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Comparative molecular mapping in *Ceratotropis* species using an interspecific cross between azuki bean (*Vigna angularis*) and rice bean (*V. umbellata*)

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Abstract A genetic linkage map was developed with 86 F_2 plants derived from an interspecific cross between azuki bean (*Vigna angularis*, 2n=2x=22) and rice bean (*V. umbellata*, 2n=2x=22). In total, 14 linkage groups, each containing more than 4 markers, were constructed with one phenotypic, 114 RFLP and 74 RAPD markers. The total map size was 1702 cM, and the average distance between markers was 9.7 cM. The loci showing significant deviation from the expected ratio clustered in several linkage groups. Most of the skewed loci were due to the predominance of rice bean alleles. The azuki-rice bean linkage map was compared with other available maps of *Vigna* species in subgenus *Ceratotropis*. Based on the lineage of the common mapped markers, 7 and 16 conserved linkage blocks were found in the interspecific map of azuki bean × *V. nakashimae* and mungbean map, respectively. Although the present map is not fully saturated, it may facilitate gene tagging, QTL mapping and further useful gene transfer for azuki bean breeding.

Key words Molecular linkage map · Comparative mapping · Azuki bean (*Vigna angularis*) · *Ceratotropis*

Introduction

The genus *Vigna* consists of seven subgenera, i.e., *Vigna, Haydonia, Plectotropis, Macroryncha, Ceratotropis, Sigmoidotropis* and *Lasiocarpa* (Maréchal et al. 1978). Among them, the subgenus *Ceratotropis* contains cultivated bean species consumed mainly in Asian countries (Smartt 1990). Based on seedling characteristics, this subgenus is conventionally divided into two groups, the azuki bean (*V. angularis*) group and the mungbean (*V. radiata*) group (Maekawa 1955; Tomooka 1991). Most of the species in these two groups are diploid (2n=2x=22); however, strong reproductive isolation barriers exists between the species in these two groups (Chen et al. 1983; Egawa 1988). Rice bean (*V. umbellata*) is another cultigen within the azuki bean group. A relatively low level of divergence between rice bean and azuki bean has been reported based on nucleotide sequences (Fatokun et al. 1993; Kaga et al. 1996b), seed proteins (D'Urzo et al. 1990; Rao et al. 1992) and isozymes (Jaaska and Jaaska 1990). Although hybrid breakdown has been observed in the crosses between rice bean and azuki bean (Ahn and Hartmann 1978a), F_1 plants can be obtained using embryo rescue, and by using subsequent generations, an interspecific linkage map between rice bean and azuki bean can be constructed.

Comparative analysis of genome evolution among reproductively isolated species has been reported for many crop species using molecular linkage maps (Tanksley et al. 1988; Bonierbale et al. 1988; Weeden et al. 1992; Devos and Gale 1997). A comparison based on a common set of molecular markers allows the identification of homologous loci and colinearity of chromosomal segments. For bean species, genomic evolution between mungbean, common bean, (*Phaseolus vulgaris*) and soybean (*Glycine max*) in the Phaseoleae tribe of the Papilionoidae subfamily was reported by Boutin et al. (1995). In *Ceratotropis* species, a molecular linkage map of mungbean (Menancio-Hautea et al. 1993) and an interspecific map of azuki bean and its wild relative, *V. nakashimae* (Kaga et al. 1996a), are available. In the study reported here, we constructed a molecular linkage map between azuki bean and rice bean and compared it with other published molecular marker maps of *Ceratotropis* species in order to clarify their genomic evolution.

