybrids between wild forms was lower than that between cultivated and wild forms. Some wild forms of *V. radiata* retain a partial affinity in seed morphology and pollen fertility with *V. mungo* (Miyazaki 1982). All these results suggest that *V. radiata* has the largest intraspecific genetic variation in the subgenus *Ceratotropis*.

The three wild species, *V. nakashimae, V. riukiuensis* and *V. reflexo-pilosa*, were studied only by RFLP analysis. They did not form clusters with cultivated species (Fig. II-6). A relatively close similarity was observed between *V. nakashimae* and *V. riukiuensis,* supporting the proposal that these two species should be rearranged into single species under the name of *V. minima* (Tomooka et al. 1991). Siriwardhane et al. (1991) reported that *V. riukiuensis* and *V. nakashimae* were cross compatible to *V. angularis* and *V. umbellata.* The degrees of genetic differentiation observed between *V. angularis*, *V. umbellata* and these wild species were almost similar. In addition, the rescued hybrid between *V. angularis* and *V. umbellata* showed

complete chromosome pairing (Ahn and Hartmann 1978; Chen et al. 1983). These results suggests that the cross incompatibility between *V. angularis* and *V. umbellata* may not be due to the large differentiation of genome structure but some of reproduction related genes might be responsible.

V. reflexo-pilosa has been thought to be a wild ancestral form of *V. grabrescens* as the only natural allotetraploid species (Tomooka et al. 1991). The two genomes of *V. glabrescence* were reported to be related to those of *V. umbellata* and *V. radiata* (Dana 1965). This concept was supported by consistent results based on isozyme patterns (Jaaska and Jaaska 1990). In contrast, Egawa et al. (1990) proposed that one genome of *V. glabrescence* was homologous to the genome of *V. umbellata*, while the other was not to that of *V. radiata,* from the observation of chromosome paring. Based on the isozyme analysis, Egawa et al. (1996) also suggested that *V. reflexo-pilosa* was derived from interspecific hybridization between *V. trinervia* and *V. minima* followed by natural chromosome doubling. In this study, *V. reflexo-pilosa* was clustered between *V. angularis* - *V. umbellata* and *V. nakashimae* - *V. riukiensis* groups (Fig. II-6), indicating that one of *V. reflexo-pilosa* genomes might have been derived from either *V. nakashimae* or *V. riukiuensis*.

RAPD analysis among azuki cultivars gave a low level of polymorphism; 0.1 polymorphic fragment per primer. Using the same primer set, Fukuoka et al. (1992) detected higher rate of polymorphism (0.4 polymorphic fragment per primer) among fourteen rice *japonica* cultivars. A low level of polymorphism was also observed between cultivated and wild forms of *V. angularis*. Based on the investigation

of ecological and morphological characteristics in wild, weed and cultivated forms of azuki bean, Yamaguchi (1992) stated that the differentiation between wild and cultivated azuki bean might be a relatively recent event. In the present study, *V. angularis* showed the lowest polymorphism level between cultivated and wild forms. The result suggests that the azuki bean is the most recently differentiated bean in the subgenus *Ceratotropis*.

Suitable parents for the linkage mapping population

The information on genetic diversity in the subgenus *Ceratotropis* is useful to select for the parents in the construction of azuki bean linkage map. RAPD analysis was applied for 12 cultivars of azuki bean collected in Japan. However, the genetic variation among cultivars were too small to construct a linkage map (Table II-4). *Ceratotropis* species except for the wild forms of *V. angularis* showed high polymorphism with azuki bean cultivar (Tables II-6 and 8). Among them, crossable species with useful genes were surveyed as follows: *V. umbellata* has been reported to have higher levels of resistance to bruchid beetle, *Callosobruchus chinensis*, and AMV (azuki bean mosaic virus) than azuki bean (Sawa and Tan 1976). Resistance to bruchid beetle was also identified in *V. riukiuensis* (Tomooka 1991). *V. nakashimae* has blown stem rot resistance (K. Murata ; personal communication). Therefore, *V. umbellata*, *V. nakashimae* and *V. riukiuensis* are the candidates of the parents for the construction of linkage maps of azuki bean.

Summary

The genetic variation among Japanese azuki bean (*Vigna angularis*) were investigated by RAPD analysis. Twelve azuki cultivars were divided into three groups, agreeing with the classification based on the hundred-seed-weight by cluster analysis. A different genetic backgrounds of Japanese 'dainagon' were confirmed by RAPD analysis.

The genetic variation among 26 accessions of 8 species in the subgenus *Ceratotropis*, genus *Vigna*, were investigated by RAPD and RFLP analysis. The subgroups recognized in both analyses were in complete agreement with taxonomic species. The ancestral wild forms were always grouped with their most closely related cultivated forms and their variations differed in each species. The largest intraspecific variation was found in *V. radiata* (mungbean), in which wild forms (*V. radiata* var. *sublobata*) were highly different from each other and from cultivated forms as well. *V. angularis* (azuki bean) showed the most limited variation and thus, was probably differentiated in relatively recent times. The wild species, *V. nakashimae, V. riukiuensis* and *V. reflexo-pilosa*, were highly divergent from other cultivated species.

Chapter III. Construction of a genetic linkage map of azuki bean with molecular and morphological markers using interspecific population (*V. angularis* x *V. nakashimae*)

Introduction

Construction of linkage map is a fundamental step forwards the efficient exploration of plant genetic potential. Recently, molecular linkage maps of many crop species have been made and applied for gene mapping, gene tagging and improved selection in breeding programs. Using RFLP markers, molecular linkage maps were constructed in several pulse species, i.e., soybean (Shoemaker and Specht 1995), lentil (Simon et al. 1993), mungbean (Menancio-Hautea et al. 1993), cowpea (Fatokun et al. 1993) and peanut (Halward et al. 1993). Random amplified polymorphic DNA (RAPD) marker also provides genetic information at DNA level (Williams et al. 1990). As compared to RFLP analysis, RAPD analysis is technically simple to construct genetic maps (Rowland and Levi 1994; Binelli and Bucci 1994) and can be easily used for marker assisted selection and breeding (Johnson et al. 1995).

In order to construct linkage map efficiently in azuki bean, we must choose an adequate combination of the parents for mapping population. Namely, high polymorphisms should be detected between the parents, and they must be crossable each other. In Chapter II, RAPD analysis was carried out to assess genetic variation among azuki cultivars and *Ceratotropis* species and a low level of polymorphism was detected within azuki species, *V. angularis*. Reproductive isolation barriers were often observed in the interspecific crosses

between *V. angularis* and other species in *Ceratotropis* (Chen et al. 1983; Kaushal and Singh 1988). However, Siriwardhane et al. (1991) reported the successful reciprocal cross of azuki bean with a wild *Ceratotropis* species, *V. nakashimae*. Based on these facts, *V. angularis* and *V. nakashimae* were chosen as the parents for producing the interspecific mapping population, and a genetic linkage map of azuki bean was constructed with RFLP, RAPD and morphological markers.

Materials and methods

Plant materials

V. angularis cv. 'Erimoshouzu' and *V. nakashimae* were used to produce mapping population. The seeds of *V. nakashimae* was kindly provided from Dr. Y. Egawa, National Institute of Agrobiological Resources, Japan. Eighty F_2 individuals from the interspecific cross (*V. angularis* x *V. nakashimae*) were separately grown in the pots of 25 cm in diameter.

DNA extraction

Total DNA was extracted with the same method described in Chapter II.

RAPD analysis

The procedure for RAPD analysis was the same as described in Chapter II. Primers used are shown in Table III-1.

Table III-1. Nucleotide sequences of the primers used in this study.

Primer code[1)]	Sequence	Primer code[1)]	Sequence
SDA08	5'-TGGACACTGA-3'	OPD02	5'-GGACCCAACC-3'
SDA09	5'-TGGCCACTGA-3'	OPD05	5'-TGAGCGGACA-3'
SDA11	5'-TGCTCACTGA-3'	OPD08	5'-GTGTGCCCCA-3'
SDA27	5'-TGGTCACTGC-3'	OPD09	5'-CTCTGGAGAC-3'
OPA07	5'-GAAACGGGTG-3'	OPD11	5'-AGCGCCATTG-3'
OPA08	5'-GTGACGTAGG-3'	OPD15	5'-CATCCGAGCT-3'
OPA17	5'-GACCGCTTGT-3'	OPD16	5'-AGGGCGTAAG-3'
OPB07	5'-GACCGCTTGT-3'	OPD20	5'-ACCCGGTCAC-3'
OPB08	5'-GTCCACACAG-3'	OPE01	5'-CCCAAGGTCC-3'
OPC01	5'-TTCGAGCCAG-3'	OPE02	5'-GGTGCGGGAA-3'
OPC02	5'-GTGAGGCGTC-3'	OPE03	5'-CCAGATGCAC-3'
OPC05	5'-GATGACCGCC-3'	OPE06	5'-AAGACCCCTC-3'
OPC06	5'-GAACGGACTC-3'	OPE09	5'-CTTCACCCGA-3'
OPC08	5'-TGGACCGGTG-3'	OPE17	5'-CTACTGCCGT-3'
OPC10	5'-TGTCTGGGTG-3'	OPF07	5'-CCGATATCCC-3'
OPC13	5'-AAGCCTCGTC-3'	OPG10	5'-AGGGCCGTCT-3'
OPC19	5'-GTTGCCAGCC-3'	OPH04	5'-GGAAGTCGCC-3'
OPD01	5'-ACCGCGAAGG-3'	OPH17	5'-CACTCTCCTC-3'

1) OP: Primers purchased from Operon Technologies, USA.

SD: Commercially synthesized primers from TOYOBO, Japan.

RFLP analysis

Total DNA of azuki bean was digested with *Hin*dIII, electrophoresed (5-10 μg per lane) using 0.8 % agarose gel, and transferred to Hybond N+ membrane (Amersham, UK). Mungbean and cowpea genomic DNA probes were kindly provided by Dr. N. D. Young, University of Minnesota, USA. Probe labeling, hybridization and band detection were carried out using ECL direct nucleic acid labeling and detection system (Amersham, UK) according to the manufacture's instruction.

Genetic analysis of morphological traits

Five morphological traits were analyzed; epicotyl color, seed testa color, hilum shape, pod color and pod shattering. These phenotypes of both parents, F_1 hybrid and F_2 individuals were evaluated to determine the manner of inheritance.

Linkage analysis

F_2 segregation data for RFLP and RAPD markers, and morphological traits were examined by chi-square tests. Linkage analysis was assessed using the MAPMAKER (Lander et al. 1987) rewritten for Macintosh computer which kindly provided by Dr. S. Tingey, Du Pont Co., USA. In this program, Twopoint/Group command was used to establish possible linkage groups with a LOD score of 3 and 0.25 recombination fraction. The order of markers in each group were determined by Multipoint/First order command (LOD=3, r=0.25). Recombination frequencies were converted into map distances (centiMorgans) using the Kosambi function (Kosambi 1944).

Results

Parental polymorphism survey and F_2 segregation of molecular markers

In order to detect RAPD markers between *V. angularis* and *V. nakashimae*, 200 decamer primers were examined. Among them, 36 primers gave at least three distinct polymorphic fragments (Table III-1). They produced in total 116 RAPDs which were also detected in the amplification products in F_1 plant, indicating their proper inheritance to next generation. Concerning RFLP markers, out of 67 mungbean and cowpea genomic probes, 42 (62.7%) gave polymorphic patterns between parents in *Hin*dIII digestion. Among them, 20 RFLP markers which gave clear hybridization bands were further used for F_2 segregation analysis.

Segregation of molecular markers (116 RAPD and 20 RFLP markers) was examined with 80 F_2 plants derived from the cross of *V. angularis* x *V. nakashimae*. Examples for segregation patterns of RAPD and RFLP markers are presented in Figs. III-1 and III-2, respectively. One hundred fifteen RAPDs (99.1%) were turned out to be dominant markers and one RAPD (0.9%) and 20 RFLP markers were codominant (Table III-2). The segregation of 23 RAPD and three RFLP markers deviated significantly from the expected ratio of 3:1 or 1:2:1 ($P<0.05$).

Morphological traits

Five morphological traits which showed polymorphisms between parents were examined with F_1 and F_2 plants (Table III-3). The F_1 hybrid showed the same characteristics with *V. nakashimae* in the

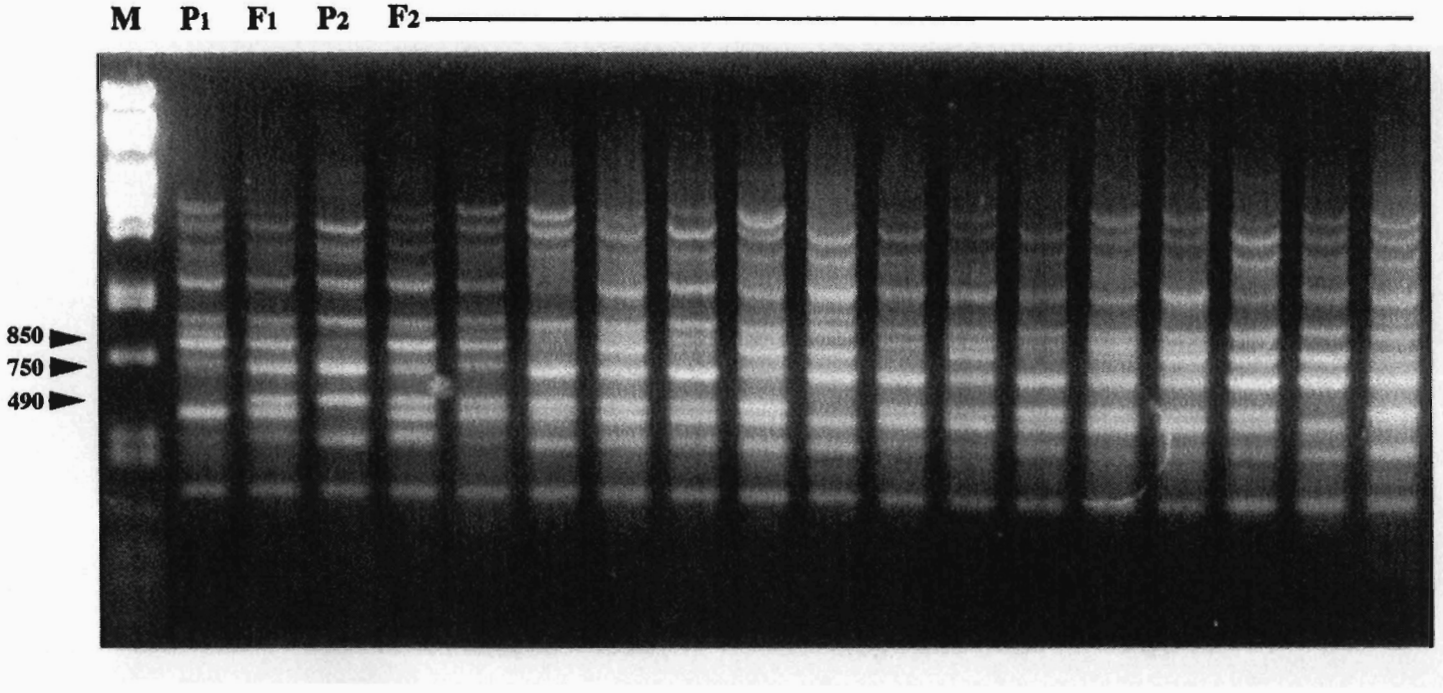

Fig. III-1. Segregation of the RAPD markers detected with a primer 'OPD08' in the F2 population derived from a cross between *V. angularis* and *V. nakashimae.* The markers are indicated by arrows with morecular size. M: Lambda DNA digested with *Pst* I. F1: F1 hybrid (*V. angularis* x *V. nakashimae).* P1: *V. angularis.* P2: *V. nakashimae.*

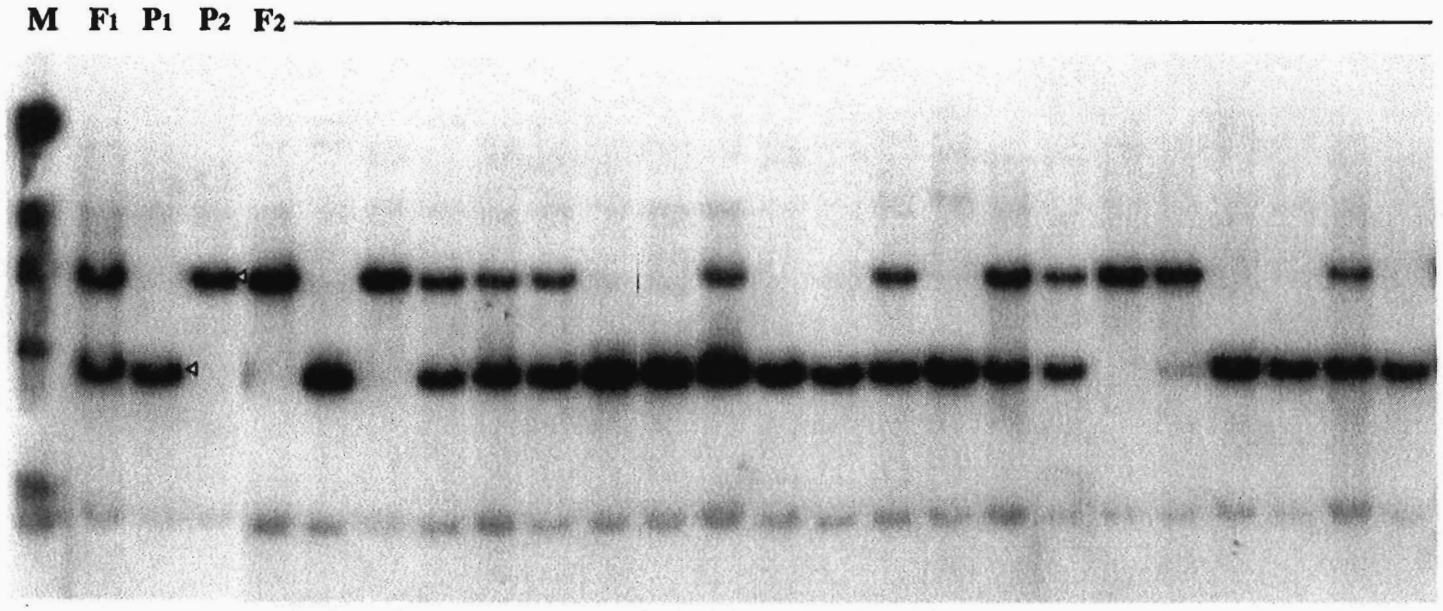

Fig. III-2. Segregation of the RFLP marker pP238 in the F2 population derived from a cross between *V. angularis* and *V. nakashimae*. *Hind* III digested DNAs were blotted onto the filter. Polymorphic bands are indicated by arrows. M: Lambda DNA digested with *Hind* III. F1: F1 hybrid (*V. angularis* x *V. nakashimae*). P1: *V. angularis*. P2: *V. nakashimae*.

Table III-2. Segregation of 116 RAPD and 20 RFLP markers in F_2 population.

Marker	No. of plants					Chi-square	Linkage group
	P_1	P_1+H	H	P_2+H	P_2		
RAPD marker							
SDA08 320		63			14	1.91	3
SDA08 640	27			50		4.16 *	8
SDA08 1010		56			21	0.21	7
SDA09 470	23			56		0.71	2
SDA09 740	19			59		0.02	-
SDA09 800		67			11	4.94 *	3
SDA11 900	16			64		1.07	1
SDA11 640		67			12	4.05 *	1
SDA27 750		66			14	2.40	6
SDA27 450	28			50		4.94 *	6
SDA27 1140	37			43		19.27 **	8
SDA27 1190	21			59		0.07	1
SDA27 1700		57			23	0.60	-
OPC01 1160		64			16	1.07	9
OPC01 370	18			61		0.21	1
OPC02 1190	16			64		1.07	7
OPC02 390		56			24	1.07	3
OPC02 860		59			19	0.02	3
OPC05 1610	19			61		0.07	2
OPC05 1160	13			67		3.27	1
OPC05 1050		59			21	0.07	4
OPC05 640		57			23	0.60	3
OPC06 1650		67			13	3.27	2
OPC06 600	15			65		1.67	3
OPC06 530	22			58		0.27	10
OPC06 390		61			19	0.07	3
OPC06 1190		62			18	0.27	2
OPC08 1020		65			15	1.67	4
OPC08 560	16			64		1.07	7
OPC08 550	24			56		1.07	4
OPC10 910	11			69		5.40 *	3
OPC10 730		60			20	0.00	14
OPC10 330		69			11	5.40 *	2
OPC10 280		60			20	0.00	3
OPC13 660		61			19	0.07	3
OPC13 550		65			15	1.67	1
OPC19 1170	19			61		0.07	6
OPC19 850	20			60		0.00	13

Table III-2. (continued)

Marker	No. of plants					Chi-square	Linkage group
	P_1	P_1+H	H	P_2+H	P_2		
RAPD markewr							
OPD01 2650	24			51		1.96	10
OPD01 1390		56			16	0.30	1
OPD01 940	16			61		0.73	3
OPD01 760	16			56		0.30	5
OPD02 910	19			61		0.07	4
OPD02 740	20			60		0.00	4
OPD02 460		56			24	1.07	6
OPD05 1510		51			29	5.40 *	6
OPD05 810	18			62		0.27	2
OPD08 490	21			59		0.07	13
OPD08 750	28			52		4.27 *	8
OPD08 850		63			17	0.60	4
OPD09 2140	13			67		3.27	6
OPD09 1350		60			20	0.00	7
OPD09 1070	19			61		0.07	4
OPD09 830	14			66		2.40	3
OPD11 1930		67			13	3.27	9
OPD11 1810	16			64		1.07	2
OPD15 1430		58			19	0.00	5
OPD15 1160		66			11	4.71 *	2
OPD15 930	10			67		5.93 *	6
OPD15 730	15			62		1.25	-
OPD16 1650	13			67		3.27	1
OPD20 1610		65			15	1.67	5
OPD20 1000		68			12	4.27 *	5
OPD20 650	16			64		1.07	3
OPE01 1140		57			19	0.00	14
OPE01 940	19			57		0.00	2
OPE01 450/430	26		31		18	3.96	2
OPE01 740	3			73		17.96 **	-
OPE02 1380		71			9	8.07 **	-
OPE02 1010	20			60		0.00	1
OPE02 530		60			20	0.00	6
OPE02 1590		67			13	3.27	5
OPE03 950		65			15	1.67	6
OPE03 650	20			60		0.00	7

Table III-2. (continued)

Marker	No. of plants					Chi-square	Linkage group
	P_1	P_1+H	H	P_2+H	P_2		
RAPD marker							
OPE06 1610		59			21	0.07	3
OPE06 580		72			8	9.60 **	2
OPE06 660		70			10	6.67 **	12
OPE06 1000		61			19	0.07	11
OPE09 1050	14			66		2.40	5
OPE09 790		70			10	6.67 **	12
OPE09 660		62			18	0.27	14
OPE09 1360		60			20	0.00	11
OPE09 2840	22			58		0.27	4
OPE17 1610		65			15	1.67	1
OPE17 1390	26			54		2.40	8
OPE17 1160	19			61		0.07	8
OPE17 370	18			62		0.27	5
OPE17 1830		69			11	5.40 *	2
OPA07 1030	20			60		0.00	5
OPA07 660	15			65		1.67	13
OPA07 510	17			63		0.60	4
OPA07 1700		67			13	3.27	9
OPA07 810		63			17	0.60	1
OPA08 1450	17			63		0.60	7
OPA08 900	32			48		9.60 **	8
OPA08 620		70			10	6.67 **	12
OPA08 510	15			65		1.67	-
OPA08 1150		63			16	0.95	5
OPA17 1390	15			65		1.67	1
OPA17 720	7			73		11.27 **	-
OPB07 1090		65			15	1.67	3
OPB07 500		51			29	5.40 *	3
OPB07 430		71			9	8.07 **	12
OPB08 1760	18			62		0.27	7
OPF07 1330	14			66		2.40	7
OPF07 700	17			63		0.60	3
OPG10 690		66			14	2.40	4
OPG10 980		66			14	2.40	5
OPG10 790	23			49		1.85	8
OPG10 720		52			20	0.30	-

Table III-2. (continued)

Marker	No. of plants					Chi-square	Linkage group
	P_1	P_1+H	H	P_2+H	P_2		
RAPD marker							
OPH04 920		63			17	0.60	5
OPH04 750	20			60		0.00	2
OPH17 680		64			16	1.07	1
OPH17 740	17			63		0.60	6
OPH17 890	16			64		1.07	6
OPH17 940	18			62		0.27	10
RFLP marker							
cM3	17		39		16	0.53	1
cM4	12		36		15	1.57	9
pM100	16		43		19	1.05	5
pM177	17		29		25	4.18	3
pM186	11		41		20	3.64	7
pM208	11		36		10	3.98	2
pM211	25		40		14	3.08	6
pM241	23		49		6	12.54 **	4
pM371	22		43		15	1.68	5
pM374	29		39		7	13.03 **	2
pM415	12		41		25	4.54	3
pO26	14		37		24	2.68	3
pO91	10		24		10	0.36	-
pO109	13		32		12	0.89	4
pP170	21		49		8	9.46 **	4
pP238	19		44		16	1.25	7
pQ62		61			18	0.21	11
pQ86	19		47		14	3.08	5
pQ117	16		40		18	0.59	6
pR48	16		44		14	2.76	1

P_1, P_2 and H indicate *V. angularis*, *V. nakashimae* and heterozygote genotypes, respectively.

* and ** show significant deviation at 5% and 1% level observed on expected segregating ratios, respectively.

Table III-3. Morphological characters of *V. angularis*, *V. nakashimae* and the F_1 hybrid, and their segregation in F_2 population

Character	*V. angularis*	*V. nakashimae*	F_1 hybrid	F_2 segregation		
				Phenotype	No. of plants	$\chi 2$ (3:1)
Epicotyl color	green	purple	purple	purple : green	61 : 19	0.07
Purple epicotyl	-	intense	intense	intense : weak	43 : 18	0.66
Black mottle on seed testa	absent	present	present	present : absent	63 : 17	0.60
Pod color	straw	dark brown	dark brown	dark brown : straw	55 : 22	0.52
Pod shattering	non-shattering	shattering	shattering	shattering : non-shattering	58 : 17	0.22
Hilum cushion	smooth	concave	concave	concave : smooth	62 : 18	0.27

following phenotypes: purple stem, black mottle on seed testa, dark brown pod, shattering pod and concaved hilum cushion. F_2 segregation of these characters showed a good fit to 3:1 ratio, indicating each of them is controlled by a single dominant gene. Therefore, in this study tentative dominant genes for these characters from *V. nakashimae* were designated as *Ps* (purple stem), *Bm* (black mottle on seed testa), *Dbp* (dark brown pod), *Sp* (shattering pod) and *Chc* (concaved hilum cushion), respectively. In addition, intensity of the stem color was also segregated in F_2 plants with purple stem, suggesting the existence of hypostatic gene for the intensity of the pigmentation. Among 61 F_2 plants with purple stems, 43 and 18 showed intense and weak purple colors, respectively. Since this segregation agreed with 3:1 ratio ($\chi 2 = 0.66$), a hypostatic gene of *Isc* (intense stem color) was also added to the tentative dominant genes from *V. nakashimae.*

Linkage analysis

Linkage analysis was carried out with 116 RAPD and 20 RFLP markers and six tentative phenotypic genes using MAPMAKER. In total, 14 linkage groups containing at least three markers were constructed, and 10 markers (7.6 %) remained unlinked (Fig. III-3). The sizes of the largest and smallest linkage groups were 196.2 and 18.8 cM, respectively. The total map size was 1250 cM and the average distance between markers was 10.6 cM. The clusters of the markers showing distorted segregation in F_2 population were located in the linkage groups, 2, 8 and 12.

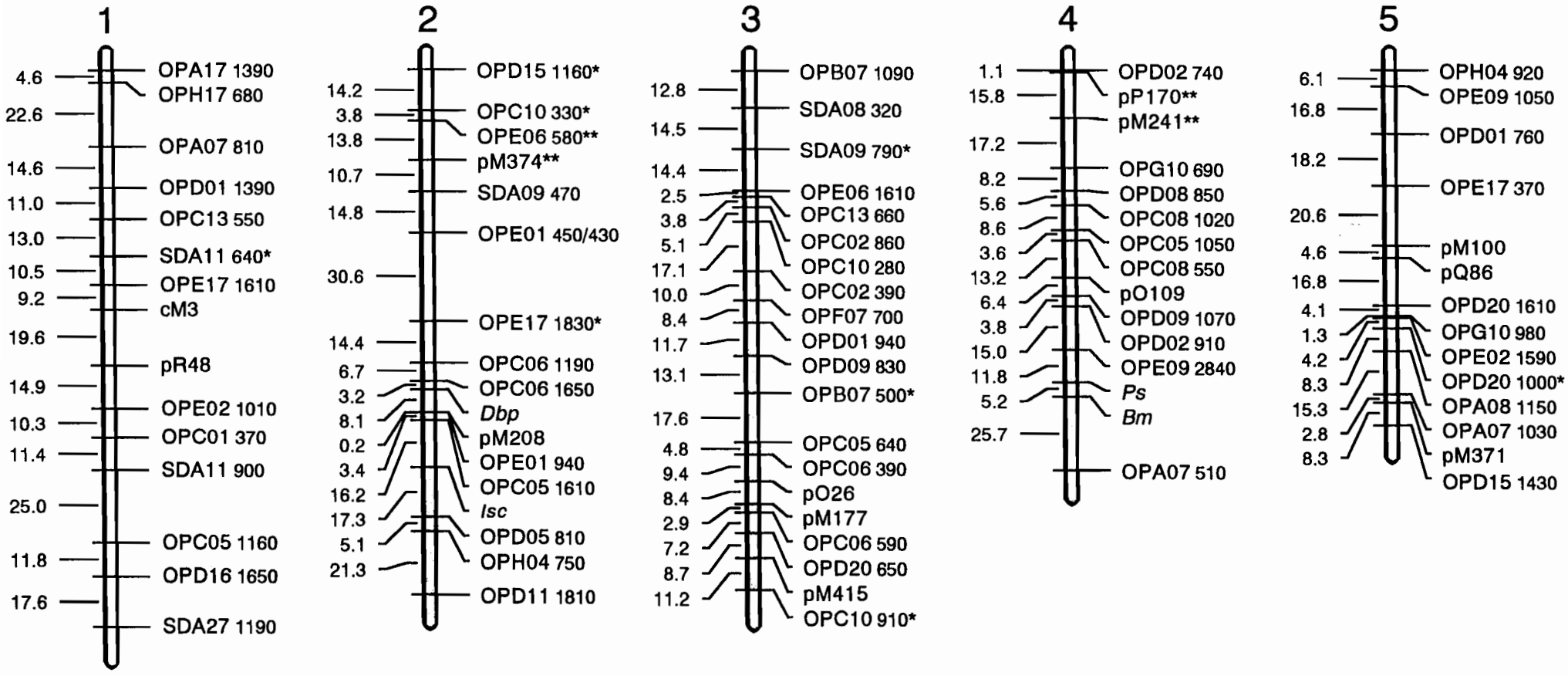

Fig. III-3. A linkage map of azuki bean constructed with a *V. angularis* x *V. nakashimae* F2 mapping population. Fourteen linkage groups are numbered in order of the size. Map distances and the marker names are shown on the left and right sides of the linkage groups, respectively. cM pM pO pP pQ and pR indicate mungbean and cowpea RFLP markers provided from Dr. N. D. Young. RAPD markers are presented with the primer codes listed in Table III-1 and the approximate molecular sizes. Markers showing significant deviations from the expected segregation ratios at 0.05 and 0.01 level are indicated with * and **, respectively.

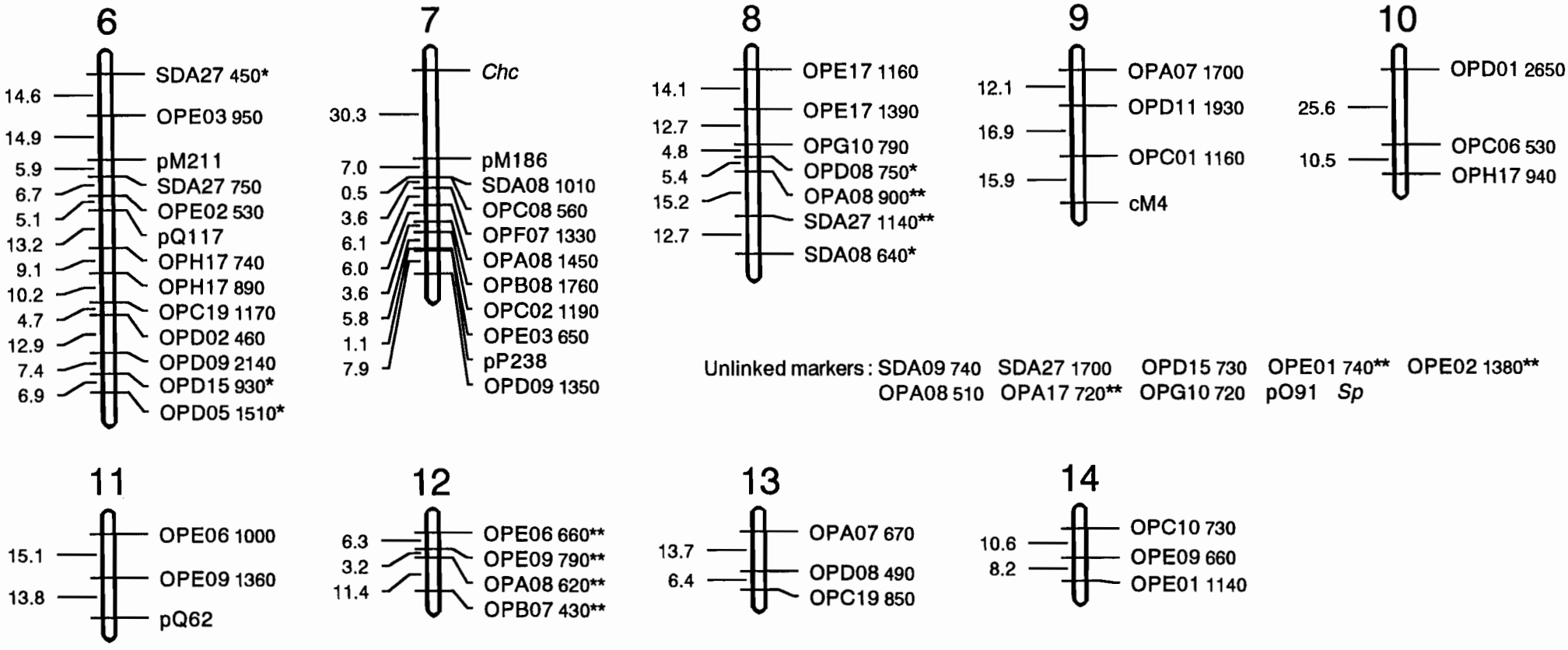

Fig. III-3. (continued)

Discussion

Linkage map of azuki bean

The first genetic linkage map of azuki bean was constructed in this study using F_2 population derived from the interspecific cross of *V. angularis* and *V. nakashimae*. In total, 132 markers (108 RAPD, 19 RFLP and five morphological markers) were included in 14 linkage groups covering 1250 cM. Since the basic chromosome number of azuki bean is eleven, highly saturated molecular map should consist of the same number of the linkage groups. In this study, azuki linkage map was mainly made with RAPD markers, because the genomic and cDNA probes from azuki bean were not available. Previously, many researchers reported that RAPD markers often arose from the repeated sequences in the genome (Devos and Gale 1992; Williams et al. 1993). Therefore in the future work, more RFLP markers should be added to this linkage map to fill the gaps, integrate linkage groups and cover the entire genome.

The clusters of markers showing distorted segregation were found in linkage groups 2, 8 and 12 (Fig. III-3). Such clusters have been commonly observed in the linkage maps derived from interspecific population (Bernatzky and Tanksley 1986; Foolad et al. 1995). Zamir and Tadmor (1986) reported that abnormal segregation may result from the expression of linked lethal genes either at the gametic or zygotic stages of plant development. In the interspecific population of *Prunus*, the interspecific nature and reproductive difference were also thought to cause abnormal segregation (Foolad et al. 1995). In this study, relatively weak F_2 plants which did not produce seeds could not be

included in F_2 mapping population. Therefore, the distorted segregation might result from the growth related factors, such as the ability of seed production.

Mapping of the genes for morphological traits

Using interspecific population of *V. angularis* and *V. nakashimae*, six morphological traits were analyzed and five tentative genes were mapped on the azuki linkage map. There are no studies available on pod shattering behavior and hilum cushion, while the mechanisms of stem, seed and pod color expression were studied by Matsuura (1933). He analyzed these characters using azuki cultivars, and identified two genes for stem color, eight for seed testa color and two for pod color. Stem color is explained by the control of the gene *P* (pigmentation of anthocyanin on stem) and the hypostatic gene *I* (intensity of pigmentation on stem). He also designated the genes *M* and *B2* for black mottle on seed testa and black pod, respectively. He further confirmed the linkage relationships between *I* and *B2*, and between *P* and *M*. In the present study, all these four characters were segregated in F_2 interspecific population and their tentative genes were mapped. Similar linkage relationships mentioned above were also observed, namely, *Isc* (intense stem color) and *Dbp* (dark brown pod) in linkage group 2, and *Ps* (purple stem) and *Bm* (black mottle on seed testa) in linkage group 4. Since these dominant genes were from *V. nakashimae*, allelism tests must be done to confirm that the genes of these common characters are located at the same locus.

Comparison of linkage maps between azuki bean and other Vigna species

Comparative linkage maps constructed with heterologous probes have been used to understand chromosomal evolution of entire genome between related species, such as tomato and pepper (Tanksley et al. 1988), tomato and potato (Bonierbale et al. 1988) and pea and lentil (Weeden et al. 1992). In the present study, 19 out of 20 RFLP markers from mungbean and cowpea genomic probes could be mapped on the linkage map of azuki bean. Table III-4 shows the comparison of linkage groups between three *Vigna* species, azuki bean, mungbean and cowpea. Although their linkage maps are not saturated with a number of markers, some sets of the markers (e.g., pO26, pM177 and pM415) were found to belong to the same linkage groups of the respective maps. This suggests some conservative linkage blocks among these *Vigna* species. In addition, one of RFLP markers, pM241, was found to be located in the cluster showing distorted segregation in azuki linkage map as well as in mungbean linkage map (Menancio-Hautea et al. 1993b), indicating the distorted segregation in this cluster might be caused by the similar mechanisms among two *Vigna* species.

Further study for azuki bean breeding

The development of a genetic linkage map in azuki bean will greatly enhance the ability of breeders to monitor the introgression of desirable traits from wild species into azuki cultivar. In the present study, dominant morphological characters from *V. nakashimae* were preliminarily analyzed. Besides them, this wild species has a resistance gene to blown stem rot which is one of serious diseases for

Table III-4. Comparison of linkage groups of 20 RFLP markers among three *Vigna* species, azuki bean, mungbean and cowpea.

Probe	Linkage group		
	Azuki bean	Mungbean[a]	Cowpea[b]
cM3	1	1	6
pR48	1	6	-
pM374	2	5	-
pM208	2	7	4
pO26	3	3	1
pM177	3	3	-
pM415	3	3	-
pP170	4	-	3
pM241	4	2	3
pO109	4	13	-
pM100	5	8	-
pQ86	5	8	-
pM371	5	1	-
pM211	6	10	8
pQ117	6	4	-
pM186	7	-	4
pP238	7	1	1
cM4	9	7	4
pQ62	11	2	-
pO91	(unlinked)	5	-

- : not examined.

a) Mapped by Menancio-Hautea et al. (1993a).

b) Mapped by Fatokun et al. (1993).

azuki bean. Once this gene is tagged and mapped with molecular markers, marker assisted selection will be applied for the improvement of azuki cultivars.

The red seed color of azuki bean is also one of the important characters for azuki breeding. In this study, a cross between an azuki cultivar and *V. nakashimae* having green seed color was carried out. However, the genetic system of red seed color expression could not be clarified because of its complicated segregation and a small number of F_2 plants analyzed. According to Matsuura (1933), the ground color of seed testa was explained by a seven-gene model with inhibitor and complementary genes. Therefore, for this character under several-gene control, the plants of advanced generation, such as recombinant inbred lines, must be analyzed.

Summary

A genetic linkage map of azuki bean (*Vigna angularis*) was constructed with molecular and morphological markers using F_2 population of interspecific cross between azuki bean and its wild relative, *V. nakashimae*. In total, 132 markers (108 RAPD, 19 RFLP and five morphological markers) were mapped in 14 linkage groups, covering 1250 cM. Ten remained unlinked. The clusters of markers showing distorted segregation were found in linkage groups 2, 8 and 12. By comparing the azuki linkage map with those of mungbean and cowpea using common 20 RFLP markers, some sets of the markers were found to belong to the same linkage groups of the respective maps, indicating that these linkage blocks are conserved among three *Vigna* species.

The map constructed should provide a tool for marker-assisted selection and studies of genome organization in *Vigna* species.

Chapter IV. QTL analysis of morphological and reproductive traits in a interspecific population of *V. angularis* x *V. nakashimae*

Introduction

Most of the agronomic characters of crops, such as plant height, seed weight, seed size, heading date, yield etc..., are inherited quantitatively. Continuous variation of these traits has been thought to arise largely from the collective effects of polygenes. Therefore, identification of these polygenes and analysis of their effects are very important to planning breeding programs for quantitative characters. In common bean, the first linkage analysis of genes controlling quantitative characters was reported by Sax (1923); the size of seeds was partially associated with seed coat pattern and pigmentation. Although many researchers tried to reveal polygenes in relation to morphological markers, the information on the quantitative traits was meager because of the limitation of morphological markers. However, the recent advent of molecular maps with large number of markers has enabled us to analyze polygenes more precisely and effectively.

