# A High-density Linkage Map of *Lotus japonicus* Based on AFLP and SSR Markers

Xinwang Wang<sup>1,\*,†</sup>, Shusei Sato<sup>2</sup>, Satoshi Tabata<sup>2</sup>, and Shinji Kawasaki<sup>1,\*</sup>

National Institute of Agrobiological Sciences, Kannon-dai 2-1-2, Tsukuba, Ibaraki 305-8602, Japan<sup>1</sup> and Kazusa DNA Research Institute, 2-6-7 Kazusa-kamatari, Kisarazu, Chiba 292-0818, Japan<sup>2</sup>

(Received 18 June 2008; accepted 18 August 2008; published online 14 September 2008)

#### Abstract

A collection of 94 F<sub>6</sub> individuals derived from crosses between *Lotus japonicus*, Gifu B-129 (G) and Miyakojima MG-20 (M) were used for mapping. By using the HEGS running system, 427 *Eco*RI/*Mse*I primer pairs were selected to generate a total of 2053 markers, consisting of 739 G-associated dominant markers, 674 M-associated dominant markers, 640 co-dominant markers, 95 SSR markers and 2 dCAPS markers. Excluding heavily distorted markers, 1588 were mapped to six chromosomes of the *L. japonicus* genome based on the 97 reference markers. This linkage map consisted of 1023 unique markers (excluding duplicated markers) and covered a total of 508.5 cM of the genome with an average chromosome length of 84.7 cM and interval distance of 0.50 cM. Fifteen quantitative traits loci for eight morphological traits were also mapped. This linkage map will provide a useful framework for physical map construction in *L. japonicus* in the near future.

Key words: Lotus japonicus; AFLP; SSR; linkage map; HEGS (high efficiency genome scanning)

#### 1. Introduction

Genetic mapping is a basic tool of eukaryotic genomic research. Molecular linkage maps provide information about the organization of the genome and can be used for genetic studies and breeding applications. A high-density genetic linkage map is essential to physical map construction and also a powerful tool for the location and map-based cloning of desired gene(s). PCR-based DNA markers make such a linkage map possible. Of these PCRbased markers, AFLP markers were demonstrated to be a powerful new class of markers making it feasible to develop linkage maps for plants with a large genome.<sup>1</sup> There are many linkage maps based on AFLP markers reported for various plants.<sup>2–6</sup>

Lotus japonicus is an autogamous diploid legume species.<sup>7</sup> As a model legume, it has many characteristics that make it a candidate for genomic research. These attributes are an autogamous diploid (2n =12) and small genome  $(432 \sim 494 \text{ Mb})$ ,<sup>8–10</sup> short life cycle and transformation ability,<sup>6,11</sup> making *L*. japonicus a model legume plant that can be used for molecular genetics and physiological studies. Genome synteny will help in marker preparation and gene cloning for other legume crops. To date, the cloning of various nodulation genes has been the subject of heated international competition. Therefore, the need for construction of a physical map covering the genome of *L. japonicus* is especially important. A primary genetic linkage map based on DAF (DNA Amplification Fingerprinting) markers with an  $F_2$  population from a cross of *L. japonicus* accessions, Gifu B-129 and Funakura B-581 has

The online version of this article has been published under an open access model. Users are entitled to use, reproduce, disseminate, or display the open access version of this article for non-commercial purposes provided that: the original authorship is properly and fully attributed; the Journal and Oxford University Press are attributed as the original place of publication with the correct citation details given; if an article is subsequently reproduced or disseminated not in its entirety but only in part or as a derivative work this must be clearly indicated. For commercial re-use, please contact journals.permissions@oxfordjournals.org

Edited by Katsumi Isono

<sup>\*</sup> To whom correspondence should be addressed. Tel. +1 865-974-1070. Fax. +1 865-974-4744. E-mail: xinwangwang@ hotmail.com (X.W.) or kawasa@nias.affrc.go.jp (S.K.)

Present address: The University of Tennessee, Knoxville, TN 37996, USA.

<sup>©</sup> The Author 2008. Kazusa DNA Research Institute.

been reported.<sup>8</sup> However, due to low polymorphism observed in this cross, the number of linkage groups did not cover all chromosomes of the genome. Crosses made with 'Miyakojima MG-20' showed the highest-level of polymorphisms relative to Gifu B-129 (>4%).<sup>12</sup> Although this recombination rate is still low when compared with other plants,  $2^{-5}$  a fast and simple high efficiency genome scanning (HEGS)<sup>10</sup> AFLP protocol system can overcome this disadvantage. Although traditional AFLP protocols simultaneously assay for large numbers of polymorphic bands on a single gel, developing more than 2000 AFLP markers is time consuming and laborious. The HEGS system allows the development of AFLP markers in a short time.<sup>10</sup> The HEGS gel running apparatus is composed of a set of  $24 \text{ cm} \times 26 \text{ cm}$ glass plates. One hundred samples can be analyzed on a two-layer gel that is composed of 13% bis: arcylamide (19:1) separating gel and 5% stocking gel at 350 V for 4 h. As a result, 800 individuals can be analyzed on eight sets of plates per day by one person. By using the HEGS/AFLP system, some linkage maps have been developed for L. japonicus.<sup>13,14</sup> For example, Hayashi et al.<sup>13</sup> constructed a linkage map consisting of 287 markers (AFLP, SSR, dCAPS and other PCRbased markers) that spanned a total length of 487.3 cM and corresponded to six chromosomes in the Lotus genome using an  $F_2$  population from a cross of 'Gifu B-129' and 'Miyakojima MG-20'. This study will use the map of Hayashi et al.<sup>13</sup> as reference to build a high-density linkage map in L. japonicus.

To construct a fine physical map, however, requires a linkage map of *L. japonicus* with sufficient high marker density (>1000 markers with less than 1 cM interval distance). This linkage map will also be used for gene cloning in future research. The resulting high-density linkage map created in this study will serve as a framework for building a genome physical map that will be suitable for mapbased cloning in *L. japonicus* genetic research.

### 2. Materials and methods

#### 2.1. Plant materials and DNA extraction

A cross of *L. japonicus* accessions 'Gifu B-129' and 'Miyakojima MG-20' was made at Kazusa DNA Institute, Japan. Gifu B-129 has a crawling habit with a red stem and Miyakojima MG-20 has erect habit with a green stem. A mapping population of 194  $F_6$  recombinant inbred lines (RILs) was obtained by single-seed descent method. A collection of 94 from the 194 RILs was randomly selected as a mapping population for convenient analysis of the HEGS/AFLP running system.