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Table 1 Morphological characters of rice bean (*V. umbellata*), azuki bean (*V. angularis*) and their F_1 hybrid

Character	Rice bean	F_1 hybrid	Azuki bean
Growth habit	Indeterminate	Indeterminate	Determinate
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 2 Morphological characters of rice bean (*V. umbellata*), azuki bean (*V. angularis*) and the F_1 hybrid, and their segregation in the F_2 population

Character	Rice bean	Azuki bean	F_1 hybrid	F_2 segregation		
				Phenotype	Number 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

Materials and methods

Plant materials

To produce a mapping population we used rice bean (*V. umbellata*) 'Kagoshima' and azuki bean (*V. angularis*) 'Erimoshouzu'. After rice bean and azuki bean were crossed, the immature pods were collected and the young embryos were removed from the seeds. These embryos were cultured on LS medium (Linsmaier and Skoog 1965) for 3 months. Seedlings obtained by embryo culture were further transferred to half-strength ($^1/_2$) MS medium (Murashige and Skoog 1962). After 2 months of secondary culture, they were transplanted to pots 25 cm in diameter. A total of 163 F_2 individuals from the interspecific cross were grown in the pots, and 10 F_3 plants from each of 86 F_2 individuals were grown in the field.

Construction of a linkage map

Total DNA was isolated from bulked leaf tissue of 10 plants of each F_3 line by the method of Draper and Scott (1988). Restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) analyses were performed as described in Kaga et al. (1996a). Mungbean and cowpea genomic DNA probes (University of Minnesota, USA), soybean genomic DNA probes (Biogenetic Services, USA) and a rice nuclear ribosomal DNA probe (National Institute of Agrobiological Resources, Japan) were used. Decamer oligonucleotide primers for RAPD analysis were purchased from Operon Technologies, USA. F_2 segregation data for RFLP, RAPD and morphological markers were examined by chi-square tests. Linkage analysis was performed with MAPMAKER EXP 3.0 program (Lander et al. 1987; Lincoln et al. 1992). Linked markers were first identified using the "Group" command with LOD>4.0, and then the "Order" command (LOD>3.0) was used to determine the most probable marker order within a linkage group. Recombination frequencies were converted into map distances (centiMorgans) using the Kosambi function (Kosambi 1944).

Results

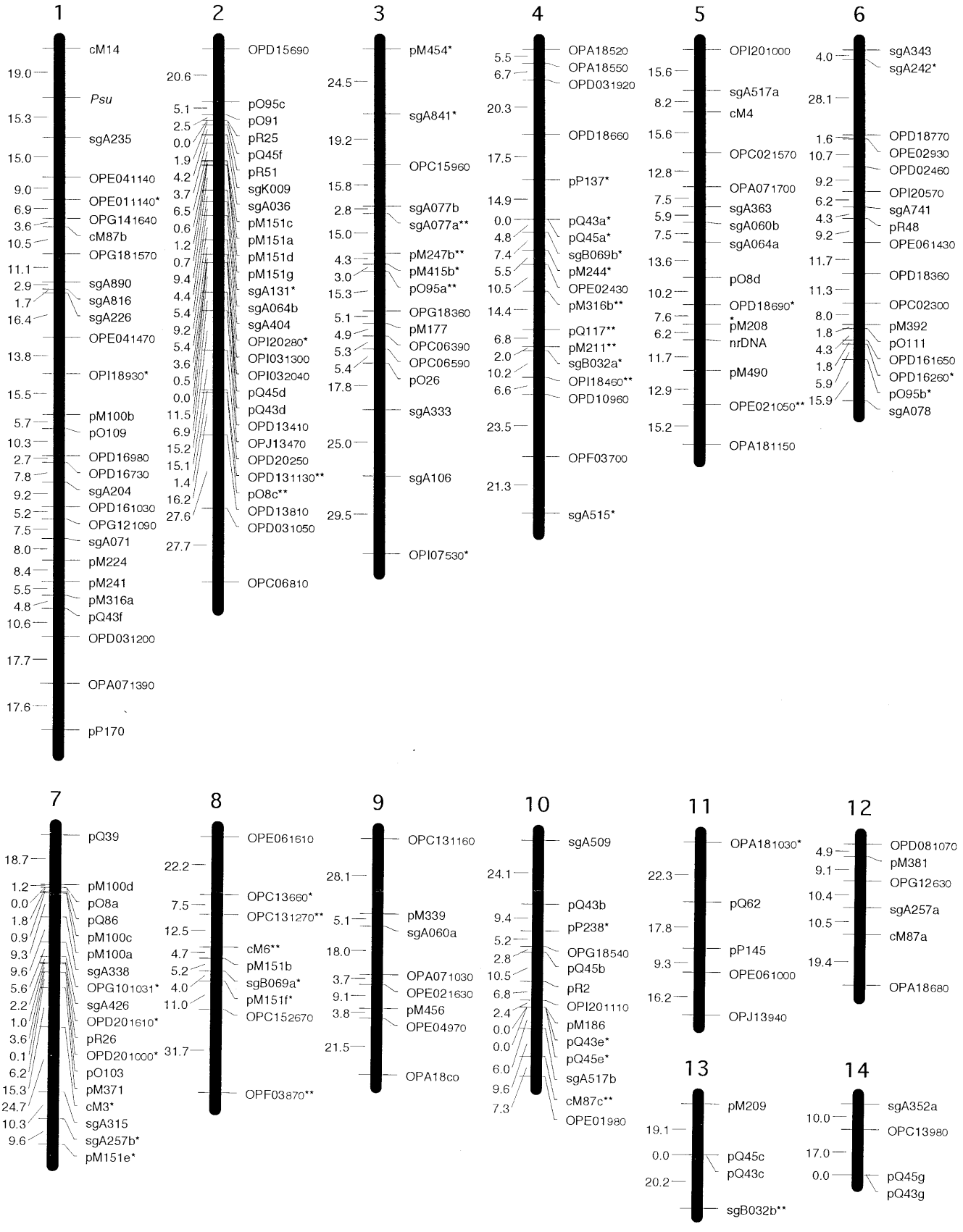
Morphological characters of F_1 hybrids between rice bean and azuki bean