Linkage map with an extensive set of marker loci helps to identify genes controlling quantitative traits. The theoretical basis for interpreting the association of maker loci with quantitative trait loci (QTLs) has been outlined (Mather and Jinks 1977; Tanksley et al. 1989). The marker loci can serves as landmarks to identify the chromosomal regions where QTLs exist. The homologous chromosomal regions in different individuals of a population having genetic variations are compared alternatively with character values of

quantitative traits. Then, the association of QTL with marker locus is measured based on statistical models. The simple models have been used to detect the makers associating with QTL, not exactly linked, by comparing for genotypic marker class means based on one way ANOVA or linear regression (Sax 1923; Rasmusson 1935; Soller and Brody 1976; Vallejos and Tanksley 1983; Osborn et al. 1987; Weller et al. 1988; Tanksley and Hewitt 1988). Theses models can be used effectively if the trait is controlled by only a few loci, though there are many limitations for estimating QTL effect and position. As compared with these methods, simple interval mapping analysis based on the maximum likelihood model using pairs of genotype of adjacent markers as the units for their interval subdivisions allows separate estimation of QTL effect and position (Lander and Botstein 1989). Recently, QTL analysis has progressed rapidly and been applied for many crop species, such as tomato (Paterson et al. 1988; deVicente and Tanksley 1993), barley (Backes et al. 1995), rice (Xiao et al. 1995; Li et al. 1995) and soybean (Mansur et al. 1993). These studies succeeded to identify many molecular markers linked to QTLs, suggesting that these markers can assist future marker-based selection in crop breeding (Johnson et al. 1995).

Azuki bean is one of the most important beans in Japan. However, only a limited genetic information is available for quantitative traits, and thus more genetic information is needed to be obtained to support azuki bean breeding. Recently, many crosses between cultivars and wild species have been made in azuki bean to transfer useful genes. For example, brown stem rot resistance has been introduced from one of the wild species, *V. nakashimae.* However, at the same time

undesirable traits from wild species might be transferred to the cultivars. Therefore, molecular markers linked to QTLs controlling wild traits must be utilized to monitor the desirable traits and eliminate the undesirable traits.

In the previous chapter, a genetic linkage map of azuki bean was constructed with RFLP, RAPD and morphological markers using a interspecific population of *V. angularis* x *V. nakashimae*. In this chapter, using the same mapping population, the inheritance of quantitative traits and the characteristics of molecular markers associated with these traits were studied. In addition, several problems on QTL mapping using dominant markers were discussed.

Materials and methods

Plant materials

V. angularis cv. 'Erimoshouzu' and *V. nakashimae* were used to produce the mapping population as described in Chapter III. Eighty F_2 individuals were separately grown in the pots of 25 cm in diameter at Kobe University in summer, 1993. Ten F_3 plants from each of 80 F_2 individual were grown in the field at Kobe University Experimental Farm in summer, 1994. Each F_3 line was planted in family groups separated by check plots of the parents (*V. angularis* and *V. nakashimae*) with the space of 15 cm between plants and 80 cm between the rows. *V. angularis* and *V. nakashimae* were also planted in the field to examine enviremental variation.

Measurement of quantitative traits

In total, 20 traits were examined using F_2 and F_3 progenies from the interspecific cross of *V. angularis* x *V. nakashimae* (Table IV-1). Of these, the following six traits were measured in both F_2 and F_3 individuals; plant height (PH), seed weight (SW), days to flowering (DF), branch number (BN), primary leaf width (PLW) and length (PLL). PH was measured in cm between shoot apex and the base of plant at maturity. SW was determined from the mean weight of 20 seeds in gram. DF was the number of days until an open flower was found, starting from August 12, 1993 for F_2 plants and August 15, 1994 for F_3 plants. BN was scored as the number of branches from the main stem at maturity. PLW and PLL were in cm when the upper leaf began to develop. One trait, epicotyl length (EL), was measured only for F_2 progeny in cm between the base of primary leaves and the epicotyl base when the upper leaf began to develop. The progeny means of each F_3 line for all these traits were calculated and used for further analysis.

Six traits were measured in F_3 individuals, and the progeny means of F_3 lines were calculated; days to ripening (DR), seed filling period (SFP), pod shattering (PS), brown seed (BS), green seed (GS), white seed (WS), red seed (RS), fertile pollen (FP) and seed germination (GES). DR was the number of days from August 15, 1994 to the day when 75 % of total pods ripened. SFP was given by subtracting DF from DR. The segregating manner of shattering pod was reexamined in F_3 progeny because *Sp* (tentative dominant gene for shattering pod) could not be located on azuki linkage map in the previous study (Chapter III). PS was recorded as the percentage of plants having shattering pods per F_3 line. The seed colors of F_4 seeds in F_3 pods were classified into four

Table IV-1. List of 20 morphological traits analyzed in this study.

Trait	Definition	Tested progeny
PH (plant height)	Length between shoot apex and stem base at maturity	F_2 and F_3
SW (seed weight)	Mean weight of 20 seeds	F_2 and F_3
DF (days to flowering)	Number of days to flower	F_2 and F_3
BN (branch number)	Number of branches on main stem	F_2 and F_3
PLW (primary leaf width)	Mean width of primary leaf in the presence of upper leaf	F_2 and F_3
PLL (primary leaf length)	Mean length of primary leaf in the presence of upper leaf	F_2 and F_3
EL (epicotyl length)	Length of epicotyl in the presence of upper leaf	F_2
DR (days to ripening)	Mean number of days for ripening 75% of total pods	F_3
SFP (seed filling period)	Mean number of seed filling days after flowering	F_3
PS (pod shattering)	Percentage of plants having shattering pod per line	F_3
BS (brown seed)	Percentage of plants with brown seeds per line	F_3
GS (green seed)	Percentage of plants with green seeds per line	F_3
WS (white seed)	Percentage of plants with white seeds per line	F_3
RS (red seed)	Percentage of plants with red seeds per line	F_3
FP (fertile pollen)	Mean percentage of stained pollens	F_3
GES (germinated seed)	Mean percentage of germinated seeds	F_3
LP (lethal phenotype)	Percentage of plants which died during development per line	F_3
SP (stunted phenotype)	Percentage of stunt plants per line	F_3
SS (sterile seed)	Percentage of plants which could not produce any seeds per line	F_3
AS (abnormal seed)	Percentage of plants which produced abnormal seeds per line	F_3

types, i.e., brown, green, white and red. On these basis, evaluations of the percentage of plants having respective seed color per line were conducted on F_3 lines. FP was examined as follows: Anthers were collected from five flowers of each F_3 individual. Dehisced anthers were tapped lightly on a drop of acetocarmine, and the red dyed pollen grains were counted as viable. Then, the mean percentage of viable pollens in each F_3 line were calculated. GES was recorded as the percentage of the germinated seed.

The percentage of respective category per F_3 line was used for evaluating four traits; lethal phenotype (LP), stunted phenotype (SP), sterile seed (SS) and abnormal seed (AS). LP and SP were the percentages of lethal and stunted plants, respectively. SS and AS were calculated as the percentages of plants with no and abnormal seeds, respectively.

Statistical analysis

Genetic variance (V_1F_2, V_1F_3 and V_2F_3), heritability in broad and narrow senses, and potence ratio ($\sqrt{H/D}$) were obtained using the estimates of variance components for *D*, *H*, *E*, *E1* and *E2* from the analysis of variance in both F_2 and F_3 individuals, and progeny means of F_3 line (Mather and Jinks 1977). Pearson correlation coefficients between quantitative traits and between F_2 individuals, and the means of F_3 lines were calculated using STATISTICA, ver 4.1J (StatSoft, OK, USA).

QTL analysis

The QTL mapping was performed with the linkage map constructed

in Chapter III. The markers were selected for the analysis to have relatively uniform spacing with an approximately 15 cM interval (Fig. IV-1). QTLs for each trait were analyzed by two models using implemented softwares in the package of 'QTLCartographer' which kindly provided by Dr. C. J. Basten, University of North Carolina, USA; LRmapqtl (single-marker analysis) and Zmapqtl model 3 (simple interval mapping). LRmapqtl detects markers which associates with QTLs by fitting the phenotypic data to the linear model $\mathbf{y_i}=\mathbf{b_0}+\mathbf{b_1}\mathbf{x_i}+\mathbf{e}$, where $\mathbf{y_i}$ is the phenotype of **i**th individual and $\mathbf{x_i}$ is an indicator variable for maker genotype (AA=2, Aa=1 and aa=0). The regression parameters $\mathbf{b_0}$ and $\mathbf{b_1}$ were estimated where **e** is assumed to have a standard normal distribution. Zmapqtl model 3, which is the same program as that of Lander and Botstein (1989), detects positions and effects of QTLs by using maximum likelihood method. The estimated genotype between adjacent pairs of markers are fitted to the model. For comparison the thresholds, F (single-marker analysis) and LOD (simple interval mapping) were transformed into likelihood ratios called 'test statistic'. A likelihood ratio (H0 : H1) of hypotheses for H0 (without QTL effect) and H1 (with QTL effect) was tested against a threshold 12.5 for a 5 % error rate. When progeny means of F_3 lines instead of F_2 individuals were used, the estimates of gene effects were calculated fitting 'T(SF_3)SF_2' model which differed from the model 'SF_2' for F_2 population. The gene action estimated were evaluated by dominant/additive ratio (Edwards et al. 1987).

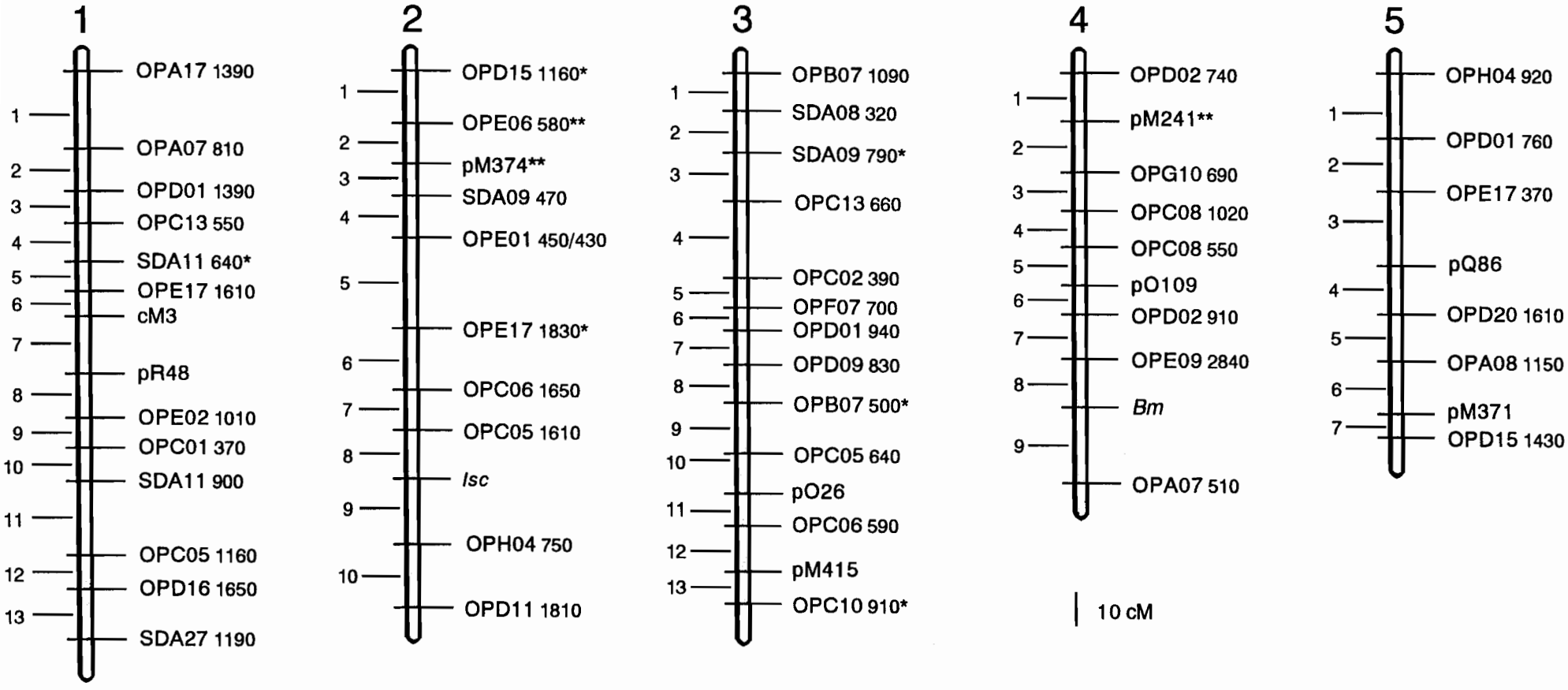

Fig. IV-1. A linkage map of azuki bean used for single-marker analysis and interval mapping. Fourteen linkage groups are numbered in order of the size. Interval numbers and marker names are shown on the left and right sides of the linkage groups, respectively. Markers showing significant deviations from the expected segregation ratios at 0.05 and 0.01 level are indicated with * and **, respectively.

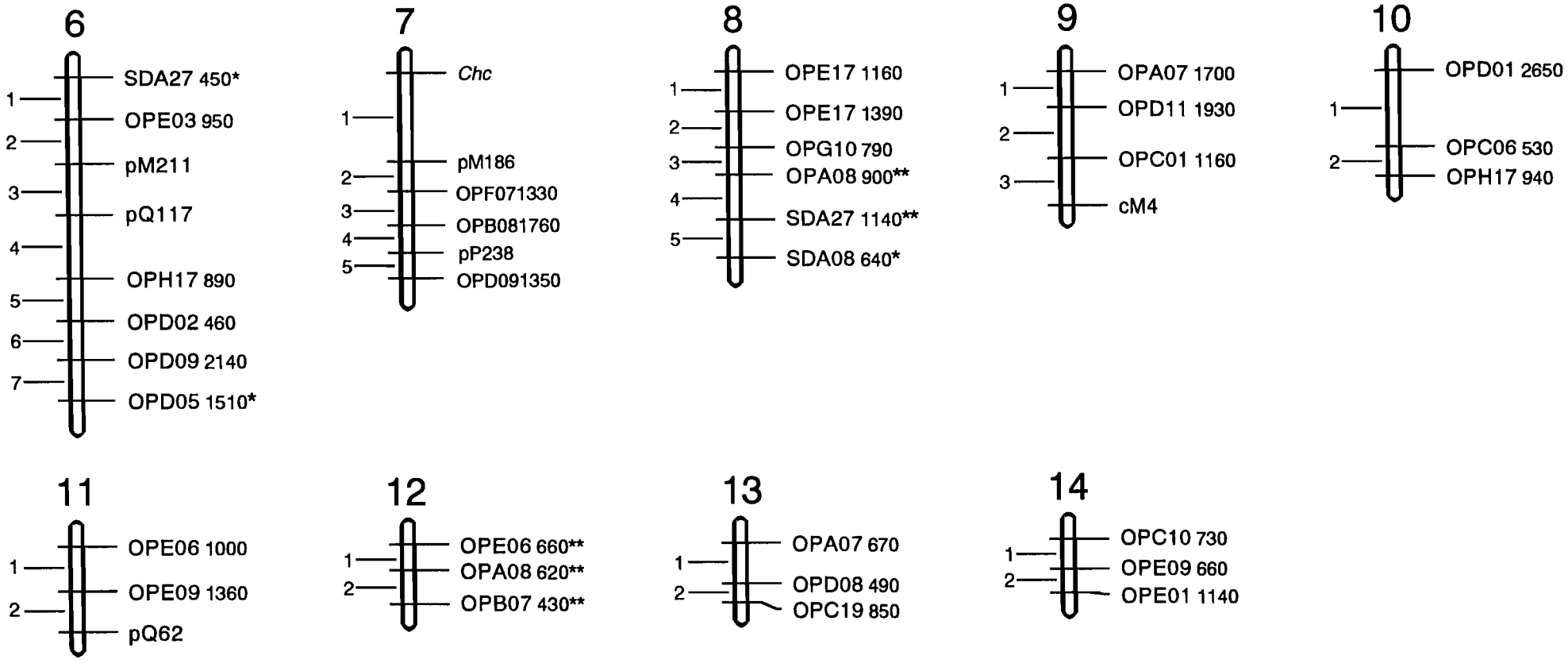

Fig. IV-1. (continued)

Results

Morphological characters of parental plants

The morphological differences between *V. angularis* and *V. nakashimae* were distinct particularly in plant structure and seed size. *V. angularis* had large red seeds and short and thick stem with large leaves. On the other hand, *V. nakashimae* had small seeds and climbing long stem with small leaves. Tables IV-2 and IV-3 summarize the average phenotypic values of two parental plants and their progenies grown in the pot and in the field condition, respectively. The morphological appearance of *V. angularis* was distinctively larger in the field cultivation than that in the pot cultivation.

QTL analysis

The markers that are significantly associated with QTLs controlling 20 traits were searched on the linkage map using single-marker analysis with probability range $p<0.05$. For each location containing significant markers, linkage group, peak, test statistic and F-test value were determined (Tables IV-4 - IV-9).

Interval mapping was also carried out for nine out of 20 traits to detect more accurate positions and estimates of gene action. At a threshold 12.5, QTLs with major effects on trait were searched. In addition, the scans of the threshold 3.84 (equivalent to probability $p= 0.05$ at single-marker analysis) were performed to detect minor effects on the trait. For each QTL identified, linkage group, interval, tested value and gene effects of QTL and the origin of allele for additive (a) and dominant (d) effects were examined (Tables IV-10 -

Table IV-2. Means for seven traits of *V. angularis*, *V. nakashimae* and their F_2 population in the pot condition.

Trait	*V. angularis*	*V. nakashimae*	Mid-parent	F_2 population		
	mean	mean		mean	maximum	minimum
PH (cm)	17.7	134.2	76.0	50.9	156	10
SW (g)	1.90	0.51	1.21	0.83	1.6	0.3
DF (day)	9.6	18.4	14.0	9.1	22	0
BN (no.)	0	2.11	1.06	1.51	4	1
PLW (cm)	3.39	2.04	2.72	2.66	3.7	1.7
PLL (cm)	3.44	2.50	2.97	2.79	4.2	1.6
EL (cm)	7.71	2.29	5.00	3.91	8.5	1.4

Table IV-3. Means for ten traits of *V. angularis*, *V. nakashimae* and their progeny means of F_3 lines in the field condition.

Trait	*V. angularis* mean	*V. nakashimae* mean	Mid-parent	Progeny means of F_3 lines		
				mean	maximum	minimum
PH (cm)	46.8	94.6	70.7	60.6	150.0	18.0
SW (g)	2.35	0.47	1.41	1.04	1.61	0.37
DF (day)	10.4	16.3	13.4	8.9	30.0	0.9
BN (no.)	4.40	6.67	5.53	4.6	10.0	0.0
PLW (cm)	3.38	1.60	2.49	2.41	4.10	1.20
PLL (cm)	3.64	1.87	2.76	2.51	4.00	1.40
DR (day)	54.7	60.3	57.5	70.6	86.0	52.0
SFP (day)	44.3	44.0	44.2	60.8	82.0	36.0
FP (%)	97.7	97.2	97.5	57.7	90.9	0.0
GES (%)	98.0	88.0	93.0	67.9	100.0	0.0

Table IV-4. Significant associations of markers for PH and EL detected by single-marker analysis.

Trait	Progeny	Linkage group	Marker	Peak	Test statistics	F(1,n-2)	pr(F)
PH	F_2	2	8--10	8	8.565	8.815	0.004 **
		3	6	6	5.254	5.295	0.024 *
		3	11--14	11	4.357	4.366	0.04 *
		4	1--2	1	16.252	17.57	0 ****
		4	6	6	4.304	4.311	0.041 *
		7	5	5	4.967	4.996	0.028 *
	F_3	2	8	8	4.143	4.146	0.045 *
		2	10	10	4.624	4.641	0.034 *
		4	1	1	3.967	3.966	0.05 *
		4	6	6	4.492	4.505	0.037 *
EL	F_2	1	3	3	5.773	5.837	0.018 *
		4	7--9	8	10.601	11.052	0.001 **
		5	1	1	4.347	4.355	0.04 *
		5	5--6	5	8.103	8.315	0.005 **
		13	3	3	4.397	4.407	0.039 *
		14	2	2	7.47	7.634	0.007 **

*, ** and **** denote significance levels of F-test at 0.05, 0.01 and 0.0001 probability levels, respectively.

Table IV-5. Significant associations of markers for SW detected by single-marker analysis.

Trait	Progeny	Linkage group	Marker	Peak	Test statistics	F(1,n-2)	pr(F)
SW	F_2	1	7	7	4.852	4.877	0.03 *
		2	1--3	1	5.295	5.338	0.024 *
		2	9	9	6.298	6.389	0.014 *
		3	6	6	5.099	5.133	0.026 *
		3	11--14	13	14.607	15.625	0 ***
		5	5--7	7	8.286	8.513	0.005 **
		7	1--5	4	8.044	8.251	0.005 **
		9	1--2	1	14.66	15.687	0 ***
		11	1	1	4.543	4.557	0.036 *
		12	1--3	1	4.998	5.029	0.028 *
		14	1	1	4.475	4.487	0.037 *
	F_3	1	1--3	3	9.082	9.377	0.003 **
		1	7--8	8	7.365	7.521	0.008 **
		2	5--6	6	6.289	6.379	0.014 *
		2	9	9	7.715	7.897	0.006 **
		3	3	3	8.815	9.086	0.003 **
		3	6--9	8	11.346	11.886	0.001 ***
		3	11--14	13	13.157	13.944	0 ***
		4	2	2	6.196	6.282	0.014 *
		4	6	6	6.536	6.64	0.012 *
		5	4--7	5	11.535	12.098	0.001 ***
		6	4--8	7	15.449	16.615	0 ***
		7	1--5	1	10.368	10.793	0.002 **
		9	1--4	1	14.015	14.934	0 ***
		10	1	1	6.682	6.795	0.011 *
		12	1--3	3	15.914	17.167	0 ****

*, **, *** and **** denote significance levels of F-test at 0.05, 0.01, 0.001 and 0.0001 probability levels, respectively.

Table IV-6. Significant associations of markers for DF, DR and SFP detected by single-marker analysis.

Trait	Progeny	Linkage group	Marker	Peak	Test statistics	F(1,n-2)	pr(F)
DF	F_2	4	1--2	1	22.701	25.593	0 ****
		5	6	6	4.859	4.884	0.03 *
		6	1--5	3	14.552	15.561	0 ***
		11	2	2	6.132	6.214	0.015 *
		13	1--2	2	11.392	11.936	0.001 ***
		14	3	3	4.284	4.291	0.042 *
	F_3	4	1--2	1	8.277	8.502	0.005 **
		6	3--7	4	7.911	8.107	0.006 **
		11	2	2	5.956	6.029	0.016 *
DR	F_3	1	1	1	7.275	7.426	0.008 **
		2	1	1	5.572	5.626	0.02 *
		4	8	8	5.674	5.733	0.019 *
		7	1--4	1	6.132	6.214	0.015 *
		8	1	1	3.972	3.971	0.05 *
		10	2--3	3	6.754	6.871	0.011 *
		13	1	1	4.414	4.425	0.039 *
SFP	F_3	1	1	1	7.357	7.513	0.008 **
		2	1	1	6.983	7.114	0.009 **
		2	8--11	8	5.065	5.098	0.027 *
		7	1--4	1	5.995	6.07	0.016 *
		8	1	1	6.227	6.314	0.014 *
		10	3	3	4.666	4.685	0.033 *
		13	1	1	7.359	7.515	0.008 **

*, **, *** and **** denote significance levels of F-test at 0.05, 0.01, 0.001 and 0.0001 probability levels, respectively.

Table IV-7. Significant associations of markers for BN, PLW and PLL detected by single-marker analysis.

Trait	Progeny	Linkage group	Marker	Peak	Test statistics	F(1,n-2)	pr(F)
BN	F_2	2	7--11	10	10.123	10.522	0.002 **
		4	1--2	1	13.253	14.054	0 ***
		4	9	9	4.831	4.855	0.031 *
		5	1--3	3	11.414	11.962	0.001 ***
		9	1	1	4.374	4.383	0.04 *
	F_3	2	1--4	3	7.959	8.159	0.005 **
		2	6	6	5.818	5.884	0.018 *
		4	1	1	8.872	9.148	0.003 **
		5	1--4	2	5.026	5.058	0.027 *
		9	1--2	1	5.787	5.851	0.018 *
		11	2	2	4.671	4.69	0.033 *
PLW	F_2	5	5	5	5.531	5.583	0.021 *
		6	5	5	6.155	6.239	0.015 *
		9	1	1	5.575	5.63	0.02 *
	F_3	3	11--13	13	5.831	5.898	0.017 *
		4	2--9	6	7.336	7.491	0.008 **
		5	5	5	4.051	4.052	0.048 *
		6	1	1	7.303	7.455	0.008 **
		14	1	1	6.932	7.06	0.01 **
PLL	F_2	5	2--3	3	5.219	5.258	0.025 *
	F_3	3	11--13	11	8.178	8.395	0.005 **
		4	6	6	5.493	5.544	0.021 *
		4	9	9	4.035	4.035	0.048 *
		6	1	1	6.947	7.076	0.009 **
		6	7	7	4.378	4.388	0.039 *
		14	1	1	11.629	12.204	0.001 ***

*, ** and *** denote significance levels of F-test at 0.05, 0.01 and 0.0001 probability levels, respectively.

Table IV-8. Significant associations of markers with PS, BS, RS, WS and GS detected by single-marker analysis.

Trait	Linkage group	Marker	Peak	Test statistics	F(1,n-2)	pr(F)
PS	2	9	9	4.301	4.309	0.041 *
	6	3	3	5.265	5.307	0.024 *
BS	3	11	11	5.078	5.112	0.027 *
	5	1	1	5.484	5.534	0.021 *
	6	5	5	5.129	5.165	0.026 *
	7	3	3	4.494	4.507	0.037 *
	9	1	1	5.512	5.564	0.021 *
	14	1--3	3	5.603	5.659	0.02 *
RS	2	10	10	4.654	4.673	0.034 *
	3	8--9	9	5.108	5.143	0.026 *
	4	2	2	5.512	5.564	0.021 *
	4	10	10	4.106	4.108	0.046 *
	7	3--4	3	19.714	21.797	0 ****
	8	1	1	19.123	21.062	0 ****
	9	1	1	4.026	4.026	0.048 *
WS	1	2	2	4.117	4.119	0.046 *
	4	2	2	7.33	7.484	0.008 **
	4	6--8	6	7.552	7.722	0.007 **
	6	6	6	4.465	4.478	0.038 *
	11	1	1	4.026	4.025	0.048 *
	14	1	1	7.743	7.926	0.006 **
GS	3	6--7	7	4.58	4.596	0.035 *
	11	2	2	4.369	4.378	0.04 *

*, **, *** and **** denote significance levels of F-test at 0.05, 0.01, 0.001 and 0.0001 probability levels, respectively.

Table IV-9. Significant associations of markers for FP, GES, LP, SP, SS and AS detected by single-marker analysis.

Trait	Linkage group	Marker	Peak	Test statistics	F(1,n-2)	pr(F)
FP	2	3--11	6	5.679	5.739	0.019 *
	5	2	2	7.003	7.136	0.009 **
GES	2	9	9	3.978	3.977	0.05 *
	3	14	14	6.358	6.452	0.013 *
LP	2	5	5	4.984	5.014	0.028 *
	3	11	11	4.114	4.116	0.046 *
SP	5	1	1	5.136	5.172	0.026 *
SS	1	9--10	9	5.654	5.713	0.019 *
	2	1--4	4	8.081	8.29	0.005 **
	2	8--10	10	16.199	17.507	0 ****
	3	11	11	4.735	4.756	0.032 *
	7	1--3	1	8.085	8.295	0.005 **
	11	2	2	6.275	6.365	0.014 *
	12	3	3	4.031	4.031	0.048 *
AS	1	7	7	5.09	5.124	0.026 *
	2	5--6	5	6.856	6.979	0.01 **
	5	4	4	4.647	4.665	0.034 *
	6	3--6	3	7.678	7.857	0.006 **
	12	3	3	4.55	4.564	0.036 *

*, **, *** and **** denote significance levels of F-test at 0.05, 0.01, 0.001 and 0.0001 probability levels, respectively.

Table IV-10. The location and parameters of QTLs affecting PH based on interval mapping.

QTL	Linkage group	Interval	Progeny	Test statistic	Additive effect	Dominance effect	D/A	Kind of effect
ph1	1	4	F_2	16.17	31.3 A	43.1 N	1.38	over d
ph2	2	8	F_2	16.29	38.3 N	28.0 A	0.73	pd or d
	2	8	F_3	11.67	21.1 N	21.8 A		
ph3	3	4	F_2	12.86	24.2 N	16.1 A	0.66	pd or d
	3	4	F_3	4.48	16.9 N	20.1 A		
ph4	4	1	F_2	23.91	45.6 N	24.2 A	0.53	pd or d
	4	1	F_3	4.96	17.7 N	16.8 A		
ph8	8	5	F_2	16.91	36.6 N	32.9 A	0.90	pd or d
ph10	10	1	F_2	20.42	32.6 N	42.6 A	1.31	over d
	10	1	F_3	5.13	19.2 N	24.1 A		
ph11	11	1	F_3	27.01	33.6 A	65.1 N	1.94	over d
	11	1	F_2	12.20	47.5 A	40.2 N		
ph12	12	2	F_2	13.60	20.6 A	53.5 N	2.59	over d
	12	2	F_3	10.60	38.2 A	44.3 N		

The number following the QTL designation corresponds to the linkage group in which QTL was located. The intervals on the linkage groups are shown in Fig. IV-1. Parameters in small letter show the effects in different progeny with more than 3.84 of the threshold. The letters next to the additive and dominance effect values (A: *V. angularis*, N: *V. nakashimae*) indicate the origin of the allele increasing trait value and the dominant allele, respectively. Gene actions of additive or partial dominance mode, partial dominance or dominance mode, and overdominance mode are shown as ad or pd, pd or d and over d, respectively.

Table IV-11. The location and parameters of QTLs affecting SW based on interval mapping.

QTL	Linkage group	Interval	Progeny	Test statistic	Additive effect	Dominance effect	D/A	Kind of effect
sw2	2	5	F_3	12.51	0.371 A	0.079 N	0.21	ad or pd
	2	5	F_2	5.249	0.126 A	0.08 N		
sw3-1	3	4	F_3	18.44	0.333 A	0.103 N	0.31	ad or pd
	3	4	F_2	3.918	0.094 A	0.173 N		
sw3-2	3	11	F_2	13.731	0.163 A	0.032 A	0.20	ad or pd
	3	11	F_3	4.62	0.110 A	0.378 A		
sw7	7	2	F_3	13.62	0.184 A	0.318 A	1.73	over d
	7	2	F_2	8.679	0.14 A	0.036 A		
sw8	8	5	F_3	13.53	0.118 A	0.584 A	4.95	over d
minor effect								
	5	7	F_2	9.73	0.132 A	0.094 A		
	5	7	F_3	3.86	0.091 A	0.203 A		
	9	1	F_2	11.223	0.227 A	0.003 N		
	9	1	F_3	5.76	0.366 A	0.023 N		
	10	2	F_2	5.338	0.145 A	0.197 A		
	10	2	F_3	4.94	0.129 A	0.440 A		

The number following the QTL designation corresponds to the linkage group in which QTL was located. The intervals on the linkage groups are shown in Fig. IV-1. Parameters in small letter show the effects in different progeny with more than 3.84 of the threshold. The letters next to the additive and dominance effect values (A: *V. angularis*, N: *V. nakashimae*) indicate the origin of the allele increasing trait value and the dominant allele , respectively. Gene actions of additive or partial dominance mode, partial dominance or dominance mode, and overdominance mode are shown as ad or pd, pd or d and over d, respectively.

Table IV-12. The location and parameters of QTLs affecting DF based on interval mapping.

QTL	Linkage group	Interval	Progeny	Test statistic	Additive effect	Dominance effect	D/A	Kind of ffect
df6	6	3	F_2	16.74	3.4 A	1.7 N	0.50	pd or d
	6	3	F_3	13.17	4.0 A	5.8 N	1.47	over d
minor effect								
	4	1	F_2	7.02	5.6 N	1.3 A		
	4	1	F_3	4.00	3.5 N	3.2 A		
	9	1	F_2	5.08	2.7 A	4.4 N		
	9	1	F_3	8.22	5.7 A	6.2 N		

The number following the QTL designation corresponds to the linkage group in which QTL was located. The intervals on the linkage groups are shown in Fig. IV-1. Parameters in small letter show the effects in different progeny with more than 3.84 of the threshold. The letters next to the additive and dominance effect values (A: *V. angularis*, N: *V. nakashimae*) indicate the origin of the allele increasing trait value and the dominant allele, respectively. Gene actions of additive or partial dominance mode, partial dominance or dominance mode, and overdominance mode are shown as ad or pd, pd or d and over d, respectively.

Table IV-13. The location and parameters of QTLs affecting BN based on interval mapping.

QTL	Linkage group	Interval	Progeny	Test statistic	Additive effect	Dominance effect	D/A	Kind of effect
bn1	1	12	F_2	17.69	0.6 N	1.7 A	2.731	over d
bn9	9	1	F_2	15.13	1.0 A	1.1 N	1.170	over d
	9	2	F_3	5.37	1.5 A	0.5 N		
bn12	12	2	F_2	15.42	0.8 A	1.4 N	1.711	over d
minor effect								
	5	1	F_2	5.81	0.7 N	0.1 N		
	5	1	F_3	8.46	1.2 N	0.1 N		
	14	1	F_2	8.81	0.6 A	1.6 N		
	14	1	F_3	4.77	1.6 A	1.9 N		

The number following the QTL designation corresponds to the linkage group in which QTL was located. The intervals on the linkage groups are shown in Fig. IV-1. Parameters in small letter show the effects in different progeny with more than 3.84 of the threshold. The letters next to the additive and dominance effect values (A: *V. angularis*, N: *V. nakashimae*) indicate the origin of the allele increasing trait value and the dominant allele, respectively. Gene actions of additive or partial dominance mode, partial dominance or dominance mode, and overdominance mode are shown as ad or pd, pd or d and over d, respectively.

13). Influencing factors of the traits were determined to exist at the position which has common allele among progenies.

At the threshold, having the most significant probability revealed by above analysis, the dominant (d) to additive (a) effects (D/A) of each QTL was calculated, and the kind of gene action was determined. Observed D/A ratios were varied from 0.01 to 4.95 (Tables IV-10 - 13). Potence ratios ($\sqrt{H/D}$) as estimates for dominant gene action were calculated from the components of variance (H and D) as measured at F_2 and F_3 progenies. These estimates indicate overall dominance ratio under the assumption that the genes having equal effect are completely associated in the parental strain and dominance is unidirectional at all loci. Observed potence ratios were varied from 0.48 for SW to 23.05 for PLL (Table IV-14).

Plant height

Plant height was measured at two different growing stages; initial elongation of epicotyl (EL) and stem elongation at maturity (PH). The parents used in this study had diverse characteristics associated with EL and PH (Tables IV-2 and IV-3). *V. angularis* was taller than *V. nakashimae* at the epicotyl elongation stage, but became shorter than *V. nakashimae* at maturity. The height of F_1 hybrid was similar to that of *V. nakashimae*. In F_2 progeny, trimodal distribution was observed for both EL and PH (Figs. IV-2 and IV-3A). The trimodal distribution of PH was also observed in the F_3 progeny (Fig. IV-3B). The variations of PH in F_2 and F_3 progenies were highly heritable and the heritability in broad sense was 0.85 and 0.80, respectively (Table IV-14). Heritability in narrow sense of PH for both F_3 families and F_3

Table IV-14. Estimates of variance components and heritabilities among F_2 and F_3 generations for seven traits.

Variance	PH	SW	DF	BN	PLW	PLL	SFP
VF_2	1389.4	0.07	25.36	1.56	0.21	0.27	-
E	204.8	0.02	4.40	0.31	0.06	0.07	-
V_a	685.2	0.103	25.32	2.50	0.104	0.127	64.35
V_i	767.9	0.075	20.66	3.28	0.201	0.288	61.18
D	896.5	0.189	41.92	2.86	0.029	0.003	97.75
H	3250.1	0.044	58.37	13.65	1.263	1.594	198.99
$\sqrt{(H/D)}$	1.90	0.48	1.18	2.18	6.60	23.05	1.43
E1	137.6	0.022	2.88	0.86	0.037	0.088	11.86
E2	33.9	0.005	0.71	0.22	0.011	0.003	3.07

Heritability	PH	SW	DF	BN	PLW	PLL	SFP
Broad sence							
F_2	0.85	0.62	0.83	0.80	0.71	0.71	-
F_3	0.80	0.78	0.89	0.66	0.65	0.31	0.82
Narrow sence							
F_3 families	0.65	0.92	0.83	0.57	0.14	0.01	0.76
F_3 individual	0.47	0.82	0.69	0.39	0.07	0.01	0.60

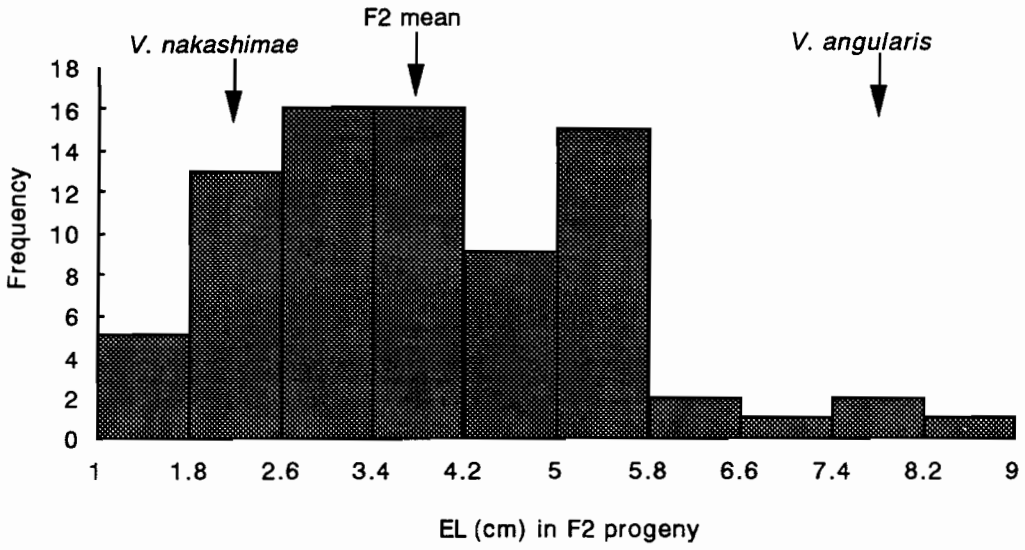

Fig. IV-2. Distribution of phenotypes for EL (epicotyl length) in F2 progeny. Means for parents and progeny are shown by arrows.

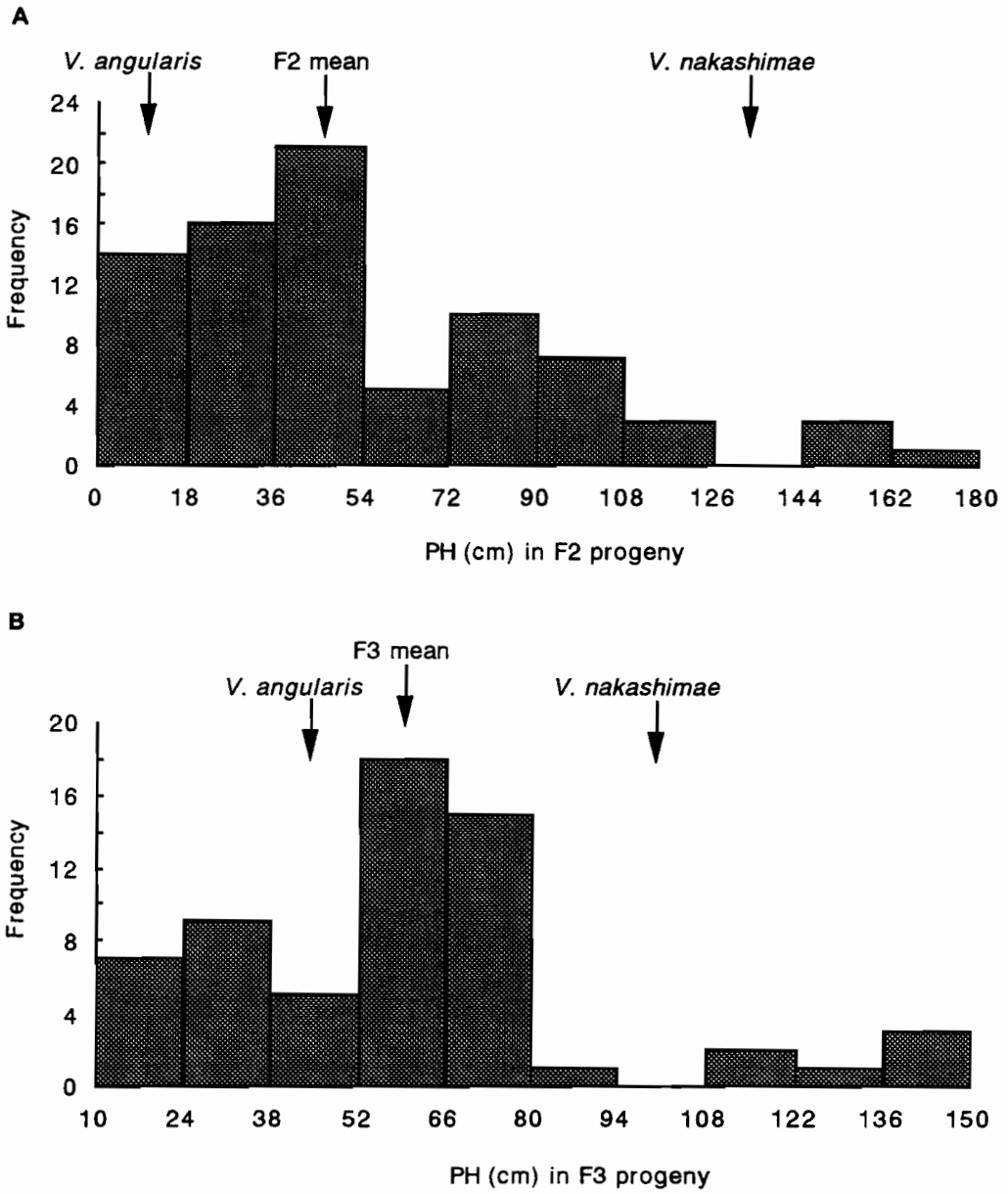

Fig. IV-3. Distribution of phenotypes for PH (plant height) in F2 progeny (A) and F3 progeny (B). Means for parents and progenies are shown by arrows.

individuals showed lower than that in broad sense.

