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ORIGINAL PAPER

Development of a black gram [*Vigna mungo* (L.) Hepper] linkage map and its comparison with an azuki bean [*Vigna angularis* (Willd.) Ohwi and Ohashi] linkage map

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Abstract The Asian *Vigna* group of grain legumes consists of six domesticated species, among them black gram is widely grown in South Asia and to a lesser extent in Southeast Asia. We report the first genetic linkage map of black gram [*Vigna mungo* (L.) Hepper], constructed using a BC_1F_1 population consisting of 180 individuals. The BC_1F_1 population was analyzed in 61 SSR primer pairs, 56 RFLP probes, 27 AFLP loci and 1 morphological marker. About 148 marker loci could be assigned to the 11 linkage groups, which correspond to the haploid chromosome number of black gram. The linkage groups cover a total of 783 cM of the black gram genome. The number of markers per linkage group ranges from 6 to 23. The average distance between adjacent markers varied from 3.5 to 9.3 cM. The results of comparative genome mapping between black gram and azuki bean show that the linkage order of markers is highly conserved. However, inversions, insertions, deletions/duplications and a translocation were detected between the black gram and azuki bean linkage maps. The marker order on parts of linkage groups 1, 2 and 5 is reversed between the two species. One region on black gram linkage group 10 appears to correspond to part of azuki bean linkage group 1. The present study suggests that the azuki bean SSR markers can be widely used for Asian *Vigna* species and the black gram genetic linkage map will assist in improvement of this crop.

The first three authors contributed equally to this research

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Introduction

Six Asian *Vigna* grain legumes have been domesticated, they are black gram [*V. mungo* (L.) Hepper], mungbean [*V. radiata* (L) Hepper], azuki bean [*V. angularis* (Willd.) Ohwi and Ohashi], rice bean [*V. umbellata* (Thunb.) Ohwi and Ohashi], creole bean [*V. reflexo-pilosa* var. *glabra* (Maréchal, Mascharpa and Stainer) N. Tomooka and Maxted] and moth bean [*V. aconitifolia* (Jacq.) Maréchal] (Tomooka et al. 2002a). The majority of the production of these grain legumes is for local consumption and the production of some of these cultigens, such as mungbean, is increasing (Tomooka et al. 2005). These six Asian *Vigna* grain legumes are closely related in the subgenus *Ceratotropis* of the genus *Vigna*. The subgenus *Ceratotropis* has been divided into three sections, *Ceratotropis*, *Aconitifoliae* and *Angulares*. Both black gram and mungbean belong to section *Ceratotropis*, moth bean to section *Aconitifoliae* and the other domesticated Asian *Vigna* to section *Angulares*. All have a haploid chromosome number of 11 except *V. reflexo-pilosa* that is tetraploid ($2n = 4x = 44$).

Black gram is mainly grown in South Asia and to a limited extent in Southeast Asia. Black gram in India,

where it is known as urd bean, is a common pulse for soups and curry dishes. In Thailand, black gram is cultivated for export. It is believed that black gram was domesticated in northern South Asia from *V. mungo* var. *silvestris* that commonly grows there (Luloki et al. 1980; Fuller 2002).

To date, linkage maps have only been developed for two of the Asian *Vigna*, mungbean and azuki bean (Kaga et al. 2005). Only the linkage map of azuki bean has resolved all the linkage groups (Han et al. 2005). Comparative genome mapping has revealed the relative order of homologous sequences along the chromosome of related species (Fatokun et al. 1992). The genetic diversity of black gram has been studied using molecular markers (Sivaprakash et al. 2004; Souframanien and Gopalakrishna 2004). However, there are no reports of the development of a genetic linkage map for black gram.

