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Providing the basis for genomics in *Lotus japonicus*: the accessions Miyakojima and Gifu are appropriate crossing partners for genetic analyses

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Abstract *Lotus japonicus* has attracted attention as a model plant legume for molecular genetic research, and several mutants defective in nodulation and mycorrhizal symbiosis have been developed from the standard accession Gifu B-129. However, as a model system, Gifu has long lacked an appropriate crossing partner for use in various genetic analyses. In a search for an appropriate partner for Gifu, we have collected plants from 15 localities throughout Japan, and analyzed their levels of DNA polymorphism (also in comparison to the African species *L. filicaulis*) by AFLP (Amplified Fragment Length Polymorphism) combined with the use of a high-throughput electrophoretic screening system termed HEGS (High-efficiency genome scanning) developed by us, using 31 primer pairs. Plants of the accession Miyakojima MG-20 showed the highest level of polymorphism relative to Gifu (over 4%). When HEGS is used for screening, this level is sufficient to permit systematic positional cloning of mutant genes. Segregation in the F_2 of the Gifu-derived symbiotic mutations *Ljsym70*, *Ljsym72*, *Ljsym74-1* (*alb1-1*) and *Ljsym78-1* from a cross with Miyakojima was normal, while the ratios seen from a cross with *L. filicaulis* were distorted. Miyakojima displays several traits that distinguish it from other Japanese accessions: low concentrations of anthocyanin in the stem and petals, few trichomes, a more upright habit, broad leaflets and petals, and large black seeds. The first two traits, which are controlled by single recessive genes, serve as useful markers for following mutant crosses.

Keywords *Lotus japonicus* · Accession Miyakojima · Crossing partner · High-efficiency genome scanning (HEGS) · Polymorphism analysis

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Introduction

The Leguminosae make up one of the largest plant families, consisting of 18,000 species, among which are included a number of agronomically important species, characterized by the specific ability to fix nitrogen through symbiosis with rhizobia. In order to establish the basis for genome analysis in this large group and to study the molecular mechanisms of nodule formation and nitrogen fixation, a systematic molecular genetic analysis with a suitable model plant appears to be the most promising approach, as demonstrated by the successful use of such an approach in *Arabidopsis* (AGI 2000; Koncz et al. 1992). In following this approach, two species, *L. japonicus* and *Medicago truncatula* have attracted the attention of researchers, as they seem to fulfill many of the requirements for model legumes (Cook et al. 1997). Handberg and Stougaard (1992) first demonstrated that *L. japonicus* Gifu B-129 has many characteristics that make it suitable for molecular and classical genetic studies. Transformability of this accession was exceptionally high among the rather recalcitrant legume plants, and its diploid genome size was reported to be about the same as that of rice (1C = 0.5 pg DNA; Borsos 1973; Cheng and Grant 1973; Bennett and Smith 1976). *L. japonicus* is naturally

self-pollinating and sets numerous seeds (up to 6000 per plant). Another merit of *L. japonicus* is that it sets globular (determinate) nodules, like the agriculturally important soybean and the common bean, while *M. truncatula* sets cylindrical (indeterminate) nodules, like pea and alfalfa. Utilizing the high transformability of *L. japonicus*, several tagging lines have been developed by inserting the *Ac* transposon (Thykjær et al. 1995) or T-DNAs (Stiller et al. 1997; Schauser et al. 1998; Martirani et al. 1999; Webb et al. 2000) into the genome. These efforts culminated in the cloning of the host gene (*Nin*) essential for nodule inception, by tagging with the *Ac* transposon (Schauser et al. 1999). However, sector-forming mutants, a key feature for the recognition of successful transposon tagging, are generally not easy to obtain. The presence of footprints, insertion hot spots, and the occurrence of several culture mutants, also hinder this approach. It was also noticed that most of the nodulation-related promoter tagged lines are difficult to obtain in homozygous form (reviewed by P. Gresshoff 1999), suggesting lethality of the large-insertion mutants.

On the other hand, positional cloning of variously mutated genes offers advantages for a systematic approach, because it can handle all the hitherto produced mutants, irrespective of the means used to generate them: chemical mutagenesis, irradiation with gamma rays, fast neutrons, or ion beams; even the culture mutations can be cloned in this way. Genes involved in natural variations are also clonable, and lesions resulting in small changes in key genes are not expected to be lethal as in the case of the long-insertion mutations. Several rich mutant libraries of *L. japonicus* have been developed (Szczyglowski et al. 1998; Wegel et al. 1998) including those created by us (Imaizumi-Anraku et al. 1997; M. Kawaguchi and H. Imaizumi-Anraku in press) from the standard accession Gifu B-129.