Genomic DNA was extracted from young leaves of the 94 individuals. In brief, 0.3 g of young leaves were collected in a 50 ml Falcon tube including five metal beads, immersed in liquid nitrogen and ground with a shaker (EYELA cute Mixer CM-100), 250 rpm, 30 s, twice. Immediately, the powder was incubated with 2 ml extraction buffer (10% CTAB: 10% SDS and sodium lanroylsarcosine = 1:2, preheated) at  $65^{\circ}C$ for 1 h with gentle shaking. About 2 ml of aqueous phase were transferred to a 6-tube strips and DNA was extracted automatically using KURABO NA-2000 (Japan) following the manufacturer's instructions. The DNA pellet was dried and dissolved in 50-100  $\mu$ l 0.1  $\times$  TE (10 mM Tris–HCl and 0.1 mM EDTA, pH 8.0). The dissolved DNA samples were treated with RNase A (50  $\mu$ g/ml) for 3 h at 37°C.

#### 2.2. SSR analysis

Microsatellite (SSRs) and dCAPS analysis were performed based on the methods of Sato et al.<sup>15</sup> with the minor modifications. Genomic DNA (0.2 ng) was used in a total volume of 5  $\mu$ l containing 0.0125 U Extaq polymerase (TaKaRa, Japan). The annealing temperature was set to 60°C. The sequence information of all SSR and dCAPS primers (written in TM prefix with serial number) can be found in Sato et al.<sup>15</sup> All the TM primers were synthesized from Invitrogen, Life Technologies, Japan.

#### 2.3. AFLP analysis

The AFLP assays were performed as described by Vos et al.<sup>1</sup> with the following modifications. Genomic DNA samples (250 ng) were digested with 8 U of EcoRI and 5 U of Msel (Biolabs Inc., New England) in a reaction volume of 25  $\mu$ l with 1  $\times$  Not I preservation buffer (10 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, pH 8.0, 0.01% BSA, 0.15% Triton X-100, 50% glycerol), mixed briefly and incubated at 37°C for 3 h. Five microliters of the digestion solution were mixed with 1  $\mu$ l *Eco*RI adapter (5 pmol/ $\mu$ l), 1  $\mu$ l *Msel* adapter (50 pmol/ $\mu$ l), 1  $\mu$ l 10 $\times$  ligation buffer and 28 U of T4 DNA ligase (TaKaRa) in a total of 10 µl of reaction volume and incubated at 37°C overnight. This digestion-ligation solution was diluted 10-fold in  $0.1 \times$  TE buffer and used for pre-amplification. Pre-amplification was conducted in 25 µl reaction volume containing 2.5  $\mu$ l diluted adaptor-ligated DNA, 1  $\mu$ l *Eco*RI+A primer (5 ng/ $\mu$ l), 1  $\mu$ l *Mse*I+C primer  $(30 \text{ ng/}\mu\text{l})$ , 2  $\mu\text{l}$  2.5 mM dNTP each, 2.5  $\mu\text{l}$  ExTaq buffer and 0.5 U ExTaq DNA polymerase (TaKaRa). The PCR profile was 94°C for 30 s, 56°C for 1 min, 72°C for 1 min and 20 cycles with a 10 min final extension at 72°C. The PCR products were diluted 100-fold in  $0.1 \times$  TE buffer and stored at  $-20^{\circ}$ C until further use. Selective amplification was performed in 5 µl

reaction volume containing 2.45  $\mu$ l diluted pre-amplification products, 0.5  $\mu$ l *Eco*RI +3 primer (7 ng/ $\mu$ l) and 1.125  $\mu$ l *Mse*I+3 primer (7 ng/ $\mu$ l), 0.4  $\mu$ l 2.5 mM dNTP each, 0.5  $\mu$ l ExTaq buffer and 0.025 U ExTaq DNA polymerase (TaKaRa). The touchdown PCR profile was one cycle at 94°C for 30 s, 68°C for 30 s and 72°C for 60 s, 17 cycles with the annealing temperature reduced 0.7°C/cycle, and 23 cycles with an annealing temperature of 56°C and with final extension at 72°C for 10 min.

# 2.4. Gel running with HEGS running system

Five microliters of PCR samples were mixed with 1  $\mu$ l loading buffer (0.25% xylene cyanol, 0.25% bromophenol blue, 1 mM EDTA, pH 8.0, 40% glycerol) and separated using the HEGS running system.<sup>5</sup> In brief, 6 µl of each sample per lane were loaded into polyacrylamide gels consisting of stacking gel (upperside gel, 2.5 cm in depth, 5% bis-polyacrylamide contained 0.5 M Tris-HCl, pH 6.8) and running gel (lower-side gel, 13% bis-polyacrylamide contained 1.5 M Tris-HCl, pH 8.8). A total of 94 F<sub>6</sub> individuals and two parents and  $\Phi X174$ -Hae III (50 ng/lane) in one gel was run in  $1 \times$  Tris-glycine buffer (25 mM Tris-HCl, pH 8.3, 1.92 M glycine) at 100 V for 80 min followed 350 V for 4.5 h. The gels were stained in 1/10 000 volume Vistra Green dye solution (Amersham Pharmacia Biotech) for 10-20 min, washed in water for 5-10 min and scanned in FluorImager 575 (Molecular Dynamics, Amersham Pharmacia Biotech). We therefore employed the HEGS/AFLP analysis with 94 F<sub>6</sub> recombination lines from a cross of 'Gifu B-129' and 'Miyakojima MG-20' to develop a large number of markers. This approach attempts to construct a high-density linkage map containing  $\sim$ 2000 markers with an average of <1 cM interval distance between markers.

# 2.5. Data collection

Clear polymorphic bands were selected using the F<sub>2</sub> generation genome analysis software (Kazusa DNA Research Institute, Japan), and the bands were confirmed visually. This software also automatically assigned molecular weights to the fragments, distinguished the single polymorphic band from parents before manually setting paternal and maternal alleles, and generated reports of fragment presence/ absence in 1/0 binary type. Only clearly visible markers were scored. For F<sub>6</sub> progeny, band presence associated with the Gifu B-129 allele was coded as A; band presence with Miyakojima MG-20 allele was coded as B, and those bands with both female and male parent were coded as H for heterozygote. Each AFLP marker was identified by a code referring to the primer combination, EM (EcoRI/Msel) and character

G (associated with Gifu B-129 allele), M (associated with Miyakojima MG-20 allele) or C (co-dominant marker), followed by the estimated size of the DNA fragment in nucleotides. The heterozygote (for the co-dominant markers) and missing data were coded as <sup>C</sup>.

Eight morphological traits, plant type (PT), stem pigment (SP), leaflet (LL), stipule (ST), petiole (PE), trichome (TR), seed color (SC) and seed size (SS), were scored as qualitative traits. We scored grade 1–5 for all traits. PT: 1—crawl stem and 5—stand stem; SP: 1 light color (green) stem and 5—dark color (red) stem; LF: 1—no LL and 5—long LL; ST: 1—no ST on the petal and 5—most STs; PE: 1—none and 5—longest; TR: 1 no TR and 5—most; SC: 1—lightest color and 5—dark (brown); SS: 1—smallest and 5—largest.