By single-marker analysis, the numbers of locations which contained markers associated with PH were six on linkage groups 2, 3, 4 and 7 for F_2 progeny and four on linkage groups 2 and 4 for F_3 progeny (Table IV-4). For EL, six significant markers were detected on linkage groups 1, 4, 5,13 and 14 using F_2 progeny (Table IV-4).

Based on the interval mapping, a total of seven QTLs controlling PH were detected using F_2 progeny, and only one was identified using F_3 progeny. Their locations and estimates of genetic effects are presented in Table IV-10. *V. nakashimae* allele caused an increase of plant height at ph2 (QTL controlling PH on linkage group 2), ph3, ph4, ph8 and ph10 (linkage group 3, 4, 8 and 10), while an increase of plant height was found at ph1, ph11 and ph12 for *V. angularis* allele. The test statistics of ph2, ph11 and ph12 were relatively high in both F_2 and F_3 progenies, indicating that they are probably stable QTLs controlling PH. Estimates of genetic effects revealed different types of gene action. The ph2, ph3, ph4 and ph8 displayed partial dominant or dominant gene action by *V. angularis* allele. Three (ph1, ph11 and ph12) out of four showing overdominant action were due to *V. nakashimae* allele. In contrast, no QTL was detected for EL. However, by searching test statistic peak under the threshold 3.84, two peaks were found in the same intervals where ph4 and ph11 exist.

Seed weight

Regarding 20-seed weight, *V. angularis* is four to five times heavier than *V. nakashimae* (Tables IV-2 and IV-3). In the F_2 and F_3 progenies, skewed distribution toward *V. nakashimae* was observed

since no seeds as heavy as *V. angularis* were produced in these segregating population (Fig. IV-4). Consequently, the mean values of SW for F_2 and F_3 progenies were smaller than those of mid-parent (Tables IV-2 and IV-3). The heritability in narrow sense was greater than that in broad sense (Table IV-14).

Based on single-marker analysis, 11 and 15 locations associated with QTLs controlling SW were detected in the linkage groups 1, 2, 3, 5, 7, 9, 11, 12 and 14 for F_2 progeny and in the linkage groups 1, 2, 3, 4, 5, 6, 7, 9, 10 and 12 for F_3 progeny, respectively (Table IV-5). Nine locations on linkage groups 1, 2, 3, 5, 7, 9 and 12 were the same using both progenies.

By interval mapping, single QTL (sw3-2) and four QTLs (sw2, sw3-1, sw7 and sw8) for SW were mapped on linkage group 3 in F_2 progeny and on linkage groups 2, 3, 7 and 8 in F_3 progeny, respectively. The locations and estimates of genetic effects of these QTLs are presented in Table IV-11. *V. angularis* alleles increased SW at all QTL positions. Additive or partial dominant gene action at the sw2 and sw3-1 were resulted from *V. nakashimae* alleles, while sw3-2 from *V. angularis* allele. Overdominance was found at sw7 and sw8 originated from *V. angularis* alleles. In addition, three factors with minor effects on SW detected at linkage groups 5, 9 and 10.

Days to flowering

By comparing both parents on flowering time, *V. angularis* flowered 6-9 days earlier than *V. nakashimae* (Tables IV-2 and IV-3). In the distribution of phenotypes for DF in F_2 and F_3 progenies, notable transgression toward early DF was observed (Fig. IV-5). The genetic

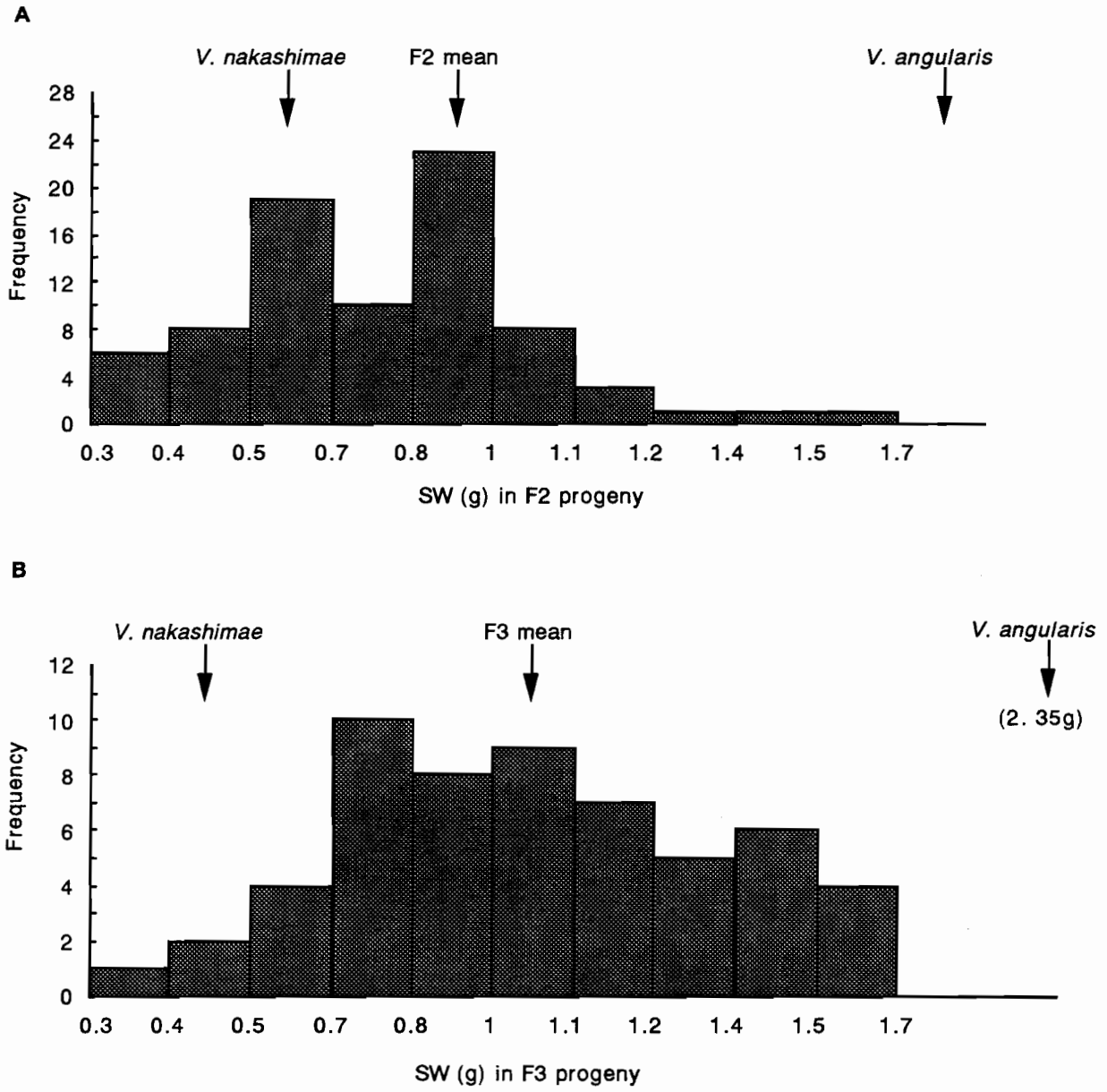

Fig. IV-4. Distribution of phenotypes for SW (20-seeds weight) in F2 progeny (A) and F3 progeny (B) Means for parents and progenies are shown by arrows.

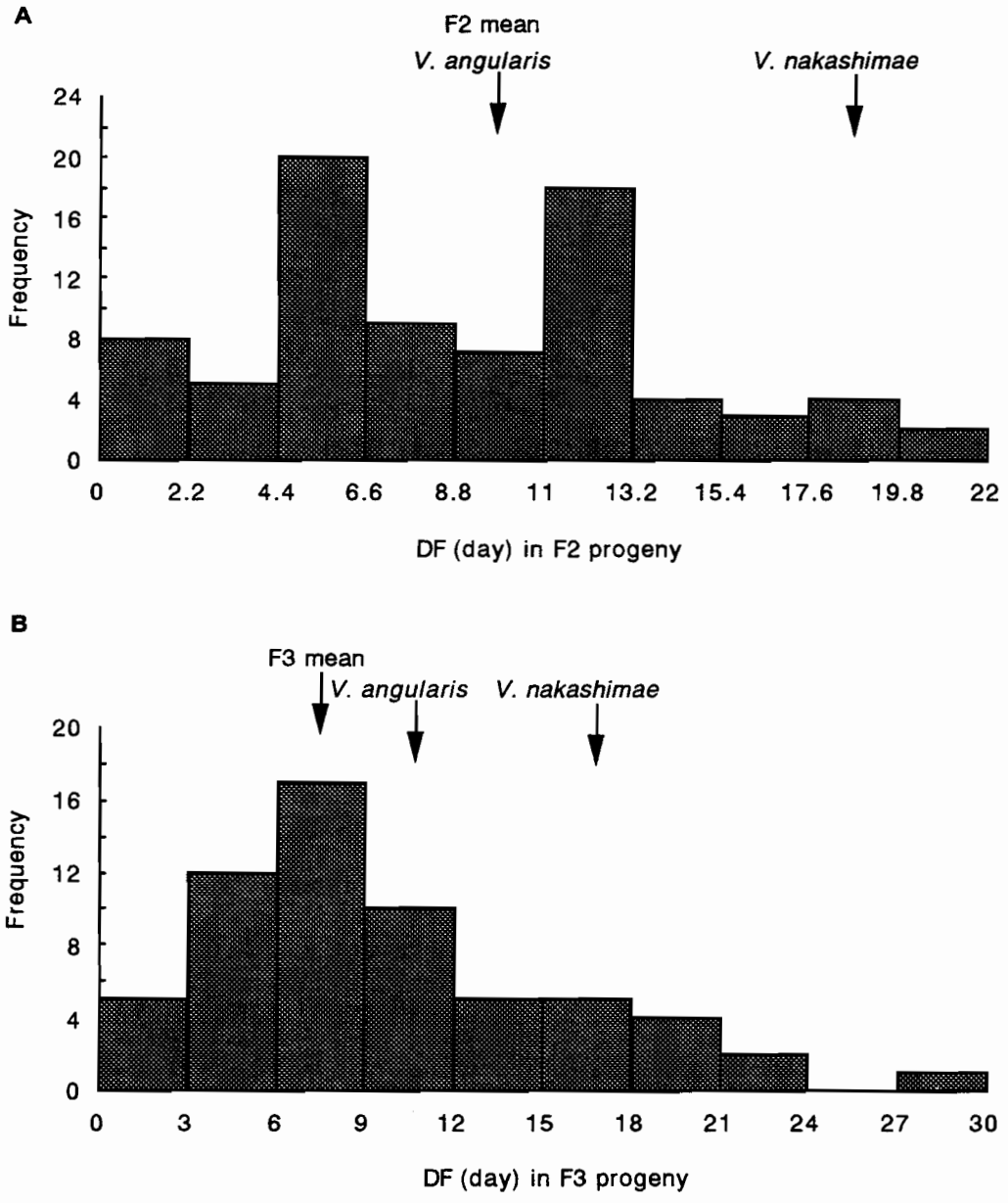

Fig. IV-5. Distribution of phenotypes for DF (days to flowering) in F2 progeny (A) and F3 progeny (B). Means for parents and progenies are shown by arrows.

variations of DF were highly heritable in broad sense and narrow sense (Table IV-14).

Six and three locations associated with QTL controlling DF were detected by single-marker analysis in linkage groups 4, 5, 6, 11, 13 and 14 for F_2 progeny and in linkage groups 4, 6 and 11 for F_3 progeny, respectively (Table IV-6). The three locations with significant markers on linkage groups 4, 6 and 11 were in common for both progenies.

By interval mapping, single QTL (df6) controlling DF was found to be located on linkage group 6 with both F_2 and F_3 progeny (Table IV-12). At this locus, *V. angularis* allele reduced about four days, and a dominant gene action by the dominant allele derived from *V. nakashimae* was observed. In addition, two factors with minor effects on DF were identified at linkage groups 4 and 9 (Table IV-12).

Reproductive period

DR (days to ripening) and SFP (seed filling period) were measured only with F_3 progeny. DR for *V. nakashimae* was 10 days more than that for *V. angularis*, but their SFP values were very close to each other (Table IV-3). For both traits in F_3 progeny, many transgressive phenotypes toward increasing days were segregated, and unimodal skewed distribution was observed (Fig. IV-6). Genetic variations of SFP were heritable in broad sense and narrow sense (Table IV-14).

Seven locations associated with QTL were respectively detected for both DR and SFP using single-marker analysis (Table IV-6). Of these, six were shared in linkage groups 1, 2, 7, 8, 10 and 13. However, no QTL was found for DR and SFP by interval mapping.

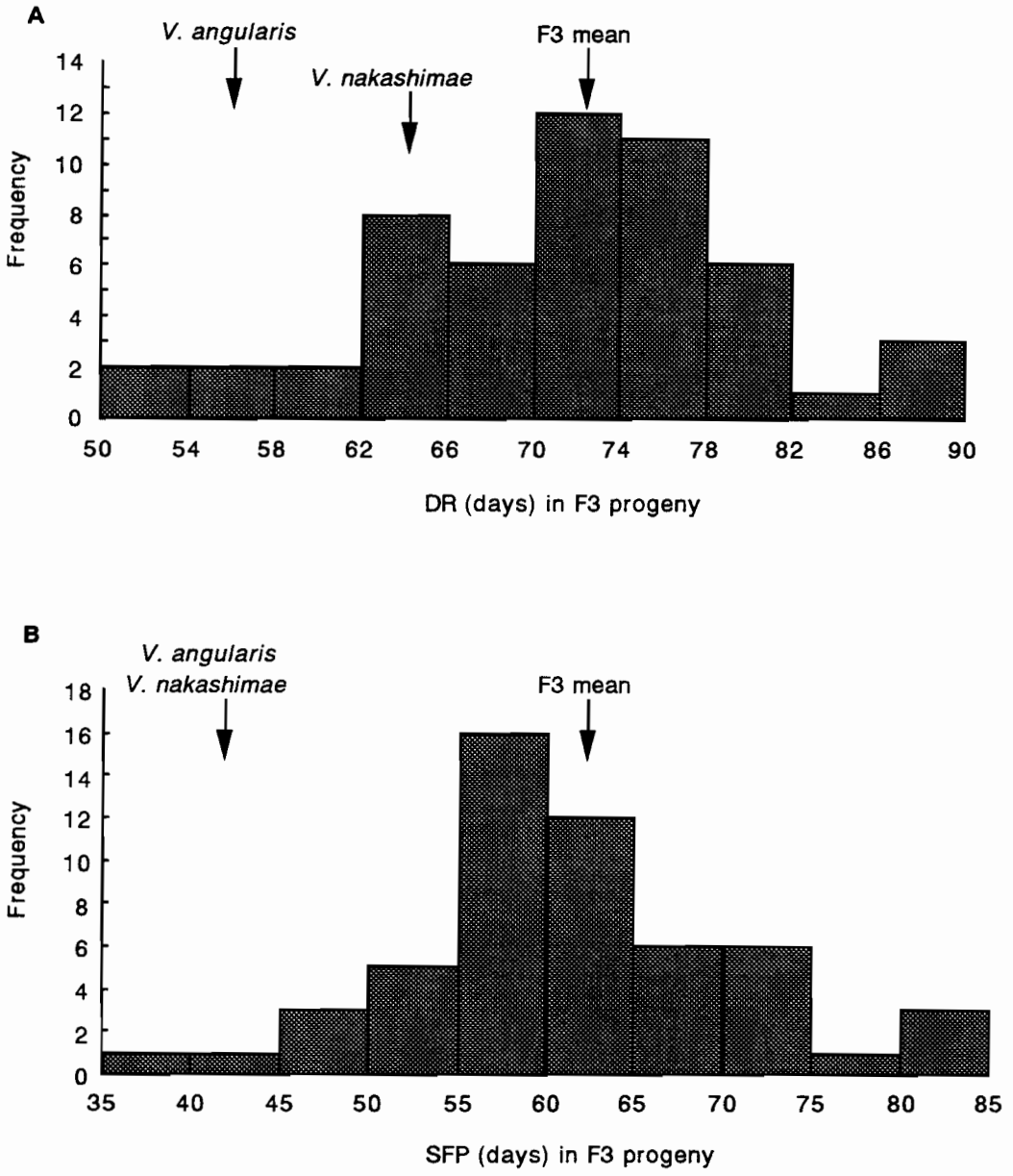

Fig. IV-6. Distribution of phenotypes for DR (days to ripening; A) and SFP (seed filling period; B) in F3 progeny. Means for parents and progeny are shown by arrows.

Number of branch

Branch numbers of both *V. angularis* and *V. nakashimae* were distinctively greater in the field condition than those in the pot condition (Tables IV-2 and IV-3). Bimodal distribution of BN was observed in the F_2 progeny as well as in the F_3 progeny (Fig. IV-7). In F_3 progeny, the several individuals transgressed toward both directions (increasing and decreasing numbers). The genetic variations were relatively heritable in broad sense and narrow sense (Table IV-14).

Five and six locations containing the markers associated with QTL controlling BN were detected in linkage groups 2, 4, 5 and 9 for F_2 progeny and in linkage groups 2, 4, 5, 9 and 11 for F_3 progeny, respectively (Table IV-7). The three locations with significant markers on linkage groups 4, 5 and 9 were in common for both progenies.

Using interval mapping, three QTLs controlling BN were localized in linkage groups 1, 9 and 12 in F_2 progeny (Table IV-13). *V. nakashimae* allele increased BN at bn1 in linkage group 1, while *V. angularis* allele increased it at bn9 and bn12. At all these locus, overdominant gene action was observed. In addition, two factors with minor effects on BN were identified in linkage groups 5 and 14 (Table IV-13).

Primary leaf characters

The shapes of primary leaves of *V. angularis* and *V. nakashimae* were cordate and ovate, respectively. The ratio of PLW to PLL of *V. angularis* was greater than that of *V. nakashimae* (Tables IV-2 and IV-

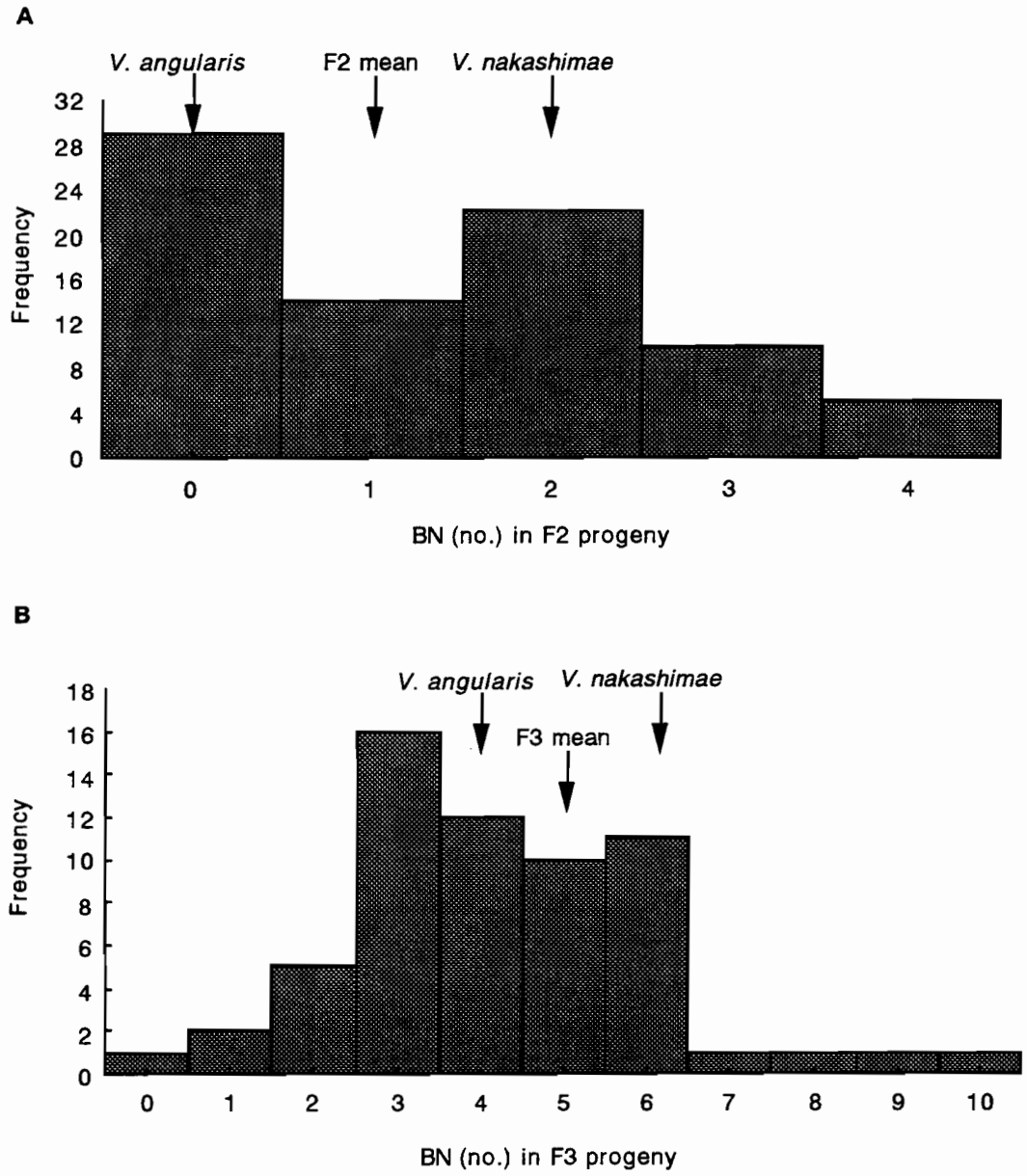

Fig. IV-7. Distribution of phenotypes for BN (number of blanch) in F2 progeny (A) and F3 progeny (B). Means for parents and progenies are shown by arrows.

3). In the F_2 population, PLW and PLL segregated with the skewed distribution toward the leaf shape of *V. nakashimae* (Figs. IV-8A and IV-9A). All of the mean values in PLW and PLL for F_2 and F_3 populations were close to those of mid-parent (Table IV-2). On the other hand, the F_3 progeny showed normal unimodal distribution (Figs. IV-8B and IV-9B). The heritabilities in narrow sense for PLW and PLL were much smaller than those in broad sense (Table IV-14).

Three and five locations containing markers associated with QTL controlling PLW were detected in linkage groups 5, 6 and 9 using F_2 progeny and in linkage groups 3, 4, 5, 6 and 14 using F_3 progeny, respectively (Table IV-7). Among them, only one on linkage group 5 was in common for both progenies. On the other hand, one and six locations with QTL controlling PLL were detected in linkage group 5 for F_2 progeny and in linkage groups 3, 4, 6 and 14 for F_3 progeny, respectively (Table IV-13). The locations for both progenies did not overlap. No QTL controlling PLW and PLL was detected using interval mapping.

Relationships among QTLs

The markers significantly associated with QTLs by single-marker analysis and the QTLs detected by interval mapping for nine traits were presented in Fig. IV-10 (F_2 progeny) and Fig. IV-11 (F_3 progeny). Among the QTLs detected by interval mapping, ph12 for plant height and bn12 for branch number on linkage group 12 were found to be located in the same interval using F_2 progeny. For single-marker analysis, many markers were found to be associated with QTLs controlling nine traits. Therefore, many locations or markers were commonly associated with

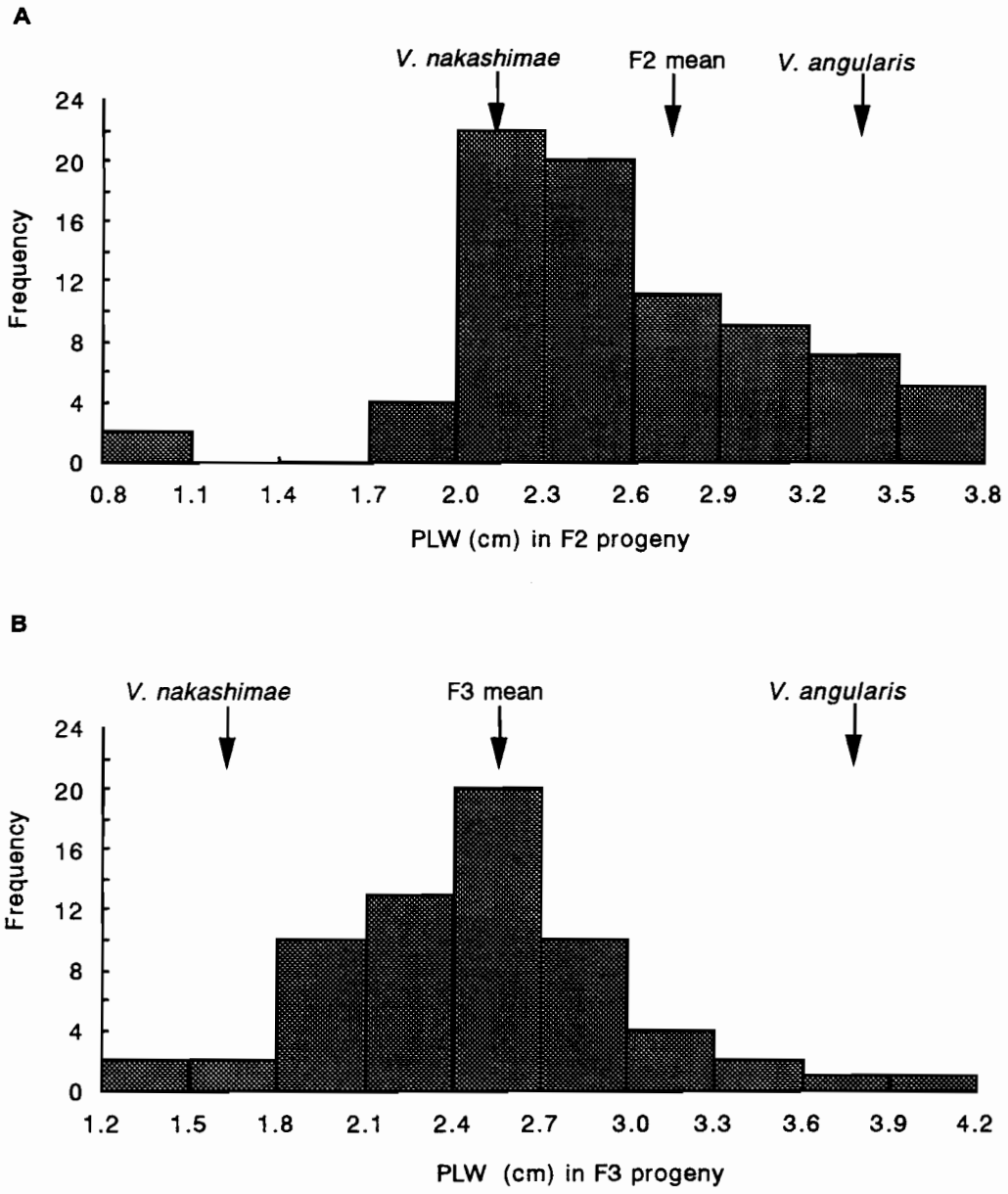

Fig. IV-8. Distribution of phenotypes for PLW (primary leaf width) in F2 progeny (A) and F3 progeny (B). Means for parents and progenies are shown by arrows.

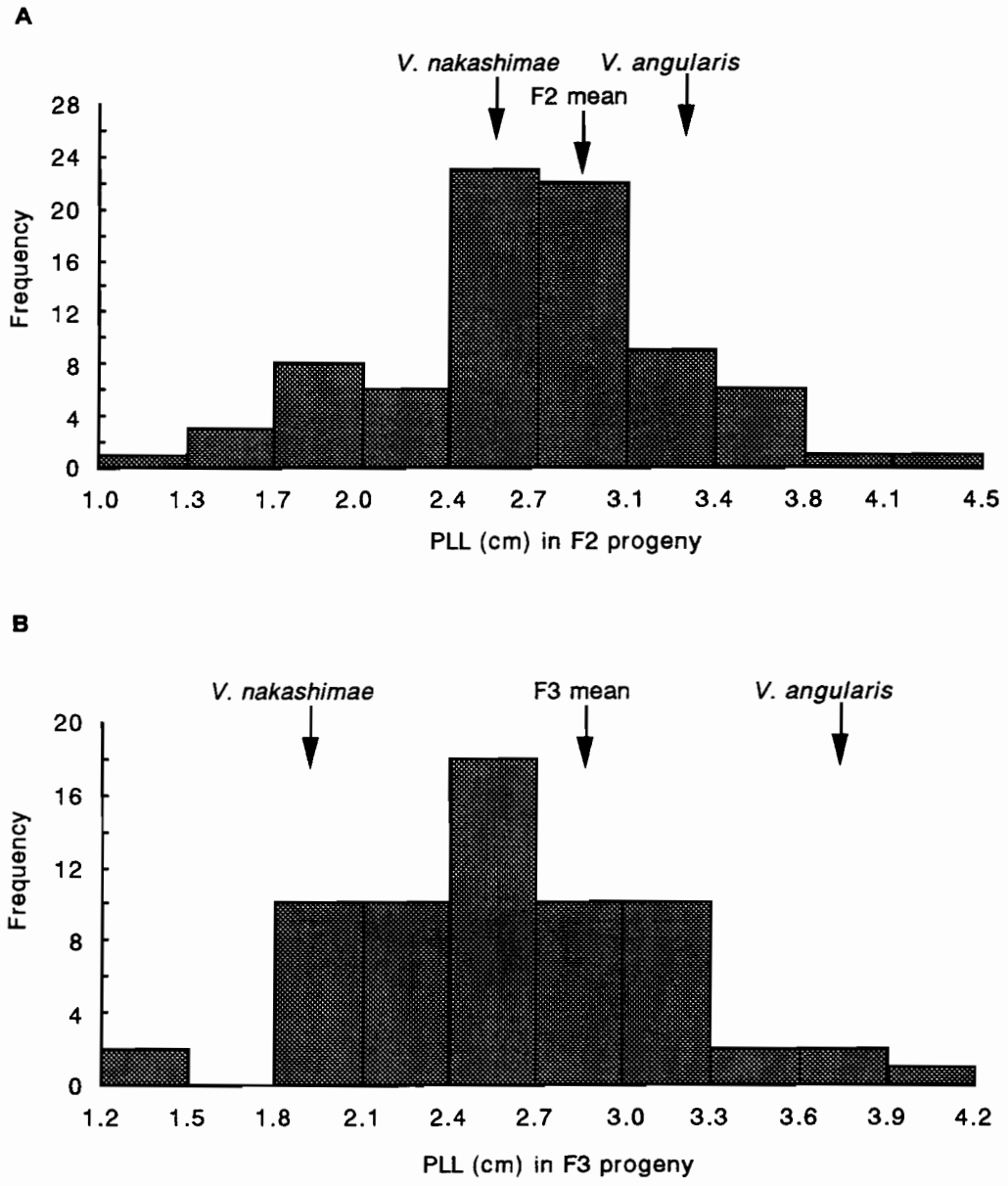

Fig. IV-9. Distribution of phenotypes for PLL (primary leaf length) in F2 progeny (A) and F3 progeny (B). Means for parents and progenies are shown by arrows.

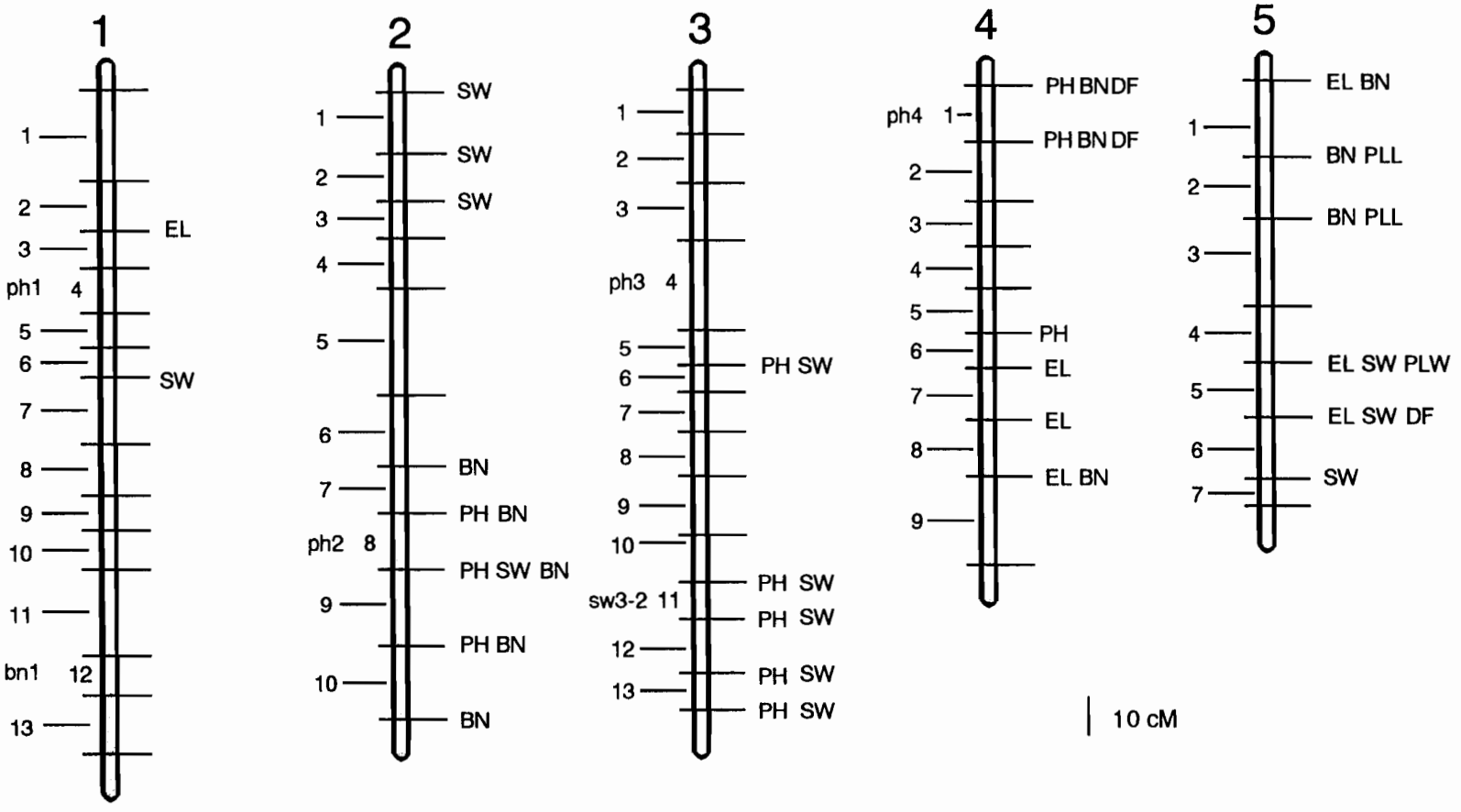

Fig. IV-10. QTLs detected by interval mapping and markers siginificantly associated with QTLs identified by single-marker analysis for nine traits using F2 progeny. Their positions are indicated on the left and right sides of linkage groups, respectively.

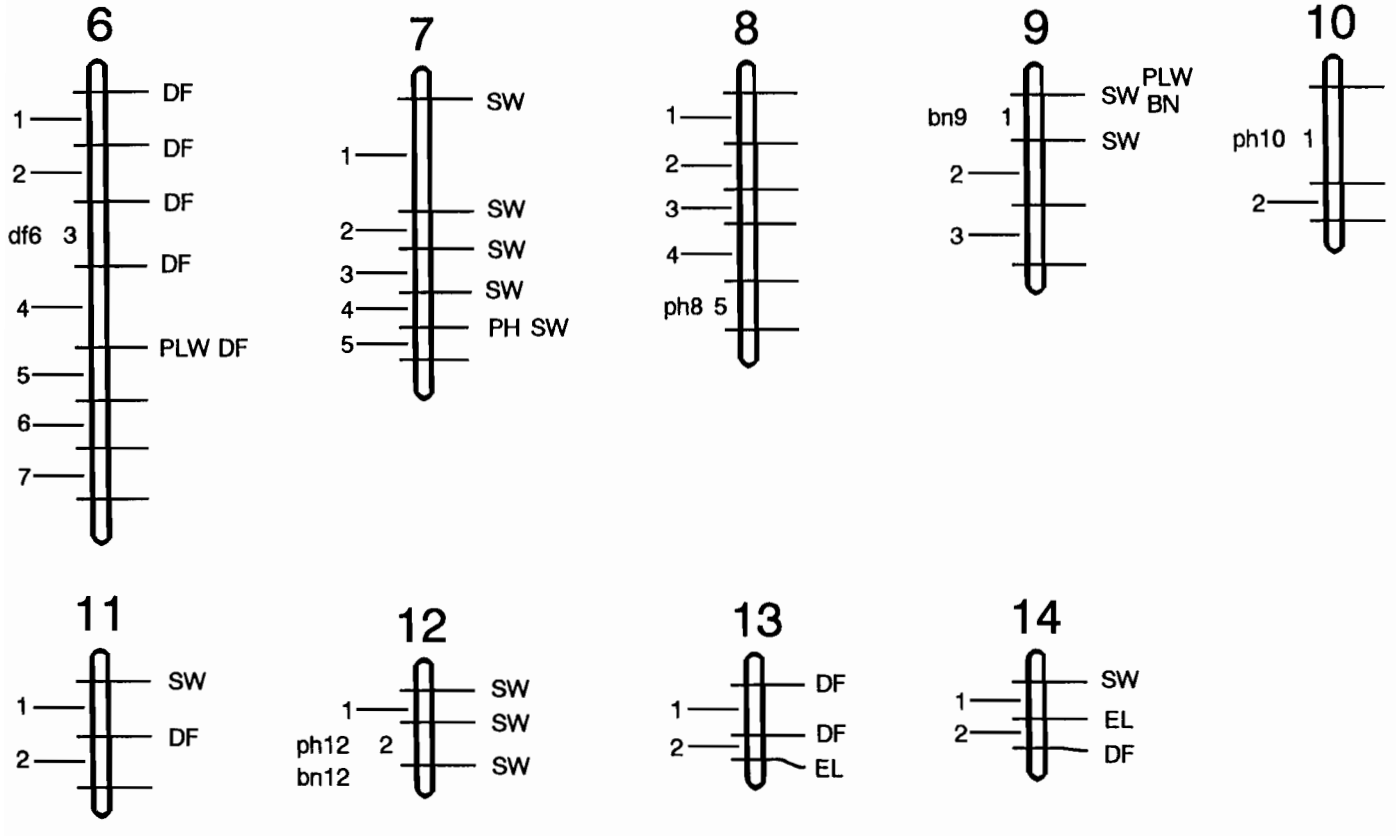

Fig. IV-10. (continued)

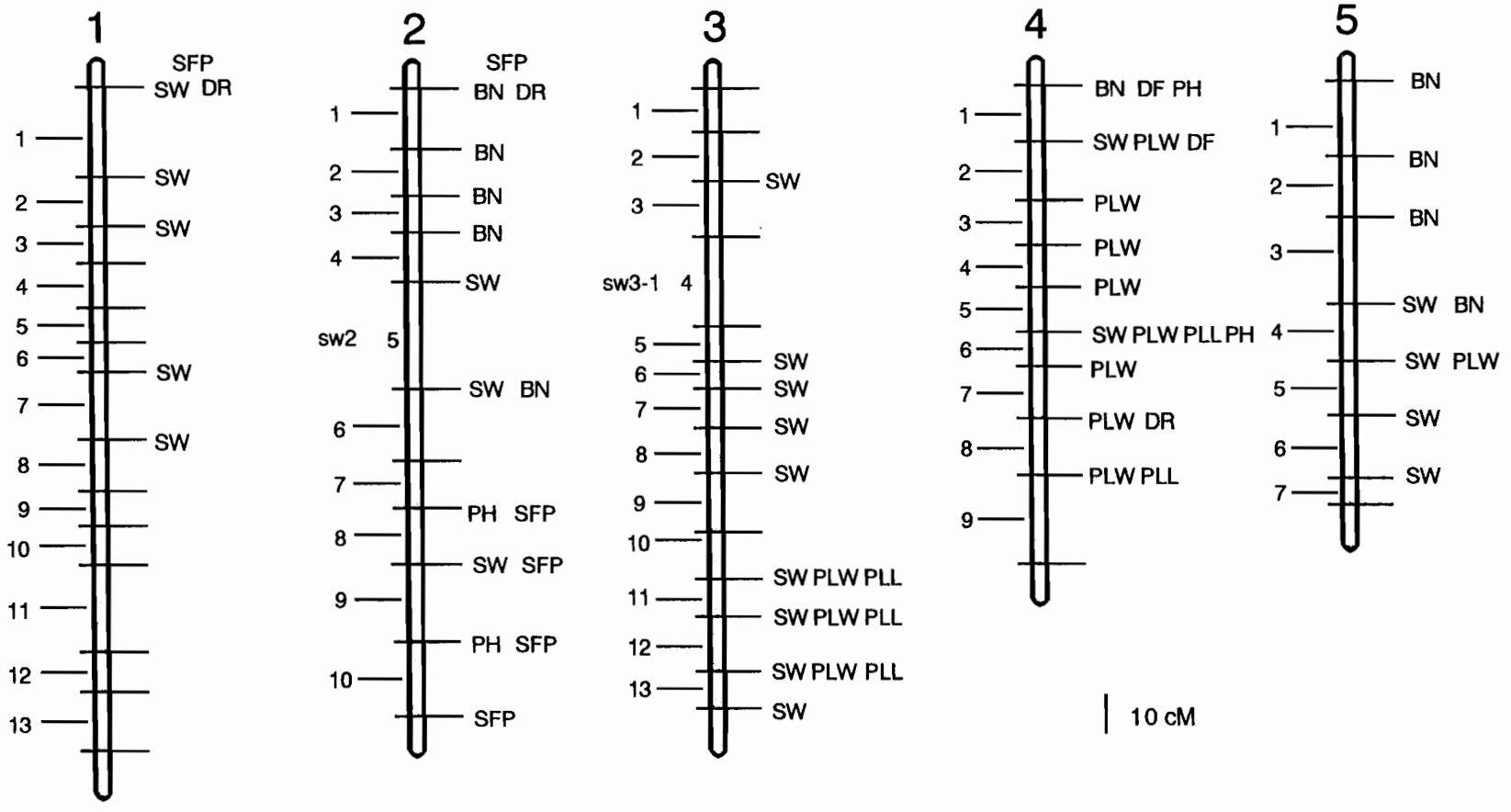

Fig. IV-11. QTLs detected by interval mapping and markers siginificantly associated with QTLs identified by single-marker analysis for nine traits using F3 progeny. Their positions are indicated on the left and right sides of linkage groups, respectively.