However, *L. japonicus* has long been lacking appropriate crossing partners for use in extensive genetic analyses. Finding an appropriate crossing partner for the accession Gifu, from which abundant mutant libraries have been developed, and the construction of a high-quality genome library, are therefore the most pressing requirements before *L. japonicus* can be established as a fully functional model legume plant. Sandal and Stougaard (1998) have proposed an African species, *L. filicaulis*, as a crossing partner, referring to an earlier report of successful interspecies crosses between *L. japonicus* and *L. filicaulis* (Grant et al. 1962). However, *L. filicaulis* is genetically quite remote from *L. japonicus* (30–50% polymorphism by RAPD and AFLP analyses), and crossing-over between homologous chromosomes could be perturbed, and segregation may be distorted in the F_2 siblings of crosses with *L. japonicus*. On the other hand, Jiang and Gresshoff (1997) have used another Japanese accession, Funakura B-581, but the grade of genetic diversity relative to Gifu has not been assessed objectively. Therefore, we initiated a thorough search for a counterpart of Gifu, with an appropriate degree of polymorphism, throughout Japan.

Here, we introduce Miyakojima as an accession characterized by several distinct morphological traits, and the highest rate of polymorphism relative to Gifu among 15 Japanese accessions of *L. japonicus* tested. HEGS (high-efficiency genome scanning), a high-throughput screening method developed by us, proved to be extremely useful for scanning of AFLP markers and polymorphic bands, and should become a standard tool for positional cloning. Furthermore, crosses between Gifu and Miyakojima gave normal segregation ratios in F_2 populations. As a consequence, it may be claimed that the infrastructure needed for the systematic genetic analysis of various nodulation mutants has now been established. Here we report on the process and the results of polymorphism evaluation.

Materials and methods

Plant materials

L. japonicus plants were collected on sampling tours, and seeds were obtained as kind gifts from several researchers. Accession numbers, original collectors, sites at which samples were collected, and donors of seeds are summarized in Table 1 and Fig. 1. Gifu B-129 which had been selfed for nine generations was kindly donated by Dr. Hiroshi Kouchi of the National Institute of Agrobiological Sciences (NIAS), Tsukuba, Japan. Miyakojima MG-20-S5 (selfed for five generations) was used for AFLP and genetic analyses. Plants were grown in 10-cm pots in Power Soil (Kanto Hiryo Kogyo) in a Biotron LH-300 (Nihon Ika) at 25°C on a 16-h light, 8-h dark cycle. Some of the plants were grown in an air-conditioned greenhouse at the NIAS, under the above conditions.

DNA preparation

Plant DNA was prepared from green leaflets using an automated DNA isolation system (Kurabo PI-50a) based on the CTAB extraction method (Rogers and Bendich 1994). Green leaflets (fresh wt. 0.4 g) were powdered with mortar and pestle, frozen in liquid nitrogen, and introduced into the apparatus as duplicate 0.2-g samples. About 14–24 μg of DNA were recovered in 2×100 μl of TE buffer (10 mM TRIS-HCl pH 7.4, 1 mM EDTA). As a control, DNA was also isolated manually by the CTAB method.

AFLP protocol

AFLP amplification was done following the protocols of Vos et al. (1995). Aliquots (150 ng) of genomic DNA were double-digested with 1.5 U each of *Eco*RI (Takara) and *Mse*I (New England Biolabs) at 37°C for 3 h in 15 μl of enzyme buffer (M buffer, Takara); then 10 μl of the digested sample was taken and immediately added to a ligation solution containing 50 U of T4 DNA ligase (Takara), 2 pmol of *Eco*RI adapter and 20 pmol of *Mse*I adapter, in a final reaction volume of 20 μl; the ligation reaction was allowed to proceed at room temperature for 8 h. After 10-fold dilution, 1 μl of the ligated sample mixture was amplified in 10 μl of PCR solution with non-selective primers and 0.2 U of Ex-*Taq* polymerase (Takara) in a PTC-100 thermal cycler (MJ Research) capable of accommodating 96 samples. A second round of amplification was carried out by adding 3 μl of the first reaction into a 12-μl reaction mixture containing +3/+3 selective nucleotide primers. The primer pairs used were (*Eco*RI/*Mse*I) ACG/CAC, ACG/CTC, ACT/CAA, ACT/CTT, ACT/CAC, AGG/CGT, AGA/CGT, AAC/CGT, AAT/CGT, AAG/CGT, AAA/CGT, ACC/CGG,