# 2.6. Linkage map construction

Before linkage analysis, chi-square tests ( $\chi^2$ ) were performed on both SSR and AFLP markers for goodness of fit to the expected Mendelian 1:1 segregation ratio of each marker. Distorted loci that deviated significantly at P <0.01 were excluded from map construction. Linkage analysis was performed with the Joinmap 3.0.<sup>16</sup> Initially, an LOD score of 14.0 was used to identify six linkage groups, corresponding to six chromosomes of *Lotus* genome based on the previously mapped SSR/ dCAPS markers<sup>13</sup> (details can also be found from http://www.kazusa.or.jp/lotus/markerdb\_index.html). The Kosambi mapping function<sup>17</sup> was used to convert recombination frequencies into map distances. Linkage maps were drawn using MapChart 2.1 software.<sup>18</sup>

A subset of markers spanned across the linkage map with even distance of 5 cM was selected and used for composite interval mapping.<sup>19</sup> Quantitative traits loci (QTL) analysis was carried out using WinQTL Cartographer 2.5.<sup>20</sup>

# 3. Results

# 3.1. AFLP markers generated in HEGS system

Ninety-four F<sub>6</sub> individuals and two parents were analyzed on a single gel (Fig. 1); 4096 EcoRI-Msel primer combinations were first screened on the parental DNAs (data not shown). Of the primer pairs (Supplement 1) that produced the most polymorphic bands, 427 were selected for further AFLP analysis for 94 random individuals of  $F_6$  population. Initially, a total of 2053 diagnostic AFLP markers, which 739 Gifu B-129 dominant, included 674 Miyakojima MG-20 dominant and 640 co-dominant markers, were scored (Fig. 2, Table 1). On average, 4.8 AFLP markers and 1.5 co-dominant markers were generated per primer combination, with a range of 2-8 visible markers (data not shown).



**Figure 1.** A sample of AFLP gel profile with a combination of E-ATG I/M-ACC primers for 94  $F_6$  individuals from a cross of *L. japonicus* accessions, 'Gifu B-129' and 'Miyakojima MG-20' with HEGS system. Lanes from left: lane 1, size marker  $\Phi$ X174-*Hae* III; lane 2, maternal 'Gifu B-129' (associated allele was assigned as 'G', showed as arrow); lane 3, paternal 'Miyakojima MG-20' (associated allele was assigned as 'M', showed as arrow); lanes 4–97,  $F_6$  individuals. Co-dominant allele was assigned as 'C', showed as arrow.



Figure 2. Distributions of 2053 AFLP alleles from 94 F<sub>6</sub> individuals derived from a cross of *L. japonicus*, 'Gifu B-129' and 'Miyakojima MG-20'.

The polymorphism ratio per primer pair combination agreed with that of Kawaguchi et al.<sup>12</sup> The electrophoresis profile (Fig. 1) scanned by the FluorImager 575 provided sufficient resolution to distinguish fragment mobility from 70 to 5000 bp, indicating a wide range of the amplification fragments with high resolution using the HEGS/AFLP-SSR running system (in ~24 cm long gel). A total of 95 SSR and 2 dCAPS markers that mapped on the previous linkage map<sup>13</sup> was also separated using HEGS running system.

An F<sub>6</sub> population is considered a RIL and presents an exception of 1:1 ratio for allele segregation among individuals, but a theoretical ratio of 3% of individuals should show as heterozygous. In this case, each segregating marker was tested with a  $\chi^2$  test for goodnessof-fit to the expected 1:1 Mendelian segregating ratio. As a result, 131 of the 2053 markers (6.4%) were distributed to a skewed segregation with significance at  $P \le 0.01$ , and 575 markers (28%) were distorted at  $P \leq 0.05$ . Most skewed segregating markers deviated to Miyakojima MG-20 alleles. In this study, the distributions of 82 of these 131 markers inclined to Miyakojima MG-20 and only 49 to Gifu B-129 (Table 1). Five SSR markers were distorted significantly at the 1% level, of which three were distorted to Gifu B-129 and two to Miyakojima MG-20. However, these five SSR markers were retained in the mapping analysis because their distribution frequencies were near to P =0.01. The AFLP markers skewed at the 1% level were discarded and excluded in the data analysis. About 24% of the SSR markers were skewed at the 5% level and included in the map analysis (Table 1). As a result, a total of 1588 AFLP markers and 97 SSR including 2 dCAPS markers were mapped on the linkage map of L. japonicus.

**Table 1.** AFLP and SSR markers generated in the 94 F<sub>6</sub> individuals from a cross between *L. japonicus* accessions, 'Gifu B-129' (G) and 'Miyakojima MG-20' (M)

	Total	Mapped markers	Distorted at $P \le 0.05$	Distorted at $P \le 0.01$
EcoRI/Msel primer pairs	427			
G associated dominant markers	739	504	194	44
M associated dominant markers	674	454	202	55
Co-dominant markers	640	533	179	32
Total AFLP markers	2053	1491	575 (28%)	131 (6.4%)
SSR markers*	97	97	23 (23.7%)	5 (5.1%)

The numbers in parentheses show the percentage of the markers stated to the total AFLP markers. \*Included two dCAPS markers.