Interspecific reciprocal crosses were carried out between rice been and azuki bean; however, the pods developed only when azuki bean was used as the pollen parent. The seeds of immature pods were germinated by means of embryo culture, and subsequently three F_1 hybrids were obtained. The morphological characteristics of rice bean, azuki bean and their F_1 plants are summarized in Table 1. Most of the morphological characteristics of the hybrids were similar to those of rice bean. The primary leaf shape of the hybrid plants showed intermediate characteristics, and the leaf shape, pod color and seed color were specific to the hybrid.

Inheritance of morphological traits

Out of 163 F_2 plants, 45 (27.6%) did not produce any seeds due to the plants either not flowering or not setting seeds. Thirty-two individuals (19.6%) did not set sufficient seeds to produce a F_3 line. Segregation among 86 normal F_2 individuals was complex; seed and pod colors exhibited many intermediate types. Only two phenotypes were observed for epicotyl color (Table 2). Since the segregation of purple stem showed a good fit to a 3:1 ratio, a dominant gene for this character from rice bean is tentatively designated as *Psu* (purple stem). In addition, since segregation for intensity of stem color in F_2 plants

Fig. 1 An interspecific linkage map constructed with the F_2 population of rice bean (*V. umbellata*) and azuki bean (*V. angularis*). Fourteen linkage groups are *numbered* in order of the size. Map distances and marker names are shown on the *left* and *right* sides of the linkage groups, respectively. *cM, pM, pO, pP, pQ* and *pR* denote mungbean and cowpea RFLP probes provided from Dr. N.D. Young; *sgA* and *sgB* indicate soybean RFLP probes. *Lowercase letters* are added to the *end* of those probes with multiple loci. RAPD markers are presented with the primer codes and the approximate molecular sizes. Markers showing significant deviations from the expected segregation ratios at 0.05 and 0.01 levels are indicated with * and **, respectively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3.6
cM87b
10.5
OPG181570
11.1
2.9
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1.7
sgA816
16.4
sgA226
OPE041470
13.8
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5.7
pM100b
pO109
10.3
2.7
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OPD16730
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sgA204
5.2
OPD161030
7.5
OPG121090
8.0