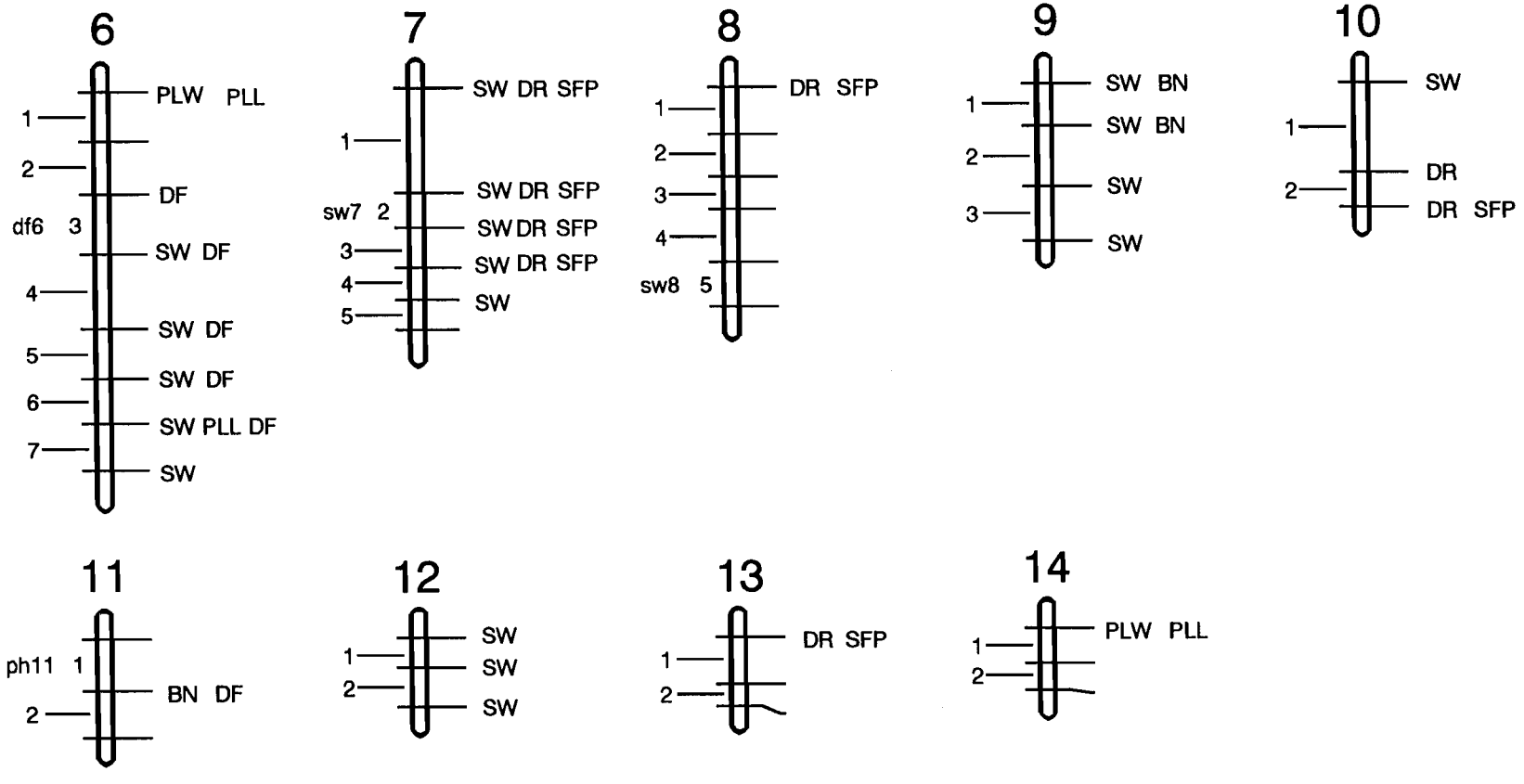

Fig. IV-11. (continued)

several traits. In order to examine the relationships between these traits, correlation coefficients was calculated and shown in Table IV-15. Between several combinations of traits, high correlation coefficients were observed, particulaly in PLW-PLL and DR-SFP.

QTL analysis of categorical traits

Pod shattering

As described in Chapter III, F_2 segregation of pod shattering (58:17) showed a good fit to 3:1 ratio, suggesting that pod shattering is controlled by a single dominant gene. The F_3 mean of pod shattering was 64.1 % (Table IV-16), however, PS varied from 20% to 90% among F_3 lines which segregated pod shattering plant (Fig. IV-12). This indicates that two or more genes were involved in controling the pod shattering. By single-marker analysis with the segregant percentages, two markers associated with QTLs controlling pod shattering were detected in linkage groups 2 and 6 (Table IV-8).

Seed color

V. angularis has red seeds, while *V. nakashimae* produced green seeds with black mottle. In F_2 progeny, plants having brown, green, white and red seeds segregated in the ratio of 56 : 13 : 1 : 10, equivalent to 70 %, 16.25 %, 1.25 % and 12.5 %, respectively (Table IV-16). The F_3 lines derived from brown or green seeds contained plants with brown, green, white and red seeds, while those derived from red seeds resulted in only red-seed plants. The percentages of each seed color in F_3 lines are shown in Fig. IV-13. As expected, the percentages of brown seed segregants were skewed to higher portion with a peak

Table IV-15. Correlation coefficients (r) between nine traits in F_2 and F_3 progenies.

Trait		SW	DF	BN	PLW	PLL	EL	DR	SFP
PH	F_2	-0.11	0.26 *	0.02	0.21	0.36 **	0.26 *	-	-
	F_3	-0.25	0.37 **	0.31 *	-0.20	0.10		0.14	-0.10
SW	F_2		0.14	0.19	0.27 *	0.03	0.13	-	-
	F_3		0.03	0.00	0.47 **	0.24		0.09	0.09
DF	F_2			0.23 *	0.01	-0.04	-0.09	-	-
	F_3			0.29 *	-0.09	0.18		0.19	-0.39 **
BN	F_2				0.39 ***	0.30 **	0.11	-	-
	F_3				-0.09	0.09		-0.04	-0.17
PLW	F_2					0.79 ***	0.60 ***	-	-
	F_3					0.76 ***		-0.06	0.02
PLL	F_2						0.58 ***	-	-
	F_3							0.00	-0.09
EL	F_2								-
DR	F_3								0.82 ***

*, ** and *** indicate correlations significant at $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

Table IV-16 . The percentage of plants with PS (pod shattering), BS (brown seed), GS (green seed), WS (white seed) and RS (red seed) found among F_2 and F_3 populations.

		F_3 lines		
Trait	F_2 population	mean	maximum	minimum
PS (%)	72.5	64.08	100	0
BS (%)	70.00	45.42	100	0
GS (%)	16.25	17.74	100	0
WS (%)	1.25	10.01	100	0
RS (%)	12.50	26.85	100	0

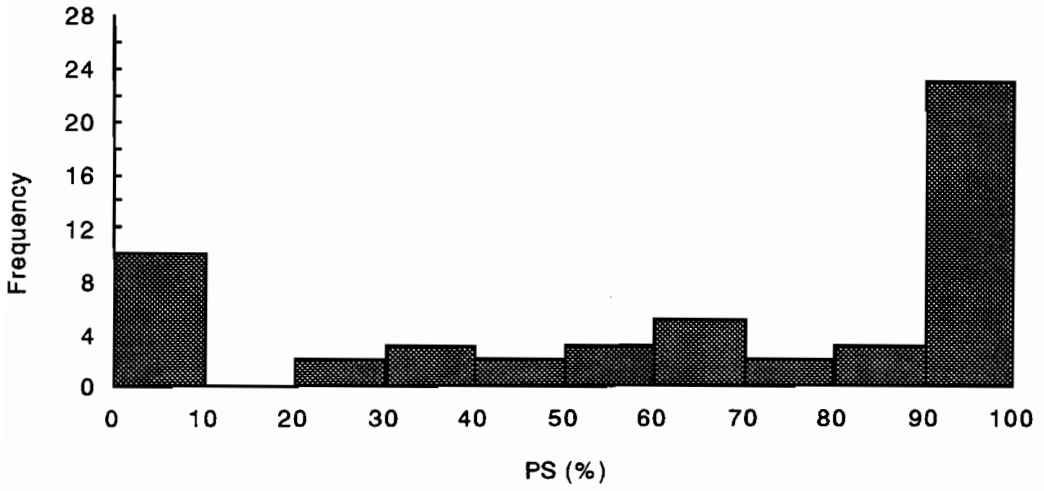

Fig. IV-12. Distribution of phenotypes for PS (percetage of plants having shattering pod per line) in F3 progeny.

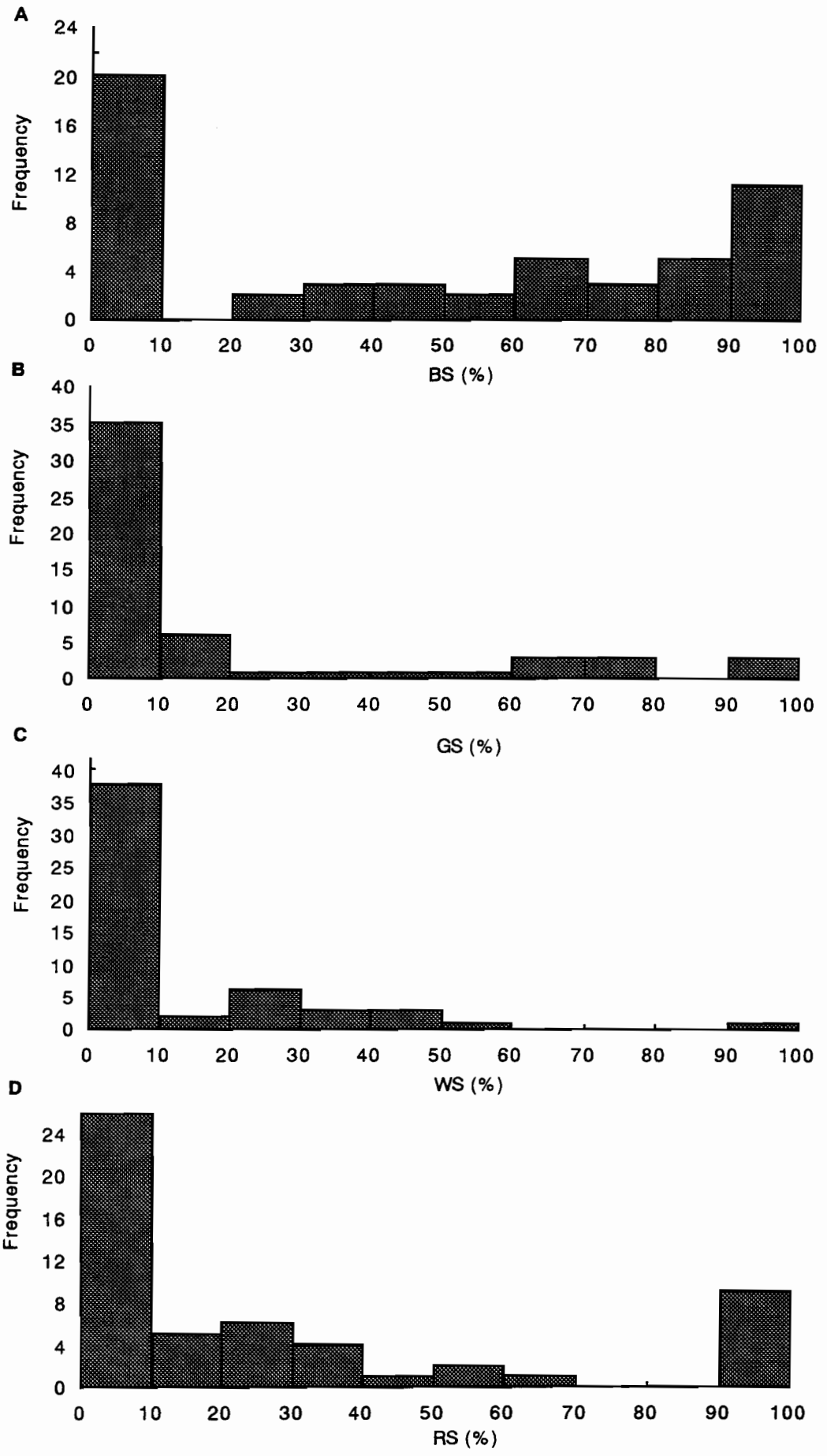

Fig. IV-13. Distribution of phenotypes for BS (percetage of plants having brown seeds per line; A), GS (percetage of plants having green seeds per line; B), WS (percetage of plants having white seeds per line; C) and RS (percetage of plants having red seeds per line; D) in F3 progeny.

around 75%, indicating that the brown seed color is partially controlled by a dominant gene. On the other hand, the segregant percentages of other seed colors were shifted to lower with a peak around 25%, indicating that the they are partially controlled by a recessive gene. Using those segregant percentages, single-marker analysis was performed. Six, seven, six and two locations in azuki linkage groups were identified to associate with QTLs controlling the percentages of brown, red, white and green seed colors, respectively (Table IV-8). The most significant peaks ($P<0.0001$) were observed in linkage groups 7 and 8 for red seed color.

Other characters

Skewed segregation of markers should occur in either gametes or zygotes. The factors related to the segregating distortion may be indirectly resolved by QTL mapping. Therefore, pollen fertility (FP), germination rate (GES), abortiveness during plant development (LP and SP), seed sterility (SS) and seed abnormality (AS) were analyzed in F_3 progeny. The distribution of FP had a peak at 50%, and low fertility (<50%) was observed at 14 out of 61 F_3 lines (Fig. IV-14A). The sterile plants (SS) was observed in 50 F_3 lines (Fig. IV-14B). There was no correlation ($r=-0.136$, $p<0.336$) between pollen fertility and seed sterility, indicating that the rate of seed fertilization is not affected by pollen fertility. The distribution of GES skewed toward high percentage, but very low germination was observed in 19 F_3 lines (Fig. IV-15A). The plants which produced abnormal seeds were observed in 23 F_3 lines (Fig. IV-15B). There were no correlation ($r=-0.044$, $p<0.754$) between GES and AS. Abnormality in plant development such

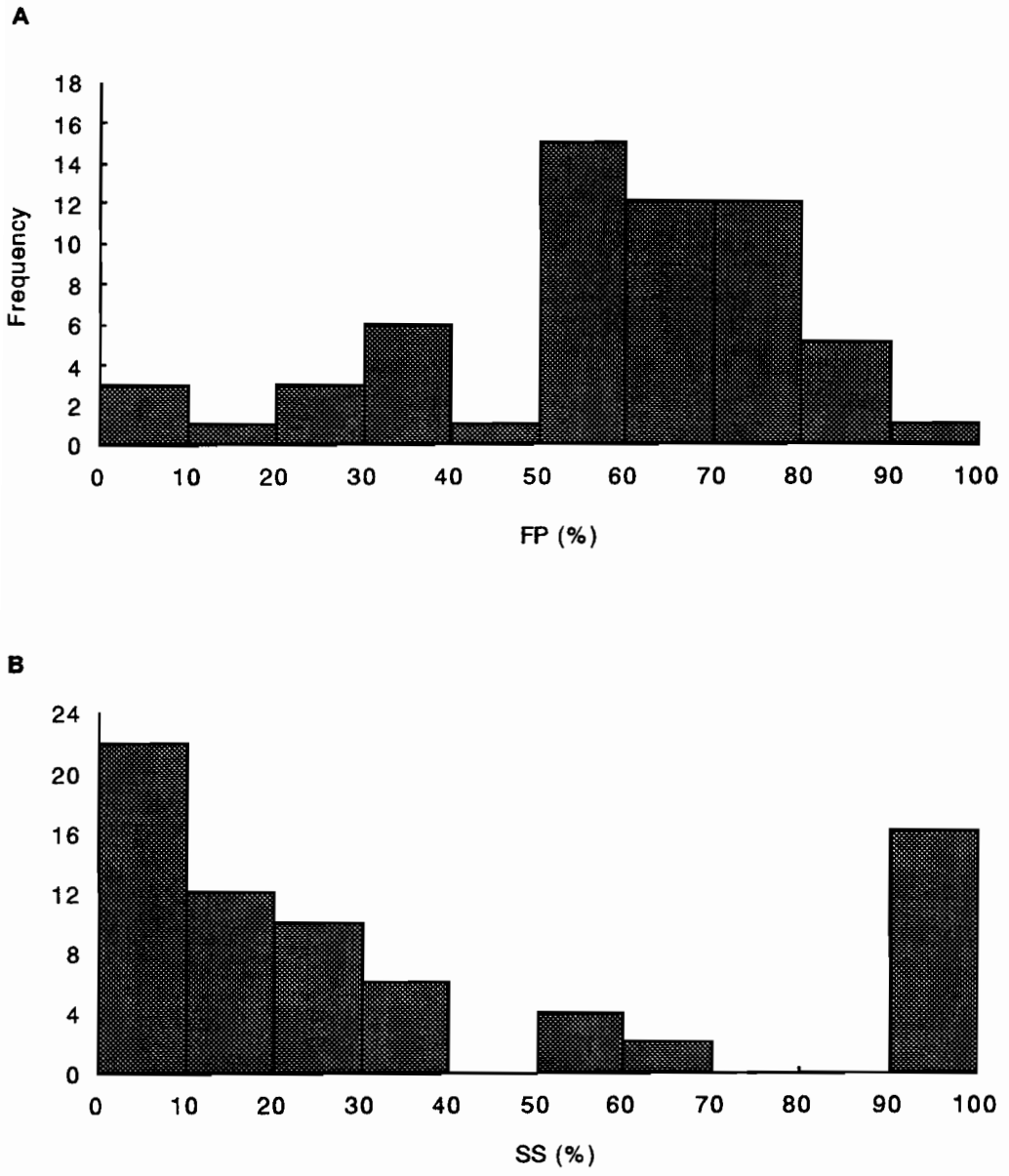

Fig. IV-14. Distribution of phenotypes for FP (mean percentage of stained pollens per line; A) and SS (percetage of plants which did not produce any seeds per line; B) in F3 progeny.

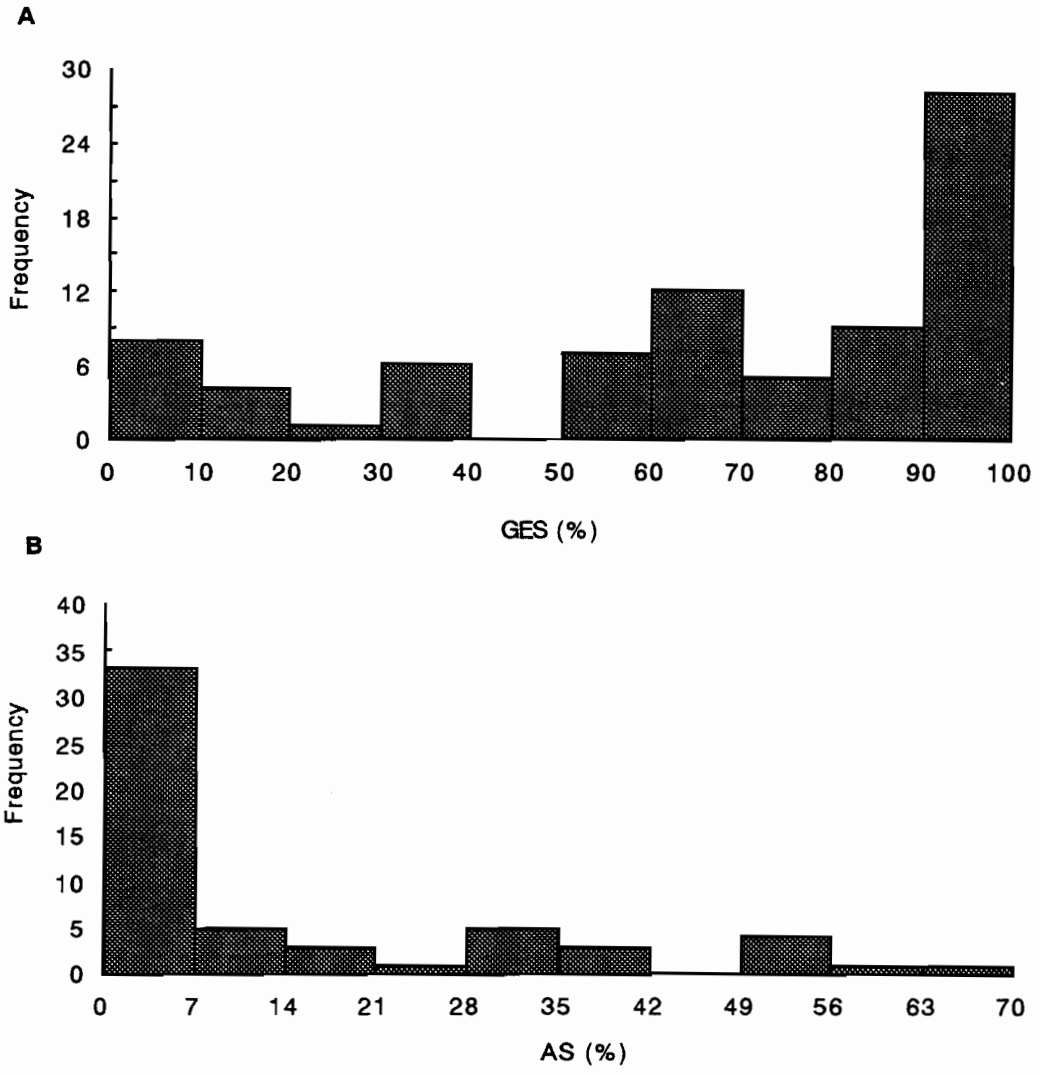

Fig. IV-15. Distribution of phenotypes for GES (mean percetage of germinated seeds per line; A) and AS (percetage of plants which produced abnormal seeds per line; B) in F3 progeny.

as lethal and stunted phenotypes were observed in 17 and 7 F_3 lines, respectively (Fig. IV-16). Four F_3 lines showed both lethal and stunted phenotype.

Significant association of markers with FP, GES, LP, SP, SS and AS based on single-marker analysis were presented in Table 9. Some of the markers associated with QTL controlling FP, LP, SS and AS were identified at the marker loci which showed abnormal segregation on linkage group 2 (Fig. IV-17). In linkage group 12, a marker associated with QTL controlling seed production, SS and AS was located. The results indicated that some factors related to the skewed segregation of markers might locate on linkage groups 2 and 12.

Discussion

Detection of QTLs

QTLs controlling agronomically important traits were analyzed in the interspecific population derived from a cross between *V. angularis* and *V. nakashimae* based on the molecular linkage map. To detect QTLs, two analytical approaches, single-marker analysis and interval mapping, were employed in this study. With large population size, these two methods are expected to provide the same results. Theoretically, the threshold value of 12.5 used in interval mapping corresponds to a 5% error rate. However, with small population size, significance threshold value for interval mapping should be established by permutation test (Churchill and Doerge 1994). In this study, putative QTL locations detected by single-marker analysis were not

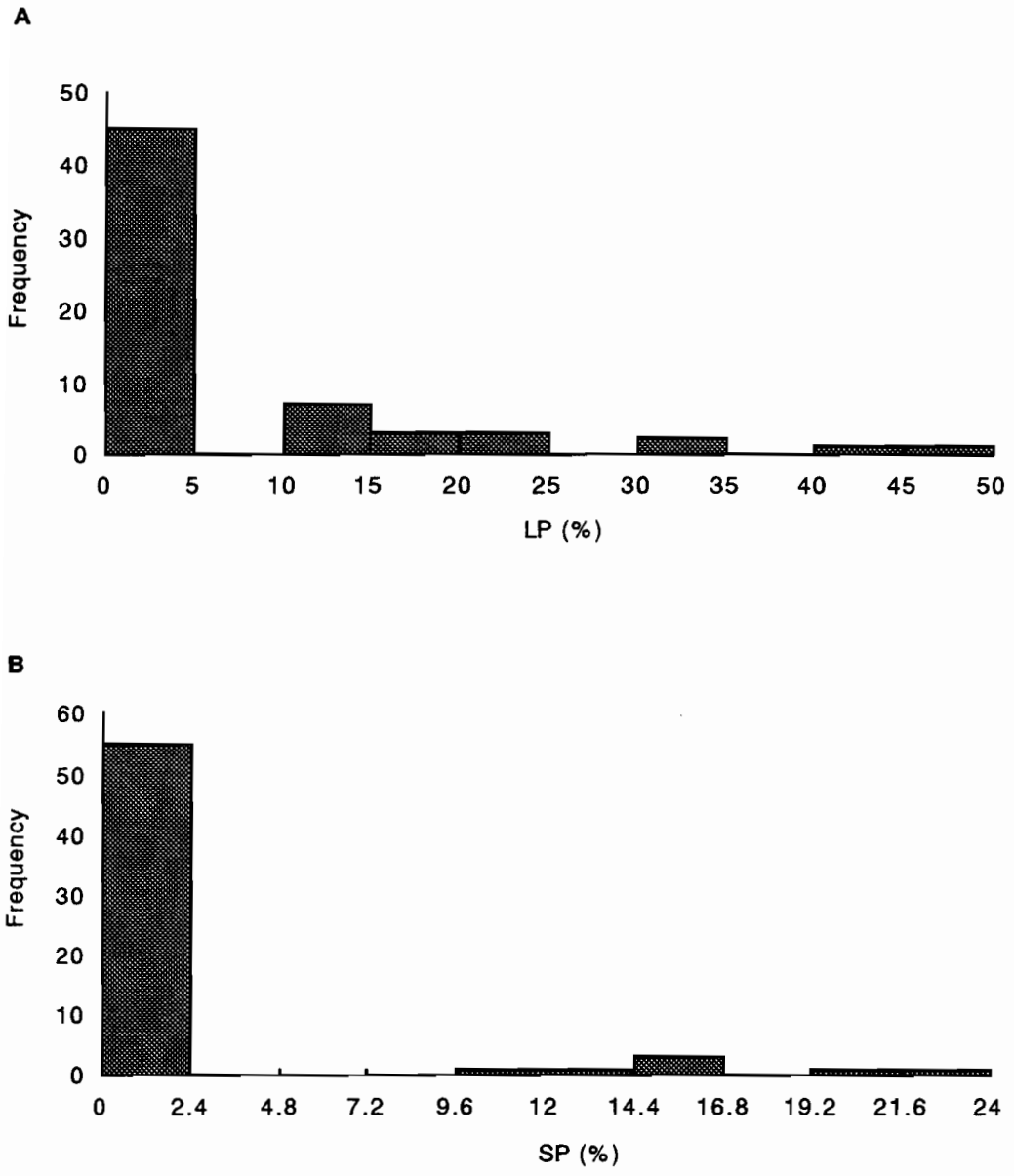

Fig. IV-16. Distribution of phenotypes for LP (percetage of lethal phenotype per line; A) and SP (percetage of stunted phenotype per line; B) in F3 progeny.

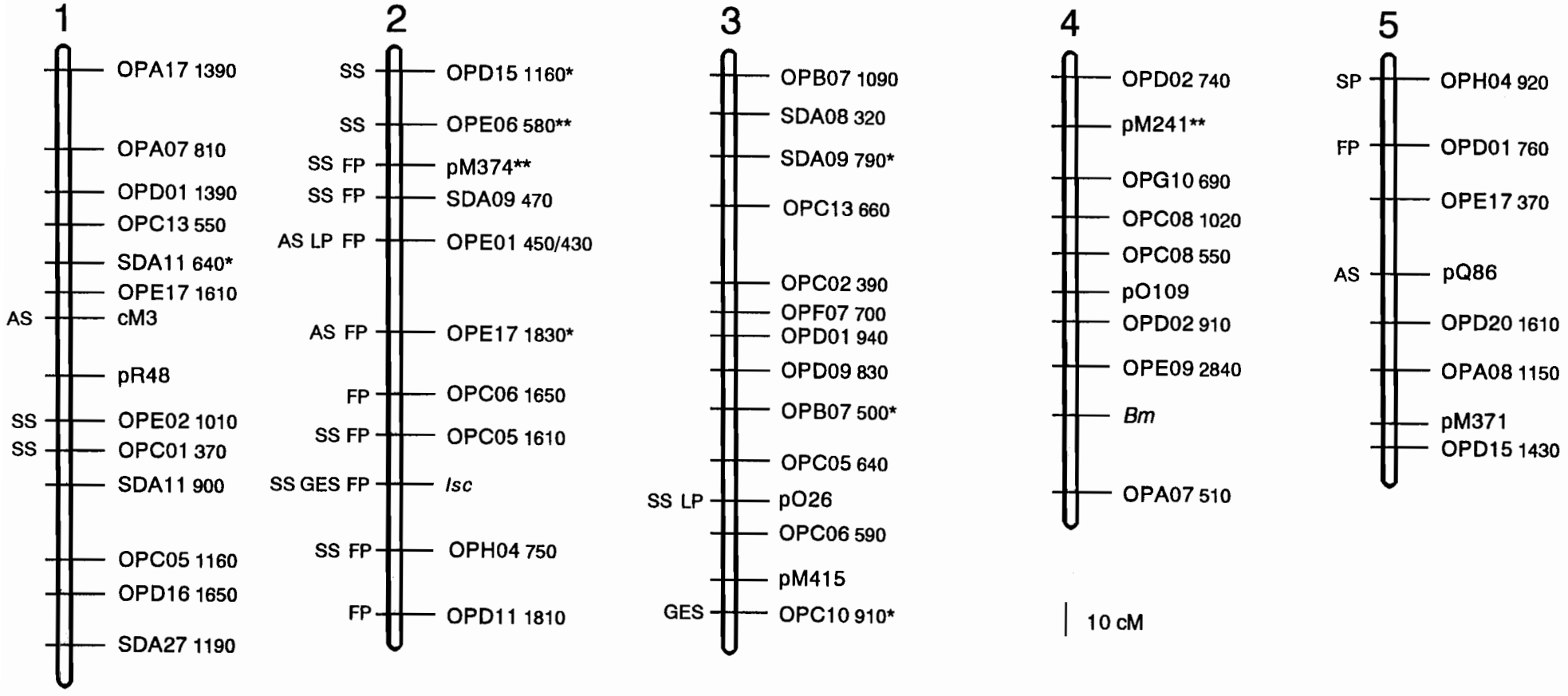

Fig. IV-17. Marker loci having significant association to abnormal segregating factors. Their positions are indicated on the left side of linkage groups.

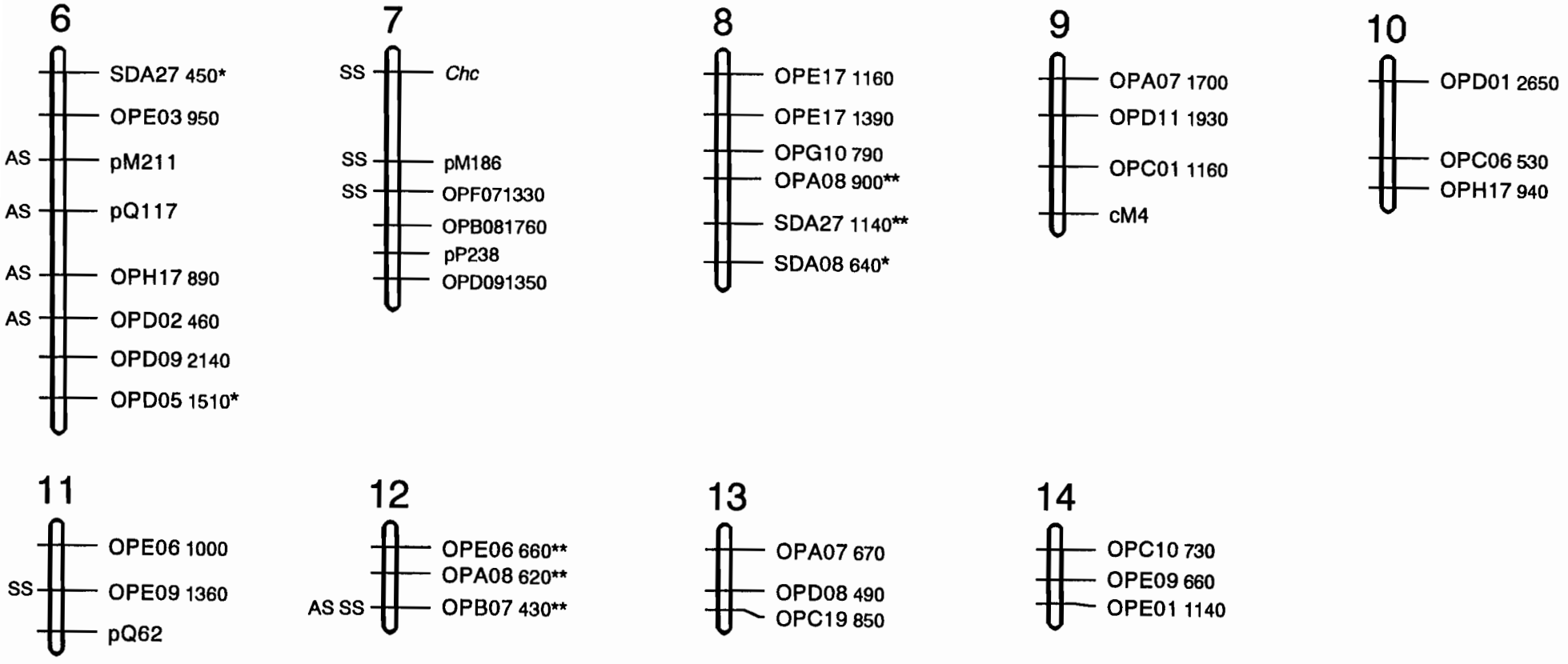

Fig. IV-17. (continued)

always detected by interval mapping. This discrepancy indicates that the threshold value for interval mapping used in the present investigation might have been higher than proper value and resulted in under-estimation. Hence, the QTLs detected by interval mapping were thought to be the most effective loci. The significant regions that were detected by single-marker analysis but not by interval mapping may correspond to QTLs with small effects. More than 1000 permutation tests performing randomization, such as bootstrapping, will be needed to confirm the QTLs with minor effects.

QTLs with stable effects

The morphological appearance of the plants in the field conditions greately differed from that in the pot conditions. Since the program could estimate the allelic effects in F_2 progeny as well as in F_3 progeny means, their test statistic values and gene effects were compared. As expected, most of the QTL locations were not in common between the two segregation populations, likely because of the effects under different environmental conditions. Only five QTLs, i.e., ph2, ph11, ph12, sw7 and df6, were identified using both progenies, suggesting their stable gene effects. Further analysis using recombinant inbred lines will help confirm the positions of QTLs.

Relationships among QTLs controlling differnt traits

QTLs for correlated traits are often observed in the same or nearby location (Abler et al 1991; Paterson et al 1991; Stuber et al 1992; Zhikang et al. 1995). In this sudy, QTLs detected by interval mapping and putative QTLs detected by single-marker analysis for

positively correlated traits, PH, BN and DF (Table IV-15), were localized at the same intervals in linkage group 4 with F_2 progeny (Fig. IV-10) and in linkage group 11 with F_3 progeny (Fig. IV-11). Among the QTLs detected by interval mapping, ph12 for plant height and bn12 for branch number, were found to be located in the same interval on linkage group 12 using F_2 progeny. The gene effects of both QTLs showed overdominance by *V. nakashimae* allele (Tables IV-10 and IV-13). These facts indicate that PH, BN and DF might be partially controlled by the same QTL, namely, a single QTL which pleiotropically affects plant growth. Using single-marker analysis, the common markers significantly associated with PH and BN were also identified in linkage group 2 (Fig. IV-10). In addition, the SW were positively correlated with PLW (Table IV-15). The markers located on linkage group 5 and 9 in F_2 progeny (Fig. IV-10) and 3, 4 and 5 in F_3 progeny (Fig. IV-11) were significantly associated with both SW and PLW. This indicates that the sizes of cotylendon, which accounts for seed weight, and primary leaf are controlled under the similar genetic mechanisms. For PLW and PLL, whose correlation coeffcient was very high, the common markers associated with them was localized on linkage groups 3 and 4 (Fig. IV-11).

Implications of QTL mapping for wild gene introgression

The positive effect of each QTL was contributed by either one of the two parental alleles. Therefore, a parent with better character than the other does not always have QTL alleles with positive effects. In tomato, deVicente and Tanksley (1993) reported similar phenomena and suggested that the combination of positive parental alleles was

important to increase the trait value. In this study, three QTLs for plant height (ph1, ph11 and ph12) were from the shorter parent, *V. angularis.* Thus, wild relatives also have potential to improve the plant height. Therefore, it may be possible to introduce selectively new wild alleles with positive effects to improve characters by use of molecular markers.

Wild relatives have been exploited as a source of disease and insect resistant genes in many crops, but their use has been limited because of the linkage drag accompanied with undesirable genes (Zeven et al. 1983). Such introgression of undesirable genes from wild species can be monitored by the use of molecular linkage maps. Even after many backcrosses, segments large enough to carry many wild undesirable genes are still linked to the objective genes (Young and Tanksley 1989). In azuki bean breeding program, brown stem rot resistant gene has been introduced from *V. nakashimae*. However, *V. nakashimae* has many undesirable characters, such as late flowering, climbing habit, pod shattering and small seed. The QTLs identified in this study for those undesirable characters will help isolate individuals with the useful genes, e.g., brown stem rot resistant gene, if the linkage with undesirable traits were broken.

QTL analysis of categorical traits

F_2 segregation of pod shattering fitted to the assumption that this character was controlled by a single dominant gene (in Chapter III). However, pod shattering gene could not be mapped on azuki linkage map. Pod shattering habit found in wild mungbean, V. *radiata* var. *sublobata* was reported to be controlled quantitatively (Singh et al. 1975). The

percentages of pod shattering segregants among F_3 lines showed continuous distribution, indicating that this trait was controlled by two or more genes. By single-marker analysis, two putative QTL locations were identified on linkage groups 2 and 6 (Table IV-8). Regarding seed color, six, seven, six and two putative QTL locations were identified using the same method for the brown, red, white and green seed color expression, respectively (Table IV-8). Some of these putative QTL locations were overlapped in linkage groups 3, 4, 6, 7, 9, 11 and 14, indicating that the seed colors may be complementary controlled by several genes. Previously, the ground color of seed testa in *V. angularis* was explained by a seven-gene interact model with inhibitor and complementary genes (Matsuura 1933). Thus, the QTL analysis using the percentage of categorical traits gave some inferences of the number of genes controlling pod shattering and seed coat color.

Factors responsible for distorted segregation

Skewed segregation of molecular markers has been observed in many linkage maps constructed with interspecific populations (Bernatzky and Tanksley 1986; McCouch et al. 1988; Halward et al. 1993; Foolad et al. 1995). There are a few studies to analyze the causes of distorted segregation of those molecular markers (Quillet et al. 1995 ; Harushma et al. 1996). The distorted segregation could partially be resolved by QTL mapping of factors which might be involved in the elimination of segregants in progeny. In this study, several factors for distorted segregation, i.e., pollen fertility (FP), germination rate (GES), abortiveness in plant development (LP and SP),

seed sterility (SS) and seed abnormality (AS) were analyzed. Based on the single-marker analysis, some QTL locations controlling FP, SS and AS were detected in the cluster of markers showing abnormal segregation on linkage group 2 (Fig. IV-17). One of the marker loci, pM374, showed homozygous distortion with normal segregation for heterozygous (in Chapter III). In rice molecular map, the markers with such homozygous segregation distortion were mapped on the region containing known gametophyte distorter genes (Harushma et al. 1996). The present result therefore suggested that the genes affecting gametophytic competition might locate on linkage group 2 of azuki map.