Comparative linkage maps based on a common set of markers have provided information on the genetic relationships among related species. The main model legumes, *Medicago truncatula* and *Lotus japonicus*, show a high degree of genome conservation but lower genome conservation with phaseoloid clade of legume crops such as soybean (*Glycine max*), common bean (*Phaseolus vulgaris*) and *Vigna* (including cowpea and Asian *Vigna*) (Choi et al. 2004). Genome comparisons among Asian *Vigna* species and between Asian *Vigna* and other closely related legumes have been reported (Menacio-Hautea et al. 1993; Boutin et al. 1995; Kaga et al. 2000; Humphry et al. 2002). These studies have revealed that differences between genomes can be accounted for by rearrangements including inversions, insertions, duplications, deletions and translocations. Moreover, the genome comparison revealed that QTL for the same or similar traits are found at syntenic regions on genetic maps from different species, for example, QTL for seed weight in mungbean, cowpea and soybean (Fatokun et al. 1992; Maughan et al. 1996).

Despite black gram being one of the most important pulses in India (Duke 1981), genome level studies of this crop are lacking. Here we report the development and analysis of a black gram genome mapping population. The specific objectives of this study were:

1. To find molecular markers that are useful for genome mapping of black gram.
2. To develop a linkage map of black gram using SSR primers and RFLP probes from related species and AFLP markers.
3. To use this linkage map for comparison with a linkage map of azuki bean (Han et al. 2005).

Materials and methods

Plant materials and DNA extraction

All plant materials used were from the Genebank collection of the National Institute of Agrobiological Sciences, Tsukuba, Japan, and have the prefix JP for accession numbers. The BC_1F_1 population of 180 individuals was derived from the cross between a mutant line selected for its large seed size (P_1 JP219132) from black gram accession "*BC48*" (*V. mungo* JP106710) and an accession "TC2210" of its wild ancestor from India (P_2 *V. mungo* var. *silvestris* JP107873). The large seeded mutant was used as recurrent parent in development of the mapping population. For SSR primer screening, an accession of each cultivar and its wild ancestor for *V. angularis* (JP109685, JP110658), *V. umbellata* (JP100311, JP109669), *V. radiata* (JP81648, JP107876) and a cultivar of *V. aconitifolia* (JP104332) were included. Total DNA was extracted from the young leaves of each individual plant using the method of Draper and Scott (1988).

SSR analysis

A total of 211 azuki bean SSR primer pairs mapped on the azuki bean linkage map (Han et al. 2005), 26 common bean SSR primer pairs (Gaitán-Soltís et al. 2002, Blair et al. 2003) and 5 cowpea SSR primer pairs (Li et al. 2001) were screened between the two *V. mungo* parents and other Asian *Vigna*. The SSR analysis was performed following the procedures of Han et al. (2005). In brief, the 5′ end of the reverse primer was labeled with one of the four fluorescent dyes, 5-Fam, VIC, NED and PET (Applied Biosystems, UK). Three or four of the differentially labeled primers were mixed into single PCR reaction mixtures containing 1 ng of total DNA, 1 U KOD-plus DNA polymerase (TOYOBO, Japan), 1× PCR buffer for KOD-plus, 0.2 mM dNTPs, 1 mM $MgSO_4$ and 5 pmol of forward and reverse primers. All PCR products were amplified using the following reaction conditions: 94°C for 2 min followed by 35 cycles of 94°C for 15 s, 45–65°C for 15 s, 68°C for 15 s. One microliter of PCR product was mixed with 10 Hi-Di formamide containing 0.13 µl of GeneScan 500 LIZ size standard and run on an ABI Prism 3100 Genetic analyzer (Applied Biosystems). Alleles were determined by their sizes using GENEMAPPER version 3 (Applied Biosystems).