Chrom	1[1]	Chror	n 1[2]	Chrom	1[3]
Chrom 0.0 0.6 1.2 2.5 1.4.1 4.9 5.7 6.0 6.4 7.3 7.4 7.5 7.6 7.7 7.8 7.9 8.1 8.3 8.4 8.5 8.8 9.9 9.4 9.6 12.4 12.5 1.4.1 1.4.9 7.3 7.5 7.6 7.7 7.8 7.8 9.2 9.4 9.2 9.4 9.2 9.4 9.2 9.4 9.2 9.4 13.1 13.2 13.4 13.7 13.4 13.7 13.4 13.7 13.4 13.7 13.4 13.7 14.1 14.1 14.1 13.4 13.7 14.1	TM0031     TM00011     TM0002     TM0132     F7M21C240     E44M10C210     TM0058     E55M63C250     E2M6M1066     E37M55M210     E18M54M310     E18M54M310     E19M49G490     E6M32G280     E38M15C1460     E19M27C3180     E17M8C1080     E50M1C400     E37M47M470     E18M3G1090     E38M35C1460     E19M27G180     E17M8C1080     E50M1C400     E37M47M470     E18M3G1090     E35M30M420     TM0145     TM0027     E43M61M840     E33M46M90     E44M56M80     E2M50G1430     E18M19M500     E59M4M210     E35M46C770     E19M60C740     E35M44C120     E38M45C130     E43M61M840     E35M44C120     E18M19M500     E59M4M210     E35M44C770     E19M60C740     E36M146180 <td><b>Chron</b> 40.0 40.5 41.5 42.0 42.9 43.1 43.4 45.6 45.7 42.9 43.1 43.4 45.6 45.7 45.6 45.7 45.6 45.7 45.7 45.6 55.7 52.8 55.5 56.3 47.5 55.5 56.3 47.5 55.5 56.3 47.5 55.5 56.3 47.5 55.5 55.5 56.3 47.5 55.5 56.3 47.5 55.5 55.5 55.5 55.5 55.5 55.5 55.5</td> <td>n 1[2] E17M35G1170 E18M3G640 E37M16G850 E18M59G1400 E8M28G230 E8M28G230 E8M28G610 E35M7C300 E35M7C300 E35M7C2260 E33M3C150 E35M32C60 E33M32C150 E35M45C500 E35M45C500 E35M45C500 E35M45C500 E19M8G700 E19M8G700 E19M8G700 E19M8G200 E56M62M135 E35M45C500 E35M51C200 E35M51C250 E33M15C310 E33M15C310 E33M15C310 E33M61G200 E33M13C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M13C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M3C230 E33M362C00 E33M362C00 E33M13C310 E33M362C00 E33M362C00 E33M362C00 E33M363C0</td> <td>82.6     82.7     84.6     85.1     85.8     86.1     86.3     86.3     86.3     86.3     86.3     86.3     86.3     86.3     86.3     86.3     86.3     86.3     87.0     90.6     91.8     92.0     92.1     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     94.9     101.7     112.3     110.7     112.3     117.3     117.6     118.9     119.2     122.5     122.7     123.6     124.1</td> <td>113]     TM0001     E35M11C245     E19M6C710     E7M41G70     E15M8C360     E25M18C330     E51M30C180     E35M61M90     E35M63G310     E35M59G230     E9M40G1090     E3M19C750     E3M190750     E3M30610     E35M25265     TM0098     E3M496240     E2M596150     E4M48C120     E18M52650     E36M52M20     E36M52M20     E36M52M20     E36M52M20     E36M52M20     E36M52M20     E36M52M20     E35M76100     E33M36M180     E17M20C740     E35M7G100     E35M7C100     E35M7C100     E35M7C100     E35M7C20     TM00142     <td< td=""></td<></td>	<b>Chron</b> 40.0 40.5 41.5 42.0 42.9 43.1 43.4 45.6 45.7 42.9 43.1 43.4 45.6 45.7 45.6 45.7 45.6 45.7 45.7 45.6 55.7 52.8 55.5 56.3 47.5 55.5 56.3 47.5 55.5 56.3 47.5 55.5 56.3 47.5 55.5 55.5 56.3 47.5 55.5 56.3 47.5 55.5 55.5 55.5 55.5 55.5 55.5 55.5	n 1[2] E17M35G1170 E18M3G640 E37M16G850 E18M59G1400 E8M28G230 E8M28G230 E8M28G610 E35M7C300 E35M7C300 E35M7C2260 E33M3C150 E35M32C60 E33M32C150 E35M45C500 E35M45C500 E35M45C500 E35M45C500 E19M8G700 E19M8G700 E19M8G700 E19M8G200 E56M62M135 E35M45C500 E35M51C200 E35M51C250 E33M15C310 E33M15C310 E33M15C310 E33M61G200 E33M13C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M13C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M15C310 E33M3C230 E33M362C00 E33M362C00 E33M13C310 E33M362C00 E33M362C00 E33M362C00 E33M363C0	82.6     82.7     84.6     85.1     85.8     86.1     86.3     86.3     86.3     86.3     86.3     86.3     86.3     86.3     86.3     86.3     86.3     86.3     87.0     90.6     91.8     92.0     92.1     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     93.4     94.9     101.7     112.3     110.7     112.3     117.3     117.6     118.9     119.2     122.5     122.7     123.6     124.1	113]     TM0001     E35M11C245     E19M6C710     E7M41G70     E15M8C360     E25M18C330     E51M30C180     E35M61M90     E35M63G310     E35M59G230     E9M40G1090     E3M19C750     E3M190750     E3M30610     E35M25265     TM0098     E3M496240     E2M596150     E4M48C120     E18M52650     E36M52M20     E36M52M20     E36M52M20     E36M52M20     E36M52M20     E36M52M20     E36M52M20     E35M76100     E33M36M180     E17M20C740     E35M7G100     E35M7C100     E35M7C100     E35M7C100     E35M7C20     TM00142 <td< td=""></td<>
39.1	<sup>L</sup> E18M62M255	02 2 J	LE38M13M120		

**Figure 3.** Linkage maps of chromosome 1–6 of *L japonicus* genome Marker names were assigned based on the combination of *Eco*RI and *Msel* AFLP primers with generated marker size (in base pair). Letter G stands for maternal 'Gifu B-129' associated marker; M for paternal 'Miyakojima' associated marker and C for co-dominant markers. All SSR/dCAPS markers assigned as TM (Hayashi et al.<sup>13</sup> and visit at http://www.kazusa.or.jp/lotus/markerdb\_index.html). Morphological QTL: SS stands for seed size; LL for leaflet; SC for seed color; ST for stipule; PE for petiole; TR for trichrome; PT for plant type and SP for stem pigment.

#### 3.2. Construction of linkage map

All AFLP markers and 95 SSR and 2 dCAPS markers were run on JoinMap<sup>®</sup> 4 to generate six groups at an LOD value of 14.0, with a maximum distance of 30 cM. Using the SSR markers on each group as references, these six groups were assigned to chromosome 1–6 of *L. japonicus.*<sup>13</sup> A high-density linkage map for *L. japonicus* was generated; 533 co-dominant AFLP markers and 97 SSR (including 2 dCAPS markers) were mapped on six chromosomes of