No factors were identified in the clusters of markers showing distorted segregation in F_2 on linkage groups 4 and 8. This suggests that the genes responsible for the elimination of genotypes were not transmitted to F_3 progeny. In this study, relatively weak F_2 plants which did not produce seeds could not be included in F_2 mapping population. Major genes responsible for hybrid weakness might have been eliminated before constructing F_2 mapping population.

Most of the putative QTL locations controlling distorting factors were identified near the markers showing the distorted segregation. However, since RAPD markers are not codominant, more information on heterozygotes are needed to be analyzed to understand the accurate mechanism of skewed segregation.

Summary

Quantitative trait loci (QTL) were mapped in F_2 and F_3 progenies from a interspecific cross of *V. angularis* x *V. nakashimae* Twenty

traits were examined including morphological, categorical and reproductive traits. A total of seventeen QTLs controlling plant height, seed weight, days to flowering and branch number were localized to nine linkage groups based on interval mapping. Of these, only five QTLs, i.e., ph2, ph11, ph12, sw7 and df6 were thought to have stable gene effects under the different circumstances. Plant height, branch numbers and days to flowering might be partially controlled by a single QTL which pleiotropically affects plant growth. Based on the single-marker analysis, putative QTL locations controlling gametophytic abnormalities were detected in the cluster of markers showing abnormal segregation on linkage group 2. One of the marker loci showed homozygous distortion with normal segregation for heterozygotes. These findings suggest that the genes affecting gametophytic competition locate in linkage group 2 of azuki map.

Chapter V. Construction of a molecular linkage map using an interspecific population (*V. umbellata* x *V. angularis*) and QTL mapping for bruchid resistance

Introduction

The azuki bean weevil, *Callosobruchus chinensis* L., is a most destructive insect pest for azuki bean. In Japan, except Hokkaido area, this insect often attacks stored azuki seeds and leads to severe damage. This insect was also reported to cause a serious damage to mungbean (*V. radiata*) seed. Fujii and Miyazaki (1987) reported that 'TC1966', an accession of wild mungbean (*V. radiata* var. *sublobata*), had complete resistance against the bruchid. Later, this resistance was shown to be controlled by a single dominant gene, *R* (Kitamura et al. 1988). Since 'TC1966' was crossable with mungbean cultivars, the bruchid resistance gene was transferred and used in a breeding program in Thailand (Tomooka et al. 1992; Watanasit and Pichitporn 1995). The bruchid resistance has also been reported on related species, *Phaseolus vulgaris, V. mungo* (Sawa and Tan 1976) and *V. unguiculata* (Piergiovanni et al. 1990). Resistance in *P. vulgaris* was explained by the mechanism that seed α-amylase inhibitor suppresses the midgut α-amylase activity in the bruchid larvae (Ishimoto and Kitamura 1989).

Resistance to azuki bean weevil was found also in rice bean (*V. umbellata*) (Sawa and Tan 1976; Tomooka 1991). Rice bean is cultivated in south and southeast Asia; India, Malaysia, China, Korea, Japan, Fiji, Mauritius and Philippines (Chatterjee and Dana, 1977; Dana et al. 1981). This bean has other valuable characteristics such as

highest yielding capacity among *Ceratotropis* species (Smartt 1990) and resistance to azuki bean mosaic virus (Sawa et al. 1984). Since azuki bean and rice bean constitute a secondary gene pool (Smartt 1981), novel and useful recombinants can be isolated from the progenies of their interspecific cross. Siriwardhane et al. (1991) showed that the valuable characteristics of rice bean could be introduced to azuki bean using one of wild relatives, *V. riukiuensis*, as a bridge species. Azuki bean in fact is crossable with rice bean with the aid of embryo rescue when azuki bean is used as a pollen parent. These hybrids showed regular meiosis with 11-bivalents formation and high pollen fertility (Ahn and Hartmann 1978; Chen et al. 1983). Therefore, it is possible to introduce the bruchid resistance from rice bean to azuki bean.

In this chapter, construction of a molecular linkage map using interspecific population between rice bean and azuki bean was reported. Further, the bruchid resistance and several morphological traits were characterized by QTL analysis based on the linkage map.

Materials and methods

Plant materials

V. umbellata 'Kagoshima' and *V. angularis* 'Erimoshouzu' were used to produce mapping population. The seeds of *V. umbellata* were kindly provided from Dr. Y. Egawa, National Institute of Agrobiological Resources, Japan. After crosses between *V. umbellata* and *V. angularis*, immature and dropped pods were collected and the immature embryos were removed from the seeds. These embryos were cultured in LB

medium (Linsmaier and Skoog 1965) for three months. Seedlings obtained by embryo culture were further transferred in 1/2 MS medium (Murashige & Skoog 1962). After two months of secondary culture, they were transplanted to the pots of 25 cm in diameter.

One hundred seventeen F_2 individuals from the interspecific cross were separately grown in the pots at Kobe University in spring, 1993. Ten plants each from 86 F_2 individuals were grown in the field at Kobe University Experimental Farm in summer, 1994. The experimental plots with the space of 15 cm between plants and 80 cm between the rows were arranged in a randomized block design with two replications. Five individuals each from parents (*V. umbellata* and *V. angularis*) and F_1 hybrids were also included in the blocks to examine environmental variation.

DNA extraction

Total DNA was extracted from bulked leaf tissue of 10 plants per F_3 line by the same method used for RFLP analysis (Chapter II).

RAPD and RFLP analyses

The methods for RAPD and RFLP analyses were the same as in Chapter II. The bulked total DNA of each F_3 line was used for genotyping parental F_2 plant. Primers used are shown in Table V-1.

Genetic analysis of morphological traits

Three morphological traits were analyzed in F_2 progeny; epicotyl color, hilum shape and pod shattering. Phenotypes of both parents, F_1

Table V-1. Nucleotide sequences of the primers used in this study.

Primer code[1)]	Sequence	Primer code[1)]	Sequence
OPA07	5'-GAAACGGGTG-3'	OPE01	5'-CCCAAGGTCC-3'
OPA17	5'-GACCGCTTGT-3'	OPE02	5'-GGTGCGGGAA-3'
OPA18	5'-AGGTGACCGT-3'	OPE04	5'-GTGACATGCC-3'
OPB01	5'-GTTTCCCTCC-3'	OPE06	5'-AAGACCCCTC-3'
OPC02	5'-GTGAGGCGTC-3'	OPF03	5'-CCTGATCACC-3'
OPC04	5'-CCGCATCTAC-3'	OPF05	5'-CCGAATTCCC-3'
OPC06	5'-GAACGGACTC-3'	OPF09	5'-CCAAGCTTCC-3'
OPC13	5'-AAGCCTCGTC-3'	OPF13	5'-GGCTGCAGAA-3'
OPC15	5'-GACGGATCAG-3'	OPG05	5'-CTGAGACGGA-3'
OPD02	5'-GGACCCAACC-3'	OPG10	5'-AGGGCCGCTC-3'
OPD03	5'-GTCGCCGTCA-3'	OPG12	5'-CAGCTCACGA-3'
OPD08	5'-GTGTGCCCCA-3'	OPG13	5'-CTCTCCGCCA-3'
OPD10	5'-GGTCTACACC-3'	OPG14	5'-GGATGAGACC-3'
OPD11	5'-AGCGCCATTG-3'	OPG18	5'-GGCTCATGTG-3'
OPD13	5'-GGGGTGACGA-3'	OPI03	5'-CAGAAGCCCA-3'
OPD15	5'-CATCCGTGCT-3'	OPI07	5'-CAGCGACAAG-3'
OPD16	5'-AGGGCGTAAG-3'	OPI18	5'-TGCCCAGCCT-3'
OPD18	5'-GAGAGCCAAC-3'	OPI20	5'-AAAGTGCGGG-3'
OPD20	5'-ACCCGGTCAC-3'	OPJ13	5'-CCACACTACC-3'

1) OP: Primers purchased from Operon Technologies, USA.

hybrid and F_2 individuals were evaluated to determine the manner of inheritance.

Construction of linkage map

F_2 segregation data for RFLP and RAPD markers, and morphological traits were examined by chi-square test. Linkage analysis and map construction were assessed with the same procedure as described in Chapter III using MAPMAKER program under LOD=3 and r=0.25 condition.

Evaluation of bruchid resistance

In order to test bruchid resistance, plastic Petri dishes (5 cm in diameter) were prepared and in each dish 16 dried seeds were placed. The dishes were kept in an environmental chamber controlled at 30 °C with about 70 % relative humidity. Azuki been weevils (*Collosobruchus chinensis*) were collected from the storage seeds and mass-reared with the cultivated azuki bean under optimal conditions. In each dish, more than ten adults were released to allow to mate and lay eggs on the seeds for a week, and then they were removed from the dishes. Five days after infestation (DAI), the number of the eggs laid on seeds was examined. For 50 DAI emerged adults were counted everyday. After counting, they were removed to avoid secondary mating and multiplication. The seeds with holes and easily crushable seeds by fingers were considered to be susceptible. Segregation manners for bruchid resistance were examined with F_2, F_3 and F_4 seeds.

Measurement of quantitative traits

Seventeen traits were investigated using F_2 and F_3 progenies from the interspecific cross of *V. angularis* x *V. nakashimae* (Table V-2). Of these, the following six traits were measured in both F_2 and F_3 individuals; plant height (PH), seed weight (SW), days to flowering (DF), branch number (BN), primary leaf width (PLW) and length (PLL). DF was the number of days until an open flower was found, starting from March 28, 1994 for F_2 plant and August 19, 1994 for F_3 plant. The other traits were measured in the same way as described in Chapter IV. The progeny means of each F_3 line were used as F_3 values of these traits.

Statistical analysis

Genetic variance (V_{1F2}, V_{1F3} and V_{2F3}), heritability in broad and narrow senses, potence ratio ($\sqrt{H/D}$) and Pearson correlation coefficients were obtained in the same way as described in Chapter IV.

QTL analysis

The QTL mapping was performed with F_3 progeny. The markers on the linkage map were selected for QTL analysis to have relatively uniform spacing (approximately 15 cM interval). QTLs for each trait were analyzed as described in Chapter III.

Table V-2. List of 17 traits analyzed in this study.

Trait	Definition	Tested progeny
PH (plant height)	Length between shoot apex and stem base at maturity	F_2 and F_3
SW (seed weight)	Mean weight of 20 seeds	F_2 and F_3
DF (days to flowering)	Number of days to flower	F_2 and F_3
BN (branch number)	Number of branches on main stem	F_2 and F_3
PLW (primary leaf width)	Mean width of primary leaf in the presence of upper leaf	F_2 and F_3
PLL (primary leaf length)	Mean length of primary leaf in the presence of upper leaf	F_2 and F_3
PS (pod shattering)	Percentage of plants having shattering pod per line	F_3
GS (green seed)	Percentage of plants with green seeds per line	F_3
WS (white seed)	Percentage of plants with white seeds per line	F_3
RS (red seed)	Percentage of plants with red seeds per line	F_3
GES (germinated seed)	Mean percentage of germinated seeds	F_3
LP (lethal phenotype)	Percentage of plants which died during development per line	F_3
SP (stunted phenotype)	Percentage of stunt plants per line	F_3
SS (sterile seed)	Percentage of plants which could not produce any seeds per line	F_3
AS (abnormal seed)	Percentage of plants which produced abnormal seeds per line	F_3
BRC (bruchid dameged seed)	Mean percentage of damaged seeds by bruchid	F_2, F_3 and F_4
BRD (bruchid developmental period)	Mean DAI for bruchids development	F_3

Results

Morphological characters of F_1 hybrids between V. umbellata and V. angularis

Three F_1 hybrids were obtained by embryo culture from the cross, *V. umbellata* x *V. angularis.* Table V-3 summarizes the average phenotypic values of *V. umbellata*, *V. angularis* and their hybrids grown in the pot condition. The size of the hybrids was smaller than both parents, while 20-seeds weight, leaf shape and pod color showed intermediate characteristic. Purple epicotyl, bright yellow flower, elongated raceme, shattering pod and concave hilum cushion of the hybrids were similar to those of *V. umbellata*, indicating that these *V. umbellata* characters are dominant. The F_1 hybrids were also compared with parents and F_3 progeny in the field condition. The morphological appearance of the F_1 hybrid was distinctively larger under the field cultivation conditions than in the pot cultivation conditions (Table V-4). The hybrid plants showed indeterminate growth with juvenile shoots and late flowering. They produced green seeds of intermediate size. The green seeds were unique to the hybrid, because both parents produce red seeds.

Inheritance of morphological traits

Three morphological traits, i.e., epicotyl color, pod shattering and hilum cushion, which showed polymorphisms between parents were examined for F_1 and F_2 plants (Table V-5). The F_1 hybrid had the same characteristics with *V. umbellata*, i.e. purple stem, shattering pod and concave hilum cushion. On the other hand, F_2 plants showed the

Table V-3. Morphological characters of *V. umbellata*, *V. angularis* and their F1 hybrid.

Character	*V. umbellata*	F1 hybrid	*V. angularis*
Plant height	38.0	14.0	30.2
20-seed weight	1.10	1.59	2.46
Shape of primary leaves	linear lanceolate	ovate	cordate
Shape of leaves	rhombic	lanceolate	rhombic
Epicotyl color	purple	purple	green
Seed color	red	green	red
Pod color	brown	dark brown	straw
Flower color	bright yellow	bright yellow	light yellow
Raceme	elongated	elongated	compact
Pod shattering	shattering	shattering	non-shattering
Hilum cushion	concave	concave	smooth

Table V-4. Means for seven traits of *V. umbellata, V. angularis* and F1 hybrids, and their progeny means of F3 lines in the field condition.

Trait	*V. umbellata* mean	*V. angularis* mean	F1 hybrid mean	Mid-parent	Progeny means of F3 lines		
					mean	maximum	minimum
PH (cm)	153.53	44.19	150.79	98.86	91.20	241.13	31.43
SW (g)	1.27	2.52	2.07	1.89	1.49	2.32	0.99
DF (day)	27.00	4.85	22.11	15.93	22.34	33.40	13.70
BN (no.)	7.78	2.90	10.78	5.34	5.06	8.86	0.25
PLW (cm)	1.34	2.96	-	2.15	2.03	3.20	1.33
PLL (cm)	4.40	2.86	-	3.63	3.29	5.43	2.40
GES (%)	98.0	90.0	-	94.0	85.6	100	50

Table V-5. Morphological characters of *V. umbellata*, *V. angularis* and F1 hybrid, and their segregation in F2 population

Character	*V. umbellata*	*V. angularis*	F1 hybrid	F2 segregation		
				Phenotype	No. of plants	χ^2 (3:1)
Epicotyl color	purple	green	purple	purple : green	60 : 25	0.88
Purple epicotyl	intense	-	intense	intense : weak	49 : 11	1.42
Pod shattering	shattering	non-shattering	shattering	shattering : non-shattering	67 : 18	0.67
Hilum cushion	concave	smooth	concave	concave : smooth	75 : 11	6.84**

** denotes significant deviation from the expected segregation ratios at 0.01 level.

segregation of these characters. The segregation of purple stem and shattering pod showed a good fit to 3:1 ratio, indicating each of them might be controlled by a single dominant gene. Therefore, tentative dominant genes for these characters from *V. umbellata* were designated as *Psu*(purple stem) and *Spu* (shattering pod), respectively. Although the segregation ratio of concave hilum cushion deviated significantly from the expected ratio, the symbol 'Chcu' for dominant gene from *V. umbellata* was used for linkage trial. In addition, intensity of the stem color segregated in F_2 plants with purple stem, suggesting the existence of hypostatic gene for the intensity of the pigmentation. Among 60 F_2 plants with purple stems, 49 and 11 showed intense and weak purple colors, respectively. Since this segregation agreed with 3:1 ratio ($c2 = 1.42$), a hypostatic gene of *Iscu* (intense stem color) was added to the tentative dominant genes from *V. umbellata.*

Survey on parental polymorphisms and segregation of molecular markers

In order to detect RAPD markers between *V. umbellata* and *V. angularis*, 200 decamer primers were examined. Among them, 38 primers gave at least three distinct polymorphic fragments (Table V-1). They produced in total 149 RAPDs which were also detected in F_1 plants, indicating their proper inheritance to next generation. Concerning RFLP markers, 67 mungbean and cowpea genomic probes and a single RAPD-derived probe were examined. The RAPD-derived probe was prepared by gel excision of 560 bp amplified product from *V. nakashimae* with a primer 'OPC08'. Parental polymorphisms were detected with 58 probes (85.3 %) using at least one out of five enzymes,

*Bam*HI, *Dra*I, *Eco*RI, *Hin*dIII and *Pst*I (Table V-6). Among them, 43 probes gave 66 clear hybridized bands. These RFLP markers were further used for F_2 segregation analysis.

Segregation of molecular markers (149 RAPD and 66 RFLP markers) was examined with 86 F_2 plants derived from the cross of *V. umbellata* and *V. angularis*. Overall genotyping of each F_2 plant was made with bulked DNA from 10 F_3 plants. Examples for segregation patterns of RAPD and RFLP markers are presented in Figs. V-1 and V-2, respectively. Based on the segregation patterns, 148 RAPD (99.3%) and 25 RFLP (37.9%) markers were turned out to be dominant markers, while one RAPD (0.7%) and 41 RFLP (62.1%) markers were codominant (Table V-7). The segregation manners of 34 RAPD and 22 RFLP markers deviated significantly from the expected ratio of 3:1 or 1:2:1 ($P<0.05$), respectively.

Linkage analysis

Linkage analysis was carried out with 149 RAPD and 66 RFLP markers and four tentative phenotypic genes using MAPMAKER. In total, 15 linkage groups containing at least two markers were constructed with 169 markers (75.9%), and 50 markers (24.1 %) remained unlinked (Fig. V-3). The sizes of the largest and smallest linkage groups were 313.1 and 4.7 cM, respectively. The total map size was 2001.4 cM and the average distance between markers was 13.9 cM. The clusters of the markers showing distorted segregation were located in the linkage groups, 1, 2, 3, 4, 5, 7, 10, 12, 13 and 14.

Table V-6. Estimation of copy numbers and polymorphic combination of enzymes for 68 RFLP probes.

Probe no.	Copy	Enzyme				
		*Bam*HI	*Dra*I	*Eco*RI	*Hind*III	*Pst*I
cM 3	L	*		*		
cM 4	S				•	*
cM 6	M		*	*	•	
cM 11	M		*		*	
cM 14	S		*		•	
cM 87	M			*	•	
cM 88	S					
cM 93	S			*		
pM 9	L				•	
pM 26	S					
pM 78	S			*		
pM 100	L			*	•	
pM 151	L		*	*	•	
pM 177	S			*	•	
pM 186	S			*	•	
pM 205	S					
pM 208	S		*		•	
pM 209	S				•	
pM 211	M			•	*	*
pM 217	S					
pM 224	S				•	
pM 228	S					
pM 236	S					
pM 241	S				•	
pM 244	S				•	
pM 245	L					*
pM 247	L				•	
pM 254	S			*		
pM 273	M	*	*	*	*	*
pM 316	S					
pM 339	S			*	•	*
pM 371	S			*		*
pM 374	L	*				*
pM 381	S				•	

Table V-6. (continued).

Probe no.	Copy	Enzyme				
		*Bam*HI	*Dra*I	*Eco*RI	*Hin*dIII	*Pst*I
pM 392	L			•		
pM 415	L	*	*	*	•	*
pM 456	L	*		*		
pM 490	S		*		•	
pO 8	M		*	*	•	*
pO 11	M				*	
pO 26	L		•		*	*
pO 34	S			*	*	*
pO 91	S				•	*
pO 95	L				•	
pO 103	L	*			•	
pO 109	S			*	•	
pO 111	S	*			•	
pP 137	S			*		
pP 145	S				•	
pP 157	L					
pP 170	S				•	
pP 238	S				•	
pQ 22	S					
pQ 39	L		•			
pQ 43	L			*	•	
pQ 45	L			*	•	
pQ 62	L		*	*	•	
pQ 66	M				*	
pQ 86	S			*	•	
pQ 117	L			•		
pR 2	S				•	
pR 25	S			*	•	
pR 26	L	*	*	*	•	*
pR 34	S					
pR 48	S	*		•		
pR 51	S			*	•	
pR 67	S		*	*		
OPC 08	M		*	*	•	

Polymorphic combinations of probes and enzymes are indicated by asterisks or black circles. The combinations marked with black circles are further used for segregation analysis. The letter in the Copy column denotes frequency of the clone in genomes (S=single locus, L=a few loci M=multiple loci).

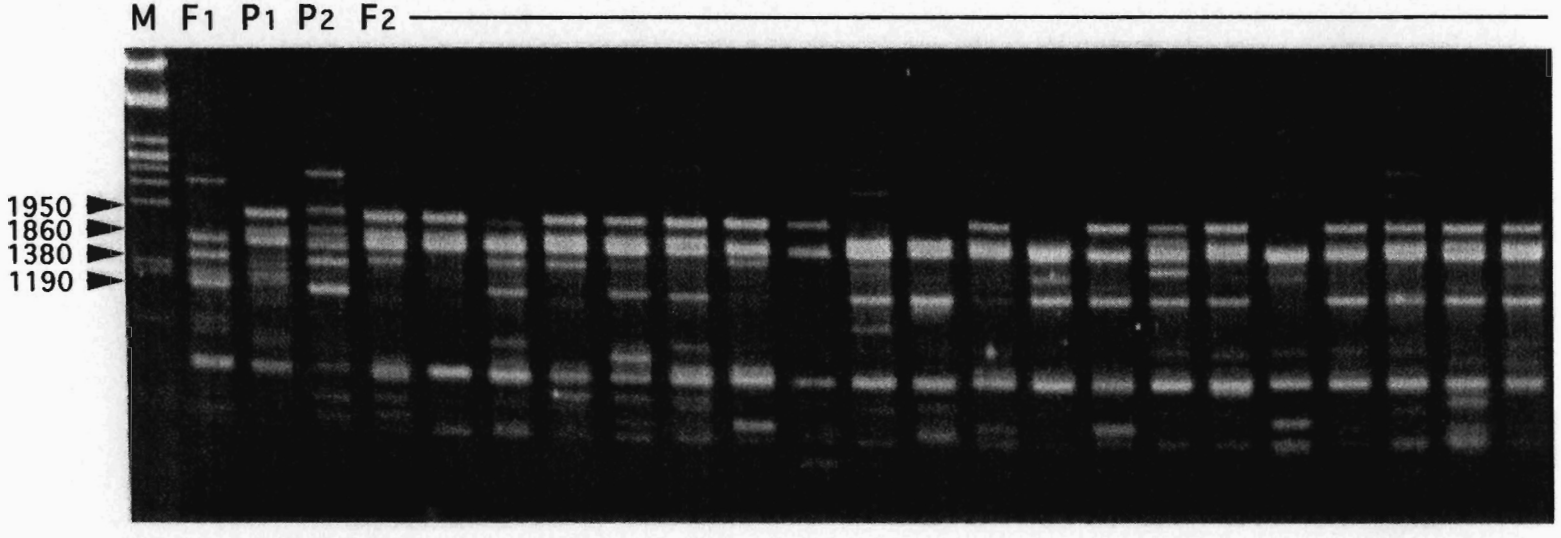

Fig. V-1. Segregation of the RAPD markers with primer 'OPA17' in the F2 population derived from a cross between *V. umbellata* and *V. angularis.* The markers are indicated by arrows with morecular size. M: Lambda DNA digested with *Pst*I. F1: F1 hybrid (*V. umbellata* x *V. angularis*). P1: *V. umbellata*. P2: *V. angularis.*

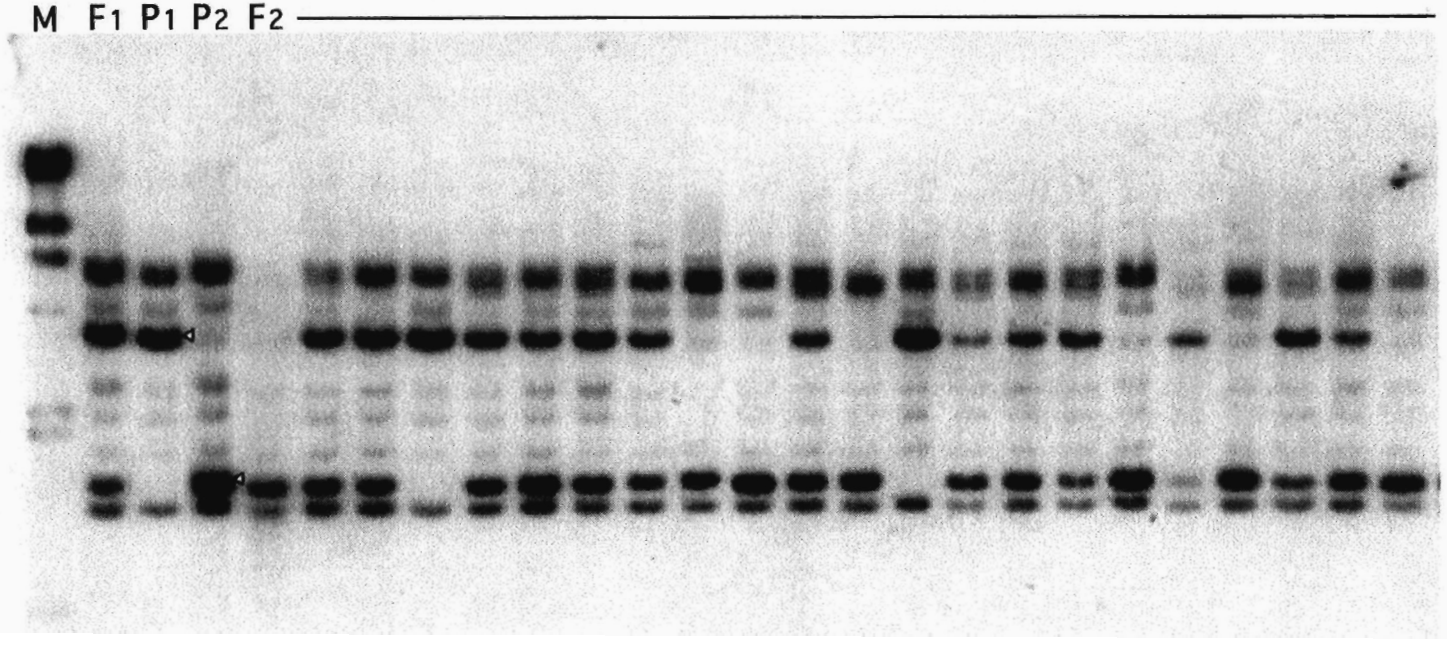

Fig. V-2. Segregation of the RFLP marker pR26 in the F2 population derived from a cross between *V. umbellata* and *V. angularis*. DNA samples were digested with *Hin*dIII and blotted onto the filter. Polymorphic bands are indicated by arrows. M: Lambda DNA digested with *Hin*dIII. F1: F1 hybrid (*V. umbellata* x *V. angularis*). P1: *V. umbellata*. P2: *V. angularis*.

Table V-7. Segregation of 149 RAPD and 66 RFLP markers in F2 population.

Marker	No. of plant					Chi-square	Linkage group
	P_1	P_1+H	H	P_2+H	P_2		
RAPD marker							
OPA07 1010		59			24	0.68	11
OPA07 1390	23			62		0.19	1
OPA07 1610	19			66		0.32	6
OPA17 1190		59			25	1.02	2
OPA17 1380		60			24	0.57	-
OPA17 1860	25			58		1.16	5
OPA17 1950	11			73		6.35 *	-
OPA18 520		69			17	1.26	2
OPA18 550		72			13	4.27 *	2
OPA18 680		64			21	0.00	9
OPA18 1030	28			58		2.62	8
OPA18 1150		62			22	0.06	6
OPA18 940/900	26		40		20	1.26	11
OPB01 510	20			53		0.22	6
OPB01 730	25			57		1.32	6
OPB01 850		63			18	0.33	7
OPB01 910	16			63		0.95	-
OPB01 960	26			60		1.26	-
OPB01 1120		58			28	2.62	5
OPB01 1160	24			62		0.39	-
OPB01 1290		63			23	0.14	-
OPB01 2640	24			59		0.68	1
OPC02 300	19			67		0.39	3
OPC02 1570		72			14	3.49	7
OPC04 810		59			23	0.41	-
OPC04 1340	17			67		1.02	-
OPC04 1630	16			67		1.45	-
OPC06 390	22			61		0.10	10
OPC06 610		71			12	4.92 *	10
OPC06 810		59			23	0.41	4
OPC13 430		73			11	6.35 *	7
OPC13 600	29			55		4.06 *	12
OPC13 980		63			21	0.00	-
OPC13 1160	17			66		0.90	-
OPC13 1270		77			7	12.44 **	12
OPC15 620		64			13	2.71	-
OPC15 750		50			32	8.60 **	11
OPC15 960	25			57		1.32	10
OPC15 1010		75			7	11.85 **	-
OPC15 2670	25			57		1.32	12
OPD02 450		70			16	1.88	3
OPD03 280		66			18	0.57	13
OPD03 530	18			64		0.41	9

Table V-7. (continued).

Marker	No. of plant					Chi-square	Linkage group
	P_1	P_1+H	H	P_2+H	P_2		
RAPD marker							
OPD03 1050	28			56		3.11	9
OPD03 1200	26			58		1.59	1
OPD03 1920	29			55		4.06 *	2
OPD08 870	18			67		0.66	-
OPD08 1070	19			62		0.10	9
OPD10 330	11			75		6.84 **	3
OPD10 960	19			67		0.39	2
OPD11 620	17			68		1.13	-
OPD11 1090	26			59		1.42	8
OPD11 1300	22			60		0.15	-
OPD11 1800	31			53		6.35 *	-
OPD11 2140		56			29	3.77	-
OPD13 410	23			63		0.14	4
OPD13 810	22			64		0.02	4
OPD13 1130		54			32	6.84 **	4
OPD15 620		64			21	0.00	-
OPD15 690	23			62		0.19	4
OPD16 260	12			74		5.60 *	3
OPD16 510	12			74		5.60 *	-
OPD16 730		69			17	1.26	1
OPD16 980	18			68		0.76	1
OPD16 1030		66			19	0.32	1
OPD16 1560		67			19	0.39	3
OPD18 360		67			19	0.39	3
OPD18 660		65			15	1.67	2
OPD18 690	29			57		3.49	6
OPD18 770	26			60		1.26	3
OPD20 250		59			27	1.88	4
OPD20 1010	12			74		5.60 *	5
OPD20 1070	19			67		0.39	3
OPD20 1460		64			20	0.06	-
OPD20 1560	11			75		6.84 *	5
OPE01 740		63			21	0.00	2
OPE01 980	22			62		0.06	13
OPE01 1100	12			74		5.60 *	1
OPE02 430		67			17	1.02	2
OPE02 550		63			21	0.00	-
OPE02 610	23			59		0.41	5
OPE02 930		67			16	1.45	3
OPE02 1050	32			51		8.13 **	6
OPE02 1410	24			60		0.57	-
OPE02 1630	18			65		0.49	11
OPE04 690		67			18	0.66	7
OPE04 750	22			63		0.04	1

Table V-7. (continued).

Marker	No. of plant					Chi-square	Linkage group
	P_1	P_1+H	H	P_2+H	P_2		
RAPD marker							
OPE04 970	17			68		1.13	11
OPE04 1140		57			28	2.86	1
OPE04 1470	25			60		0.88	1
OPE06 1060	22			61		0.10	8
OPE06 1130		62			21	0.00	-
OPE06 1430		69			14	2.93	3
OPE06 1550	15			68		2.12	-
OPF03 700	21			63		0.00	2
OPF03 870	47			37		42.92 **	12
OPF05 390		55			31	5.60 *	-
OPF05 420	26			60		1.26	-
OPF05 580		60			26	1.26	-
OPF05 710	36			49		13.65 **	-
OPF05 750		69			16	1.73	-
OPF05 930		68			16	1.59	-
OPF05 980	23			61		0.25	-
OPF05 1130	15			69		2.29	-
OPF09 690		64			20	0.06	-
OPF09 720	25			59		1.02	4
OPF09 830		51			34	10.20 **	5
OPF09 920	6			79		14.59 **	-
OPF09 950		67			18	0.66	-
OPF13 680		82			4	18.99 **	-
OPF13 750	28			58		2.62	-
OPF13 830		74			11	6.59 *	-
OPF13 890	24			62		0.39	-
OPF13 1130		53			31	6.35 *	-
OPF13 1190	21			64		0.00	1
OPF13 1300		58			26	1.59	-
OPF13 1380		63			21	0.00	3
OPF13 1450	25			61		0.76	8
OPF13 1570	23			62		0.19	-
OPF13 1660		58			26	1.59	-
OPG05 850	17			67		1.02	-
OPG05 1120		66			17	0.90	-
OPG05 2650	25			58		1.16	-
OPG10 1030	12			74		5.60 *	5
OPG12 630		71			15	2.62	9
OPG12 1090	17			69		1.26	1
OPG13 690		63			19	0.15	2
OPG14 1640		61			25	0.76	1
OPG18 360	27			59		1.88	10

Table V-7. (continued).

Marker	No. of plant					Chi-square	Linkage group
	P_1	P_1+H	H	P_2+H	P_2		
RAPD marker							
OPG18 540		70			16	1.88	13
OPG18 1570		63			21	3.96 *	-
OPI03 280		61			25	0.76	-
OPI03 1110		53			30	5.50 *	-
OPI03 1300	23			60		0.33	4
OPI03 2040	21			62		0.00	4
OPI07 530	31			51		7.17 **	10
OPI07 2050		63			18	0.33	-
OPI18 460		78			8	11.30 **	2
OPI18 620	23			63		0.14	-
OPI18 930		55			30	4.80 *	1
OPI18 1260	17			69		1.26	1
OPI20 280		56			30	4.48 *	4
OPI20 570		70			15	2.45	3
OPI20 600		58			27	2.07	13
OPI20 1000		64			22	0.02	7
OPI20 1110		71			15	2.62	13
OPJ13 470		61			25	0.76	4
OPJ13 650	7			77		12.44 **	-
OPJ13 940	27			57		2.29	-
RFLP marker							
cM4	18		47		19	1.21	7
cM6	34		38		11	13.34 **	12
cM14	15		45		23	2.13	1
cM87a	23		39		21	0.40	9
cM87b	14			69		2.93	1
cM87c	26		54		3	20.28 **	13
pM9		61			22	0.10	-
pM100a	14			72		3.49	15
pM100b	19		44		22	0.32	-
pM100c	13		51		21	4.91	15
pM100d	15		48		17	3.30	15
pM151a	17			66		0.90	4
pM151b	33		36		14	10.16 **	12
pM151c		57			26	1.77	4
pM151d	16			67		1.45	4
pM151e	10		48		25	7.46 *	-
pM177	27		42		14	4.08	10
pM186	20		52		12	6.29 *	13
pM208	26		41		17	1.98	6
pM209	25		41		18	1.21	8
pM211	31		39		11	9.99 **	2
pM224	22		39		22	0.30	1

Table V-7. (continued).

Marker	No. of plant					Chi-square	Linkage group
	P_1	P_1+H	H	P_2+H	P_2		
RFLP marker							
pM241	21		41		22	0.07	1
pM244	33		37		12	11.54 **	2
pM247a		74			9	8.87 **	14
pM247b	37		34		12	17.77 **	10
pM339	18		38		28	3.14	11
pM381	20		51		12	5.89	9
pM392	13		49		21	4.25	3
pM415a		74			9	8.87 **	14
pM415b	34		38		12	12.29 **	10
pM490	21		46		16	1.58	6
pO8a	15		50		18	3.70	5
pO8b	20			63		0.04	-
pO8c	9			73		8.60 **	4
pO8d	22			56		0.43	6
pO26	19		49		16	2.55	10
pO91	18		40		25	1.29	4
pO95a	35		36		12	14.20 **	-
pO95b	13			70		3.86 *	3
pO95c	17		46		20	1.19	4
pO103	12		45		26	5.31	5
pO109	19		41		22	0.22	1
pO111	13		47		23	3.87	3
pP145	21			56		0.21	8
pP170	20			61		0.00	1
pP238	14		56		14	9.33 **	13
pQ39	25		46		15	2.74	5
pQ43a		73			10	7.43 **	2
pQ43b		66			17	0.90	13
pQ43c		62			21	0.00	8
pQ43d		56			27	2.51	4
pQ43e		72			11	6.11 *	13
pQ45		57			26	1.77	4
pQ62	17			66		0.90	8
pQ86	18		46		18	1.22	5
pQ117	39		34		10	22.98 **	2
pR2	23		46		14	2.93	13
pR25	17		40		26	2.06	4
pR26	12		43		29	6.93 *	5
pR48	29		38		12	7.43 *	-
pR51	19		39		25	1.17	4
OPC08bNb	30			49		7.09 **	7
OPC08bNc	13			67		3.27	5
OPC08bNd		60			20	0.00	3
OPC08bNe	13			70		3.86 *	7

P_1, P_2 and H indicate *V. umbellata*, *V. angularis* and heterozygote genotypes, respectively. * and ** show significant deviation at 5% and 1% level from the expected segregating ratios, respectively.

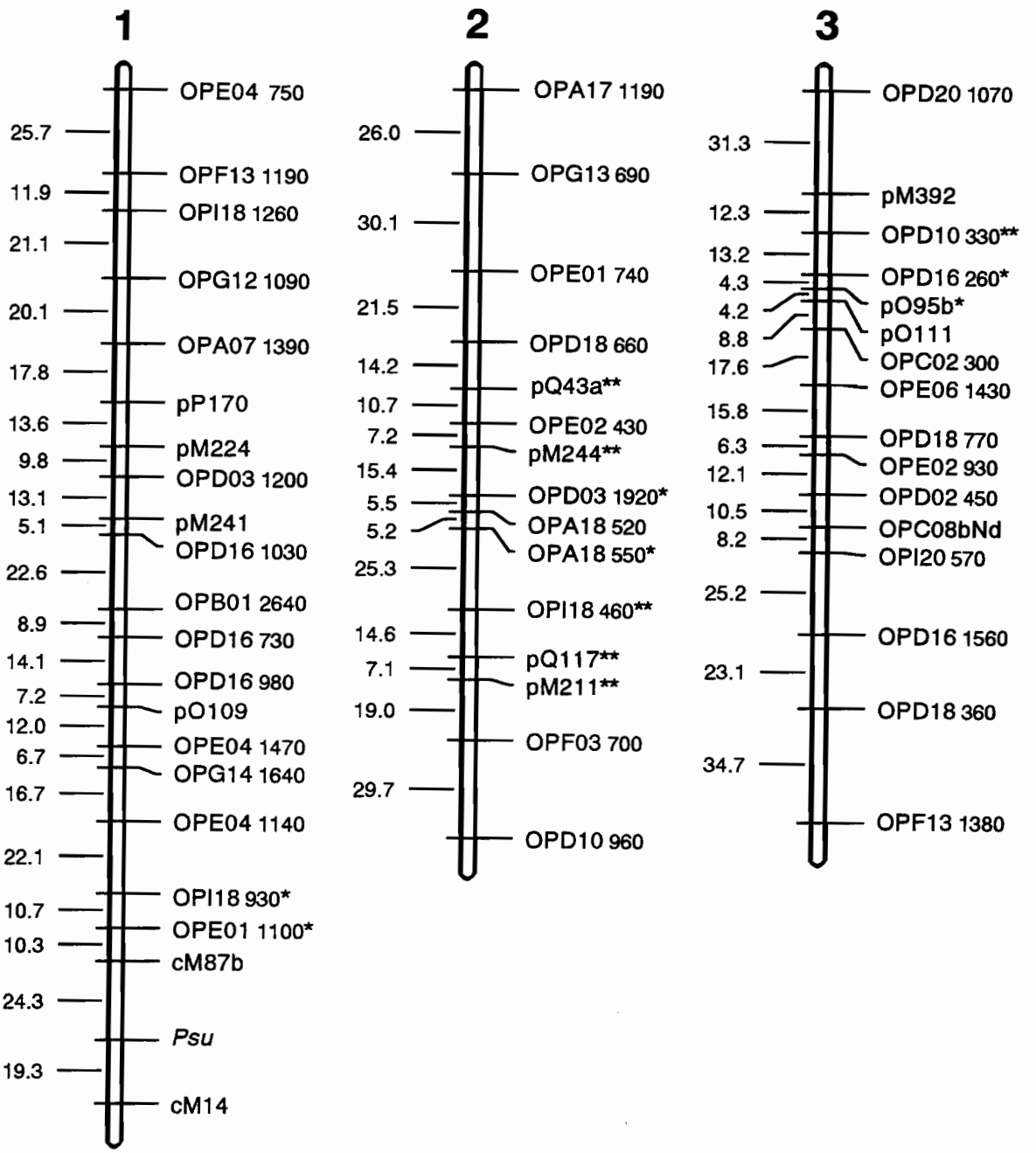

Fig. V-3. A linkage map of azuki bean constructed with a *V. umbellata* x *V. angularis* mapping population. Fifteen linkage groups are numbered in order of the size. Map distances and the marker names are shown on the left and right sides of the linkage groups, respectively. RAPD markers are presented with the primer codes (Table V-1) and the approximate molecular sizes. Markers showing significant deviations from the expected segregation ratios at 0.05 and 0.01 level are indicated by * and **, respectively.