RFLP analysis

DNA from P_1, P_2 and BC_1F_1 individuals was digested with *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Swa*I and

*Xba*I restriction enzymes and separated on 1% agarose gel. The DNA was denatured by 0.4 M NaOH and 1.5 M NaCl, fixed by UV cross linker at 60 mJ/cm^2 (Amersham Pharmacia Biotech, UK) and transferred onto Hybond N+ membrane. Total of 122 RFLP probes from mungbean, cowpea, common bean and soybean were screened between the two *Vigna mungo* parents. Of these 53 were also used to develop the azuki bean genome map (Han et al. 2005) (Table 1). These probes are designated mc, mgM, mgQ, mgR and mgS, cgO and cgP for mungbean and cowpea, Bng for common bean and sgA, sgB and sgK for soybean. DNA hybridization was carried out by ECL direct nucleic acid labeling and detection systems according to the manufacturer's instructions (Amersham Biosciences, UK).

AFLP analysis

AFLP analysis was performed using AFLP analysis system II kit (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturers instructions. In brief, total DNA (250 ng) from the two parents and the 180 individuals of BC_1F_1 population was digested with 2.5 U each of *Eco*RI and *Mse*I in a 25 µl reaction mixture and incubated at 37°C for 2.5 h. Twenty-four microliters of adapter/ligation solution [*Eco*RI and *Mse*I adapters, 0.4 mM ATP, 10 mM Tris–HCl (pH 7.5), 10 mM Mg acetate, 50 mM K acetate] and 1 µl of T4 DNA ligase [1 unit/µl in 10 mM Tris–HCl (pH 7.5), 1 mM DTT, 50 mM KCl, 50% (v/v) glycerol] were added. The ligation mixture was incubated at 20°C for 2 h. Five microliters of a tenfold diluted ligation mixture was used as a template DNA for the pre-selective amplification using primer pairs based on the sequence of the *Eco*RI and *Mse*I adapters, without selective nucleotide at the 3′ end. Sixty-five AFLP primer combinations with three selective nucleotides at the 3′ end were examined in a polymorphic survey between parents. Five microliters of a 50-fold diluted PCR product was used as DNA template for the selective amplification using the *Eco*RI and *Mse*I primers with three additional selective nucleotides at the 3′ end. Denatured selective amplification products were run on 6% denaturing polyacrylamide gel (19:1) and stained according to the silver sequence DNA sequencing system (Promega, USA). The polymorphic bands were named according to the name of primer pair and numbered serially in descending order of fragment size.

Linkage map construction

JoinMap ver. 3.0 (Van Ooijen and Voorrips 2001) was used to test the segregation ratio of the markers in the BC_1F_1 population and construct a linkage map. The goodness-of-fit to a 1:1 segregation ratio of each marker was analyzed using the Chi-square test at 5 and 1% significance levels. First, the pair-wise SSR and RFLP loci that showed a recombination frequency smaller than the REC threshold of 0.4 and a LOD value larger than the LOD threshold 3 was used to create linkage groups and the recombination frequencies were converted into map distances (cM) using the Kosambi mapping function (Kosambi 1944). Then crossover between marker loci in a linkage group was verified manually and some doubtful data were reconfirmed by additional reexamination. After building the framework map, linkage analysis including AFLP markers were repeated. Numbering of linkage groups follows that for azuki bean (Han et al. 2005).

Results

Polymorphism and markers for mapping

SSR primer pairs developed from azuki bean were initially used to determine their usefulness in other domesticated Asian *Vigna* (Table 2). As expected, the highest success of PCR amplification by the SSR primer pairs were attained with *V. angularis* (100%), the origin of the primer sequences. Amplification of the other species tested was high. In *V. umbellata*, most closely related to *V. angularis*, amplification was 90%. For the

Table 1 Polymorphisms between *Vigna mungo* var. *mungo* and *V. mungo* var. *silvestris* by RFLP probes from other legumes

Marker	Probes common with azuki bean map[a]		Other probes screened		Total (% polymorphic)
	Probes hybridized[b]	Polymorphic	Probes hybridized[b]	Polymorphic	
Mungbean (mg)	18	11	18	6	36 (47.2)
Cowpea (cg)	4	4	6	2	10 (60)
Phaseolus (Bng)	17	11	16	7	32 (56.3)
Soybean (sg)	14	3	30	12	44 (34.1)
Total	53	29	70	27	123 (45.5)

