

Asian *Vigna* Genome Research

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Abstract

To make progress in genome analysis of the Asian *Vigna* cultigens, genetic linkage map for azuki bean (*Vigna angularis*), mungbean (*V. radiata*), black gram (*V. mungo*) and rice bean (*V. umbellata*), among six fully domesticated *Vigna* species in Asia, have been constructed using mapping populations between cultigens and their presumed wild ancestors mainly based on azuki bean genomic SSR markers. Newly developed cowpea genomic SSR markers and soybean EST-SSR markers have been integrated into the mungbean linkage map. Simultaneously, we have constructed a detailed comparative genome map across four Asian *Vigna* species based on these linkage maps. Comparison of the order of common azuki bean SSR markers and RFLP markers on the linkage maps allowed detection of high level macro-synteny between genomes of the four Asian *Vigna* species.

The azuki bean, mungbean, black gram and rice bean have been domesticated from 4 different wild species in different locations in Asia. These cultigens have parallel remarkable morphological and physiological changes the so-called domestication syndrome. The traits related to the domestication syndrome are controlled by several major and some minor QTLs. These QTLs have a tendency to be clustered and non-randomly distributed across the genome. Based on the comparative genome map, the genomic regions of the QTL clusters in mungbean and black gram are different from those of azuki bean and rice bean. Many orthologous QTLs for seed size, seed shattering and plant height were identified. In contrast, different sets of alleles on QTLs controlling seed dormancy and flowering time appears to be used in each species. The present studies on comparative analysis of domestication or adaptation traits that played a major role on the evolutionally changes from wild relative into crop offer further evolutionary potential for Asian *Vigna* cultigens.

The Asian *Vigna* comparative map is being used to develop comparative map between Asian *Vigna* and soybean. Preliminary comparative approaches using sequence information of azuki bean, cowpea and soybean SSR markers on the mungbean linkage map could infer presumed syntenic regions between mungbean and soybean. Although much more information is required to test co-linearity of markers, segmentations of soybean linkage block are frequently observed at most of mungbean linkage groups. Further efforts are need to make steady progress on the establishment of a genomic base for the Asian *Vigna* by collaboration in order to utilize gene and sequence information of soybean in Asian *Vigna* through comparative genome analysis.

Importance of the development of genomic resources and collaborative initiatives for the Asian *Vigna*

The genus *Vigna* is a large genus with about 90 species distributed worldwide in warm and tropical regions. The genus includes 13 cultigens of these cowpea, mungbean and azuki bean, are the most important. Perhaps due to *Vigna* cultigens being mainly crops in the developing world molecular marker and genomic studies have lagged behind other major crops. However, there has recently been considerable progress in cowpea genome studies. Cowpea Genome Initiative was set up in September 2000 by funding from the Kirkhouse Trust, a UK-based charity, and now provides cowpea sequence database (<http://cowpeagenomics.med.virginia.edu/>) from University of Virginia (Timko *et al.* 2008). A database for cowpea physical map will be avail-

able from University of California (<http://phymap.ucdavis.edu:8080/cowpea/>), as an outcome of the CGIAR Generation Challenge Program which has been supported since May 2007. This group is preparing a consensus genetic map containing more than 1000 SNP markers to generate a BAC based cowpea physical map. Cowpea EST database is also available from University of California (<http://harvest.ucr.edu/>). This research is supported by USDA Plant Genome program earlier and CGIAR Generation Challenge Program now.

We frequently use phrase “Asian *Vigna*” conspicuously discriminate species originated in Asia from species in Africa such as cowpea or “African *Vigna*”. Asian *Vigna* and African *Vigna* belong to two different subgenera, *Ceratotropis* and *Vigna*, respectively. Azuki bean (*V. angularis*), mungbean (*V. radiata*), black gram (*V. mungo*) and rice bean (*V.*

umbellata) are taken up as the four major domesticated Asian *Vigna* in the present paper. Besides of these domesticated pulses, moth bean (*V. aconitifolia*) and creole bean (*V. reflexo-pilosa* var. *glabra*) have been fully domesticated and several other *Vigna* species are being domesticated or cultivated across Asia. Comparative studies of these independently domesticated crop from different species such as understanding the evolutionary changes from wild species to crops might offer possible further evolutionary changes of current crop.