Chrom	2[1]	Chrom	2[2]
0.0 ı	E1M7G670	8.7	- E38M27G120
0.2	E34M2C500	88	- E37M24G130
0.3	E7M41M70	8.9	- E3M28C700
0.6	E15M8C350	9.0	E6M62M120
0.7 -	E7M2C2500	9.1	- E33M37G320
0.8	E35M9C300	9.2	- E31M39G1500
0.9	E38M4G260	9.3	- E2M18C1200
1.0 -	E35M36C1080	9.4 -	E19M12G270
1.2	E34M3C580	9.5	- E37M56C230
1.37	E17M8M610	9.6	E36M52G1230
1.7	- E51M30M1560	9.7	E TOWIZUG000
1.9	- E37M54G180	9.9	- E37M62C250
2.0	- E20M18G290	10.0 -	- E17M18C250
2.1	- E36M23G180	10.1	- E3M22C200
2.2	E1M25M190	10.2	E62M7C750
2.3	- E24M59C1440	10.3	E8M20C1100
2.4 1 =	E35M46G200	10.4	E36M/C2/40
2.5	E19M16G500	10.5	E/W090200
27	- E5M55G120	10.7	E7M49C640
2.8	- E37M35M130	10.8	E36M3C2760
2.9	E35M58G700	10.9	E33M64C700
3.0 -	E37M26G150	11.0	E37M56C850
3.1	E59M31G290	11.1	E18M55M100
3.2	E33M12G1900	11.2	E6M62G1080
3.3 -	- E33M63G500	11.3	- E35M36C230
3.4 1	E4M24C630	11.4	E24M47G580
3.5	E190490270	11.5	E 10101120700
3.7	E35M30M380	11.7	E43M61C2000
3.8-	E7M2C720	11.8	E38M23C1240
3.9 -	E38M23C290	12.1	E37M4C130
4.0	E33M13C680	12.3	E20M8C550
4.1	E18M58M1000	12.6	E37M3G260
4.2	- E57M33M450	12.8	E19M13C1380
4.3	E19M4/M1/0	13.0	E1/M9G6900
4.41	E35M03C200	13.3	E18M57C120
4.5	E34M4M200	14.0	E13M52G350
47-	E7M30G11080	15.8	E55M54G210
4.8	E11M23C200	16.0	E33M42G200
4.9-	E5M30M1088	16.3	E2M6G1115
5.0	E7M52G210	16.6	E18M58C330
5.2	E33M39G1120	16.8	E18M55C550
5.31	E31M39C2330	17.01	E21VI0C320
5.5	E6M7G1730	17.3	E17M36C1360
5.6	E35M14C600	17.5	E2M29C870
5.7 -	E34M32C1330	17.7	E19M45C170 SP 1
5.8 -	E9M64C620	17.9	E17M35G128
5.9 -	E37M42C310 SS_2	18.1	- TM0065
6.0	E34M3M150	18.5	E7M1G380
6.1	E19M31C440	22.8	E14M52G600
6.21	E38M/G420	24.5	E3510154G250
6.4 -	E35M42G300	25.5	E17M27G1140
6.6-	- TM0134	27.0	E9M55M370
6.8 -	E8M55C490	27.6	E33M37M1450
6.9	E33M41C700	28.8	E38M24M355
7.0	E34M59M110	29.8	E33M24M150
7.1	E7M6C1240	32.1	TM0124
7.2	E11M29M150	33.5	E15M51M140
7.3	E7M29G550	36.91	E3/1000330
7.4	E34M55G240	38.4	E35M60C110
7.6	E33M2G1090	39.9	E34M63M1470
7.7	E33M42M260	40.7	E36M38C410
7.8	E36M53G150	41.1	E2M18C1350
7.9	E34M8G780	42.2	E17M31C310
8.0	E5M42C590	43.3	- I M0076
8.1	E34M51M80	45.8	E1 MOCOUU
8.2	E38M/G2/5	47.8	TM0020
84	E35M8C220	48.2	E1M18C290
8.5	E33M15M270	48.9	E33M21M470
8.6	E37M14C610	50.1	E42M59M1500

#### Figure 3. Continued

*L. japonicus*, with an additional 958 AFLP markers (504 Gifu associated and 454 Miyakojima associated markers, Table 2). A total of 1588 markers were mapped on the six chromosome maps (Supplement 2). This linkage map consisted of 1013 unique markers (excluded duplicated markers) and spanned a total length of 508.5 cM with an average of 0.50 cM between markers. The range of the length of each chromosome varies from 50.1 cM

(chromosome 2) to 131.7 cM (chromosome 1), with a mean of 84.7 cM (Table 2, Fig. 3).

Eight morphological traits were recorded as QTL. After the map of each chromosome for each parent was constructed, several markers with almost an equal distance interval of <10 cM were used as frame markers to scan eight phenotypic markers within the linkage map of each chromosome. As a result, 14 QTLs were detected on six chromosomes for the eight morphological traits. There were six QTLs detected on chromosome 4, only one QTL on chromosomes 3, 5 and 6 (Table 2). SS showed five QTLs and was distributed to five chromosomes except for chromosome 5. LL and SC presented two QTLs. SP, PT, ST, PE and TR showed only one QTL, indicating control by a single gene (Table 2, Fig. 3).

The duplicated markers presented in six chromosomes, resulting in significant clustering in the whole genome, especially in the center region of each chromosome. To decrease the numbers of markers, all duplicated markers will be excluded on the each chromosome map (Fig. 3). So Fig. 3 presented only unique markers. All requirements about the details of mapped markers should be address to the corresponding authors.

#### 3.3. Characterization of the linkage map

Chromosome 1 had the largest number of markers (373) and longest genetic distance (131.7 cM). Chromosome 5 had the fewest number of markers (184) and chromosome 2 has shortest genetic distance (50.1 cM). The average interval distance between markers (excluded the duplicated markers) was 0.50 cM for all chromosomes. Chromosome 1 (0.64 cM) had the longest and chromosome 2 (0.33 cM) had the shortest average interval distance between markers. The other four chromosomes had similar average interval distances (Table 2). The distorted markers were mainly mapped on distal parts of chromosome 5 (marked with asterisk on chromosome 5 in Fig. 3). Chromosome 6 also showed some distorted markers, dispersed through the chromosome. The clustering of markers occurred on the center region of all six chromosomes (Fig. 3).

#### 4. Discussion

# 4.1. Determination of the linkage map of each chromosome

Before determining the chromosome of the genome, all AFLP and SSR markers were combined and analyzed with JoinMap<sup>®</sup> 3.0.<sup>16</sup> At the LOD of 14.0, six big groups were generated. On the basis of the distribution of SSR markers<sup>13</sup> within each group, we were able to locate six groups to six corresponding