[a] Han et al. 2005

[b] Low copy number

other species, *V. mungo*, *V. radiata* and *V. aconitifolia*, amplification of SSR primer pairs was between 67 and 73%. Polymorphism between cultivated and wild accessions of black gram was lower than between other cultigens and their wild ancestors. Of the 205 azuki SSR primer pairs screened, 55 (26.8%) showed polymorphism between cultivated and wild black gram parents and were used for mapping. Other SSR primer pairs developed from common bean and cowpea were tested for PCR amplification in the black gram parents. Of 26 common bean SSR primer pairs tested, 22 (84.6%) successfully amplified fragments but only 4 revealed polymorphisms between parents. Four of the five cowpea SSR primer pairs amplified fragments but only two revealed polymorphisms between parents and these were used for mapping (Supplementary Table 1).

About 122 RFLP probes and 7 restriction enzyme combinations were analyzed for polymorphisms between the two *V. mungo* parents. Fifty-six probes (45.9%) detected polymorphic bands between parents at, at least one restriction enzyme.

Results of AFLP analysis revealed that among the 65 primer combinations tested between the two *V. mungo* parents, 52 primer pairs showed polymorphic bands. The number of polymorphic fragments per primer pair ranged from 1 to 6. Ten primer pairs were selected that generated more than one band between the two parents. In total, 27 polymorphic bands were used for linkage map construction.

Construction of black gram linkage map

The segregation pattern of SSR, RFLP and AFLP markers was analyzed in the 180 BC_1F_1 individuals and all of these followed the expected Mendelian ratio (1:1). Of 56 RFLP probes, three probes, mgS003, Bng151 and sgK472, detected two polymorphic loci and these were named (a) or (b), with (a) denoting the locus with the strongest signal strength (Fig. 1). In addition to molecular markers one morphological trait was recorded, twisted and curly leaf (TC) a characteristic trait of the mutant black gram parent.

A total of 148 marker loci comprised of 59 RFLP, 61 SSR, 27 AFLP and 1 morphological marker were grouped by JoinMap ver. 3 at the LOD score of 3.0. All the 148 marker loci could be assigned to 11 linkage groups covering a total of 783 cM of the black gram genome at an average marker density of 5.7 cM (Fig. 1). The number of markers per linkage group ranged from 6 to 23. The average distance between markers varied from 3.5 to 9.3 cM. Numbering of linkage groups follows that for azuki bean (Han et al. 2005). Linkage group 8 was the longest linkage group with a length of 112 cM, whereas, linkage group 6 was the shortest linkage group (53 cM). Linkage group 2 had most markers with an average distance between markers of 3.5 cM. Linkage groups 1, 4 and 8 had gaps greater than 20 cM. The longest gap of 26 cM was on linkage group 1.

Comparison of black gram linkage map with azuki bean linkage map

The black gram linkage map was compared with azuki bean linkage map on the basis of common markers (Han et al. 2005). Of 119 RFLP and SSR markers loci on the black gram linkage map, 80 marker loci were common loci with azuki bean. The order of common markers on linkage groups revealed a high level of conservation between black gram and azuki bean. Marker order of 70 (88%) of these loci (25 RFLP and 45 SSR)

Table 2 A summary of the application of "Erimo–Shozu" SSR markers to other species in the Asian *Vigna*

Species	Accession	Origin	Number of SSR markers		
			Screened	Amplified (%)	Polymorphic between (c) and (w) (%)
Section *Angulares*					
V. angularis (c)[a]	JP109685	Japan	223	223 (100)	123 (55.2)
V. angularis (w)	JP110658	Japan	223	223 (100)	
V. umbelata (c)	JP100311	Nepal	205	185 (90.2)	97 (53.6)
V. umbellata (w)	JP109669	Thailand	205	185 (90.2)	
Section *Ceratotropis*					
V. mungo (cult)	JP219132	Thailand	211	165 (78.2)	68 (49.6)
V. mungo (wild)	JP107873	India	205	138 (67.3)	
V. radiata (cult)	JP81648	Thailand	205	149 (72.7)	88 (63.3)
V. raditata (wild)	JP107876	Australia	205	143 (69.8)	
Section *Aconitifoliae*					
V. aconitifolia	JP104332	Pakistan	205	141 (68.8)	