The potential use of genetic diversity in the wild species of Asian *Vigna* has been other focus of our studies. The Asian *Vigna* show wide adaptation in relation to the climates in which they grow, from cool and wet temperate to hot and dry tropical climates. Germplasm collection of Asian *Vigna* has resulted in a comprehensive collection of germplasm from several countries in Asia conserved in the Genebank at NIAS. These wild species, collected from various natural habitats, possess potentially useful traits related to environmental stress resistances and insect and disease pest resistances. In spite of the rich genetic resources available for the Asian *Vigna*, establishment of a germplasm evaluation system and genomic tools have lagged behind other crop groups. Genomic studies on Asian *Vigna* are proceeding gradually but as they are not mandated crops of the

CG system have not received the research attention their importance deserves. International collaborative research on Asian *Vigna* will be a powerful motivation to discover and utilize efficiently genes responsible for important economic and adaptive traits.

Development of molecular marker and genetic linkage map of the Asian *Vigna*

To make progress in genome analysis of the Asian *Vigna* cultigens we have developed azuki bean SSR library construction involved an oligo-primed second-strand synthesis enrichment procedure on single strand genomic library (Wang *et al.* 2002). The enriched library enabled low redundant SSR containing clones to be identified and SSR primers revealed a high percentage of successful single-locus amplification. These SSR markers have successfully involved in trait mapping (Chaitieng *et al.* 2006; Isemura *et al.* 2007; Gupta *et al.* 2008; Somta *et al.* 2008; Kaga *et al.* 2008) and evaluation of other Asian *Vigna* germplasm (Sangiri *et al.* 2007; Gupta and Gopalakrishna 2009). The SSR marker information is available from the NIAS Genebank database

(http://www.gene.affrc.go.jp/databases-marker_information_en.php).