~ ~ · · · ·

Chrom	3[1]	Chrom	3[2]	Chrom	3[3]
0.0 <sub>1</sub>	「E38M3C349	41.5	- E38M6M105	60.8	- E4M48M212
1.1	E17M31C608	41.9	E37M42G1714	60.9	E28M53M137
2.5	E37M60C1253	42.3	E37M44M146	61.2	E38M13M295
4.1	E37M4M865	42.7	E19M46M615	61.4	E34M26M607
5.4 -	E34M56G180	42.9	E33M63M652	61.8	E13M52G530
6.11	E351V1211V1134	43.5	E11M32C100	62.1	E21VI30C440
7.0	E3310210040	43.0	TM0155	62.5	E5M42C190
8.61	TM0106	44.1	E35M54C1077	62.9	E61M60G187
8.9 -	E38M3C699	44.5	E2M6M737	63.0	E4M51G278
10.0 -	E17M18M600	45.0 -	E17M31C261	63.1 -	E59M38G198
10.4 -	r E19M60M236	45.2	E35M28C570	63.3	E55M63C508
11.1	F 1M0059	46.01	E3310132101510	62 7	E30100G192
12.1	FE30104310129	46.2	E5M30C510	64.1	E36M23C266
13.5	E25M12C815	46.5	TM0022	64.2 —	E32M18G1023
14.5	E37M46M138	46.9	E34M51C2246	64.3 -	E5M28G270
15.1 - 🗐	TM0436	47.2	E37M52C428	64.4	E5M22G200
15.9	E37M23G114	47.3	E1/M2/C812	64.7	E38M27G460
17.0		47.4	E34M64C675	65.4	E19M20C309
18.0	F F37M14C271	47.6	E35M46C553	65.8	E36M5C628
20.7	E35M35M792	47.7	- TM0159	66.8 -	E19M16C408
22.1	r E35M61G340	48.1	E37M21G252	67.2	E56M8G138
22.8 -	E34M49C106	48.2	E39M62C3363	67.4	E38M6G160
23.1	E37M3G186	48.3	E4410110101507	60.5	E341003G453
24.5	E33M22C645	40.4	F19M28M400*	69.5	E7M59M2600
24.7	E 181/1540409	49.0	E1M25C781	70.3	E4M48C306
25.4	F37M4C175	49.4	E8M12G750	70.6 -	- TM0115
25.7	- E37M4M385	49.5	E33M8M126	71.3	E33M37C840
25.9	~ E37M48M128	49.61	E35M44C191	71.6	E62M/M487
26.5	- E8M63C220	49.7	TM0005	72.8	E2M19M700
27.3	E11M30G200	50.2	E20M18C236	74.0	E32M17C479
20.0	~ E24M62C260	50.3	E57M33C560	74.2	- TM0160
32.8	~ E5M23M134	50.8	E18M62G123	75.2	E35M63C519
33.2	- E35M21G588	50.9	E7M59G3000	/6.5	E15M51M580
33.5	E38M24M578	51.2	E37M53G125	80.2	E38M6C544
33.7	E33M42M590	51.6	E35M36G217	81.2	E9M55C240
33.9		52.0	E37M4M635	83.3 -	E34M51C1019
34.5	E31M18M845	52.4	E37M4M68	84.0	E35M19G116
34.9	E34M4M101	52.5	E33M36M1737	84.71	E18M54C318
35.1	E19M35M585	52.0 52.8	E34M51C1900	85.7	E190310810
35.2	E33M52M316	53.1	E37M19C249	85.9 -	E48M56G523
35.3	E35M03M352	53.5	E46M36G595	86.2	E33M64G196
36.0	E18M13C419 SS 3	53.7	E6M58C379	86.6 -	E33M63G102
36.2	E33M3C638	54.1	E2M59M97	86.9	E33M53C524
36.5	E18M27G175	54.2	E36M56C132	07.3 87.6 -	E18M25C527
36.9	E40M26M68	54.5	E34M10M431	88.3 -	E12M51M125
37.0	E2M34M418	55.3 -	- TM0083*	89.3 -	E2M17C870
37.41	E341004G200	55.9	- TM0129	90.6	E3M28C160
38.2	E11M29C400	56.0	E/M24C3500	91.0	E/M28M620
38.4	- TM0070	57.0	E18M56M268	91.2	E36M42M139
38.5	- TM0035	58.5	E26M28M101	92.1	E33M64G266
38.9	E37M59C400	58.7	E15M8M133	92.6	E35M25M120
39.4	E35M29G221	58.8	E18M12C466	93.1	E35M27C459
39.0	E36M42M2610	58.9	E15M8M133	95.1	F I M0135
40.1	E35M17M587	59.0	E37 10031000	96.1	E171VI200413
40.2	E9M64C421	59.5	E4M51C480	96.6	E10M18G260
40.5	E42M59M360	59.6	TM0142	97.3	E38M3M1262
40.7	E11M32M210	60.1	E15M22M90	98.2	E36M15C296
41.01	E3/1042101422	60.2	E5M24C679	99.0 -	· E38M7M1196
41.4	E34M46M608	00.7 -	- E341/1331/1230		

Figure 3. Continued

chromosomes of L. japonicus. After running the JoinMap<sup>®</sup> 3.0 program for each group, a linkage map on each chromosome was determined. The orders of these SSR markers on each chromosome were the same as those in the map of created by Hayashi et al.<sup>13</sup> except for some minor differences for some markers. Therefore, we confirmed the six 4.2. The length of linkage map This linkage map spans a total genetic distance of 508.5 cM in *L. japonicus* genome, slightly longer

groups correspond to the six chromosomes of Lotus genome.

#### Linkage Map of Lotus japonicus

Chrom 4[1]	Chrom 4[2]	Chrom 5[1]	Chrom 5[2]
Chrom 4[1] 0.0 0.2 1.6 0.2 0.8 1.6 1.6 1.6 1.6 1.6 1.6 1.6 1.6	Chrom 4[2] 34.8 = CTM5M610 35.0 = E18M220705 35.2 = E19M36324 35.5 = E19M36324 35.5 = E19M36324 35.5 = E19M36324 35.5 = E19M36324 35.5 = E19M36324 35.5 = E19M36713 35.8 = TM6100 35.9 = E36M57M213 36.0 = TM0131 36.0 = E18M5C2021 36.6 = E18M5C2021 36.6 = E18M5C2021 36.6 = E18M5C2021 36.8 = TM0087 37.2 = E38M57M213 37.2 = E38M57M213 37.2 = E18M5C2021 36.6 = E18M5C420 37.4 = E3M39G178 37.9 = E38M28C1804 38.2 = E15M22C734 38.5 = E15M22C734 38.5 = E15M22C734 38.5 = E15M22C734 38.5 = E33M39G178 39.0 = E5M24604 39.0 = E1M22M450 39.2 = E3M12M97 39.4 = E18M10M252 39.9 = E5M24604 39.0 = E1M22M450 39.2 = E3M12M97 39.4 = E18M10M829 39.6 = E52M28C206 39.9 = E3M39G49 40.5 = E56M42C738 42.5 = E3M17C263 42.5 = E3M17C263 42.5 = E3M17C263 42.5 = E3M47C406 45.2 = E3M47C406 45.2 = E3M47C406 45.2 = E19M50G751 43.4 = E19M50G751 43.4 = E19M50G751 43.4 = E19M50G751 43.4 = E19M50G751 43.4 = E19M50G751 43.4 = E19M50G751 $53.0$ = E17M34G798 LL_2 52.4 = E3M22M603 55.3 = E12M54M2100 55.5 = E3M22M63 55.3 = E12M54M2100 55.5 = E3M22M63 55.4 = E3M42C103 57.2 = E10M19G1400 57.4 = E1M22G415 57.2 = E10M19G1400 57.4 = E13M26C1351 51.1 = TM024 59.2 = E3M43G185 56.3 = TM0244 59.2 = E3M43G185 56.3 = E12M54M2100 56.5 = E3M43G185 56.3 = E12M54M2100 56.5 = E3M42C1686 50.7 = E1M22G415 57.8 = E11M25043 58.6 = TM024415 57.2 = E10M19G1400 57.4 = E1M22G435 58.6 = TM02443 59.9 = E3M43G1065 57.2 = E3M43G1065 57.2 = E10M19C403 77.0 = E3M43G1065 57.1 = TM10243 59.9 = E3M43G1065 57.1 = TM10243 59.9 = E3M43G1065 57.1 = TM104438 59.9 = E3M43G1065 57.1 = TM0047 57.1 = TM0047 57.1 = TM0047 57.1 = TM0047 57.1 = E1M459243 57.1 = TM0047 57.1 = TM0047 57.1 = TM00	Chrom 5[1] 0.0 2.5 3.4 4.9 4.2 4.9 5.9 5.9 5.9 5.9 5.9 5.9 5.9 5	Chrom 5[2] 54.8 55.0 55.2 55.2 55.2 55.5 57.1 57.5
32.9 EIMITSOT400 33.0 E33M64C243 33.1 E36M3G88 33.2 E36M3G88 33.3 EE5M22C1575 33.5 E5M22C1575 33.5 E37M52C110 33.6 E37M25G1591 33.7 E5M24C1485 33.8 E5M24C1485 33.9 E34M46C242	b1.b     1 INU162 S1_1       62.6     - TM0046       76.3     - E7M19C403       77.0     - E35M43G1065       78.1     - HTM097 TM0073       78.5     - E1M28G100       79.0     - E1M28G100       79.0     - E1M28G100       79.3     - E33M46C1167       79.4     - E34M46C268	50.3 - E37M46G222 50.8 - E11M24G580 50.9 - E33M28M146 51.1 - E38M8M65 52.4 - E6M21C1700 52.5 - E37M55C982 52.8 - E19M46M245 53.0 - E18M63M547 53.1 - E34M32M577	65.3     = E18061G1361       65.5     = E18061G1361       65.9     = E56042069       66.2     = E3501363495       67.6     = TM0906*       68.2     = E10096*       68.2     = E10096*       68.2     = E10036648       69.9     = E43061C129       70.8     = TM0146*
34.0   E38M13C476     34.1   E38M7680     34.2   E18M43M1149     34.3   E59M31M103     34.4   E18M55C737     34.5   E33M61C603     34.6   E17M27G1086     34.7   E46M36C434	79.5 E35M46C268   79.6 E35M43C263   79.8 E17M27C856   80.5 E37M25G247   81.0 E3M26750   81.5 TM069   82.2 E3M17C825   82.5 E3M46C253	53.3 - E36M4C190 53.4 - E17M13C497 53.6 - E1M18C2878 53.8 - E37M35G207 53.9 - E18M54C296 PT_1 54.0 - E2M56G500 54.1 - E35M40C572 54.3 - E38M15C781 64.5 - E2M56M222	71.1     E35M10C341*       71.4     E37M35G459*       71.6     E37M4C537*       71.9     E35M10M423*       72.6     E17M25G422*       73.3     E37M43M190*       73.4     E38M27M290*       73.8     E3M16C773*       74.0     E5M31C1112*
re 3. Continued		54.6 E19M50M252	75.2 E35M47G99*