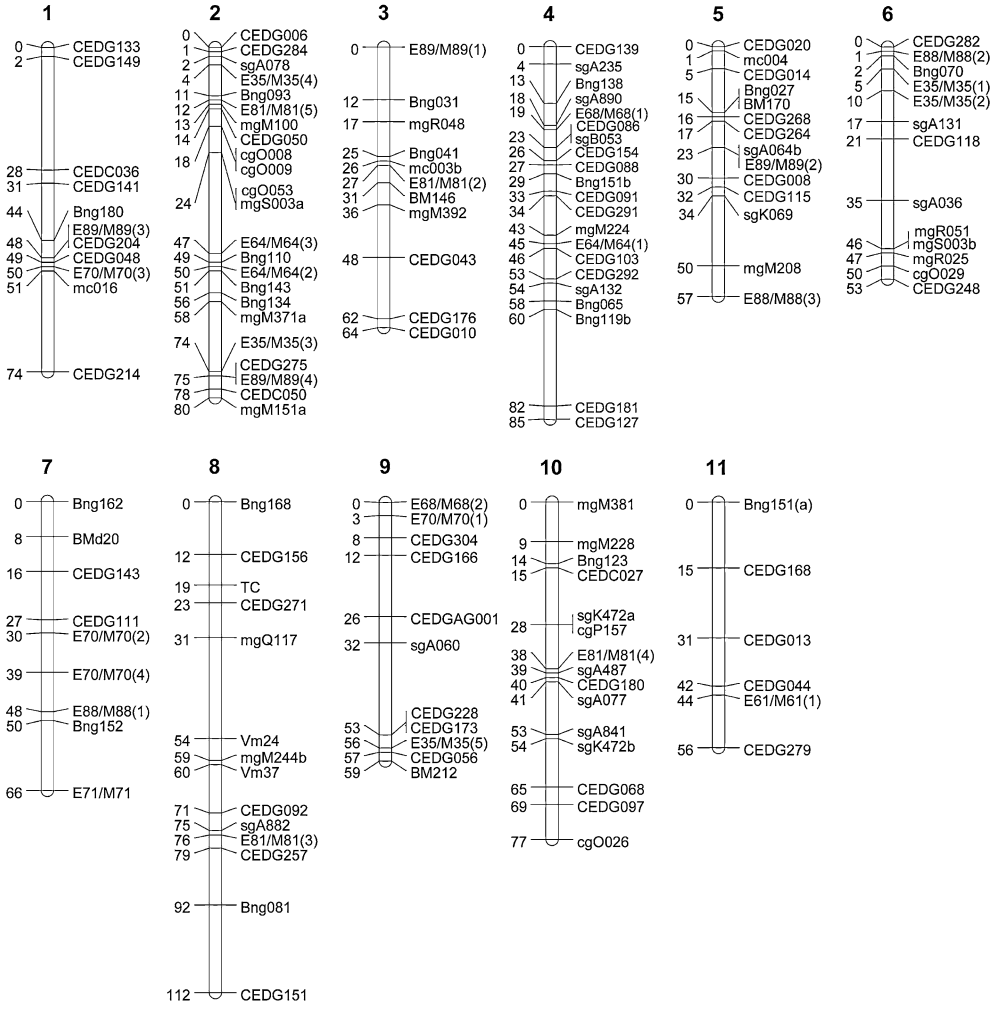

Fig. 1 A genetic linkage map of black gram based on SSR, RFLP, AFLP markers and a morphological marker. This map was constructed from 180 BC_1F_1 individuals of a cross (*V. mungo* var. *mungo* × *V. mungo* var. *silvestris*) × *V. mungo* var. *mungo*. Linkage groups are numbered based on their similarity to the azuki linkage map (Han et al. 2005). Map distances and marker names are shown on the left and right side of the linkage groups, respectively. SSR markers with prefix CED, BM, and Vm indicate the marker origin from azuki bean, common bean and cowpea, respectively. RFLP markers showing multiple loci are followed by *lower case letters* indicating signal strength of the fragment: *a* (*strong*), *b* (*weak*). Prefix mgM, mgQ and mgR and mc indicate mungbean probes; cgO and cgP label cowpea probes; Bng identifies common bean probes. AFLP markers E, M were named according to the primer combination followed by the number serially in descending order of molecular size

was colinear with the azuki bean linkage map. These genetic markers were present on every linkage group of black gram and the number ranged from 3 to 11 markers. Black gram linkage group 8 had the longest genomic region with colinear marker loci of 112 cM (nine markers). On linkage group 4, colinear marker loci covered 85 cM (11 markers) (Fig. 2).

Three internal inversions were found between the black gram and azuki bean map. These inversions were on linkage group 1 at markers CEDG204, CEDG048 and mc016, linkage group 2 at markers CEDG006 and CEDG284 and linkage group 5 at markers Bng 027 and CEDG264. A long linkage block of three markers, mgM381, pM228 and CEDC027, on black gram linkage group 10 corresponds to the distal end of azuki bean linkage group 1 (Fig. 2).

Probes Bng180, cgO008, cgO009, Bng031, sgA064, mgM208, cgO029 and mgM381 detected a single locus each on the black gram linkage map. In contrast, these probes could detect at least two loci each on the azuki bean linkage map (Table 3). Among these probes, cgO008 detected a single copy on black gram linkage group 2, whereas this probe could detect two loci on azuki bean linkage group 1 and 2. Furthermore, probe mgM208 and SSR primer CEDG115 detected one locus each at different positions, 16 cM apart on black gram linkage group 5. However, these two markers detected two loci at similar locations on the azuki bean