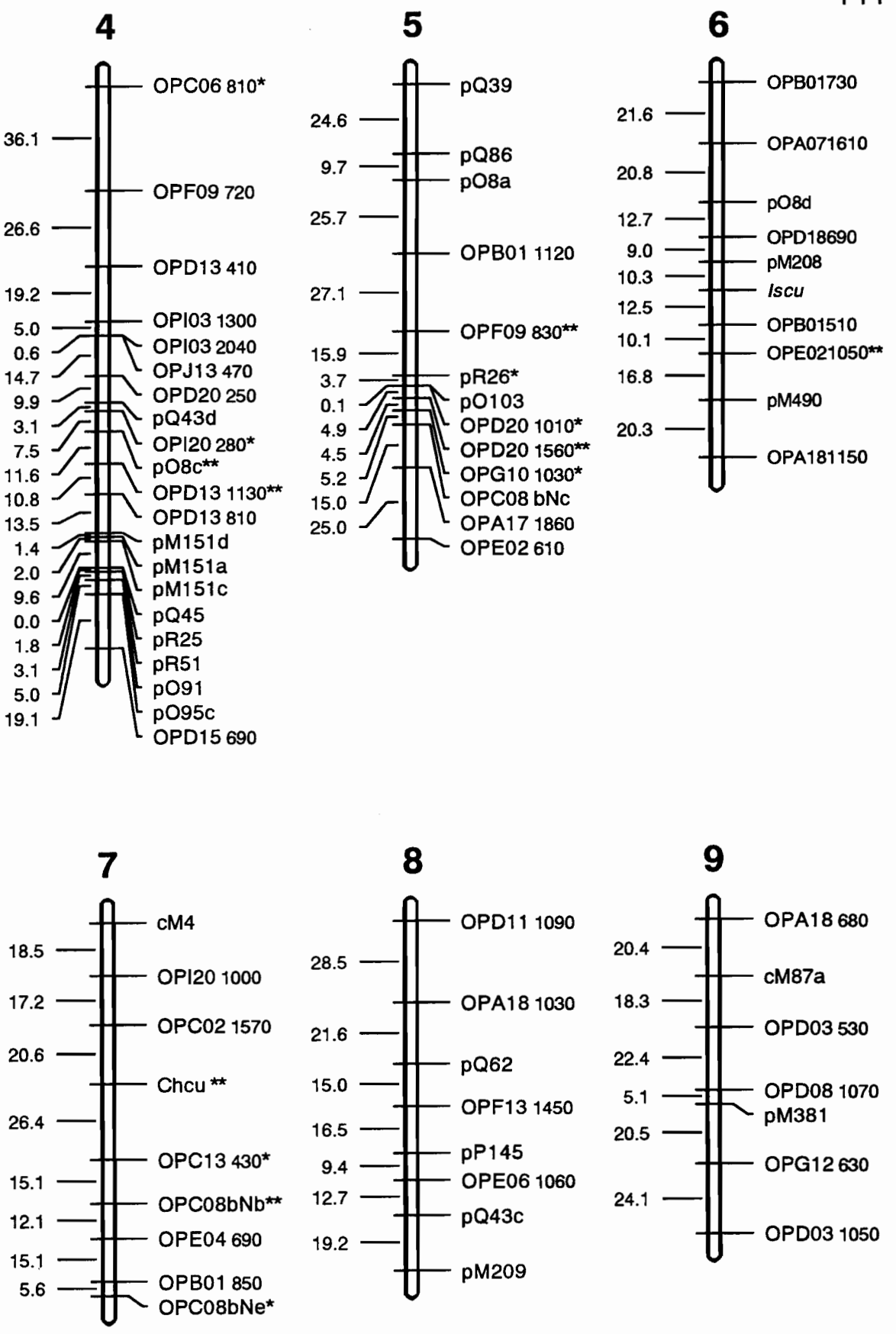

Fig. V-3. (continued).

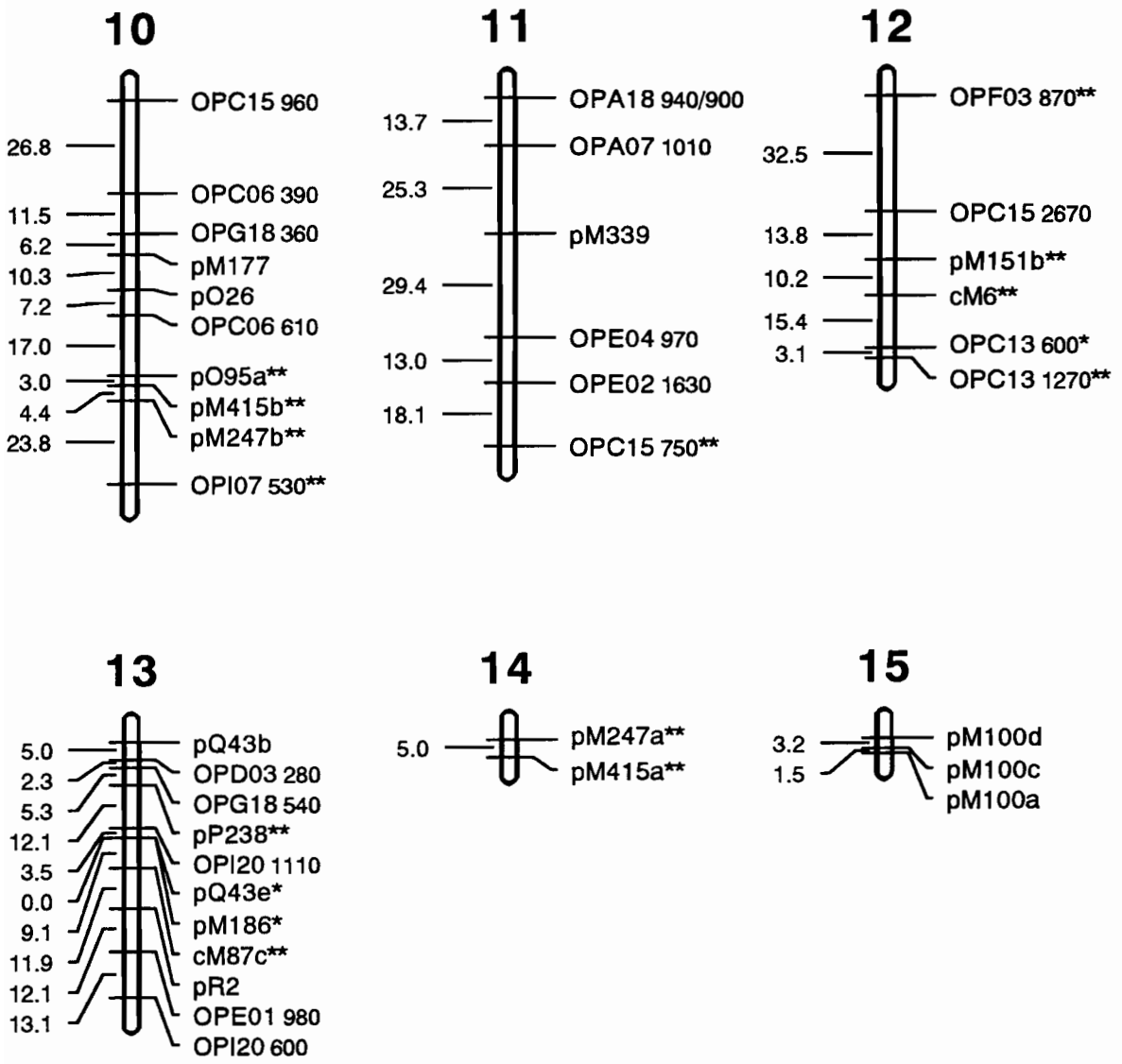

Fig. V-3. (continued).

Bruchid resistance

Test for bruchid resistance of *V. umbellata* and *V. angularis*

Five different numbers (approximately. 10, 20, 30, 40 and 50) of *C. chinesis* were released for oviposition of eggs on seeds of parental lines, *V. umbellata* and *V. angularis.* The oviposition frequency for *V. umbellata* was one-third lower than that for susceptible *V. angularis* (Table V-8). The number of eggs laid and oviposition on both parental seeds increased in proportion to the numbers of the bruchid released up to over 50, whereas they decreased when more than 50 bruchids were released (Table V-8 and Fig. V-4). Although the bruchids laid sufficient number of eggs on the seeds of *V. umbellata*, they failed to get into the seeds. On the other hand, all the seeds of *V. angularis* were damaged by the bruchids. These results indicate that *V. umbellata* has strong resistance against bruchid. The developmental period of adults bruchids in the seeds of *V. angularis* varied from 27 DAI (days after infestation) to 33 DAI depending on the density of the eggs on the seed (Table V-8).

Inheritance of bruchid resistance

Segregation of resistance to *C. chinensis* was examined with F_2 seeds obtained from a single F_1 hybrid of *V. umbellata* and *V. angularis.* Among 179 F_2 seeds tested, 140 (83.2%) were susceptible, while 39 (16.8%) were resistant. Since F_2 seeds set on F_1 plant were segregating for the bruchid resistance, the genotypes of F_2 generation might reflect these resistant characters of F_2 seeds. The segregation of F_2 seeds gave a good fit to 3:1 ratio (c^2 =0.99, 0.25<P<0.5). The inheritance of the bruchid resistance was further investigated with F_3

Table V-8. Oviposition and development of bruchids with 16 seeds of *V. umbellata* and *V. angularis*

Species	No. released bruchids	No. laid eggs	No. eggs per seed	Damaged		Mean days after infestation for adult emergence
				seed	%	
V. umbellata	10	28	1.8	0	0	-
	20	29	1.8	0	0	-
	29	57	3.6	0	0	-
	40	68	4.3	0	0	-
	56	48	3.0	0	0	-
V. angularis	10	89	5.6	16	100	32.5
	23	118	7.4	16	100	31.4
	32	171	11.4	16	100	27.9
	39	275	17.2	16	100	26.6
	50	161	10.1	16	100	29.1

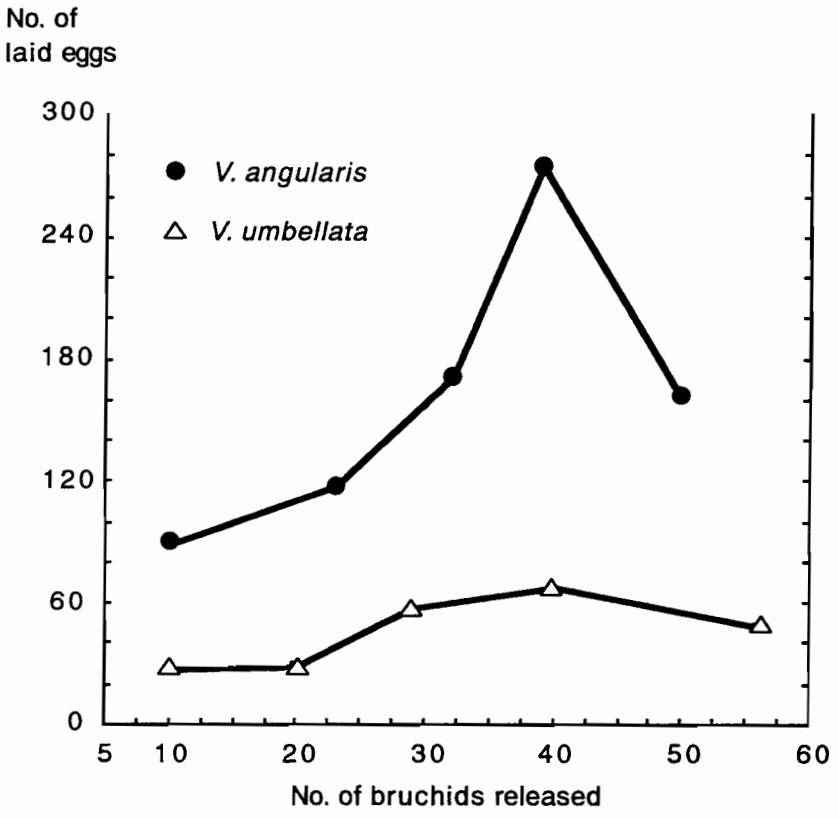

Fig. V-4. Relationships between number of bruchids released and laid eggs on 16 seeds of *V. angularis* and *V. umbellata*.

seeds from each F_2 plant; the proportion of susceptible seeds was examined for each F_3 line. The tetramodal continuous distribution with four peaks around 5%, 45%, 75% and 95% was observed for the percentages of damaged F_3 seeds (BRC) (Fig. V-5A). Developmental period of bruchid (BRD) on susceptible seeds showed unimodal distribution with a peak around 25 DAI (Fig. V-6). In addition, the percentages of damaged F_4 seeds were investigated based on the nine classes (1 - 9) shown in Fig. V-5A. F_4 seeds derived from highly resistant (1 and 2) and highly susceptible classes (7, 8 and 9) had a tendency to maintain the similar characteristics (Fig. V-5B). However, those from intermediate classes (3, 4, 5 and 6) showed various degrees of resistance. Especially, F_4 seeds from class 4 had trimodal distribution with three peaks around 5 %, 35 % and 75 %, and those from classes 5 and 6 showed two peaks around 65% and 85%. These facts indicate that besides a recessive gene there are minor genes with additive effects.

QTL analysis

Based on the linkage map constructed from the cross between *V. umbellata* and *V. angularis*, QTLs controlling six morphological traits (PH, SW, DF, BN, PLW and PLL) and two bruchid resistance related factors (BRC and BRD) were analyzed in F_3 progeny by single-marker analysis and interval mapping. Fig. V-7. shows the markers and intervals selected from the linkage map for QTL analysis.

Factors related to bruchid resistance

To identify putative QTLs for bruchid resistance, the proportion

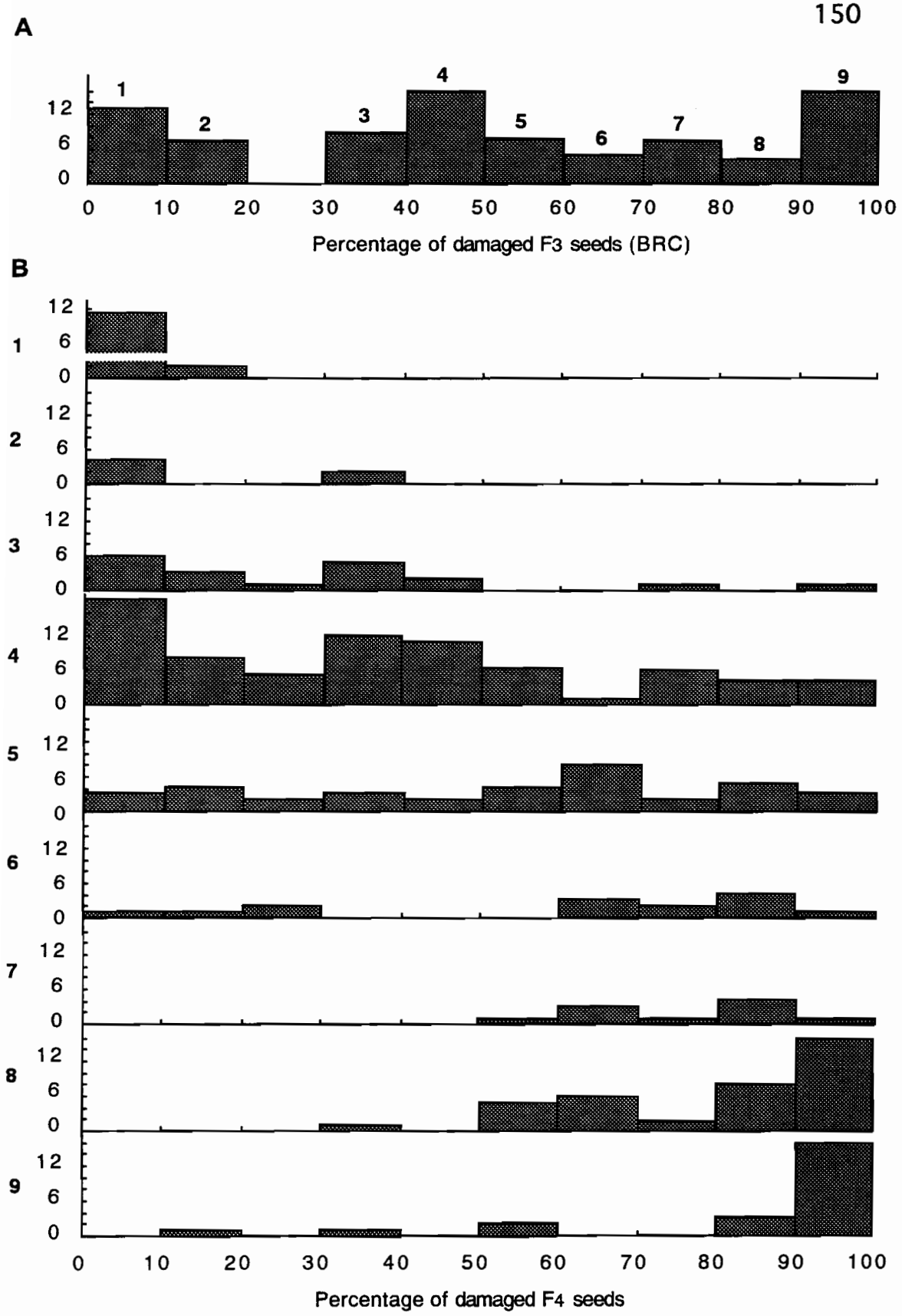

Fig. V-5. (A) Segregation of percetage of damaged F3 seeds. (B) Segregation of percetage of damaged F4 seeds derived from nine classes based on the percentages of damaged seeds in F3 generation.

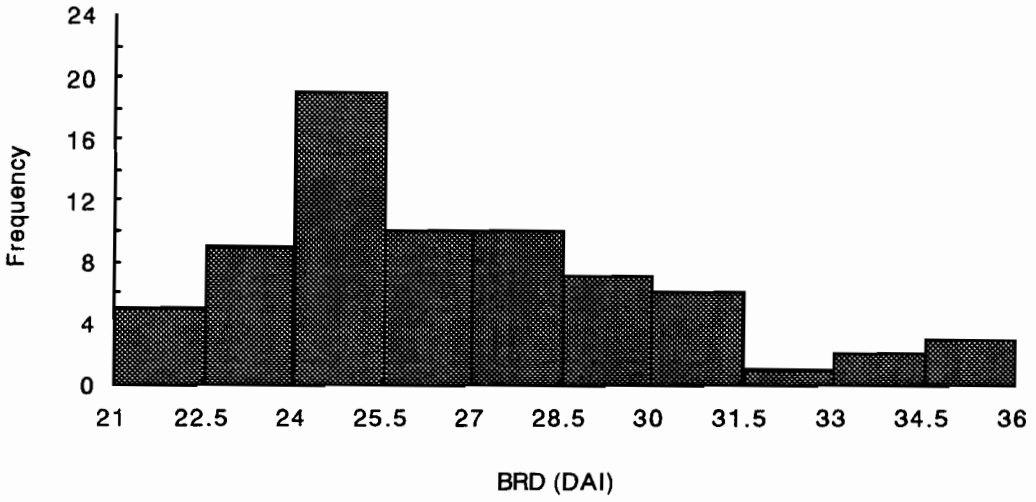

Fig. V-6. Distribution of phenotypes for BRD (developmental period of bruchid) in F3 progeny.

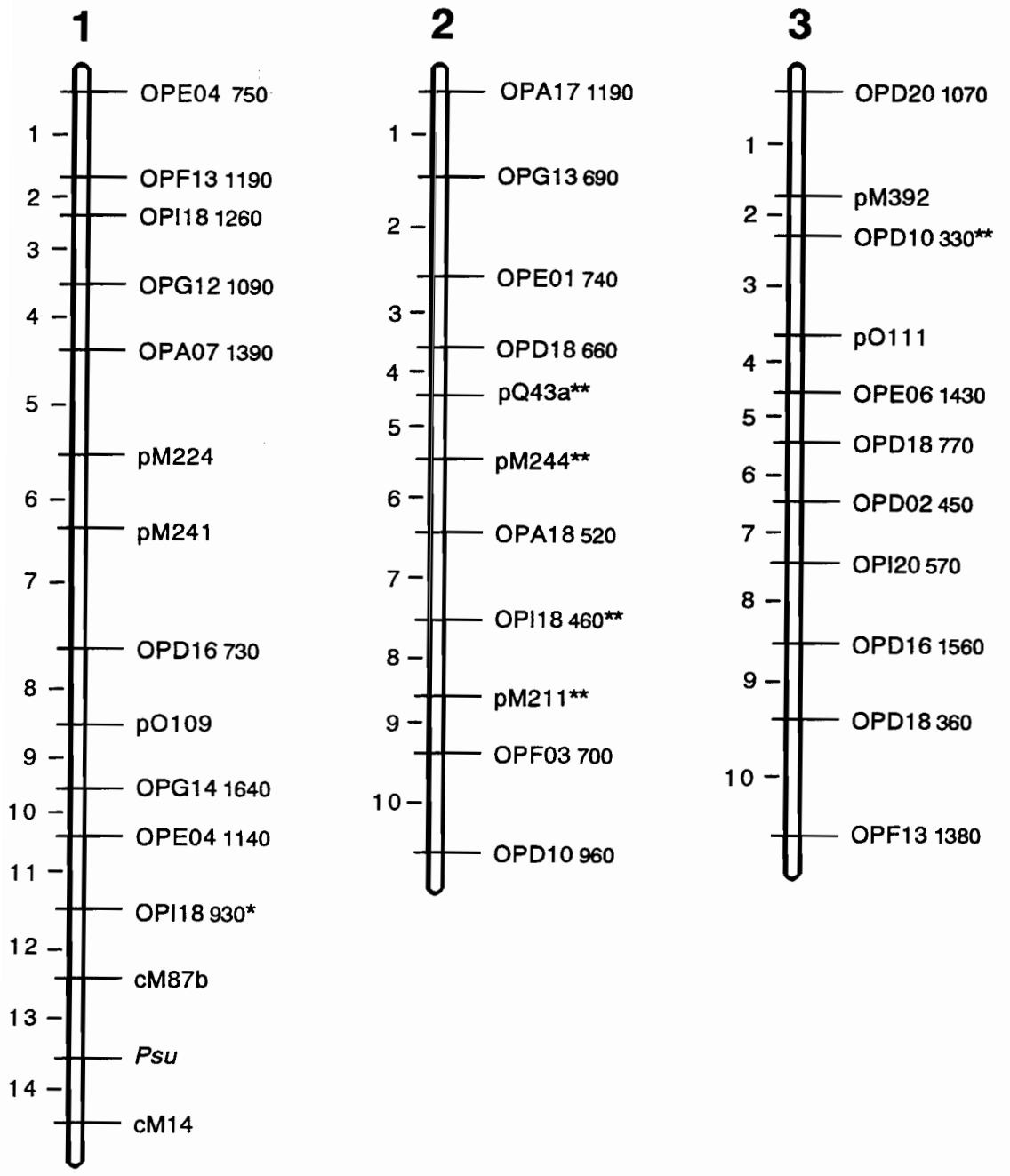

Fig. V-7. A linkage map of azuki bean used for single-marker analysis and interval mapping. Fifteen linkage groups are numbered in order of the size. Interval numbers and marker names are shown on the left and right sides of the linkage groups, respectively. Markers showing significant deviations from the expected segregation ratios at 0.05 and 0.01 level are indicated with * and **, respectively.

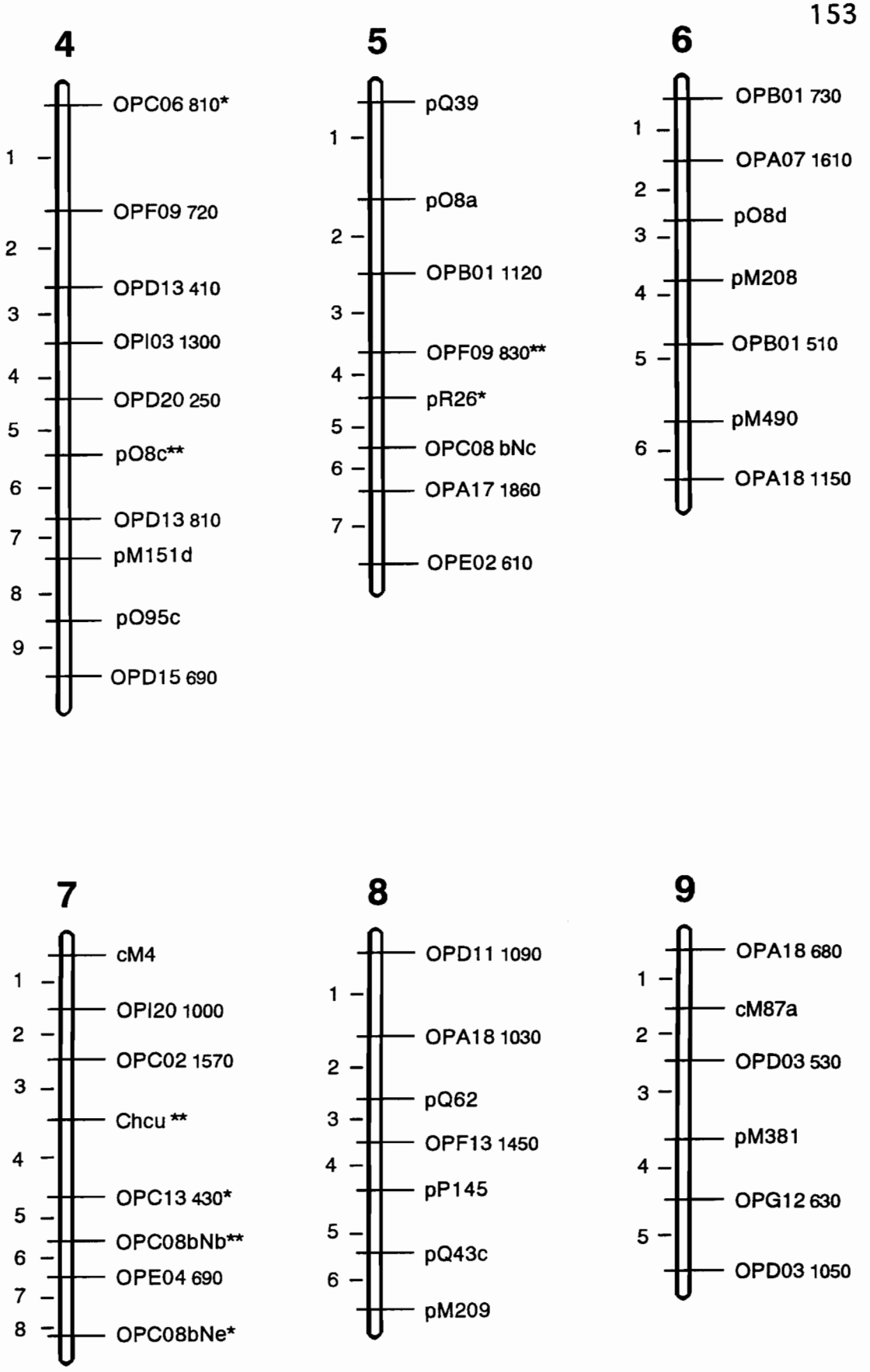

Fig. V-7. (continued)

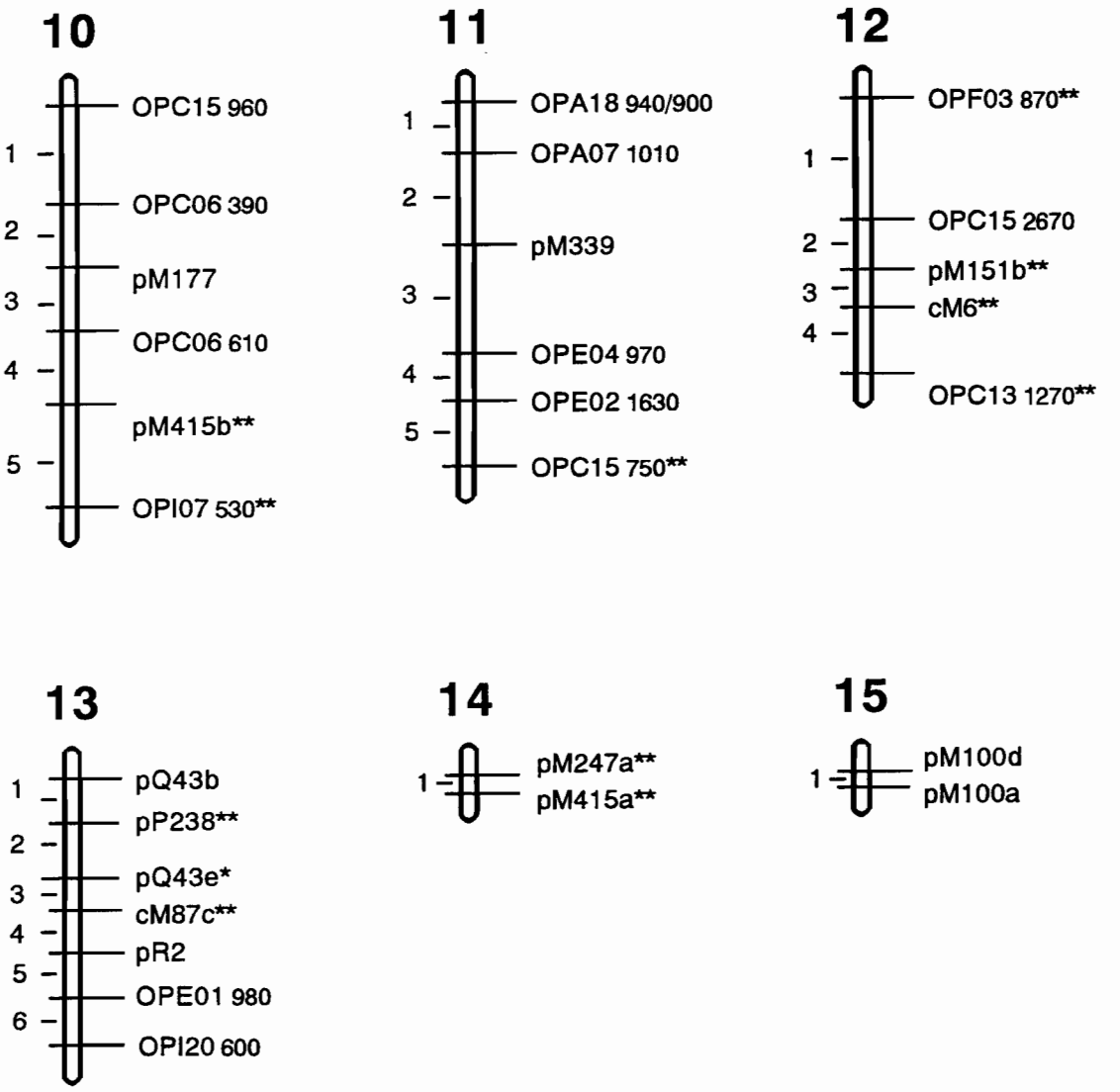

Fig. V-7. (continued)

of damaged F_3 seeds (BRC) and the developmental period of bruchid (BRD) were analyzed. By single-marker analysis, 12 locations for BRC and one for BRD were detected in linkage groups 1, 2, 3, 4, 5, 6, 7, 9, 10 and 13 and in linkage group 3, respectively (Table V-9). Using interval mapping, four QTLs (brcu1-1, brcu1-2, brcu3 and brcu5) controlling BRC were found to be located in linkage groups 1, 3 and 5. The locations and estimates of genetic effects of these QTLs are presented in Table V-10. *V. angularis* allele increased the degree of the damages by the bruchids at brcu1-1, brcu1-2 and brcu5, while *V. umbellata* allele increased at brcu3. Distinct overdominance was observed at brcu3 originated from *V. umbellata* allele. The brcu5 had the largest additive effect which accounted for 40.8% of damage increase.

Plant height

Concerning plant height, *V. umbellata* was much taller than *V. angularis* (Tables V-4 and V-11). Especially in the field conditions, *V. umbellata* was much larger than that in the pot conditions. The height of F_1 hybrid was similar to that of *V. umbellata*. In both F_2 and F_3 progenies, the unimodal distribution of PH was observed (Fig. V-8). Heritabilities in broad sense for PH estimated from F_2 and F_3 progenies were 0.99 and 0.60, respectively (Table V-12). Heritability in narrow sense for PH estimated from F_3 families was higher than that from F_3 individuals.

Based on single-marker analysis, eight locations associated with QTLs controlling PH were detected in the linkage groups 1, 3, 4, 5, 7, 9, 10 and 11 for F_3 progeny (Table V-9). By interval mapping, single QTL (phu11) was mapped on linkage group 11. The location and estimate of

Table V-9. Significant associations of markers for BRC, BRD, PH, SW and DF detected by single-marker analysis.

Trait	Linkage group	Marker	Peak	Test statistics	F(1,n-2)	pr(F)
BRC	1	3--9	3	14.654	15.605	0 ***
	1	12	12	8.524	8.752	0.004 **
	2	11	11	5.742	5.799	0.018 *
	3	6	6	5.938	6.004	0.016 *
	4	6	6	4.409	4.418	0.039 *
	5	2--6	5	38.026	46.71	0 ****
	6	2	2	7.997	8.186	0.005 **
	7	4	4	9.419	9.722	0.002 **
	9	6	6	4.94	4.966	0.029 *
	10	3	3	4.735	4.754	0.032 *
	13	4--5	4	5.219	5.256	0.024 *
	15	1--2	1	7.785	7.959	0.006 **
BRD	3	1--6	3	5.642	5.696	0.019 *
PH	1	3--7	5	7.758	7.93	0.006 **
	3	3--6	6	7.79	7.964	0.006 **
	4	9	9	4.374	4.383	0.039 *
	5	3	3	4.678	4.696	0.033 *
	7	4	4	8.672	8.912	0.004 **
	9	6	6	14.26	15.15	0 ***
	10	5--6	6	6.887	7.004	0.01 **
	11	1--5	5	11.792	12.345	0.001 ***
SW	1	3--4	4	7.327	7.471	0.008 **
	5	2--6	5	15.256	16.305	0 ***
	7	6	6	4.961	4.988	0.028 *
	11	1	1	5.642	5.696	0.019 *
	11	4		4.067	4.068	0.047 *
	15	1	1	7.693	7.86	0.006 **
DF	1	5--9	9	8.659	8.898	0.004 **
	1	13	13	6.432	6.523	0.012 *
	2	8	8	4.691	4.71	0.033 *
	3	6	6	5.518	5.567	0.021 *
	5	1--6	2	12.831	13.516	0 ***
	6	3	3	4.483	4.495	0.037 *
	7	6--8	8	4.479	4.491	0.037 *
	8	7	7	19.624	21.531	0 ****
	10	3	3	4.437	4.448	0.038 *
	10	6	6	4.297	4.304	0.041 *
	11	3	3	4.257	4.263	0.042 *
	13	4	4	4.223	4.227	0.043 *

*, **, *** and **** denote significance levels of F-test at 0.05, 0.01, 0.001 and 0.0001 probability levels, respectively.

Table V-10. The locations and parameters of QTLs based on interval mapping.

QTL	Linkage group	Interval	Test statistic	Additive effect	Dominance effect	D/A	Kind of effect
brcu1-1	1	3	21.004	41.14 A	1.369 U	0.03	ad or pd
brcu1-2	1	7	14.565	19.519 A	30.543 A	1.56	over d
brcu3	3	10	18.672	18.605 U	60.793 U	3.27	over d
brcu5	5	5	67.379	40.76 A	1.057 U	0.03	ad or pd
phu11	11	1	15.194	16.957 U	7.967 A	0.47	ad or pd
swu5	5	5	13.166	0.139 A	0.107 U	0.77	pd or d
dfu5	5	5	20.043	3.236 U	3.417 A	1.06	over d
dfu8	8	6	27.011	3.472 U	2.92 A	0.84	pd or d
plwu2	2	3	12.685	0.217 A	0.055 U	0.25	ad or pd
pllu2	2	5	15.373	0.472 A	0.22 U	0.47	ad or pd
pllu5	5	4	22.144	0.396 U	0.194 A	0.49	ad or pd

The number following the QTL designation corresponds to the linkage group in which QTL was located. The intervals on the linkage groups are shown in Fig. V-7. The letters next to the additive and dominance effect values (A: *V. angularis*, U: *V. umbellata*) indicate the origin of the allele increasing trait value and the dominant allele, respectively. Gene actions of additive or partial dominance mode, partial dominance or dominance mode, and overdominance mode are shown as ad or pd, pd or d and over d, respectively.

Table V-11. Means of six traits in *V. umbellata*, *V. angularis* and their F2 population in the pot conditions.

Trait	*V. umbellata*	*V. angularis*	Mid-parent	F2 population		
	mean	mean		mean	maximum	minimum
PH (cm)	31.66	14.33	23.00	31.82	145.00	8.50
SW (g)	1.026	2.163	1.595	1.180	1.830	0.710
DF (day)	45.66	26.00	35.83	16.29	52.00	0.00
BN (no.)	0	0	-	0	0	0
PLW (cm)	1.883	4.300	3.092	3.050	4.100	1.800
PLL (cm)	5.467	4.133	4.800	4.640	7.450	1.350

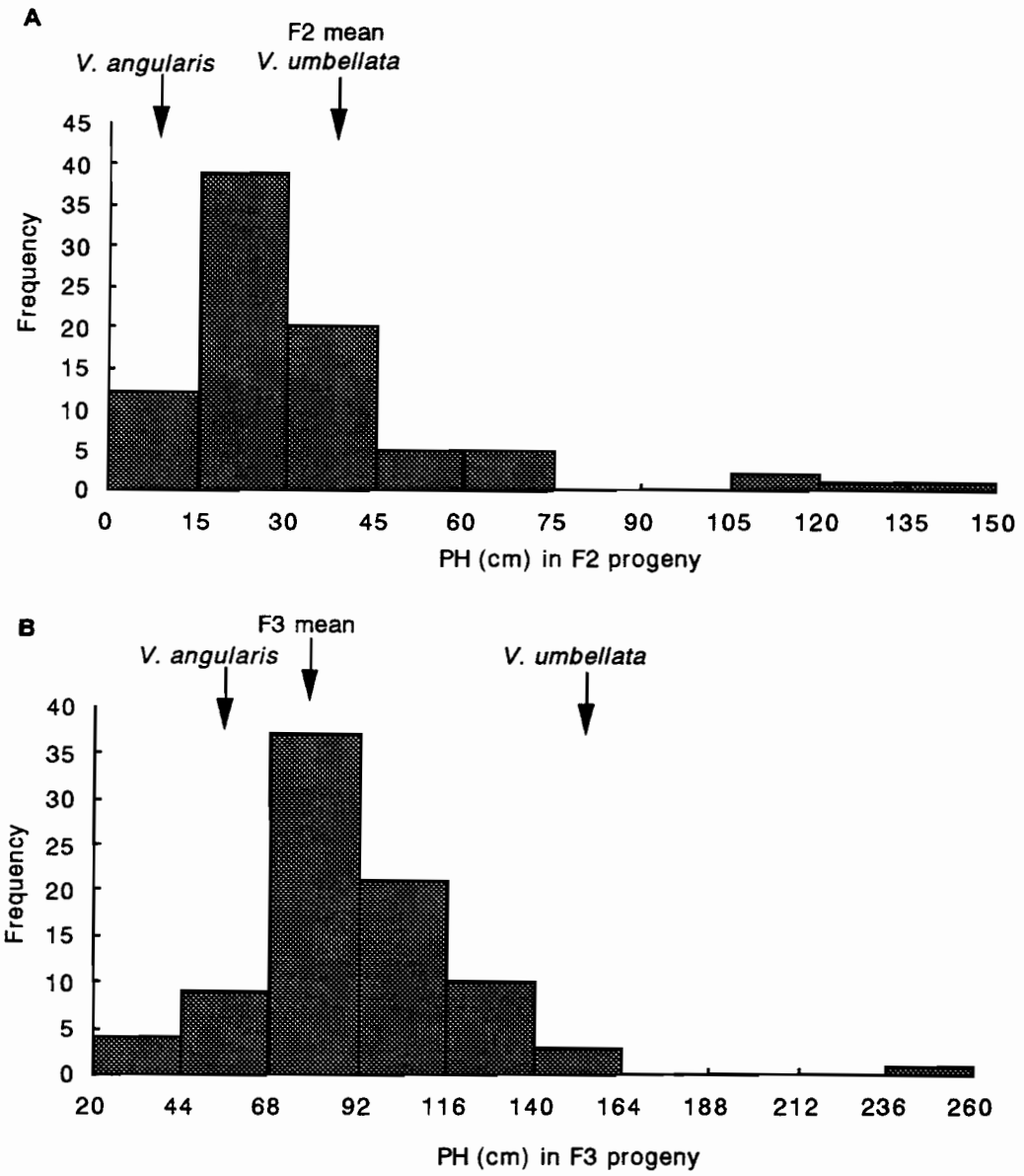

Fig. V-8. Distribution of phenotypes for PH (plant height) in F2 progeny (A) and F3 progeny (B). Means for parents and progenies are shown by arrows.

Table V-12. Estimates of variance components and heritabilities among F_2 and F_3 generations for six traits.