Figure 3. Continued

than those of previous maps.<sup>13,14</sup> This can be explained by the marker numbers dramatically increased in the present mapping population. The larger number of markers within one linkage group may enlarge the genetic intercrossing value between markers. Additionally, the small size of the F<sub>6</sub> population (94 individuals) compared with 127  $F_2$  individuals in Hayashi et al.<sup>13</sup> may not be enough for allele segregation and cause allele partial distribution.

The lengths of chromosome 3–6 were very close to the lengths of Hayashi's results.<sup>13</sup> However, they were

# 54.7 Figure 3. Continued

54.6

E19M50M252

E35M59G412

nearly 30 cM longer for chromosome 1 and 30 cM shorter for chromosome 2 than the reference map.13 Hayashi et al.13 reported that there was a translocation region between the chromosome 1 and 2 in both parents' map. This translocation could have caused unequal crossover in the second generation and inherited to sixth generation. Fragment deletion may also occur during the translocation. In the

Chrom 6[1]	Chrom 6[2]
0.0     E18M63G2773       0.3     E34M35G170       0.4     E34M35G170       1.4     E35M14M101       1.9     E77M28G470       2.4     E16M60C340       2.7     E38M22G198       2.9     E18M41M1095       3.2     E6M7M675       3.5     F35M14M11095       3.2     E16M7M675       3.5     F35M27M252       3.7     E36M38M202       4.8     F23M27M245       6.0     E14M2C106       6.6     E14M2626       6.7     E36M38M202       4.8     F23M24M385       6.0     E14M2C268       7.4     E36M36667       8.9     E35M440385       9.0     E33M401983*       9.0     E33M401983*       9.0     E33M401983*       9.1     E34M901488       10.1     E34M801383       9.9     E21M37M349       10.1     E34M801383       9.9     E21M37M349       10.1     E34M801383	19.8   E33M19G251     19.9   E34M46C147     20.0   E33M12G425     20.1   E33M12G425     20.3   E36M13G172*     20.4   E36M13G172*     20.6   E24M47C5244     20.7   E33M12G286     20.6   E37M26639     20.6   E37M12G246     21.0   E33M12C246     21.7   E33M13C281     21.8   E33M4645576     21.3   E33M13C281     21.4   E33M24M5576     23.3   E19M13C255     24.7   E34M38M75     24.5   E36M3G443     28.8   E34M38M77     29.4   E34M38M771     29.4   E34M38M721     29.5   E34M38M721 <td< td=""></td<>

Figure 3. Continued

present map, chromosome 1 is 131.7 cM in length and chromosome 2 is only 50.1 cM in length. The difference of 30 cM is supposed to be the translocation fragment, compared with Hayashi et al.<sup>13</sup> chromosome 1 and 2 map. However, the map length of chromosome 1 and 2 are very close to that of Sandal et al.<sup>14</sup> map of *L. japonicus* from a cross of *L.filicaulis* × *L. japonicus*.

To further confirm the reliability of this linkage map, we developed a total of 300 AFLP co-dominant markers with the same primer-pair combinations and 97 SSR markers with 94  $F_9$  individuals derived from same  $F_6$  individuals. With the same calculation, these 397 markers were located on six chromosomes and the order was generally the same within each chromosome, although there is a slight difference for some distances between markers. So this linkage map is reliable to use as a framework for physical map construction and map-based cloning in *L. japonicas*.

## 4.3. *Clustering of the markers*

The clustering of markers on each chromosome occurred significantly in this study. AFLP markers characteristically cluster in centromeric and/or telomeric regions in plant species with large genome.<sup>21-24</sup> Clustering of markers occurred mainly at heterochromatin-rich centromeric regions that ascribed to the great portions of repetitive sequences frequently present, and these repetitive sequences suppressed recombination between chromosomes.<sup>21</sup> Also, a high degree of clustering of markers in the AFLP map is much more pronounced than in the RFLP map.<sup>25</sup> There may be some very small variation, possibly, 1 bp deletion/insertion in repetitive sequences that can be detected by the AFLP technique, but not by RFLP technique. Thus, AFLP markers can be relatively easy to generate in highly repetitive regions near centromere.<sup>22</sup> In the present study, of the 1588 mapped markers, 575 duplicated markers will be excluded in the Fig. 3.