Azuki bean linkage map: The azuki bean SSR

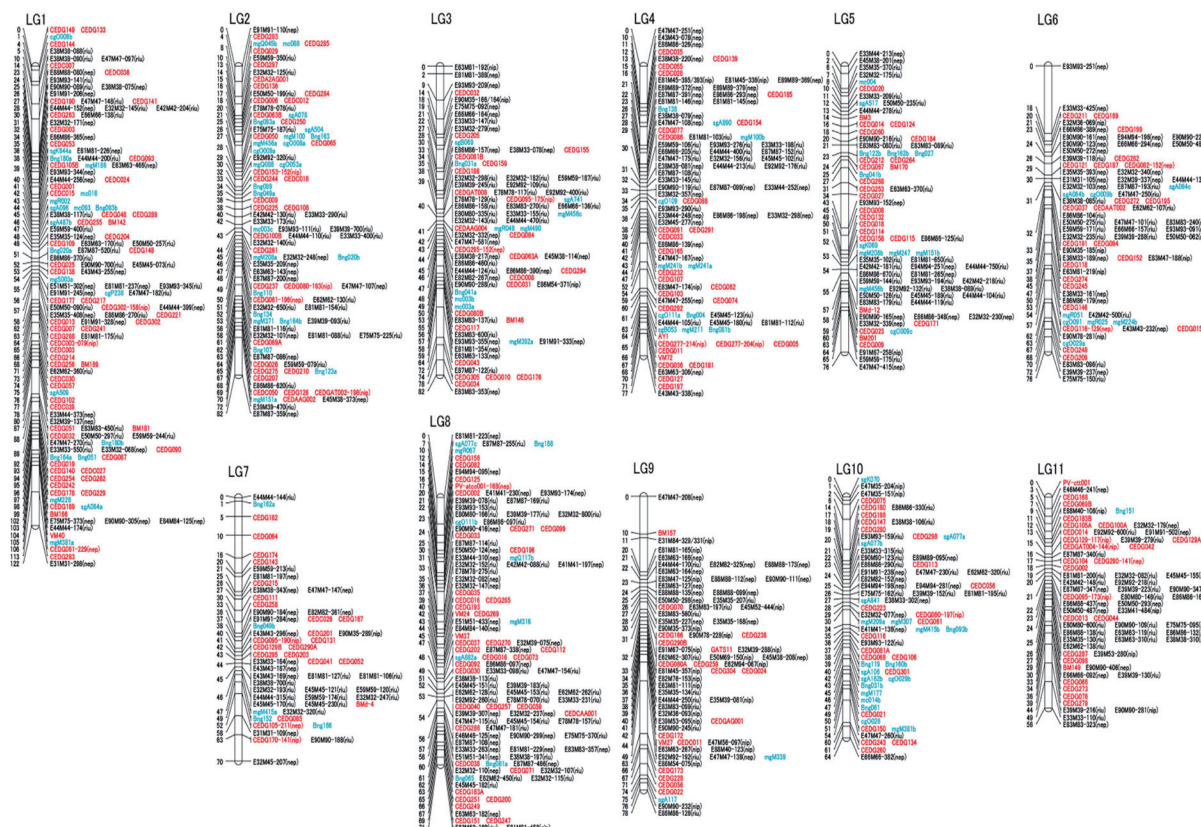


Fig.1. Consensus genetic linkage map of azuki bean (*Vigna angularis*). Azuki bean SSR, AFLP and RFLP markers are indicated by red, black and blue, respectively.

markers in addition to AFLP and RFLP markers from other legumes were used to develop the most saturated genome map for Asian *Vigna* (Han *et al.* 2005). This map has 205 SSR, 93 RFLP and 187 AFLP markers. Further, we integrated marker information from three different mapping populations, (*V. nepalensis* x *V. angularis*) x *V. angularis* BC₁F₁ population (Han *et al.* 2005), *V. angularis* var. *angularis* x *V. angularis* var. *nipponensis* F₂ population (Kaga *et al.* 2008) and (*V. angularis* x *V. nepalensis*) x *V. angularis* BC₁F₁ population (Kaga unpublished), into single consensus linkage map. Currently this map consists of 896 markers and spans a total length of 854 cM. The average distance between SSR markers is 3.1 cM and this is the standard linkage map for azuki bean (Fig.1).

Black gram linkage map: The first genome map was developed by Chaiteng *et al.* (2006) using the azuki bean SSR and RFLP makers from other legumes. Recently a more saturated map consisting of 254 AFLP, 47 SSR, 86 RAPD and 41 ISSR markers is reported by Gupta *et al.* (2008). Relatively lower amplification or polymorphisms of the azuki bean SSR markers in black gram than the other Asian *Vigna* has force the use PCR-based anonymous dominant markers. Development of SSR markers is prerequisite to obtain more informative markers for black gram.

Rice bean linkage map: Isemura *et al.* (2010) developed the first genome map for rice bean by using 172 azuki bean SSR markers, 103 AFLP markers, 55 SSR markers from other legumes. The linkage map has been successfully resolved into 11 linkage groups. Among the 55 SSR markers, 44 are newly designed cowpea SSR markers. Since gene Thresher sequence database, containing mostly gene-rich genomic sequence, has become available from <http://cowpeagenomics.med.virginia.edu/CGKB/> (Chen *et al.* 2007), we expected the SSR containing 30,877 sequences deposited in the database might provide a good marker resources for Asian *Vigna*. After removal of redundant sequences, 6,580 sequences can be used to design SSR primer pairs. Approximately 480 cowpea SSR markers containing mainly di-nucleotide repeat with more than 25 repeat have been tested for amplification and polymorphisms among the parents of the rice bean mapping population, but only half of the markers produce a single reliable PCR product, suggesting the cowpea genome sequence is well diverged from rice bean. Only 44 cowpea SSR markers have been integrated into rice bean linkage

map.

Mungbean linkage map: Isemura *et al.* (paper in preparation) have constructed a linkage map that initially resolved into 11 linkage groups. The map has been constructed with 141 SSR markers from azuki bean, 30 SSR markers from mungbean, 196 EST-SSR makers from soybean, 59 cowpea SSR markers described above and 9 common bean SSR markers. All of the markers on the map are highly informative because these have sequence information. Although stable and single locus PCR amplification of 7,000 soybean EST-SSR markers developed by Hisano *et al.* (2007) was very difficult in Asian *Vigna*, 196 of 7000 markers have successfully mapped. These soybean EST-SSR markers are very useful for identifying syntenic regions such that new candidate EST-SSRs were able to fill a large gap at one problematic linkage group, 4, of mungbean.