sgA071
8.4
pM224
5.5
pM241
4.8
pM316a
10.6
pQ43f
OPD031200
17.7
OPA071390
17.6
pP170
2
OPD15690
20.6
5.1
pO95c
2.5
pO91
0.0
pR25
1.9
pQ45f
4.2
pR51
3.7
sgK009
6.5
sgA036
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pM151c
1.2
pM151a
0.7
pM151d
9.4
pM151g
4.4
sgA131*
5.4
sgA064b
9.2
sgA404
5.4
OPI20280*
3.6
OPI031300
0.5
OPI032040
0.0
pQ45d
11.5
pQ43d
6.9
OPD13410
15.2
OPJ13470
15.1
OPD20250
1.4
OPD131130**
16.2
pO8c**
27.6
OPD13810
OPD031050
27.7
OPC06810
3
pM454*
24.5
sgA841*
19.2
OPC15960
15.8
2.8
sgA077b
sgA077a**
15.0
4.3
pM247b**
3.0
pM415b*
15.3
OPG18360
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5.3
OPC06590
5.4
pO26
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4.8
pQ45a*
7.4
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pM211**
10.2
sgB032a*
6.6
OPI18460**
OPD10960
23.5
OPF03700
21.3
sgA515*
5
OPI201000
15.6
sgA517a
8.2
cM4
15.6
OPC021570
12.8
OPA071700
7.5
sgA363
5.9
sgA060b
7.5
sgA064a
13.6
pO8d
10.2
OPD18690*
7.6
pM208
6.2
nrDNA
11.7
pM490
12.9
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6
4.0
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sgA242*
28.1
1.6
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10.7
OPE02930
OPD02460
9.2
OPI20570
6.2
sgA741
4.3
pR48
9.2
OPE061430
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8.0
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1.8
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4.3
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1.8
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5.9
pO95b*
15.9
sgA078
7
pQ39
18.7
1.2
pM100d
0.0
pO8a
1.8
pQ86
0.9
pM100c
9.3
pM100a
9.6
sgA338
5.6
OPG101031*
2.2
sgA426
1.0
OPD201610*
3.6
pR26
0.1
OPD201000*
6.2
pO103
15.3
pM371
24.7
cM3*
10.3
sgA315
9.6
sgA257b*
pM151e*
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22.2
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OPC131270**
12.5
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cM6**
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11.0
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OPC152670
31.7
OPF03870**
9
OPC131160
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5.1
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OPA071030
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OPE021630
3.8
pM456
OPE04970
21.5
OPA18co
10
sgA509
24.1
pQ43b
9.4
pP238*
5.2
OPG18540
2.8
pQ45b
10.5
pR2
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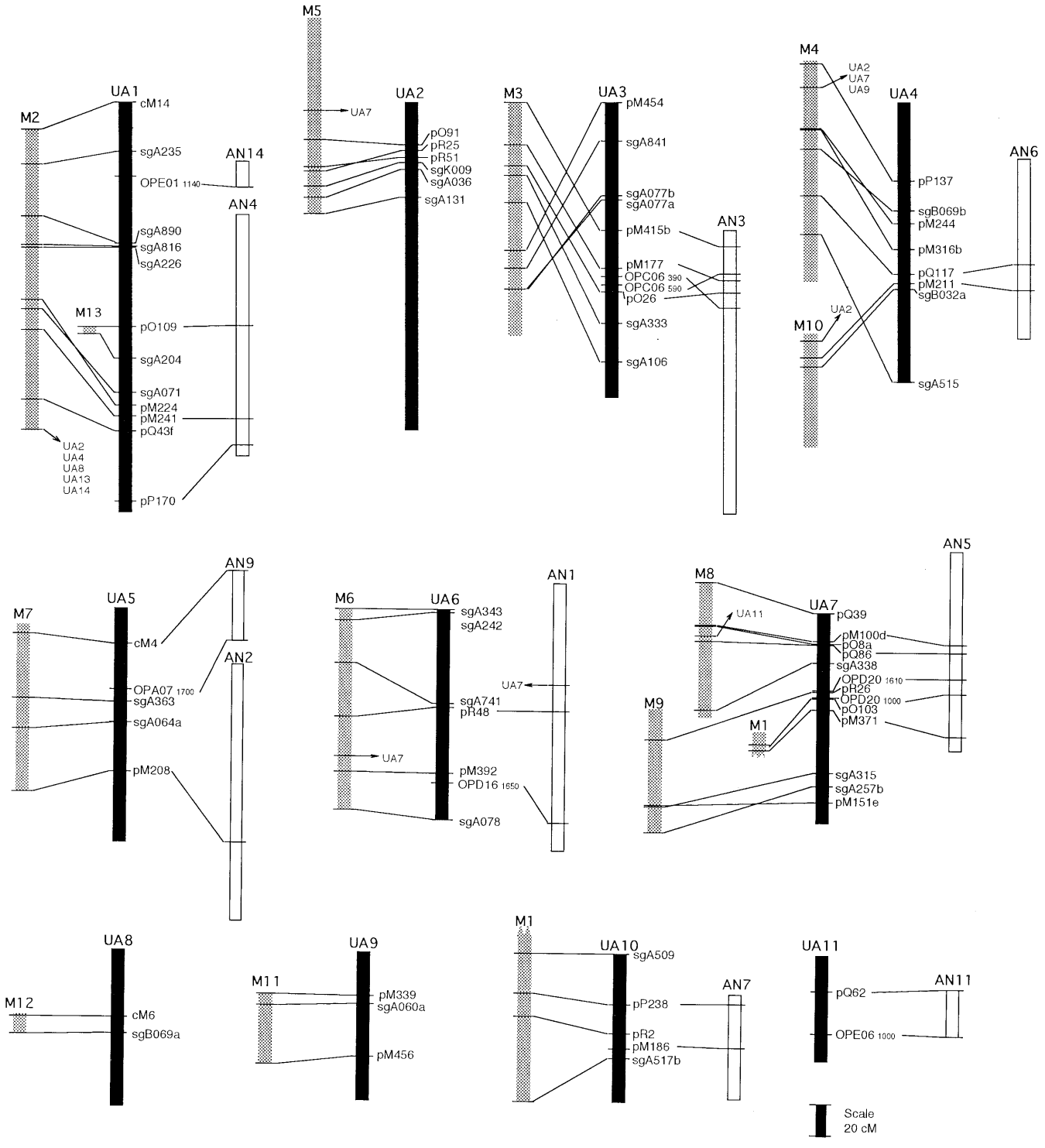