Variance	PH	SW	DF	BN	PLW	PLL
VF_2	643.8	0.05	202.04	-	0.36	1.48
E	3.3	0.02	76.17	-	0.06	0.19
V_a	890.3	0.0553	20.73	2.728	0.1136	0.2615
V_i	1103.7	0.0466	25.91	5.768	0.1140	0.3062
D	1327.5	0.097	24.9	0.57	0.176	0.331
H	2607.2	0.289	121.7	36.60	0.311	1.389
$\sqrt{(H/D)}$	1.40	1.73	2.21	8.01	1.33	2.05
E1	446.0	0.0133	4.48	1.051	0.0312	0.0499
E2	63.6	0.0022	0.67	0.156	0.0064	0.0093

Heritability	PH	SW	DF	BN	PLW	PLL
Broad sence						
F_2	0.99	0.70	0.62	-	0.83	0.87
F_3	0.60	0.68	0.66	0.50	0.58	0.62
Narrow sence						
F_3 families	0.75	0.82	0.60	0.10	0.77	0.63
F_3 individual	0.52	0.65	0.41	0.05	0.60	0.44

genetic effects are presented in Table V-10. *V. umbellata* allele increased PH with additive gene action.

Seed weight

Regarding 20-seeds weight, *V. angularis* is twice heavier than *V. umbellata* (Tables V-4 and V-11). In F_2 and F_3 progenies, skewed distribution toward *V. umbellata* was observed, and no seeds were as heavy as *V. angularis* in the population (Fig. V-9). Consequently, the mean values of SW for F_2 and F_3 progenies were smaller than those of mid-parent. The heritability in narrow sense estimated from F_3 families was greater than that in broad sense (Table V-12).

Based on single-marker analysis, six locations associated with QTLs controlling SW in F_3 progenies were detected in linkage groups 1, 5, 7, 11 and 15 (Table V-9). By interval mapping, single QTL (swu5) for SW was mapped on linkage group 5 (Table V-10). At the QTL position, *V. angularis* allele increased SW with additive gene effect.

Days to flowering

As for flowering time, *V. angularis* flowered 20 and 22 days earlier than *V. umbellata* (Tables V-4 and V-11). In the distribution of phenotypes for DF in F_2 and F_3 progenies, notable transgression toward early DF was observed (Fig. V-10). The genetic variations of DF were heritable in broad sense and narrow sense (Table V-12).

Twelve locations associated with QTL controlling DF in F_3 progeny were detected by single-marker analysis in linkage groups 1, 2, 3, 5, 6, 7, 8, 10, 11 and 13 (Table V-9). Using interval mapping, two QTLs (dfu5 and dfu8) controlling DF were found to be located on linkage

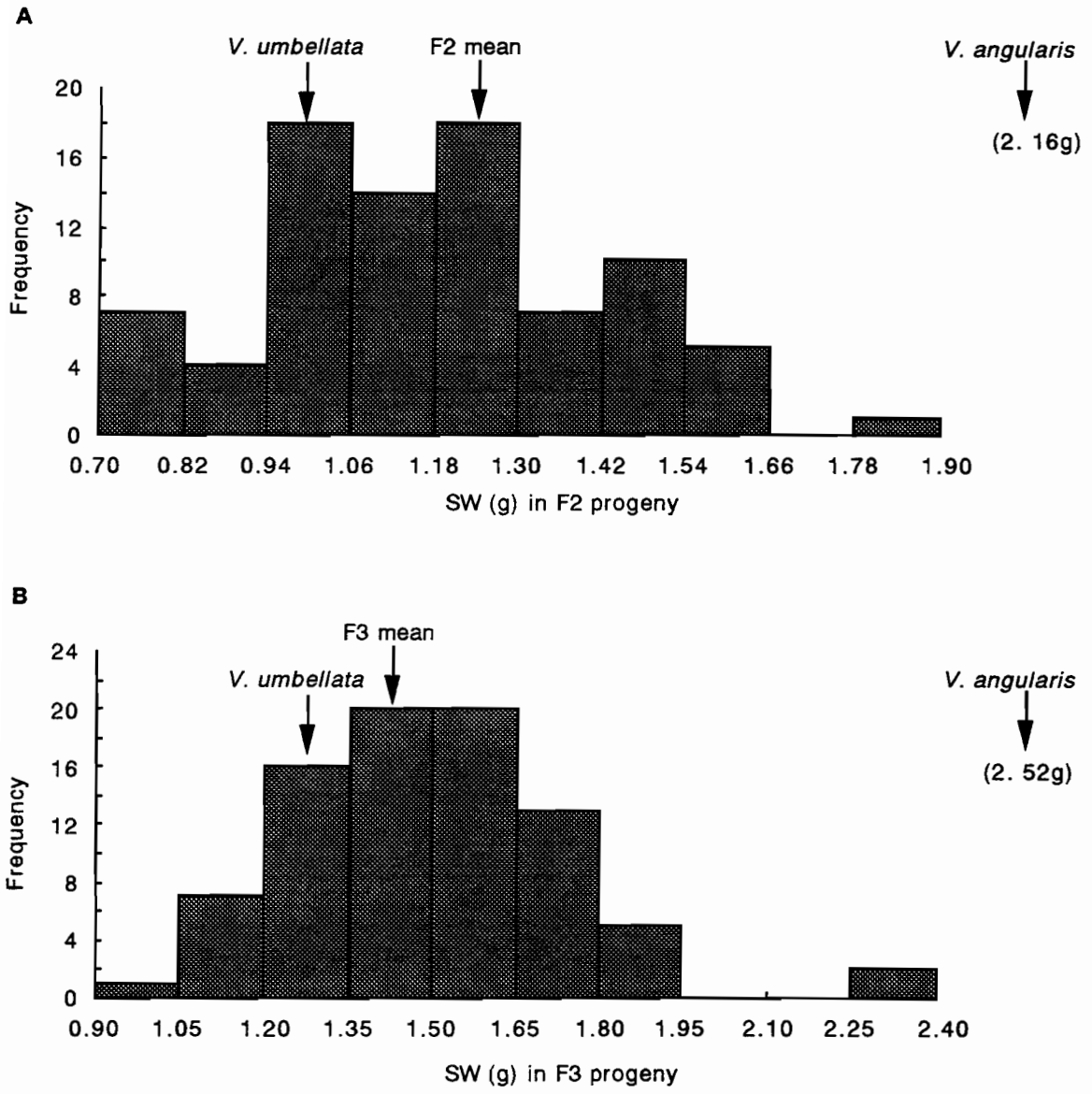

Fig. V-9. Distribution of phenotypes for SW (20-seeds weight) in F2 progeny (A) and F3 progeny (B). Means for parents and progenies are shown by arrows.

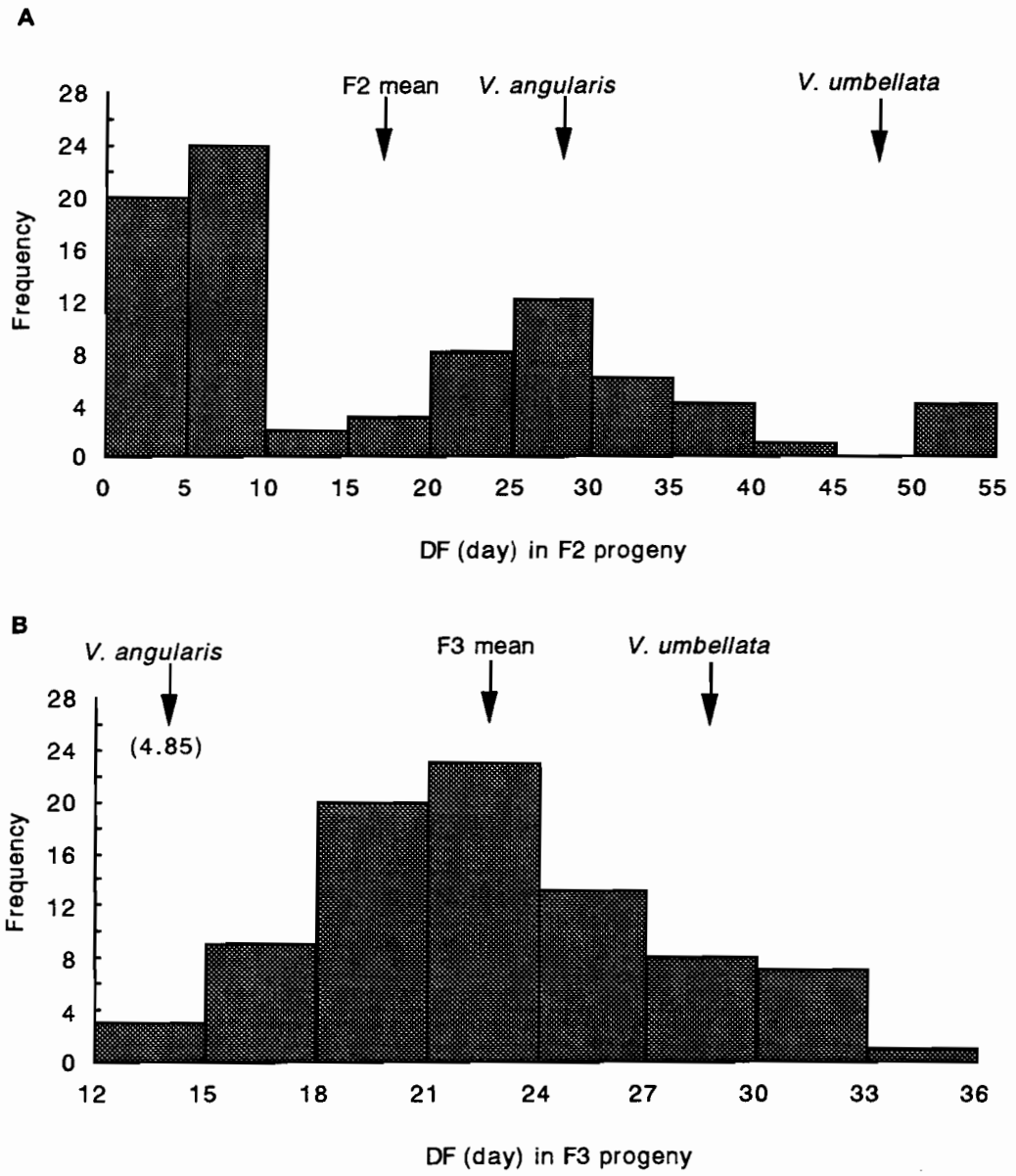

Fig. V-10. Distribution of phenotypes for DF (days to flowering) in F2 progeny (A) and F3 progeny (B). Means for parents and progenies are shown by arrows.

groups 5 and 8. The locations and estimates of genetic effects of these QTLs are presented in Table V-10. *V. umbellata* allele increased about three days at both QTL position. Overdominance was observed at dfu5 originated from *V. angularis* allele.

Number of branch

Both parents, *V. umbellata* and *V. angularis*, had no branch in pod condition. In the field condition, branch numbers of both parents increased distinctively (Tables V-4 and V-11). Trimodal distribution of BN was observed in F_3 progeny (Fig. V-11). In F_3 progeny, several phenotypes transgressed toward both directions (increasing and decreasing numbers). The heritability in narrow sense for BN was much smaller than those in broad sense (Table V-12).

Eight locations containing the markers associated with QTL controlling BN were detected by single-marker analysis in linkage groups 2, 3, 5, 8, 9, 12, 14 and 15 for F_3 progeny (Table V-13). No QTL controlling BN was detected using interval mapping.

Primary leaf characters

The shapes of primary leaves of *V. umbellata* and *V. angularis* were linear lanceolate and corodate, respectively. The ratio of PLW to PLL of *V. angularis* was greater than that of *V. umbellata* (Tables V-4 and V-11). The trimodal distributions of PLW were observed in F_2 progeny, while F_3 progeny showed unimodal distributions (Fig. V-12). On the other hand, both F_2 and F_3 progenies showed unimodal distributions of PLL (Fig. V-13). In F_2 progeny, several phenotypes appeared to transgress toward both directions. The genetic variations

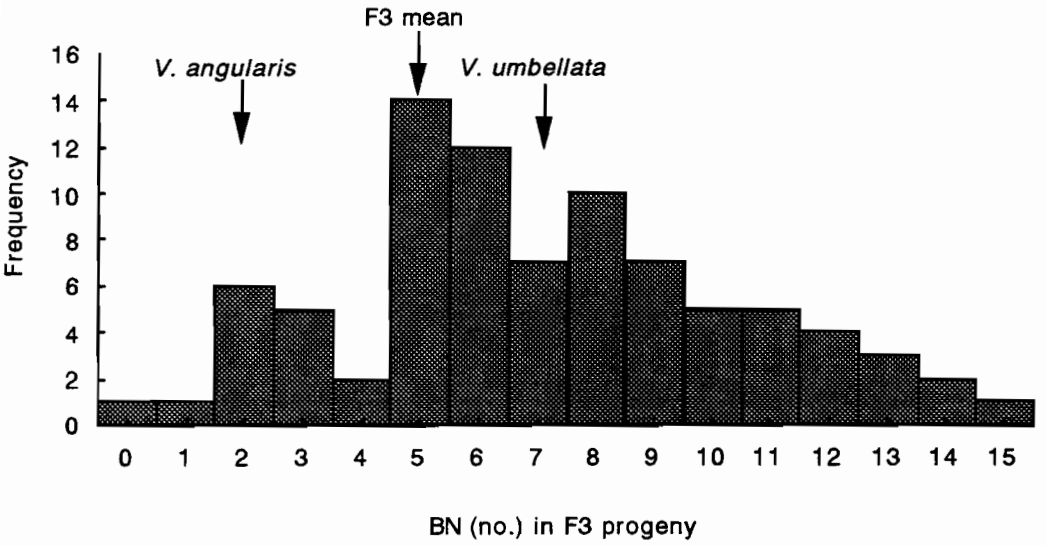

Fig. V-11. Distribution of phenotypes for BN (number of branches) in F3 progeny. Means for parents and progenies are shown by arrows.

Table V-13. Significant associations of markers for BN, PLW and PLL detected by single-marker analysis.

Trait	Linkage group	Marker	Peak	Test statistics	F(1,n-2)	pr(F)
BN	2	9	9	4.936	4.963	0.029 *
	3	6	6	8.097	8.293	0.005 **
	5	3--5	3	9.884	10.231	0.002 **
	8	6--7	7	6.499	6.594	0.012 *
	9	6	6	5.893	5.957	0.017 *
	12	2	2	6.906	7.024	0.01 **
	14	1	1	6.606	6.706	0.011 *
	15	1--2	2	6.661	6.765	0.011 *
PLW	1	1--4	4	7.707	7.876	0.006 **
	2	1--6	6	17.705	19.203	0 ****
	2	9--11	9	9.942	10.295	0.002 **
	5	2	2	5.493	5.54	0.021 *
	5	5	5	4.752	4.772	0.032 *
	8	3	3	5.239	5.276	0.024 *
	10	3--5	12	12.239	12.846	0.001 ***
	11	4	4	4.435	4.445	0.038 *
PLL	1	2--3	3	5.694	5.75	0.019 *
	1	6--9	7	5.974	6.043	0.016 *
	2	2--6	5	10.46	10.864	0.001 **
	3	6	6	10.424	10.824	0.001 **
	4	8	8	4.522	4.536	0.036 *
	5	2--6	4	17.557	19.025	0 ****
	7	5--8	6	5.883	5.947	0.017 *
	9	2	2	7.855	8.034	0.006 **
	9	6	6	4.861	4.885	0.03 *
	10	6	6	5.925	5.991	0.016 *
	13	2--4	2	9.034	9.304	0.003 **
	15	1	1	4.431	4.442	0.038 *

*, **, *** and **** denote significance levels of F-test at 0.05, 0.01, 0.001 and 0.0001 probability levels, respectively.

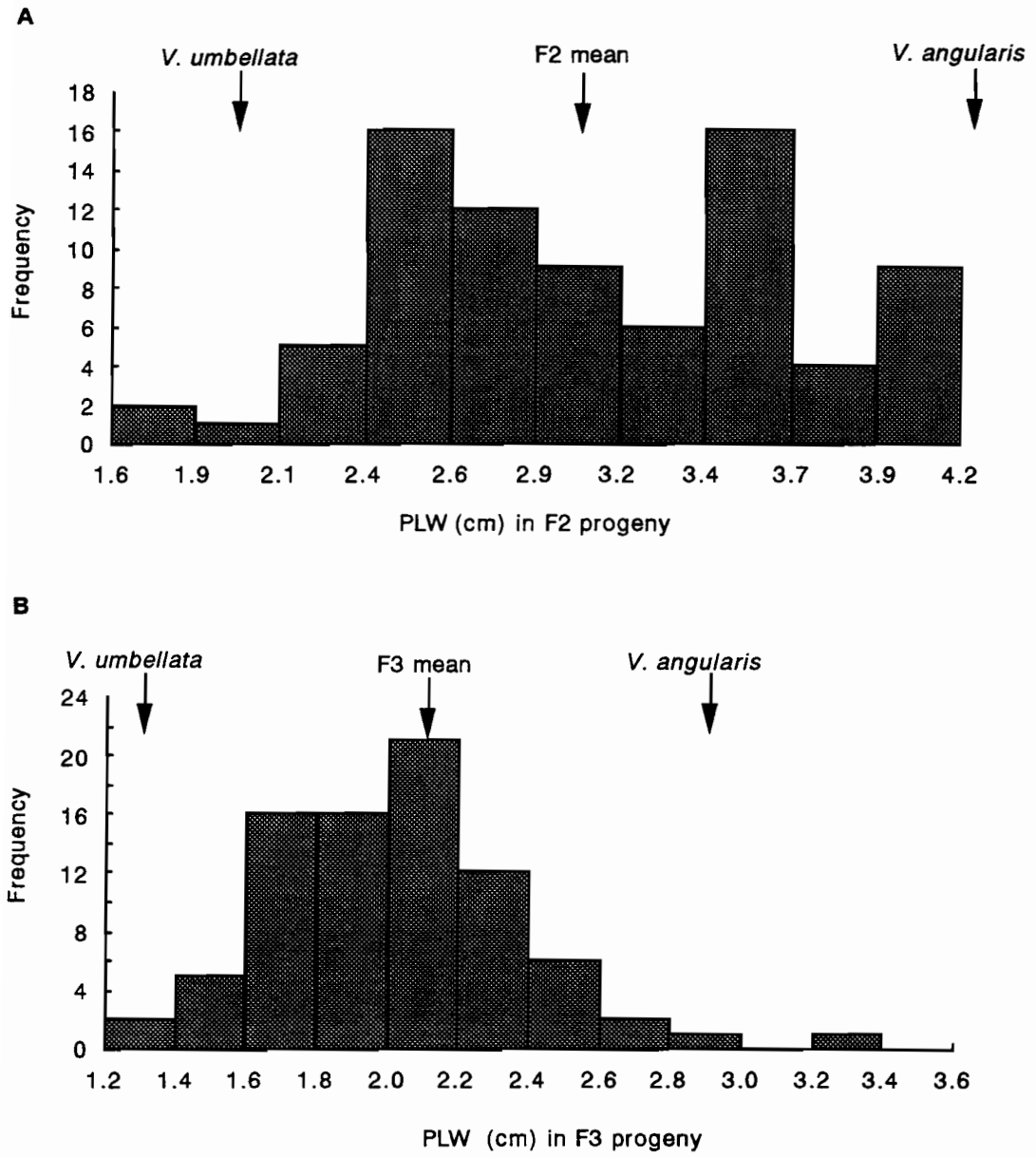

Fig. V-12. Distribution of phenotypes for PLW (primary leaf width) in F2 progeny (A) and F3 progeny (B). Means for parents and progenies are shown by arrows.

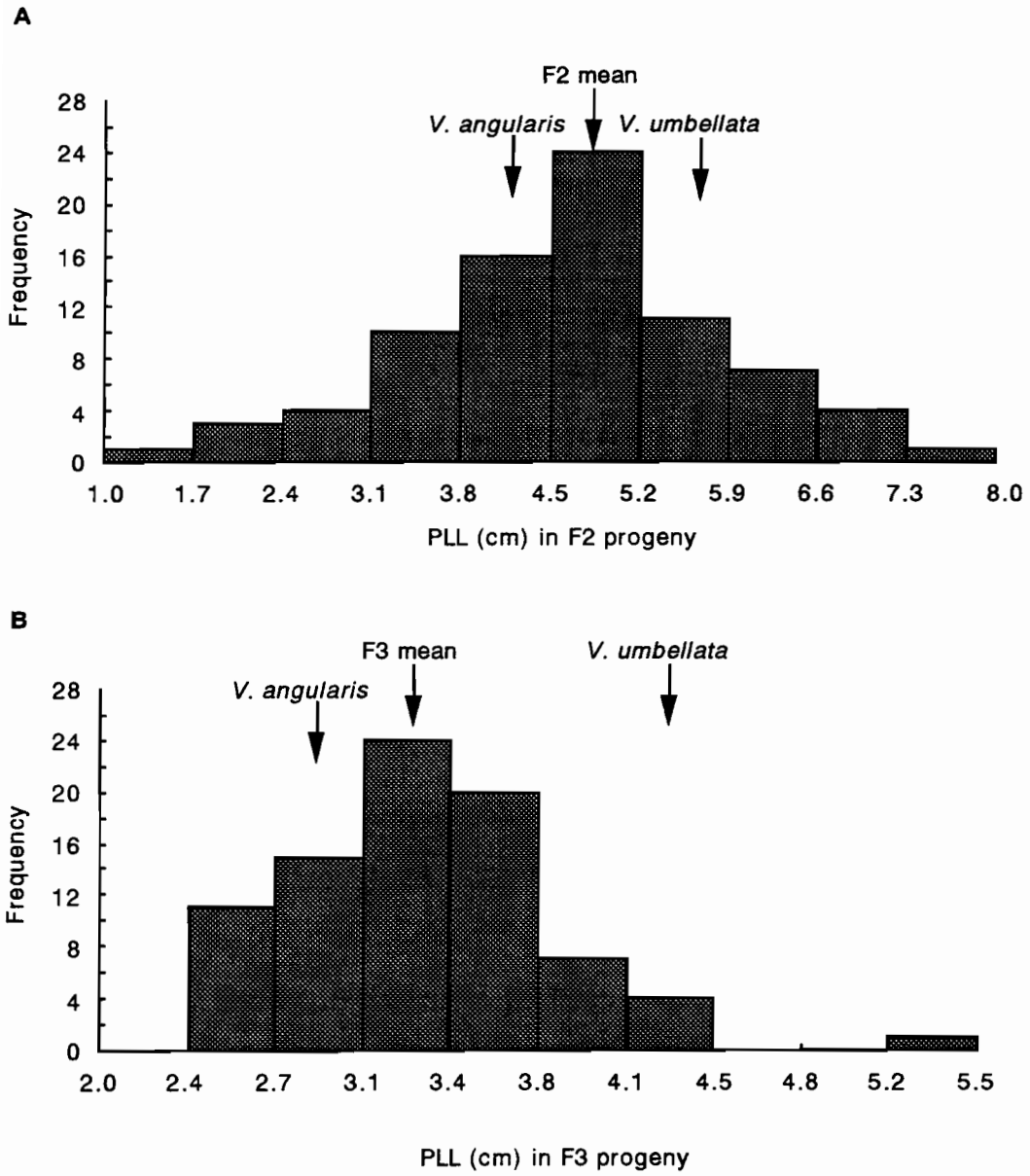

Fig. V-13. Distribution of phenotypes for PLL (primary leaf length) in F2 progeny (A) and F3 progeny (B). Means for parents and progenies are shown by arrows.

of PLW and PLL were highly heritable in broad sense and narrow sense (Table V-12).

By single marker analysis, eight locations associated with QTLs controlling PLW were detected in linkage groups 1, 2, 5, 8, 10 and 11 (Table V-13). On the other hand, 12 locations with QTLs controlling PLL were detected in linkage group 1, 2, 3, 4, 5, 7, 9, 10, 13 and 15. By interval mapping, single QTL (plwu2) for PLW and two QTLs (pllu2 and pllu5) for PLL were mapped on linkage group 2 and on linkage groups 2 and 5, respectively. The locations and estimates of genetic effects of these QTLs are presented in Table V-10. Additive or partial dominant gene action at plwu2 and pllu2 were resulted from *V. angularis* alleles, while that at pllu5 were from *V. umbellata* allele.

Relationships among QTLs

The markers significantly associated with QTLs by single-marker analysis and the QTLs detected by interval mapping for six morphological traits and two bruchid resistance related factors in F_3 progeny were presented in Fig. V-14. Among the QTLs detected by interval mapping, brcu5 for percentage of damaged seeds by bruchid, swu5 for seed weight and dfu5 for days to flowering on linkage group 5 were found to be located in the same interval. Similarly, putative locations of QTLs controlling for percentage of damaged seeds by bruchid, days to flowering and seed weight revealed by single-marker analysis were frequently overlapped on linkage group 1. High correlation coefficients were observed between percentage of damaged seeds by bruchid (BRC) and other two traits, days to flowering (negative) and 20-seeds weight (positive) (Table V-14).

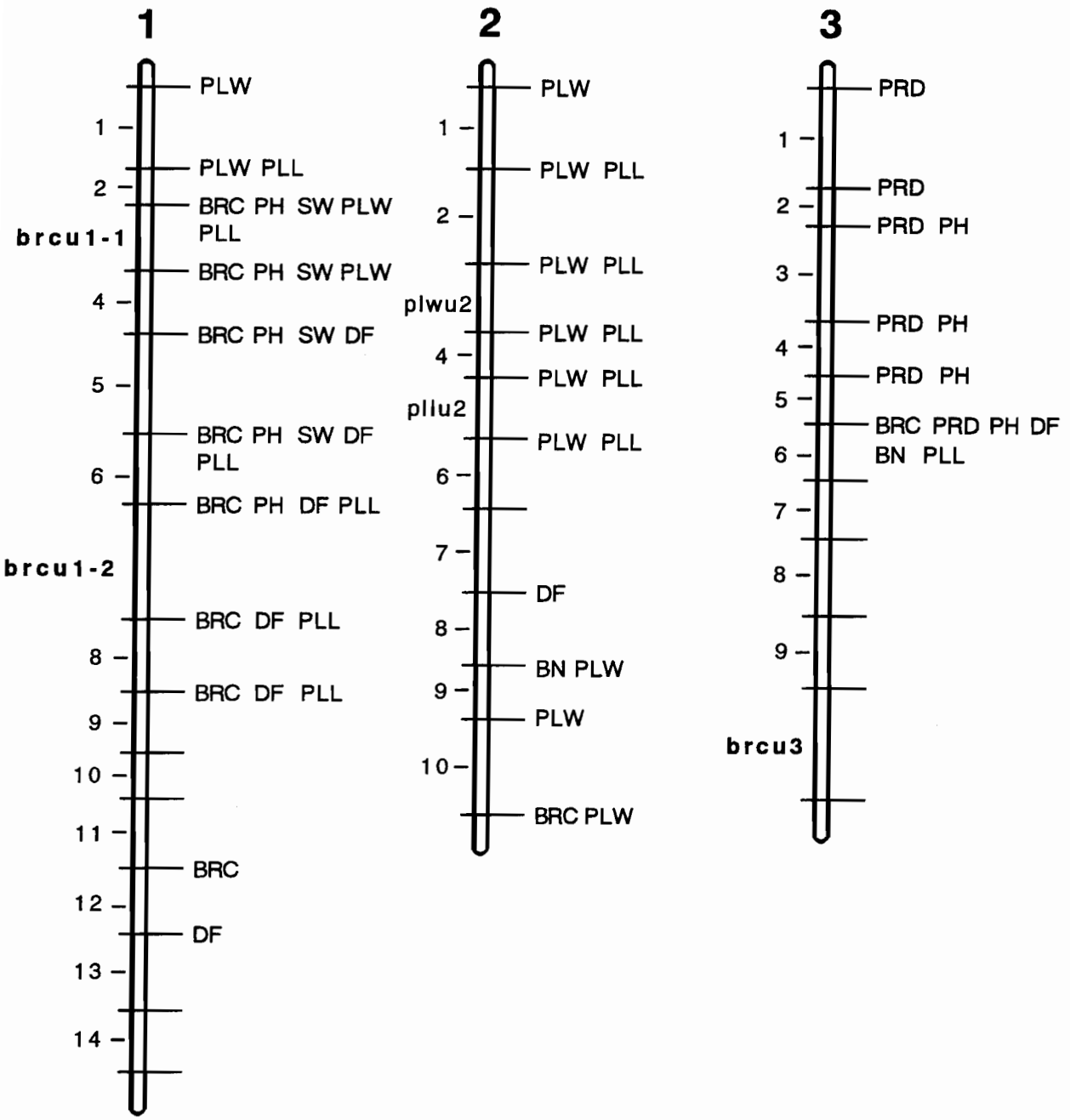

Fig. V-14. QTLs detected by interval mapping and markers siginificantly associated with QTLs identified by single-marker analysis for eight traits using F3 progeny. Their positions are indicated on the left and right sides of linkage groups, respectively.

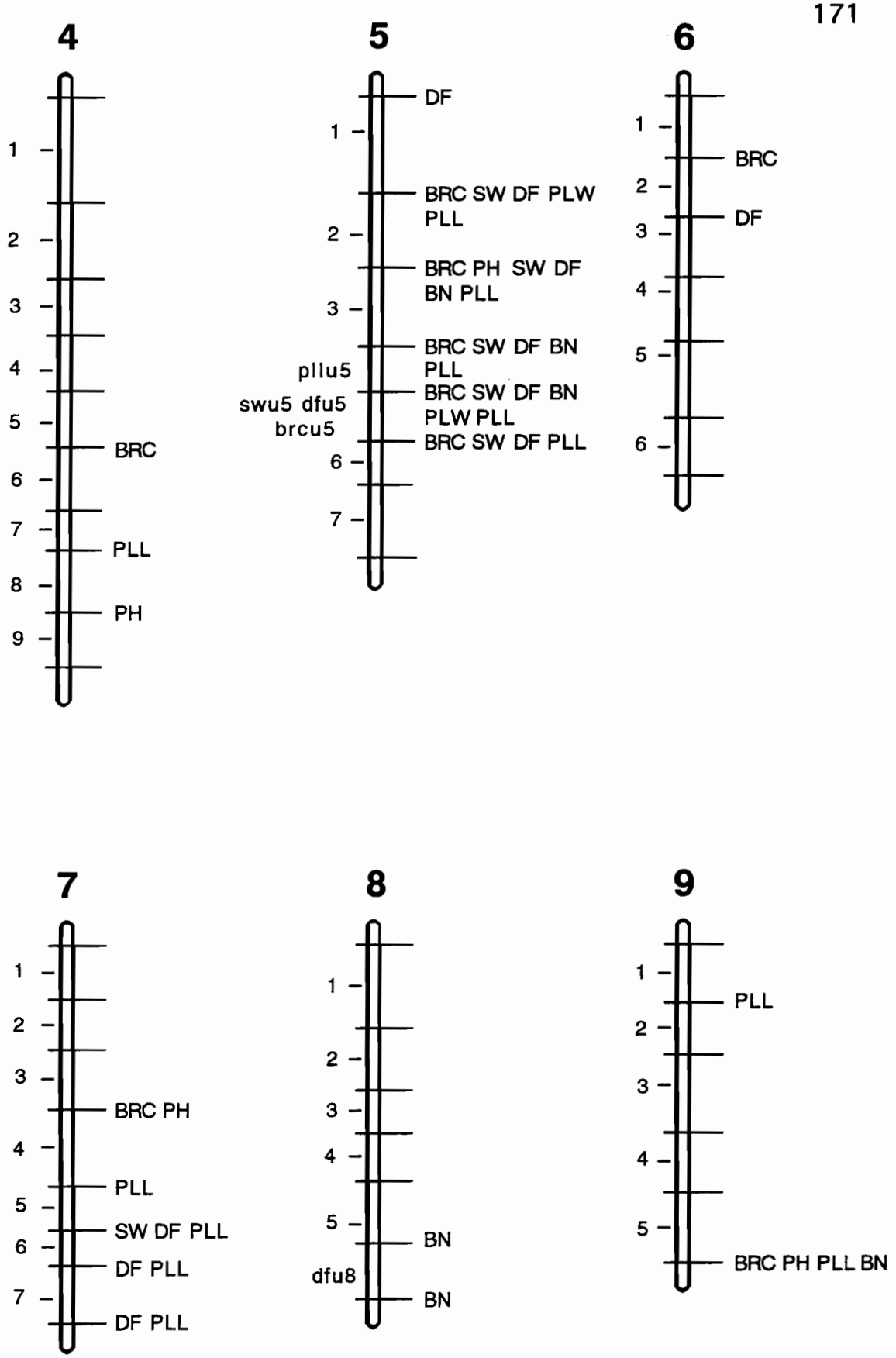

Fig. V-14. (continued)

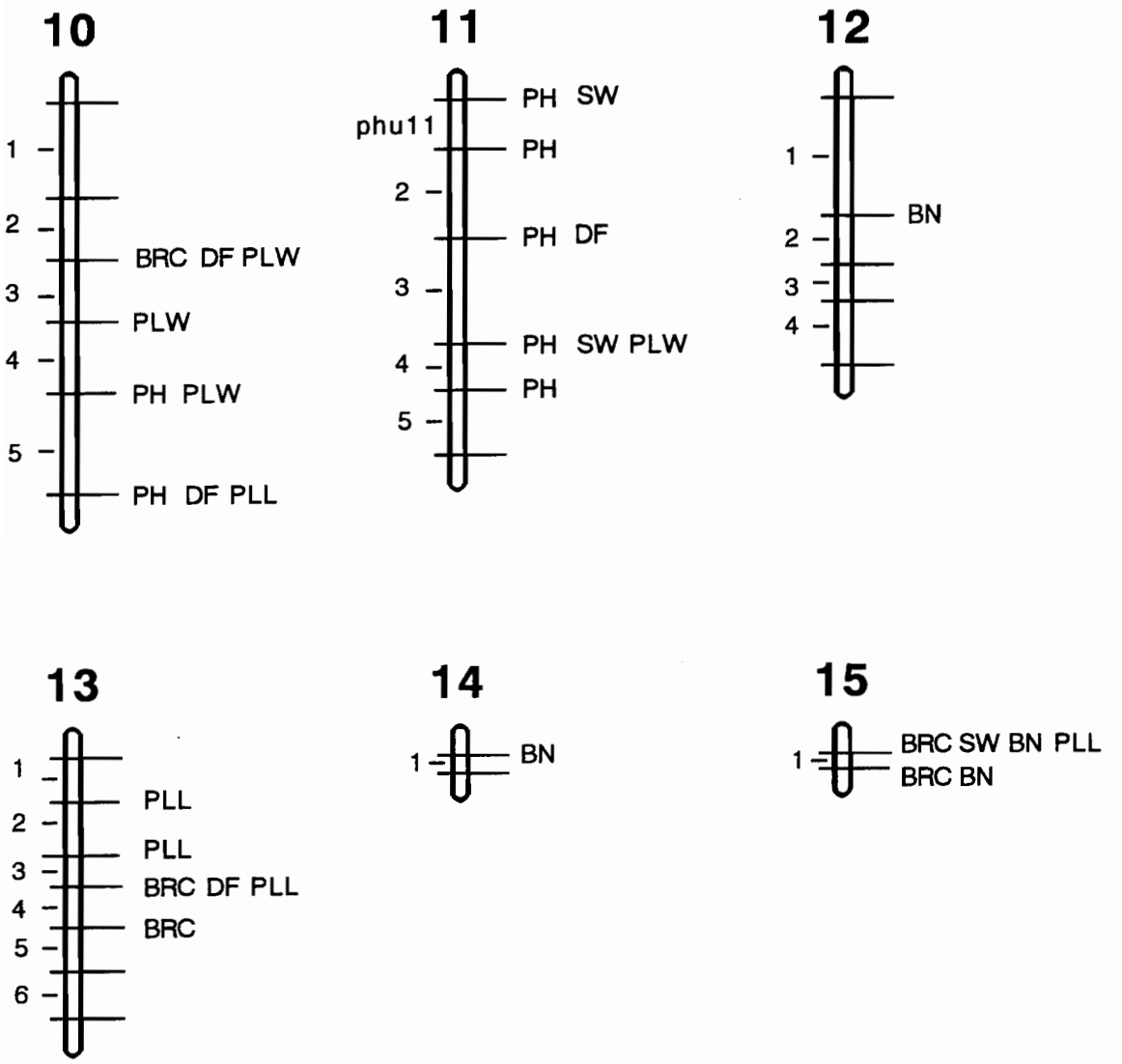

Fig. V-14. (continued)

Table V-14. Correlation coefficients (r) between eight traits in F2 and F3 progenies. *, ** and *** indicate correlations significant at p<0.05, p<0.01 and p<0.001, respectively.

Trait		SW	DF	BN	PLW	PLL	BRC	BRD
PH	F_2	0.15	0.22	-	0.15	0.12	-	-
	F_3	-0.17	-0.03	0.33 **	-0.05	0.38 **	-0.34 **	0.20
SW	F_2		-0.21	-	0.52 **	0.66 **	-	-
	F_3		-0.07	0.03	0.64 **	0.30 **	0.45 ***	-0.26 *
DF	F_2			-	-0.18	-0.23 *	-	-
	F_3			0.08	-0.21	0.25 *	-0.52 ***	0.24 *
BN	F_2				-	-	-	-
	F_3				0.07	0.12	-0.29	0.20
PLW	F_2					0.44 **	-	-
	F_3					0.26 *	0.19	-0.05
PLL	F_2						-	-
	F_3						-0.32	0.27 *
BRC	F_3							-0.42 ***

QTL analysis of categorical traits

Pod shattering

F_2 segregation of pod shattering showed a good fit to 3:1 ratio (Table V-5). The F_3 mean of pod shattering was 57.2 %, varying from 20% to 90% among F_3 lines (Fig. V-15). These results indicate that two or more genes are controlling pod shattering. By single-marker analysis using the segregant percentages, five intervals associated with QTLs controlling pod shattering were detected in linkage groups 1, 6, 8, 9 and 12 (Table V-15).

Seed color

V. umbellata and *V. angularis* had red seeds, while their F_1 plants produced green seeds. In F_2 progeny, plants having green, white and red seeds were in the ratio of 62 : 1 : 23, equivalent to 72.1 %, 1.16 % and 26.7 %, respectively. F_3 lines derived from green seeds contained plants producing green, white and red seeds, while those derived from red seeds resulted in only red-seed plants. The percentages of seed color in F_3 lines are shown in Fig. V-16. As expected, the percentages of green seed segregants were skewed to higher portion with a peak around 75%, indicating that the green seed color is partially controlled by at least one dominant gene. On the other hand, the segregant percentages of other seed colors were shifted to lower levels with a peak around 25%, indicating that the they are partially controlled by recessive genes. Using those segregant percentages, single-marker analysis was performed. Eight, six and nine locations were identified to associate with QTLs controlling the percentages of green, white and red seed colors, respectively (Table V-15). For red and green seed

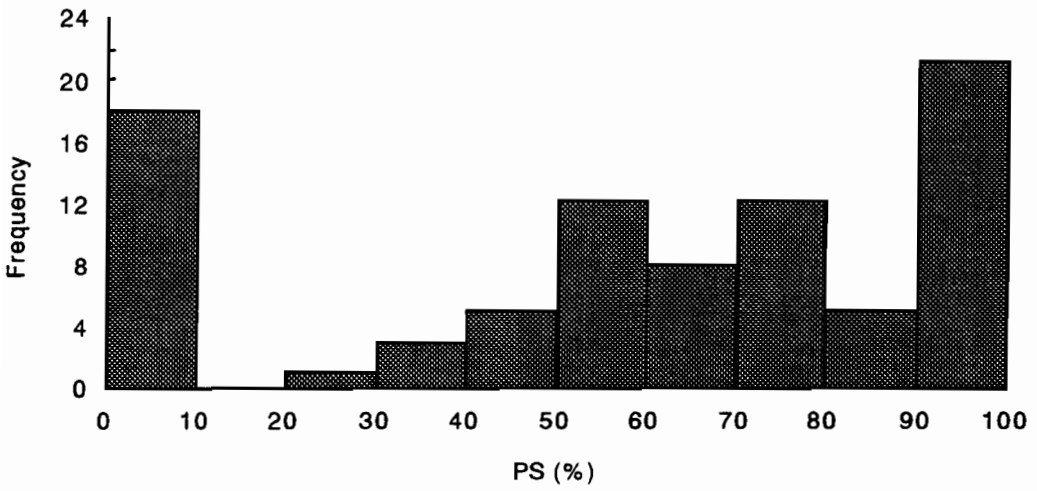

Fig. V-15. Distribution of phenotypes for PS (percetage of plants having shattering pod per line) in F3 progeny.

Table V-15. Significant associations of markers for SP, RS, GS, and WS detected by single-marker analysis.

Trait	Linkage group	Marker	Peak	Test statistics	F(1,n-2)	pr(F)
SP	1	1--4	4	5.361	5.403	0.023 *
	6	3	3	3.963	3.961	0.05 *
	8	6	6	10.211	10.589	0.002 **
	9	3	3	6.535	6.632	0.012 *
	12	3--4	3	9.201	9.485	0.003 **
RS	1	8	8	5.296	5.336	0.023 *
	1	12	12	4.159	4.163	0.044 *
	2	9	9	4.086	4.087	0.046 *
	4	6	6	9.15	9.43	0.003 **
	5	1	1	4.478	4.49	0.037 *
	7	3--8	6	76.417	120.258	0 ****
	10	6	6	4.08	4.081	0.047 *
	12	1	1	3.987	3.986	0.049 *
	13	1--7	4	76.352	120.104	0 ****
GS	1	8	8	5.266	5.305	0.024 *
	2	9	9	5.666	5.721	0.019 *
	4	6--7	6	7.375	7.521	0.007 **
	5	1	1	4.155	4.158	0.045 *
	7	4--8	6	77.161	122.032	0 ****
	11	2	2	3.977	3.975	0.049 *
	12	1--2	1	6.913	7.031	0.01 **
	13	1--7	4	73.177	112.708	0 ****
WS	1	1	1	5.641	5.694	0.019 *
	3	11	11	6.176	6.255	0.014 *
	7	2--3	3	10.281	10.667	0.002 **
	8	4	4	4.646	4.663	0.034 *
	12	2--4	4	6.155	6.232	0.014 *
	15	1	1	5.427	5.471	0.022 *

*, **, *** and **** denote significance levels of F-test at 0.05, 0.01, 0.001 and 0.0001 probability levels, respectively.