Genome synteny among Asian *Vigna*

Comparison order of common azuki bean SSR and RFLP markers on the linkage maps constructed above with each other allowed the detection of a high level macro-syntenly between the genomes of four different Asian *Vigna*. The high level synteny ensures that chromosome nomenclature of Asian *Vigna* species other than cowpea can be standardized by cytogenetical studies to

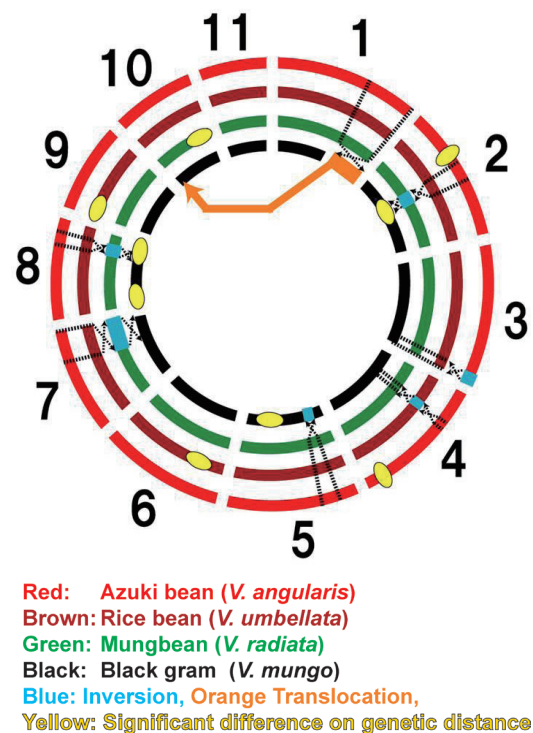


Fig.2. Comparative linkage map among four major Asian *Vigna* species. Numbers aside circumferential map indicate name of linkage group.

determine chromosome length and is certainly useful for future research. Major difference is a chromosomal translocation where a block on linkage group 1 in black gram, the most divergent species among four species, has moved to linkage group 10 (Fig.2, orange). Azuki bean SSR markers that can be compared are gradually reduced as genetic divergence increased from azuki bean, several inversions (Fig.2, blue) and many changes on genetic distance (Fig.2, yellow) are inferred in some species. Azuki bean and rice bean belongs to the same section *Angulares* of the subgenus *Ceratotropis* and their speciation appears to be recent as interspecific genetic divergence is not so great. As expected, rice bean shared a highly conserved genome organization with azuki bean as well as highly successful transferability of azuki bean SSR markers and supported well results of previous cytogenetical studies. Mungbean belongs to section *Ceratotropis*, including black gram, but showed genome similarity to azuki bean and rice bean in the section *Angulares* rather than black gram. The comparative map provides adequate syntenic context of domestication related traits among Asian *Vigna* discussed below.

Domestication syndrome of Asian *Vigna*

Four major Asian *Vigna* cultigens, azuki bean, mungbean, black gram and rice bean, have been domesticated from 4 different wild species in different location of Asia (Fig. 3).

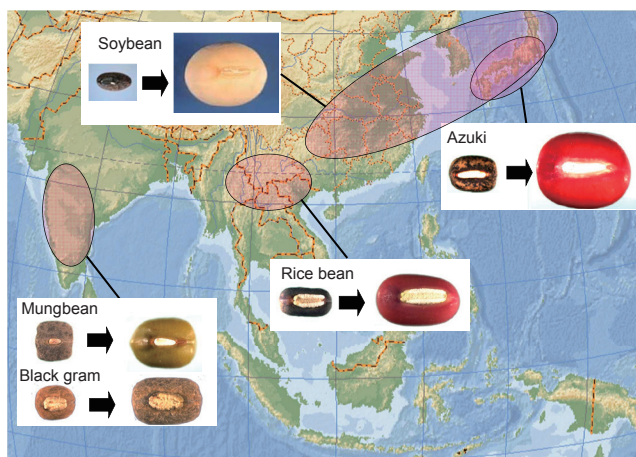


Fig 3. The putative domestication center of major legumes in Asia. Right and left of seed pictures indicate the cultigen and its presumed wild ancestor, respectively.

physiological changes such as the increase in organ size of various plant parts, loss of dormancy and loss of pod shattering, the domestication syndrome (Hammer 1984). We have been collected QTL information on the domestication or adaptation related traits in azuki bean (Kaga *et al.* 2008, Isemura *et al.* 2007), mungbean (Isemura *et al.* paper in preparation), black gram (Chaitieng *et al.* 2010) and rice bean (Isemura *et al.* 2010), and now these detailed comparative maps of four Asian *Vigna* facili-

tates comparison of major QTLs for the domestication related traits across these species.