Fig. 2 Comaprative linkage maps of *Ceratotropis* species based on common RFLP and RAPD markers. Linkage groups of the interspecific map between rice bean and azuki bean were aligned with the corresponding linkage groups of the mungbean linkage map (*M1–13*, *left*) (Menancio-Hautea et al. 1993a) and the interspecific map between azuki bean and *V. nakashimae* (*AN1–14*, *right*) (Kaga et al. 1996a). Linkage groups with *notched ends* indicated the separated linkage blocks. The positions of common marker loci are connected by the *lines* between linkage groups. *Arrows* indicate the locations of the loci on other linkage groups.

with purple stem agreed with a 3:1 ratio (χ^2=1.42), a hypostatic gene is tentatively designated as *lscu* (intense stem color).

Parental polymorphisms and segregation of molecular markers

Thirty-nine mungbean, 29 cowpea and 40 soybean genomic probes and one rice nuclear rDNA clone were

Table 3 Conserved linkage blocks detected between UA (rice bean×azuki bean) and AN (azuki bean×*V. nakashimae*) linkage maps, and between UA and mungbean linkage maps, based on the lineage of common molecular markers

Between UA and AN maps			Linkage group	Between UA and mungbean (M) maps		
Segment	Size (cM)			Segment	Size (cM)	
	UA	AN[a]			UA	M[a]
pO109–pP170	115	72	UA1	cM14–sgA226	95	73
				pM241–pQ43f	10	47
				pO109–sgA204	21	5
			UA2	sgK009–sgA131	13	17
			UA3	pM454–sgA841	25	11
				pM415b-sgA106	82	65
pQ117–pM211	7	18	UA4	pM211–sgB032a	2	9
cM4–OPA07$_{1700}$	28	45	UA5	cM4–pM208	81	97
pR48-OPD16$_{1650}$	46	73	UA6	sgA343–pR48	64	69
				pM392–sgA078	30	24
pM100d–pM371	42	58	UA7	pQ39–pO8a	20	28
				pQ86–sgA338	13	50
				pO103–pM371	6	3
			UA8	cM6–sgB069a	10	11
			UA9	pM339–pM456	36	42
pP238–pM186	28	34	UA10	sgA509–sgA517b	66	94
pQ62–OPE06$_{1000}$	27	29	UA11			