Table 2. The mapping characterization of six chromosomes of L. japonicus geno	me
---	----

	SSR	AFLP markers			Total	Length	Unique	Mean	QTL
		Co- dominant	G Dominant	M Dominant	markers	(cM)	markers*	intervals	
Chromosome 1	41	137	95	100	373	131.7	204	0.65	3
Chromosome 2	7	102	83	67	259	50.1	154	0.33	2
Chromosome 3	17	115	92	114	338	98.9	206	0.48	1
Chromosome 4	12	71	100	58	241	82.5	157	0.53	6
Chromosome 5	10	60	65	49	184	75.2	146	0.52	1
Chromosome 6	10	48	69	66	193	70.1	146	0.48	1
Total III	97	533	504	454	1588	508.5	1013	0.50	14

\*The duplicated markers were excluded.

Most significant clustering of markers located near the centromeric region.

Although the significant clusters presented, map gaps were found in whole genome, but much smaller than previous map. The biggest gap of 13.7 cM was found on chromosome 4. The gaps on other chromosomes ranged from 3.8 to 7.7 cM.

In this study, the rapid and efficient development of the linkage map with high resolution of *L. japonicus* was facilitated by the HEGS/AFLP system, by which a total of 1588 AFLP markers was mapped on the *L. japonicus* genome in 6 months. This map created a framework for anchoring EST, SSR and other sequence-based markers, and built the foundation for physical map construction in *L. japonicus* and gene cloning in other legume crops.

**Supplementary Data:** Supplementary data are available online at www.dnaresearch.oxfordjournals.org.

#### Funding

This work was supported by the fund for the Promotion of Basic Research Activities for Innovative Biosciences (BRAIN), Japan.

#### References

- Vos, P., Hogers, R., Bleeker, M., et al. 1995, AFLP: a new technique for DNA fingerprinting, *Nucleic Acids Res.*, 23, 4407–4414.
- Mackill, D. J., Zhang, Z., Redona, E. D. and Colowit, P. M. 1996, Level of polymorphism and genetic mapping of AFLP markers in rice, *Genome*, **39**, 969–977.
- Keim, P., Schupp, J. M., Travis, S. E., et al. 1997, A highdensity soybean genetic map based on AFLP markers, *Crop. Sci.*, 37, 537–543.
- 4. Remington, D. L., Whetten, R. W., Liu, B. H. and O'Malley, D. M. 1999, Construction of an AFLP genetic map with nearly complete genome coverage in *Pinus taeda*, *Theor. Appl. Genet.*, **98**, 1279–1292.
- Katengam, S., Crane, J. M. and Knapp, S. J. 2002, The development of a genetic map for meadowfoam comprised of amplified fragment length polymorphisms, *Theor. Appl. Genet.*, **104**, 92–96.
- 6. Kriegner, A., Cervantes, J. C., Burg, K., Mwanga, R. O. M. and Zhang, D. 2003, A genetic linkage map of sweetpotato [*Ipomoea batatas* (L.) Lam.] based on AFLP markers, *Mol. Breed.*, **11**, 169–185.
- Handberg, K. and Stougaard, J. 1992, *Lotus japonicus*, an autogamous, diploid legume species for classical and molecular genetics, *Plant J.*, 2, 487–496.
- 8. Jiang, Q. and Gressholf, P. M. 1997, Classical and molecular genetics of the model legume *Lotus japonicus*, *Mol. Plant Microbe Interact.*, **10**, 59–68.
- 9. Ito, M., Miyamoto, J., Mori, Y., Fujimoto, S., Uchiumi, T., Abe, M., Suzuki, A., Tabata, S. and Fukui, K. 2000,

Genome and chromosome dimensions of *Lotus japonicus*, *J. Plant Res.*, **113**, 435–442.

- 10. Kawasaki, S. and Murakami, Y. 2000, Genome analysis of *Lotus japonicus*, *J. Plant Res.*, **113**, 497–506.
- Stiller, J., Martirani, L., Tuppale, S., Chian, R. J., Chiurazzi, M. and Gresshoff, P. M. 1997, High frequency transformation and regeneration of transgenic plants in the model legume *Lotus japonicus*, *J. Exp. Bot.*, 48, 1357–1365.
- Kawaguchi, M., Motomura, T., Imaizumi-Anraku, H., Akao, S. and Kawasaki, S. 2001, Providing the basis for genomics in *Lotus japonicus*: the accessions Miyakojima MG-20 and Gifu B-129 are appropriate crossing partners for genetic analyses, *Mol. Gen. Genet.*, **266**, 157–166.
- 13. Hayashi, M., Miyahara, A., Sato, S., et al. 2001, Construction of a genetic linkage map of the model legume *Lotus japonicus* using an intraspecific  $F_2$  population, *DNA Res.*, **8**, 301–310.
- Sandal, N., Krusell, L., Radutoiu, S., et al. 2002, A genetic linkage map of the model legume *Lotus japonicus* and strategies for fast mapping of new loci, *Genetics*, **161**, 1673–1683.
- Sato, S., Kaneko, T., Nakamura, Y., Asamizu, E., Kato, T. and Tabata, S. 2001, Structural analysis of a *Lotus japonicus* genome. I. Sequence features and mapping of fifty-six TAC clones which cover the 5.4 Mb regions of the genome, *DNA Res.*, **8**, 311–318.
- Van Ooijen, J. W. and Voorrips, R. E. 2001, JoinMap<sup>®</sup> 3.0, Software for the calculation of genetic linkage maps, Plant Research International, the Netherlands, Wageningen.
- 17. Kosambi, D. D. 1944, The estimation of map distances from recombination values, *Ann. Eugen.*, **12**, 172–175.
- 18. Voorrips, R. E. 2002, MapChart: Software for the graphical presentation of linkage maps and QTLs, J. Hered., 93, 77–78.
- 19. Zeng, B. 1994, Precision mapping of quantitative trait loci, *Genetics*, **136**, 1457–1466.
- 20. Wang, S., Basten, C. J. and Zeng, B. 2006, Windows QTL Cartographer 2.5, Department of Statistics, North Carolina State University, Raleigh, NC, http://statgen. ncsu.edu/qtlcart/WQTLCart.htm.
- 21. Tanksley, S. D., Ganal, M. W., Prince, J. P., et al. 1992, High-density molecular linkage maps of the tomato and potato genomes, *Genetics*, **132**, 1141–1160.
- 22. Qi, X., Stam, P. and lindhout, P. 1998, Use of locusspecific AFLP markers to construct a high-density molecular map in barley, *Theor. Appl. Genet.*, **96**, 376–384.
- 23. Gedil, M. A., Wye, C., Berry, et al. 2001, An integrated RFLP-AFLP linkage map for cultivated sunflower, *Genome*, **44**, 213–221.
- 24. Strommer, J., Peters, J., Zethof, J. and de Keukekeire, P. 2002, AFLP maps of *Petunia hybrida*: building maps when markers cluster, *Theor. Appl. Genet.*, **105**, 1000–1009.
- Devos, K. M., Atkinson, M. D., Chinoy, C. N., Liu, C. J. and Gale, M. D. 1992, RFLP-based genetic map of the homoeologous group-3 chromosomes of wheat and rye, *Theor. Appl. Genet.*, 83, 931–939.