Previous genetic studies have revealed that traits related to the domestication syndrome in various crops are controlled by several major and some minor genes with non-random distribution across genomes (Gepts 2004). A similar situation exists in the four Asian *Vigna*. QTLs for various domestication or adaptation related traits of azuki bean were clustered on linkage groups 1, 4, 7 and 9. Such clusters were found at similar locations on rice bean linkage groups 1 and 7. The genomic regions of the QTL clusters in mungbean and black gram were different from azuki bean and rice bean. These are distributed mainly on mungbean linkage groups 2 and 6 and on the black gram linkage groups 8 and 9. Further studies are required to determine whether QTLs for traits related to the domestication syndrome are clustered at locations that have highly suppressed recombination or by pleiotropic effects of some key gene with large effect. Since pericentrometric genome regions have been elucidated in the soybean genome, alignment *Vigna* genome with soybean will be very useful to clarify the former hypothesis.

Distribution of QTL locations on the Asian *Vigna* comparative map are shown for seed size, seed dormancy and seed shattering (Fig. 4), and plant height and

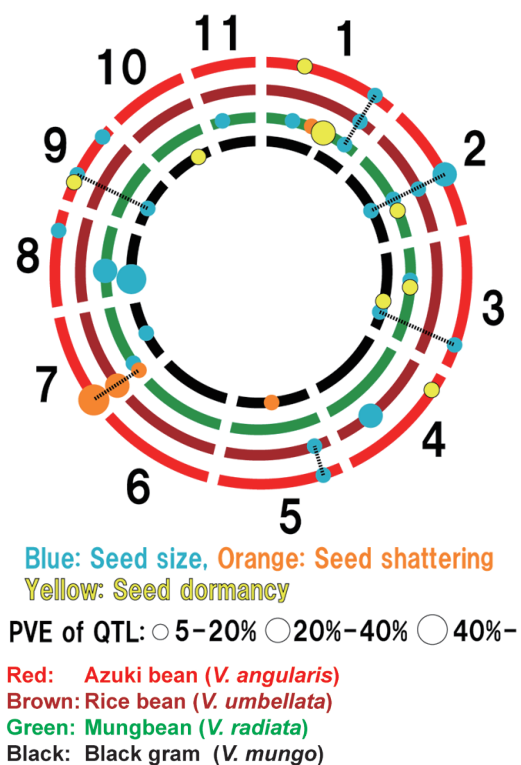


Fig.4. Distribution of major QTLs for seed size, shattering and seed dormancy on a comparative map for Asian *Vigna*.

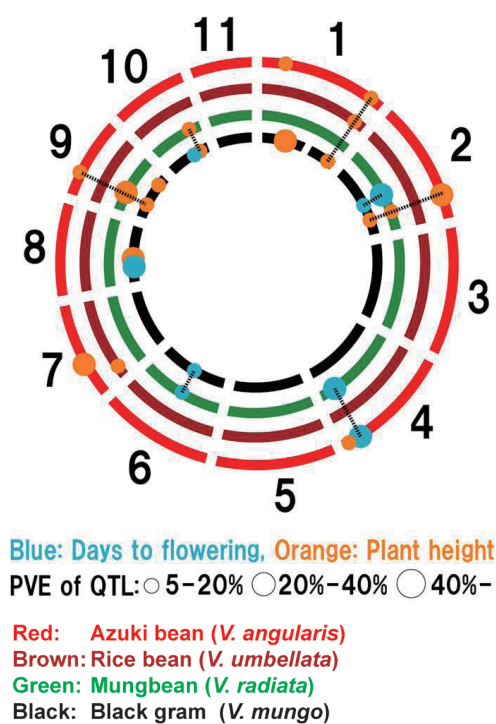


Fig.5. Distribution of major QTLs for flowering and plant height on comparative map for Asian *Vigna*.

flowering (Fig. 5). Despite the limitations of QTL information results from a single specific cross combination in each species and the extent of morphological and physiological differences between cultigens and the wild ancestors are not the same across four species, some QTLs were identified in all species and some were specific to one or several species.