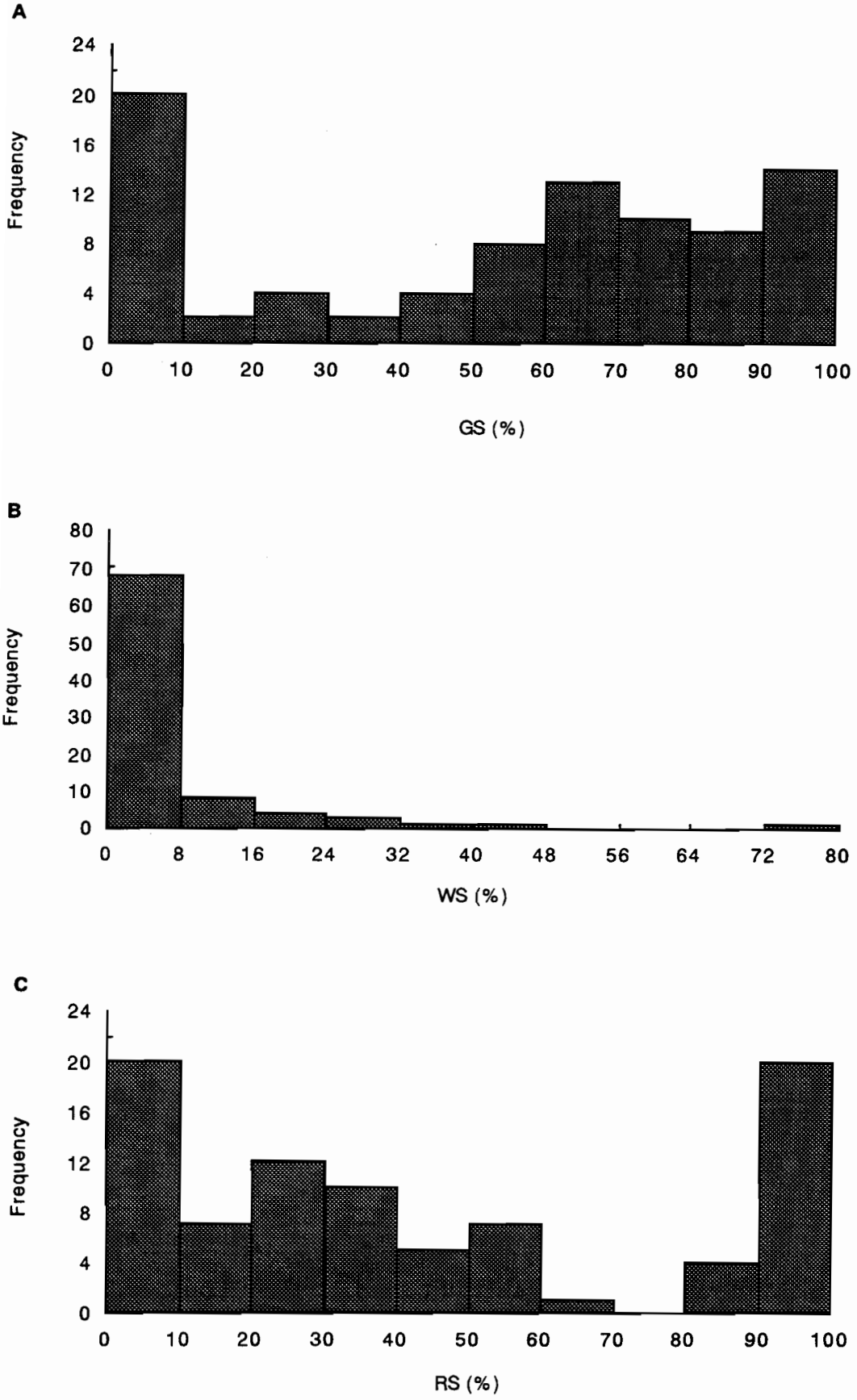

Fig. V-16. Distribution of phenotypes for GS (percetage of plants having green seeds per line; A), WS (percetage of plants having white seeds per line; B) and RS (percetage of plants having red seeds per line; C) in F3 progeny.

colors, the most significant peaks ($P<0.0001$) were observed in the same linkage groups 7 and 13.

Other characters

The germination rate (GES), abortiveness in plant development (LP and SP), sterility (SS) and seed abnormality (AS) were analyzed in F_3 progeny. The distribution of GES skewed toward high percentage, and no F_3 line showed less than 50 % germination rate (Fig. V-17A). The sterile plants (SS) and seed abnormality (AS) were observed in 31 and 17 F_3 lines, respectively (Fig. V-17B and C). Nine F_3 lines showed both sterile and abnormal seed phenotypes. Regarding abnormality in plant development, lethal (LP) and stunted phenotype (SP) were observed in 33 and 31 F_3 lines, respectively (Fig. V-18). Among them, 15 F_3 lines showed both phenotypes.

Significant associations of markers for GES, LP, SP, SS and AS based on single-marker analysis were presented in Table V-16. Some QTLs controlling AS were found at the marker loci which showed abnormal segregation on linkage group 5, 7, 13 and 14 (Fig. V-19). Putative QTLs for LP were found to be located in common on linkage groups 2 and 13.

Discussion

Bruchid resistance

In this study, foreign genes of rice bean (*V. umbellata*) including bruchid resistance were introduced to azuki bean by interspecific hybridization. Some of their progenies showed the bruchid resistance

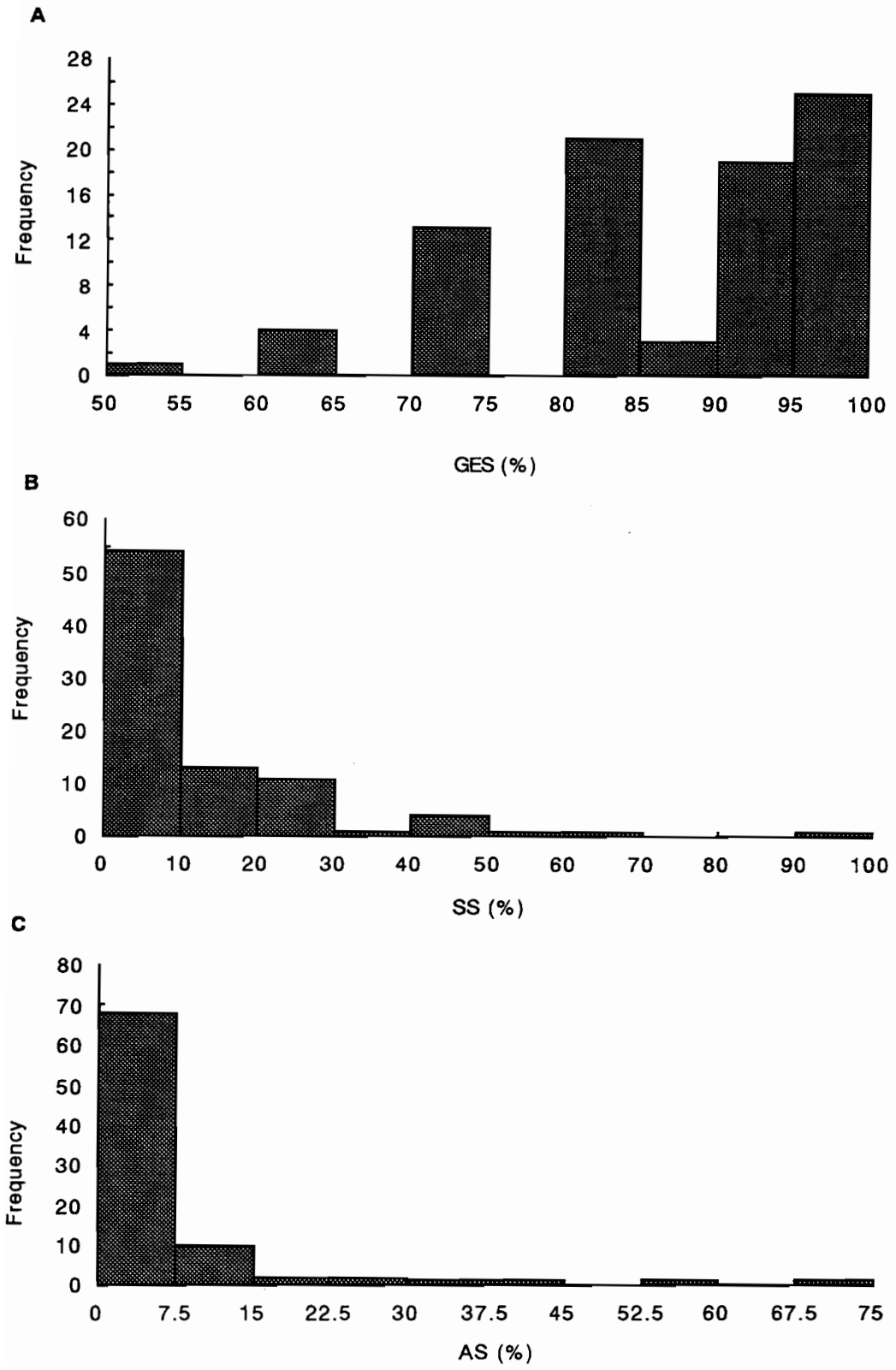

Fig. V-17. Distribution of phenotypes for GES (mean percetage of germinated seeds per line; A), SS (percetage of sterile plants per line; B) and AS (percetage of plants which produced abnormal seeds per line; C) in F3 progeny.

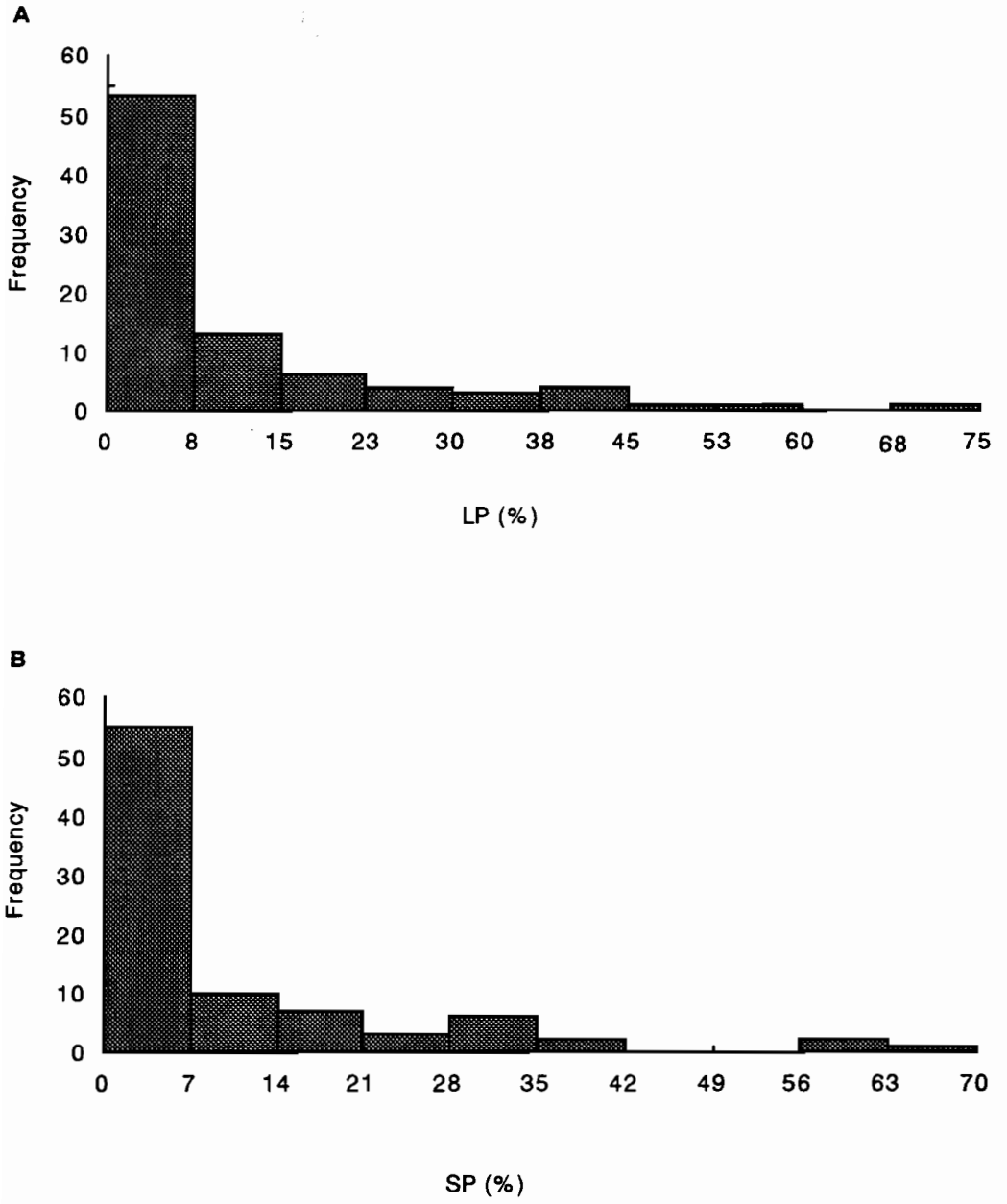

Fig. V-18. Distribution of phenotypes for LP (percetage of lethal phenotype per line; A) and SP (percetage of stunted phenotype per line; B) in F3 progeny.

Table V-16. Significant associations of markers for GES, LP, SP, SS and AS detected by single-marker analysis.

Trait	Linkage group	Marker	Peak	Test statistics	F(1,n-2)	pr(F)
GES	1	6	6	4.707	4.726	0.033 *
	1	9	9	6.1	6.174	0.015 *
	8	6--7	7	5.306	5.346	0.023 *
	10	4	4	10.691	11.119	0.001 **
	12	3	3	7.066	7.193	0.009 **
LP	1	4	4	4.448	4.459	0.038 *
	2	1	1	5.445	5.491	0.021 *
	3	11	11	7.933	8.117	0.006 **
	7	5--6	6	5.935	6.002	0.016 *
	11	2--3	2	8.173	8.374	0.005 **
	13	1--7	1	10.88	11.329	0.001 **
	15	1	1	8.104	8.301	0.005 **
SP	3	11	11	4.462	4.473	0.037 *
	5	3	3	4.906	4.932	0.029 *
	9	6	6	5.126	5.159	0.026 *
	11	1--5	2	10.914	11.366	0.001 **
SS	1	15	15	4.096	4.098	0.046 *
	6	1--4	1	5.504	5.551	0.021 *
	7	4	4	4.14	4.142	0.045 *
	9	6	6	5.826	5.888	0.017 *
	11	1--2	1	10.074	10.44	0.002 **
	12	5	5	4.518	4.531	0.036 *
AS	2	1--4	2	14.676	15.63	0 ***
	2	7--8	8	9.788	10.125	0.002 **
	3	8	8	5.158	5.192	0.025 *
	5	5--6	5	5.352	5.393	0.023 *
	6	7	7	5.281	5.32	0.024 *
	7	4	4	7.9	8.082	0.006 **
	13	1--2	2	4.953	4.979	0.028 *
	14	1--2	2	4.878	4.902	0.03 *

*, **, *** and **** denote significance levels of F-test at 0.05, 0.01, 0.001 and 0.0001 probability levels, respectively.

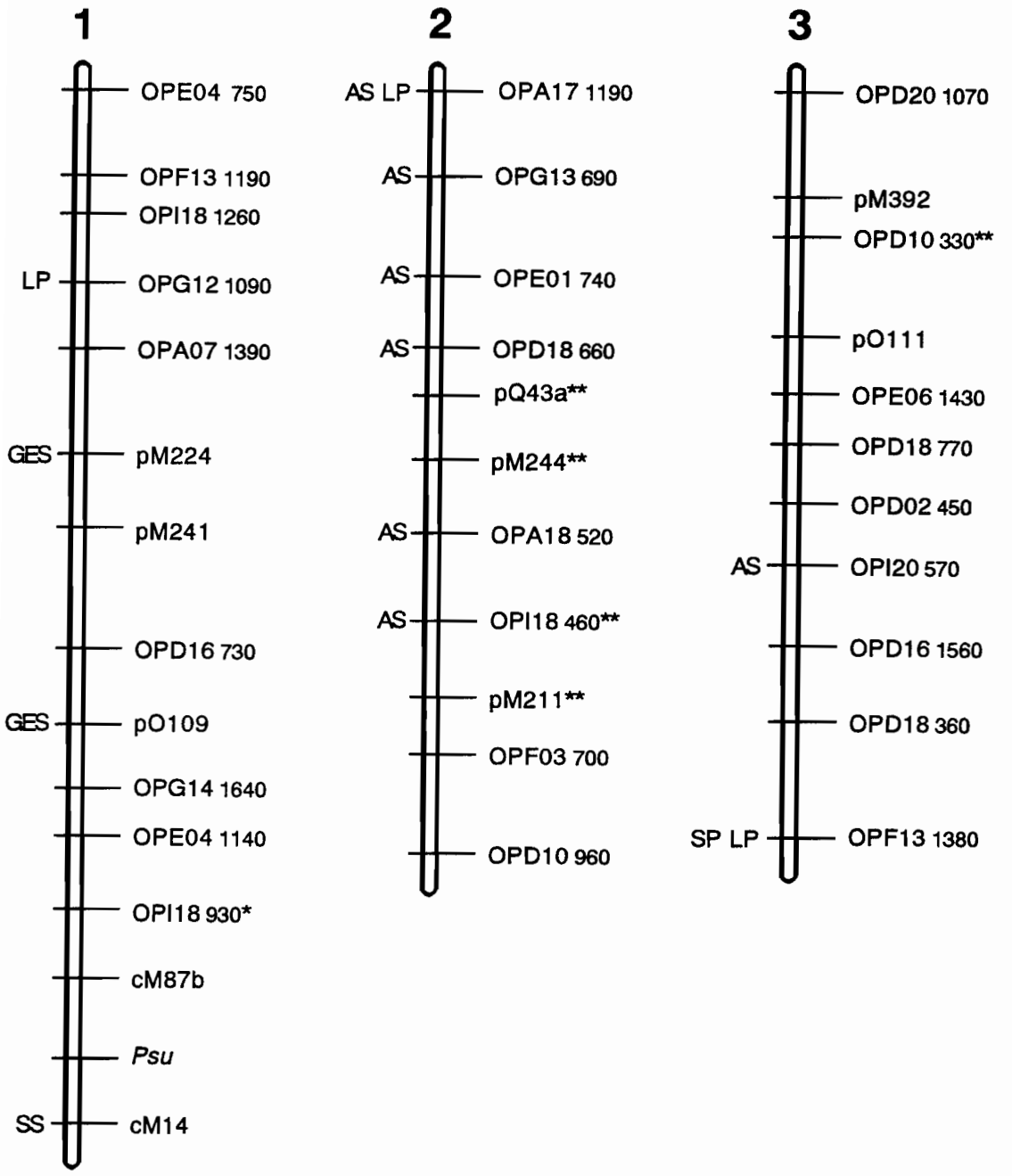

Fig. V-19. Marker loci having significant association to abnormal segregating factors based on the single-marker analysis. Their positions are indicated on the left side of linkage groups. Markers showing significant deviations from the expected segregation ratios at 0.05 and 0.01 level are indicated with * and **, respectively.

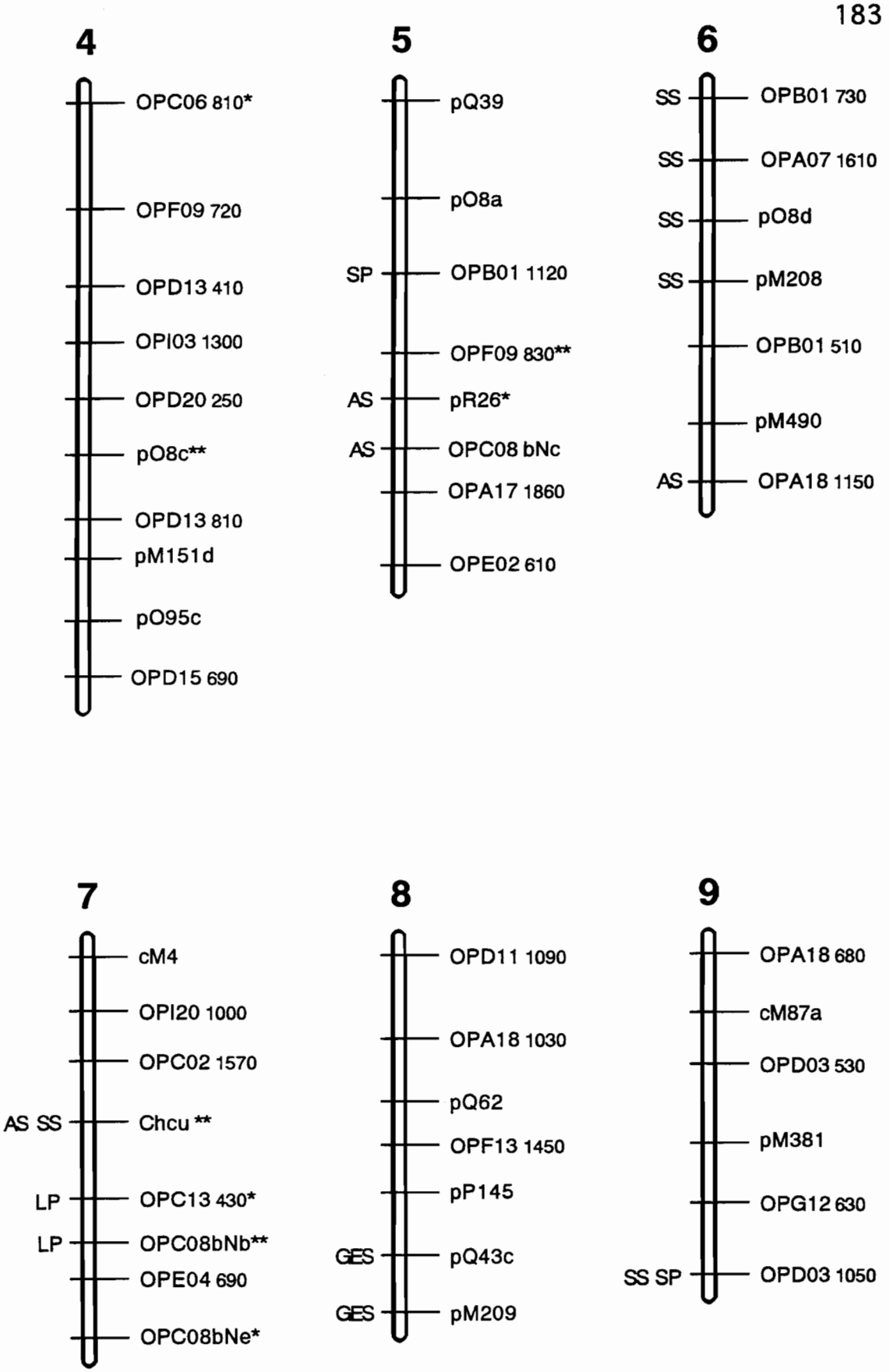

Fig. V-19. (continued)

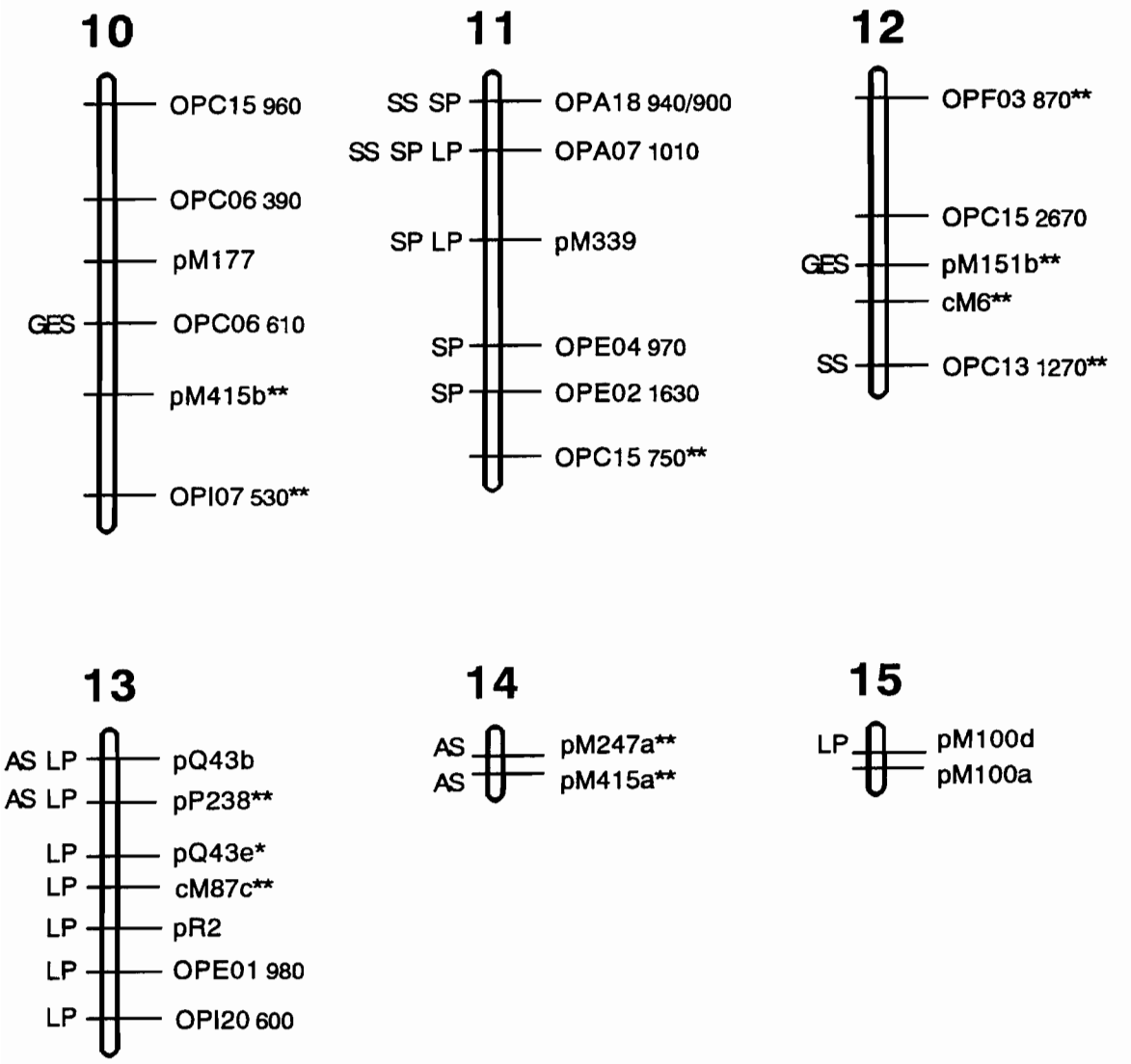

Fig. V-19. (continued)

which was governed by polygenes with additive effects (Fig. V-5). By using QTL mapping, the additive effects was shown to be mainly due to the *V. umbellata* allele of major QTL, brcu5 (Table V-10). The well-known bruchid resistance of wild mungbean accession 'TC1966' is controlled by a single dominant gene (Kitamura et al. 1988), while there is a bruchid resistance of mungbean accession 'V2802' which is controlled by polygenes (Watanasit and Pichitporn 1995). The dominant gene for bruchid resistance of wild mungbean was found to be linked with one mungbean RFLP marker, pR26, which was also used in this study. Interestingly, brcu5 from *V. umbellata* was found to be located in the interval adjacent to pR26. This suggests these resistant genes might be in the same locus and have similar defense mechanisms.

In this study, the factors that affecting the bruchid resistance were analyzed and correlation between the proportion of damaged seed (BRC) and other traits was also examined (Table V-14). The seed damage rate by the bruchids was positively correlated with 20-seeds weight (SW) and days to flower (DF). Based on the single-marker analysis, several putative QTLs controlling BRC, SW and DF were found to be in the same locations (linkage groups 1 and 5). By interval mapping, brcu5, swu5 and dfu5 were mapped in the same interval of linkage group 5 (Table V-10). These results indicate that the linkage among these traits were complicated. In order to establish the best combination with desirable characters, further investigation will be needed around these QTL regions using more markers with large size of population.

Linkage map construction

With interspecific population of *V. umbellata* and *V. angularis*, a genetic linkage map was constructed (Fig. V-3). It was composed of 15 linkage groups including 95 RAPD, 61 RFLP and three morphological markers. This linkage map covered 2001.4 cM with average distance of 13.9 cM between markers. Although the number of linkage groups did not coincide with 11 of basic chromosome number of azuki bean and rice bean, the total size is larger than those of the other linkage maps of *Vigna* species (Menancio-Hautea et al. 1993b; Fatokun et al. 1993). Since one third of RAPD markers (54 out of 149 markers) remained unlinked, there are still gaps or uncovered region in the genome. In this study, most of RFLP markers (61 out of 66) could be localized in azuki linkage map. However, they were previously mapped on mungbean or cowpea linkage maps which did not cover their whole genome. Therefore, in order to integrate some linkage groups and to construct saturated map, new RFLP markers or other molecular markers should be added.

Homologous genes

Since two azuki molecular linkage maps (*V. umbellata* x *V. angularis* and *V. angularis* x *V. nakashimae* linkage maps) were constructed mainly with RAPD markers, it is difficult to compare the entire regions of their maps. However, part of the maps could be compared based on the information on common markers, such as RFLP markers and RAPD markers from *V. angularis*. In several regions, the order of the markers was consistent between two maps (Table V-17). Especially, linkage groups 5, 11 and 15 of *V. umbellata* x *V. angularis*

Table V-17. Comparison of linkage groups of RFLP and RAPD markers among four *Vigna* linkage maps.

Marker	Linkage group			
	V. umbellata x *V. angularis*	*V. angularis* x *V. nakashimae* [a]	Mungbean[b]	Cowpea[c]
OPE01 1100*	1	14	-	-
cM14	1	-	2	-
pM224	1	-	2	-
pM241	1	4	2	3
pO109	1	4	13	-
pP170	1	4	-	3
Psu	1	4 (Ps)	-	-
pM211	2	6	10	8
pQ117	2	6	4	-
pM244	2	-	4	-
pQ43a	2	-	2	-
OPC02 300*	3	3	-	-
OPD02 450*	3	6	-	-
OPD16 1560*	3	1	-	-
pM392	3	-	6	-
pO95b	3	-	-	5
pM151a	4	-	9&10	5
pM151c	4	-	9&10	5
pM151d	4	-	9&10	5
pO95c	4	-	-	5
pO91	4	unlinked	5	-
pR51	4	-	5	-
pR25	4	-	5	6
pQ43d	4	-	2	-
pQ45	4	-	2	-
pO8c	4	-	8	-
OPD20 1010*	5	5	-	-
OPD20 1560*	5	5	-	-
OPG10 1030*	5	5	-	-
pQ86	5	5	8	-
pQ39	5	-	8	-
pO8a	5	-	8	-
pO103	5	-	1	2
pR26	5	-	9	-

Table V-17. (continued).

Marker	Linkage group			
	V. umbellata x *V. angularis*	*V. angularis* x *V. nakashimae* [a]	Mungbean[b]	Cowpea[c]
OPA07 1610*	6	9	-	-
Iscu	6	2 (Isc)	-	-
pM208	6	2	7	4
pO8d	6	-	8	-
Chcu	7	7 (Chc)	-	-
cM4	7	9	7	4
OPE06 1060*	8	11	-	-
pQ62	8	11	8	-
pM209	8	-	-	8
pP145	8	-	-	3&4
pQ43c	8	-	2	-
pM381	9	-	1	-
OPC06 390	10	3	-	-
OPC06 610	10	3	-	-
pM177	10	3	3	-
pM415b	10	3	3	-
pO26	10	3	3	1
pM247b	10	-	7	-
pO95a	10	-	-	5
OPA07 1010*	11	5	-	-
OPE02 1630*	11	5	-	-
pM339	11	-	11	-
OPC13 600*	12	1,3	-	-
cM6	12	-	12	-
pM151b	12	-	9&10	5
pM186	13	7	-	4
pP238	13	7	1	1
pR2	13	-	1	-
pQ43b	13	-	2	-
pQ43e	13	-	2	-
pM247a	14	-	7	-
pM415a	14	3	3	-
pM100a	15	5	8	-
pM100c	15	5	8	-
pM100d	15	5	8	-

* : RAPD marker. - : not examined.

a) Mapped in Chapter III. b) Mapped by Menancio-Hautea et al. (1993a).

c) Mapped by Fatokun et al. (1993).

map included sets of the markers mapped on linkage group 5 of *V. angularis* x *V. nakashimae* map, indicating that these three linkage groups may belong to the same linkage group. The disagreement of the marker locations was still found in some linkage groups. However, this might be due to the insufficient number of markers in the *V. angularis* x *V. nakashimae* linkage map.

Two pairs of genes from different origins with similar function were mapped in both azuki molecular maps. The first pair, i.e. two dominant genes, *Ps* from *V. nakashimae* and *Psu* from *V. umbellata,* controlling the expression of purple pigmentation on epicotyl, had linkage with a common RFLP marker 'pO109' on the respective maps (Table V-17). Regarding the genes for antocyanin pigmentation, similar relationships were found in pea and lentil by Weeden et al. (1992). In addition, they showed that these genes were derived from the homologous regions. The second pair of morphological genes were *Isc* from *V. nakashimae* and *Iscu* from *V. umbellata,* which have hypostatic effect for intensity of pigmentation on stem. Both genes were mapped in the position adjacent to common RFLP marker 'pM208' (Table V-17). These results suggest that these two pairs of genes might be located in homologous region.

Similarly, the locations of QTLs controlling the same traits were compared using the two azuki linkage maps. Since the morphological appearance varied under the different planting conditions, the comparison was carried out only for the marker locations associated with QTLs using F_3 progenies under the field conditions. Several markers were detected to associate with the same QTLs using two different interspecific populations (Table V-18). One of the

Table V-18. RFLP and RAPD markers associated with QTLs controlling common traits in both interspecific populations of azuki bean.

Marker	Linkage group		Trait					
	V. umbellata x *V. angularis*	*V. angularis* x *V. nakashimae*	PH	SW	DF	BN	PLW	PLL
pM241	1	4			*			
pP170	1	4	*		*			
pO109	1	4						*
OPD20 1010	5	5		*			*	
OPD20 1560	5	5		*			*	
OPG10 1030	5	5		*			*	
pQ86	5	5		*			*	
OPC06 610	10	3					*	
pM177	10	3					*	
pM415b	10	3					*	
pO26	10	3					*	
pM100 a,b,c	15	5		*		*		

agromonically important characters analyzed in this study was 20-seeds weight (SW). In both populations, QTLs controlling SW were observed in the location including common RFLP marker 'pQ86' and three RAPD markers 'OPD20$_{1010}$', 'OPD20$_{1560}$' and 'OPG10$_{1030}$'. At all these loci, an increase of seed weight was controlled by *V. angularis* alleles.

Comparison of linkage maps between azuki bean and other Vigna species

Azuki linkage map constructed with interspecific population of *V. umbellata* and *V. angularis* was compared with mungbean and cowpea linkage maps (Table V-17). Several sets of the markers were found to belong to the same linkage groups of the respective maps. However, not all the markers in the same linkage group of azuki map were located in a single linkage group of different maps. They were usually located in two or more linkage groups. Previously, Menancio-Hautea et al. (1993a) reported the genome rearrangement between mungbean and cowpea. As compared with their results, the orders of the mungbean RFLP markers were less conservative in azuki map than in cowpea. This might indicate that more extensive genome rearrangement occurred between azuki and mungbean than between mungbean and cowpea.

Factors responsible for distorted segregation

The clusters of the markers showing distorted segregation were located in the linkage groups, 2, 5, 7, 10, 12, 13 and 14 (Fig 19). The elimination of *V. angularis* alleles occurred at many loci in the linkage groups 2, 7, 10, 12 and 14, while lower elimination frequency of *V.*

umbellata allele and both of them was observed in the linkage group 5 and linkage group 13, respectively (Table 7). As a whole, strong selective pressure against *V. angularis* alleles existed in the mapping population. In the cross between *V. umbellata* and *V. angularis,* the rescue of immature hybrid embryos is needed to obtain hybrids (Ahn and Hartmann 1978; Chen et al. 1983). F_1 rescued hybrid usually show lower seed set than the parental species. In this study, almost all pods of the hybrids contained abnormal seeds. Based on the single-marker analysis, many QTLs controlling abnormal seed (AS) were found around the marker loci which showed abnormal segregation on linkage groups 2, 5, 7, 13 and 14 (Fig. V-19). Therefore, one reason for the elimination of allele may be due to an interruption of embryo development in the hybrid.

Summary

Foreign genes of rice bean (*V. umbellata*) including bruchid resistance were introduced to azuki bean by interspecific hybridization through embryo culture. Some of their progenies showed the bruchid resistance which was governed by a major gene with additive effect. Using F_2 population, a genetic linkage map was constructed with molecular and morphological markers. In total, 169 markers (95 RAPD, 61 RFLP and three morphological markers) were mapped in 15 linkage groups covering 2001.4 cM, and 50 markers remained unlinked. Based on the linkage map, QTL analysis for 17 traits were performed. Using interval mapping, the additive effects of the bruchid resistance was mainly due to the *V. umbellata* allele of major QTL, brcu5. However,

swu5 and dfu5 controlling seed weight and days to flowering, respectively, were mapped to the same interval in that brcu5 located, indicating that further investigation will be needed around these QTL regions in order to establish the best combination with desirable characters. By comparing this map with those of mungbean, brcu5 from *V. umbellata* was located in the interval adjacent to pR26, suggesting these resistant genes might be in the same locus and have similar defense mechanisms.

Chapter VI. Conclusion

Despite the fact that azuki bean is one of the most important beans in Japan, only a limited genetic information is available. To support azuki bean breeding, therefore, more genetic information is needed to be obtained. Molecular linkage maps have provided bases for gene mapping, gene tagging and efficient selection in breeding programs. In the present studies, construction of linkage maps of azuki bean and their application for QTL analysis of some agronomically important traits were carried out.

Genetic variations of azuki bean and its relatives were investigated using RAPD and RFLP markers. The genetic variation among Japanese azuki bean cultivars was small, while *Ceratotropis* species, except for the wild forms of azuki bean, had larger genetic variations and showed considerable differentiation from azuki cultivars. The low genetic diversity not only within azuki cultivars but also between cultivars and wild forms of azuki bean in Japan indicates the necessity of introgression of other genetic resources to expand the genetic variation in azuki bean.

In *Ceratotropis* species, *V. nakashimae* and *V. umbellata* are the most attractive resources because they have blown stem rot resistance and bruchid resistance, both causing serious damages to azuki bean. They also possess large enough DNA polymorphisms as compared with azuki bean, the feature being helpful to construct linkage maps. Therefore, two linkage maps were constructed using interspecific populations between azuki bean and species in *Ceratotropis* in order to introduce their desirable genes effectively into azuki cultivars. One

linkage map was constructed using F_2 population of the cross between *V. angularis* and *V. nakashimae*, and the other using F_2 population of the cross between *V. umbellata* and *V. angularis*. These two maps partially allowed to predict the position of markers and estimate the homologies of genes based on the information on common markers. However, in order to understand the genomic structure of azuki bean and its related species, many more additional common RFLP markers should be located on these linkage maps.

Since *V. nakashimae* has many wild undesirable characters, such as late flowering, climbing habit, pod shattering and small seed, individuals with undesirable characters segregated frequently in the F_2 and F_3 populations. Based on the linkage map of *V. angularis* and *V. nakashimae*, QTL analysis of several important traits were performed. Many QTLs for such traits were mapped and the markers linked to QTLs helped elucidating whether the allele has desirable or undesirable effects. In azuki bean breeding program, brown stem rot resistance gene has been introduced from *V. nakashimae*. The QTLs identified in this study for those undesirable characters will be effectively used to isolate individuals with brown stem rot resistance gene, provided the linkage with undesirable traits could be broken.

Bruchid resistance of *V. umbellata* was successfully introduced into azuki bean by the interspecific cross. The bruchid resistance was shown to be governed by a major gene with additive effect and some minor genes. QTLs controlling bruchid resistance were successfully mapped using the linkage map of *V. umbellata* x *V. angularis*. This suggests that the interspecific population will provide a source material to breed desirable azuki cultivars with bruchid resistance

using the markers linked to the QTLs. Since one each of QTL controlling seed weight and days to flowering were mapped to the same interval where the major gene for bruchid resistance located, resistant individuals must have small seed and late flowering habit. In future, selection for recombinants around these QTL regions will be needed to develop resistant azuki cultivars with desirable genetic backgrounds.

Molecular linkage maps constructed using the interspecific populations between azuki bean and its relatives offer new opportunities for their application in genetics and breeding of azuki bean. In addition, desirable genes introgressed from related genetic sources can be characterized by QTL mapping, thus expand the genetic variation of azuki bean. The ability to monitor QTLs controlling important agronomic traits by their linkage to molecular markers will increase the efficiency of their introgression into desirable genetic backgrounds of azuki bean.

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