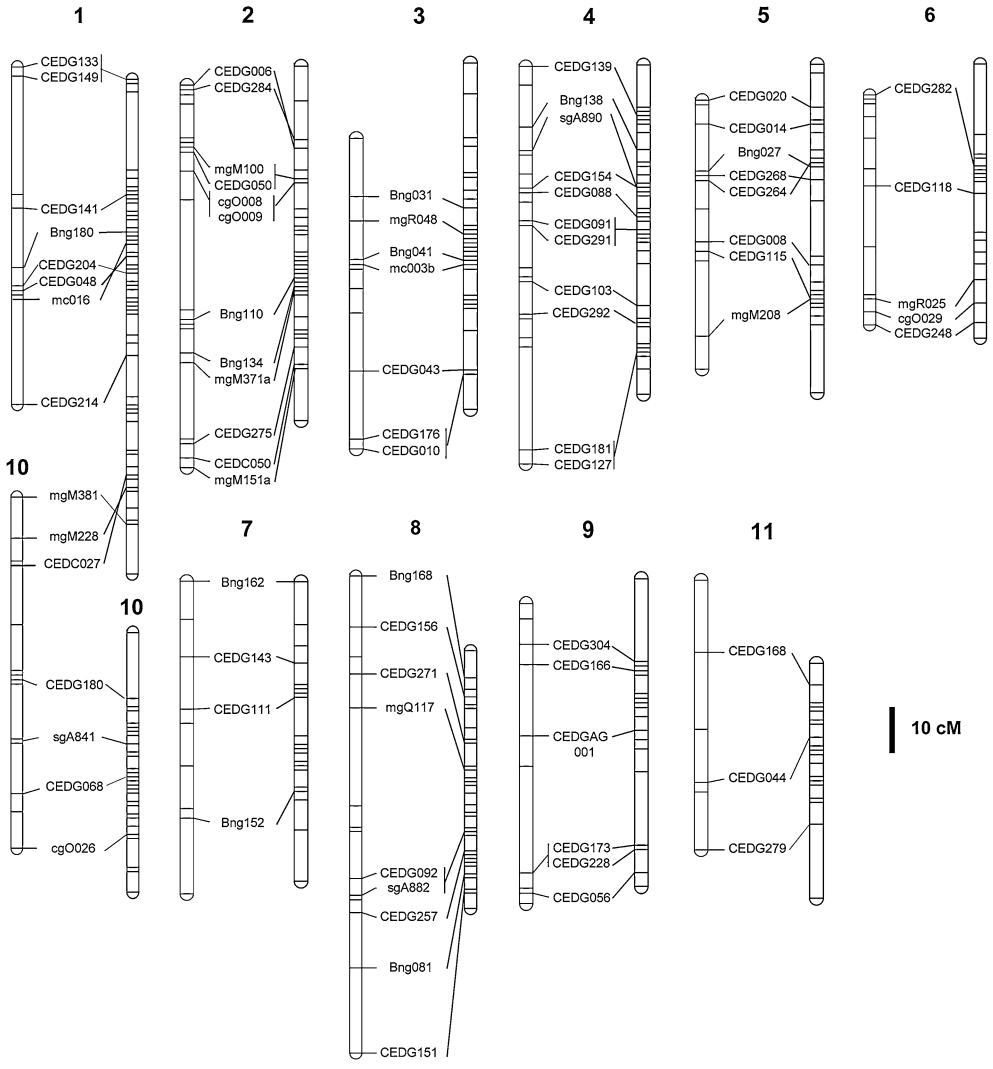

Fig. 2 A comparative genetic linkage map between black gram (*left*) and azuki bean (*right*) based on common RFLP and SSR markers. Linkage groups of the black gram map were aligned with the corresponding linkage groups of the azuki linkage map (Han et al. 2005). The number of the linkage group corresponds to that used for the azuki linkage map. The lines between linkage groups connect the positions of common marker loci

Table 3 RFLP probes detecting a single copy in black gram genome and two or more copies in the azuki bean genome

Probe	Black gram	Azuki bean	
	Linkage group	Copy number	Linkage group
Bng180	1	2	1
cgO008	2	2	1, 2
cgO009	2	3	2, 5, 6
Bng031	3	2	3, 10
sgA064	5	3	1, 6
mgM208	5	2	2, 5
cgO029	6	2	6, 10
mgM381	10	2	1, 10

linkage group 5. This suggests that there has either been duplication or deletion at these sites during the evolutionary divergence of black gram and azuki bean.

Discussion

Since genome level studies of black gram have not previously been conducted it was necessary to use SSR primer pairs and RFLP probes developed for other related species. The high proportion of azuki SSR primer pairs that amplify DNA fragments in other Asian *Vigna* indicates their close relationship (Table 2). Approximately 90% of the SSR primer pairs amplified fragments in *V. umbellata*, of section *Angulares*; whereas in species of section *Ceratotropis* and *Aconitifoliae* 67–78% primer pairs succeeded in amplification (Table 2). This points to the close phylogenetic relationship among the Asian *Vigna*. In addition, azuki bean SSR markers are useful in helping to improve the linkage maps of other Asian *Vigna*. Of the amplified DNA fragments in black gram, 39.8% were able to

detect polymorphism in the black gram mapping population developed here (Table 2).

RFLP probes from mungbean, cowpea, common bean and soybean were all useful for detecting polymorphism in the black gram mapping population. The percentage of useful probes for black gram mapping from mungbean, cowpea and common bean were quite similar, between 47 and 60%, reflecting their close phylogenetic relationship (Table 1). The percentage of useful soybean probes was lower (34%) reflecting the more distant relationship of this species from black gram.