Among variation between cultigens and their wild ancestors, seed size is conspicuous and the extent differ across four species. For example, mungbean has seeds approximately 5 fold as heavier than its wild ancestor, and azuki bean has seeds approximately 10 fold as heavier than the wild ancestor. However, there was no difference in the number of QTL between mungbean and azuki bean. Seed size QTL, probably species specific, with a large increase effect was identified in rice bean on linkage group 4 and in black gram on linkage group 8. In contrast, orthologous QTLs across all four cultigens and three cultigens were found on linkage groups 2 and 1, respectively.

Seed shattering is an important characteristics to prevent seed loss before harvesting. Orthologous QTL with a large effect for loss of seed shattering for azuki bean and rice bean was found in linkage group 7. Seed shattering of mungbean is controlled by partially the same QTL with minor effect and another QTL on linkage group 1. In contrast, black gram had a different QTL with minor effect on the linkage group 5. Most seed shattering QTL were clustered with QTL for pod length. Large variation in pod length are observed among the

four wild ancestors; pod length of wild azuki bean and wild rice bean are longer than those of wild mungbean and wild black gram. Each wild species may have evolved different physiological mechanism and/or gene for pod dehiscence.

Seed dormancy QTLs were completely different among the *Vigna* species. The parallel phenomena of loss of dormancy appear to involve different genes in mungbean, black gram and azuki bean. There are different seasonal changes of climate where cultivated and wild species are distributed; mungbean and black gram (dry and rainy season) and azuki bean (cold and rainy warm season), might be one of factors that resulted in the different physiological mechanisms underlie seed dormancy in *Vigna*.

A different set of alleles on QTL controlling flowering time appears to be used in each species. Since the presumed wild ancestors of mungbean and black gram are distributed in lower latitudes, different genes from azuki bean distribute in higher latitude might have involved different photothermal responses which are important for flowering response. However, an orthologous QTL between mungbean and azuki bean was identified on linkage group 4. Since significant progress in the identification of major genes which control flowering time in soybean, has been made, comparative analysis of a conserved syntenic region between soybean and Asian *Vigna* will bring about greater understanding of the flowering mechanism in Asian *Vigna*.

The wild ancestors of Asian *Vigna* possess an indeterminate habit in which the plant continues to grow in length throughout its life whereas most domesticated Asian *Vigna* have a determinate growth habit. Only domesticated rice bean has indeterminate growth habit. Two orthologous QTLs for plant height on the linkage group 2 and 9 were found in mungbean, black gram and azuki bean mapping populations and the allele of the cultigens reduced plant height, suggesting that these two QTL might be important for transition of plant architecture into determinate habit.

Morphological and physiological changes from wild ancestors into cultigens might be consciously selected by the farmer preference and their agricultural activities. However, it is interesting that several orthologous genes underlying domestication appear to have been unconsciously selected across four Asian *Vigna* based on an interplay of photoperiod sensitivities (long and short day), seasonal changes of climate (dry, rainy, hot and cold), etc. In addition, the present studies on comparative analysis of domestication or adaptation traits that played a major role on the evolutionally changes from wild species to crop offer further evolutionary potential to Asian *Vigna* cultigens. These four major cultigens are

Table 1. Presumed syntenic regions between mungbean and soybean. Each mungbean linkage group is divided into three parts. Letter in parenthesis and after inequality sign indicate soybean linkage group with second highest score based on similarity search and changes to another linkage groups in the middle of partition, respectively.

Mungbean linkage group (MLG)	Corresponding soybean linkage group			No.of makers compared
	Top of MLG	Middle of MLG	Bottom of MLG	
1	A1(A2) > M	A1 > A2 > J	A1 > D2(E,F)	36
2	C2(C1)	C2(C1)	E(K)	30
3	B2(D1b)	B2(D1b)	B2(D2)	24
4	G(A2) > L	J > L(N)	nd	21
5	E or K	E or K > L(N)	G	23
6	nd	B1 or H	B1 or H	13
7	E or F > F or H	nd	nd	14
8	I or O	I or O	O > D1b	21
9	N	nd	A2(M)	10
10	D1a	D1a > K or B1	K or B1	13
11	L	L > K	K > K(M)	17

within each others secondary genepool, so verification of this hypothesis will be possible by the cross breeding. For example, introduction of azuki bean QTLs controlling plant height on linkage group 2 and 9 into rice bean and introduction of rice bean QTL controlling seed size on linkage group 4 into azuki bean will assist breeding varieties with determinate habit in rice bean and increased seed size in azuki beans.