[a] Map distances of the AN and mungbean linkage maps were from Kaga et al. (1996a) and Menancio-Hautea et al. (1993), respectively

used to survey polymorphisms between rice bean and azuki bean with four restriction enzymes: *Dra*I, *Eco*RI, *Eco*RV and *Hin*dIII. Of these, 26 mungbean probes (66.7%), 22 cowpea probes (75.9%), 32 soybean probes (80.0%) and one rDNA probe gave polymorphisms using at least one out of the four enzymes. For RAPD analysis, 200 decamer primers were used to detect polymorphisms between rice bean and azuki bean. In total, 120 RFLP and 105 RAPD loci were identified based on the banding patterns of the F_2 plants. Among them, the segregation of 67 molecular markers (29.8%) showed significant deviation from the expected ratio of 1:2:1 or 3:1 ($P<0.05$). Most of these skewed loci were due to the predominance of rice bean alleles.

Linkage analysis

Linkage analysis was carried out with 120 RFLP and 105 RAPD markers and two tentative phenotypic genes using MAPMAKER. In total, 14 linkage groups, each containing more than 4 markers, were constructed with 114 RFLP markers (95.0%), 74 RAPD markers (70.5%) and one tentative phenotypic gene (Fig. 1). The total map size was 1701.9 cM, and the average distance between markers was 9.7 cM. Clusters of skewed marker loci were distributed in linkage groups 3, 4, 7, 8 and 10 (Fig. 1). Tentative phenotypic gene *Psu* was localized to linkage group 1. The hypostatic gene for the intensity of stem pigmentation, *Iscu*, was excluded from the linkage map but showed linkage to RFLP marker pM208 (linkage group 5) with a low LOD score (3.39). The nuclear ribosomal RNA gene was mapped as a single locus on linkage group 5.

Identification of homologous segments among *Ceratotropis* species

The interspecific linkage map of rice bean × azuki bean (UA linkage map) was compared with other available maps of *Ceratotropis* species, the interspecific linkage map of azuki bean × *V. nakashimae* (AN linkage map) (Kaga et al. 1996a) and the mungbean linkage map (Menancio-Hautea et al. 1993). The linkage conservation among the three maps based on the lineage of common markers is shown in Fig. 2. Although there are not so many common markers between the UA and AN linkage maps (18 RFLP and 8 RAPD markers), UA linkage groups 1, 3, 4, 6, 7, 10 and 11 seem to correspond to AN linkage groups 4, 3, 6, 1, 5, 7 and 11, respectively. Seven conserved linkage blocks were found between the UA and AN linkage maps (Table 3). The sizes of the segments are similar in both maps.

Sixty-six RFLP markers from 10 UA linkage groups were found to be common between UA and mungbean linkage maps (Fig. 2). A total of 16 segments were found to be conserved linkage blocks without regions of inversion or translocation (Table 3). Based on the lineage of the marker loci, UA linkage groups 1, 2, 3, 4, 5, 6, 8, 9 and 10 might be orthologous to mungbean linkage groups 2, 5, 3, 4, 7, 6, 12, 11 and 1, respectively. UA linkage groups 1 and 4 contained small insertions of mungbean linkage blocks, and 2 clusters of markers on UA linkage group 3 were exchanged in the mungbean linkage group 3. The markers on UA linkage group 7 were located on 3 different mungbean linkage groups, 1, 8 and 9.

Discussion

The construction of linkage maps is of fundamental importance for the efficient exploitation of crop genetic re-

sources. In the genus *Vigna* subgenus *Ceratotropis*, a molecular linkage map of mungbean (Menancio-Hautea et al. 1993) and an interspecific map of azuki bean and its wild relative *V. nakashimae* (Kaga et al. 1996a) have been constructed. Since both azuki bean and rice bean have low levels of within-species polymorphism (Kaga et al. 1993, 1996b), their interspecific molecular map was constructed in this study. A total of one phenotypic, 114 RFLP and 74 RAPD markers were assorted into 14 linkage groups covering 1702 cM. Of these, 66 RFLP loci were common to the mungbean linkage map, covering 1351 cM (86.1%) in the latter; therefore, these linkage maps may contain many syntenic regions conserved among the subgenus *Ceratotropis* species.