Previously, seven inter and intra-specific linkage maps have been reported for Asian *Vigna* (Han et al. 2005; see Kaga et al. 2005 for review). Most previous linkage maps have used a combination of RFLP and/or RAPD markers for the development of linkage maps. In addition, most mapping populations have consisted of less than 90 individuals. To date, large SSR marker libraries have only been developed for azuki bean among Asian *Vigna* species (Wang et al. 2004) and enabled a saturated genome map for azuki bean to be developed (Han et al. 2005). Here azuki markers have enabled the second Asian *Vigna* linkage map to be developed that has resolved all linkage groups.

The black gram genome map had several gaps of >20 cM. Compared to the azuki bean map some of these gaps corresponded with regions where azuki bean either had many mapped markers (chromosomes 1 and 2) or few mapped markers (chromosomes 1 and 9) (Fig. 2). Locations where azuki had many mapped markers compared with black gram may reflect greater polymorphism due to the genetic distance between parents from which the two maps were developed. The azuki bean map was based on an interspecific cross whereas the black gram map was based on an intraspecific cross. Other locations on the black gram map with gaps correspond to much sorter segments on the azuki bean map (chromosome 4 and 8) and this suggests that there have been genome changes at these locations.

Black gram is classified in section *Ceratotropis* and azuki bean in section *Angulares*. Divergence between sections *Ceratotropis* and *Angulares* is presumed to be high based on chloroplast and nuclear DNA variations (Kaga et al. 1996; Doi et al. 2002; Tomooka et al. 2002b). Here the comparison of the linkage maps of black gram and azuki bean, while showing a high degree of marker colinearity, also reveals genome rearrangements. Some markers detected only a single copy in black gram but could detect two copies in the azuki bean linkage map (Table 3). Duplicated loci (markers) were distributed on more than one linkage group in both the black gram and azuki bean linkage maps. These results indicated that the two species have apparently accumulated a number of deletions/duplications after they diverged. The inversions, insertions, deletions, duplications and translocation that have all been detected between black gram and azuki bean linkage maps indicate that these chromosomal rearrangements played a role in the divergence of these two species.

Among the three Asian *Vigna* sections, section *Ceratotropis*, which includes black gram, it is considered morphologically intermediate between the other two sections (Tateishi 1996). Recent studies of species in the section *Angulares* points to a close relationship among species in this section and their current evolutionary dynamism (Seehalak et al. 2006). The evolutionary relationships among the three sections of the Asian *Vigna* require clarification but the genome level differences between black gram and azuki bean suggest that duplication and deletion events have occurred in azuki bean. Since the nuclear 2C DNA content of black gram is 5% more than azuki bean (Parida et al. 1990) it is likely that there are other genome level differences between these two species that were not detected here.

Black gram linkage map developed here is the first linkage map for black gram. The development of this map has been possible by using genetic markers of related legume species. The black gram and azuki bean linkage map constructed using a common set of markers have revealed a high proportion of genome colinearity. However, due to the presence of gaps in the linkage map of >20 cM, additional markers are required for enhanced resolution of the black gram genome. Ouédraogo et al. (2002) used AFLP markers to improve map density of the cowpea (*V. unguiculata*) linkage map and to identify the markers linked to the parasitic weed *Striga* (*Striga gesnerioides*) and other pathogen and pest resistance genes. The advantages of a high-density linkage map are that it provides a tool for map based cloning and marker assisted selection (Sanchez et al. 2000; Ouédraogo et al. 2002). Black gram accessions have a variety of useful traits such as virus tolerance, bruchid resistance and very large seed size that may be useful for black gram improvement programs or transfer to other *Vigna* (Duke, 1981; Chinchest and Nakeerak, 1990; Tomooka et al. 2000). Integration of more molecular markers and morphological traits to the black gram linkage map will enhance its usefulness for marker-assisted selection. The genetic linkage map of black gram developed here is currently being used to analyze QTL for seed weight, yield related and developmental traits.

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