Genome synteny between Asian *Vigna* and soybean

Soybean EST-SSR markers have been integrated into the *Vigna* linkage maps to efficiently utilize soybean sequence data via synteny. Since December 2008, the chromosome-scale assembly of the soybean genome, Glyma 1.0, has been available from Soybean Genome Project, DoE Joint Genome Institute (<http://www.phytozome.net/soybean.php>). Initially, synteny was examined comparing locations 196 soybean EST-SSR markers on the mungbean linkage map with the soybean linkage map. However, the number of mapped markers common to both the mungbean and the soybean linkage map was inadequate to find syntenic region by direct comparison. Many integrated *Vigna* SSR markers, azuki bean and cowpea SSR, are expected to provide sequence data that can identify similar soybean chromosomal location as well as soybean EST-SSR markers. Alternatively, similarity search using blastn program with a cut off E-value of E-20 between the Glyma 1.0 sequence database and sequence of the mapped *Vigna* SSR markers was performed and location of the top hit sequence from 171 *Vigna* SSR markers has been

used for comparison.

The result was very complex all query sequences from each marker matched to several soybean chromosomes within the database and mungbean linkage group was composed of several soybean chromosomes. Since extensive sequence differences exist between soybean and *Vigna* so far as genomic sequences around SSR compared, similarity search with a cut off E-value of E^{-4} , a lower threshold, increased to 222 marker comparisons between mungbean and soybean linkage groups.

Table 1 indicates presumed syntenic regions between mungbean and soybean. This shows similar soybean linkage groups with a highest and second highest sequence similarity, but further comparison using more information may alter this result. On mungbean linkage group 5, 6, 7, 8 and 10, we could not determine single syntenic soybean linkage group because two soybean similar chromosome blocks, may be paralogous blocks, that revealed top hit with the sequences around *Vigna* SSR tend to jumble together. Although much more information is required to test co-linearity, segmentations of soybean linkage block are also observed for most of the mungbean linkage groups. Only one mungbean linkage group revealed high similarity to a soybean linkage group (B2), mungbean linkage group 3. When multiple comparisons that include second hit information are tested, the top to central part of mungbean linkage group 3 is similar to soybean linkage group D1b. Such segmental duplication among soybean chromosomes has been reported by Shoemaker *et al.* (2006) and Hisano *et al.* (2007).

Other instances are A1 and A2, C1 and C2,

Bland H and these are supported in mungbean linkage group 1, 2 and 6, respectively. Soybean is well known as a diploid species having 20 chromosomes, but these chromosomes are thought to be derived from at least two rounds of genome wide duplication and many rearrangements, a paleopolyploid species (Shoemaker *et al.* 2006, Walling *et al.* 2006). These events might prevent finding clear syntenic relationships between mungbean and soybean as was observed between soybean and the model legume, *Lotus japonicus* (Hisano *et al.* 2007). In future, extensive investigation into distribution and extent of paralogous genomic segments within soybean genome sequence is essential to utilize soybean sequence information simply in other legumes.

Future perspectives

We plan to combine genome map information of four different Asian *Vigna* into single *Vigna* consensus map to integrate marker information bridging the soybean sequence. The soybean sequence information has the potential to serve as a landmark for gene identification through comparative genome analysis. However, it might be too complex for direct use of such information in marker development in the Asian *Vigna*. Since a high level of macro-syteny between the genomes of four different Asian *Vigna* are observed, development of the genomic foundation with sequence information, for example in azuki bean as a representative species, would be an effective approach for various *Vigna* species. There is a growing tendency for sequencing various organisms by read length improvements of next generation sequencing technologies. As with the success of the soybean genome sequence assembly based on information of a high density linkage map and FPC (Finger Printed Contigs) of BAC clones, there is a need to make steady progress on the establishment genomic base by collaboration until embarking on genome sequencing of Asian *Vigna*. To this end use of gene and sequence information from soybean in Asian *Vigna* through comparative genome analysis will be most helpful.

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