The azuki-rice bean map was constructed with the F_2 population derived from the interspecific cross of these reproductively isolated cultigens. Although their F_1 hybrids had normal chromosome pairing at meiosis (Ahn and Hartmann 1978a), 67 loci (29.8%) showed distorted segregation in the F_2 population. This phenomenon is common among plant species. Using the data of 14 interspecific mapping populations Xu et al. (1997) reported that the average percentage of markers showing segregation distortion was 25.3%. They further suggested that the skewed segregation mainly arose from gametophytic selection. Since the clusters of skewed marker loci were distributed in several azuki-rice bean linkage groups, some of them might be associated with the gametophyte or sterility genes.

Comparison between two azuki interspecific maps, the UA linkage map (*V. umbellata* × *V. angularis*) and the AN map (*V. angularis* × *V. nakashimae*), revealed that UA linkage groups 1, 3, 4, 6, 7, 10 and 11 appeared to correspond to AN linkage groups 4, 3, 6, 1, 5, 7 and 11, respectively. Tentative dominant genes for purple stem color, *Psu* from rice bean and *Ps* from *V. nakashimae*, were mapped at the distal end of UA linkage group 1 and AN linkage group 4, respectively. Although there are no common markers adjacent to these genes, they seem to be orthologous genes. In addition, *Iscu*, a hypostatic gene for the intensity of pigmentation from rice bean, and *Isc* from *V. nakashimae* showed linkage to the common RFLP marker pM208, also suggesting they might be orthologous.

While mungbean (*V. radiata*), belonging to the subgenus *Ceratotropis*, has the same number of the chromosomes (2n=2x=22) as azuki bean and rice bean, a strong reproductive isolation barrier exists between them. Cytological studies have revealed irregular chromosome pairing at meiosis in interspecific hybrids between mungbean and rice bean and between mungbean and azuki bean. Egawa et al. (1988) and Ahn and Hartmann (1978b) reported average bivalent numbers to be only 4.7 and 2.4 in these two interspecific hybrids, respectively. This indicates that a relatively wide differentiation has taken place between mungbean and the other two cultigens, rice bean and azuki bean. A molecular linkage map of mungbean has already been constructed with RFLP markers (Menancio-Hautea et al. 1993). Of these, 66 markers were mapped on the azuki-rice bean linkage groups. The lineage of these common loci revealed conserved blocks distributed in most of the linkage groups; however, some of their locations were inconsistent between the two maps: small insertions of mungbean blocks in UA linkage groups 1 and 4 and exchanged clusters in UA linkage group 3. Although there is no clear evidence, chromosomal rearrangements between mungbean and azuki-rice bean could explain the inconsistency of the marker locations. The marker loci on UA linkage group 7 were located on three different mungbean linkage groups, 1, 8 and 9. Using genus *Phaseolus* species, which is a closely related genus to *Vigna*, Boutin et al. (1995) studied genome conservation between the molecular linkage maps of mungbean and common bean (*P. vulgaris*, 2n=22). They also reported that mungbean linkage groups 8 and 9 and part of linkage group 1 corresponded to linkage group K of common bean. These facts suggest that three parts of the mungbean linkage groups probably belong to a single linkage group.

Azuki bean is the second most important legume in Japan. Its annual production is unstable because of damage from cold weather and many pests. Rice bean has many useful characteristics, such as bruchid resistance (Sawa and Tan 1976; Kitamura et al. 1988) and resistance to azuki bean mosaic virus (Sawa et al. 1984), and has the highest yielding capacity among *Ceratotropis* species (Smartt 1990). Here we constructed an interspecific molecular map of azuki bean and rice bean. Although it is not fully saturated, we hope this map will facilitate gene tagging and QTL mapping and further useful gene transfer for azuki bean breeding.

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