Table 1 Accessions of *L. japonicus* and *L. filicaulis* and their sources

Accession *Lotus japonicus* (Regel) Larsen	Accession number	Collection site (Prefecture)	Collector[a]	Donor[a]
Gifu	B-129	Gifu	I. Hirayoshi	H. Kouchi (National Institute of Agrobiological Sciences)
Funakura	B-581	Ishikawa	Satomi	N. Sandal (Arrhus University)
Ashizuri	MG-1	Kouchi	T. Shimada (Obihiro University of Agriculture and Veterinary Medicine)	T. Shimada
Taiki	MG-2	Hokkaido	T. Sato and M. Kawaguchi (Tokyo University)	[b]
Totsuka	MG-3	Kanagawa	K. Syono (Nihon Woman's University)	K. Syono
Kaseda	MG-4	Kagoshima	J. Masunaga	M. Abe (Kagoshima University)
Ninomiya	MG-5	Kanagawa	M. Kawaguchi (Tokyo University)	[b]
Towa	MG-7	Iwate	T. Aoki and M. Aoki-Komoto (Nihon University)	T. Aoki
Kameoka	MG-8	Kyoto	K. Syono	K. Syono
Oga	MG-9	Akita	R. Matsuki (Iwate Biotechnological Research Center)	R. Matsuki
Tono	MG-10	Iwate	T. Aoki and M. Aoki-Komoto	T. Aoki
Arasaki	MG-14	Kanagawa	T. Aoki and M. Kawaguchi	[b]
Bishamon	MG-16	Kanagawa	T. Aoki and M. Aoki-Komoto; M. Kawaguchi	[b]
Miyakojima	MG-20	Okinawa	M. Kawaguchi	[b]
Aomori	MG-23	Aomori	Unknown	T. Nemoto (Tohoku University)
L. filicaulis	B-37	Algeria (obtained from the Botanical Gardens, Madrid, Spain)	N. Sandal	N. Sandal

[a]The affiliation of the collector or donor is indicated in *parentheses*
[b]Seeds owned by M. Kawaguchi

ACT/CGG, ACG/CGG, ACA/CGG, ACG/CAC, ACG/CTT, AGA/AGT, AGC/AGT, AGC/CGT, AGC/CTG, AGG/AGT, AGG/CAG, AGG/CTG, AGG/CTT, AGT/AGT, AGT/CGT, ATA/AGT, ATC/AGT, ATG/AGT, and ATT/AGT. Adapters and primers were custom-synthesized and open column-purified by Sawady Technology.

Analysis of polymorphism

The amplification products were electrophoresed on the HEGS system – four gels, each 1 mm thick, cast between 18-cm square glass plates and with special 66-lane combs (Nihon-Eido) – basically using the discontinuous polyacrylamide gel system developed for non-denaturing protein electrophoresis (Davis 1964). The separating gel solution (25 ml) consisted of 0.375 M TRIS-HCl pH 8.8, 0.35% Bis (N,N'-methylenebisacrylamide), 13% acrylamide, and 0.1% TEMED (N,N,N',N'-tetrametylethylenediamine), and was polymerized with 0.042% ammonium persulfate (APS). The stacking gel solution (6 ml) consisted of 0.13 M TRIS-HCl pH 6.8, 0.13% Bis, 5% acrylamide, and 0.1% TEMED, and was polymerized with 0.08% APS. The running buffer contained 0.25 M TRIS and 1.92 M glycine (all chemicals were from Nakarai Tesque).

Each gel accommodates 64 sample lanes and two lanes for size markers (ϕX174 DNA digested with *Hae*III, and/or λ DNA digested with *Hin*dIII). Four gels were simultaneously silver-stained with the Silvest Stain protein kit (Nakarai Tesque) in a plastic box . Where samples had not been properly amplified (mostly due to malfunction of the PCR machine), amplification with the same collection of primer combinations was repeated until a clear set of 16 lanes was obtained. The gels were dried by heating to 70°C with a gel drier and archived with great convenience and compactness in a photo pocket album of cabinet size (Verjour Album, Konica), and also stored as digital files after scanning.

The polymorphic bands were detected by direct observation of the gel, and a complete matrix of the polymorphism rates between all pairs of accessions, including *L. filicaulis*, was constructed. The half matrix data for the DNA polymorphism rate were processed by the NEIGHBOR program, which uses the UPGMA method, in PHYLIP (Phylogeny Inference Package) Version 3.5c (Felsenstein 1993; see the PHYLIP home page: http://evolution.genetics.washington.edu/phylip.html).

Segregation analysis of the F_2 from crosses of Miyakojima with Gifu mutants

Wild-type Miyakojima plants were fertilized with pollen from Gifu mutants [*Ljsym70*, *Ljsym72*, *Ljsym74-1* (*alb1-1*) and *Ljsym78-1*], and *Ljsym 74-1* Gifu (female) was also crossed with wild-type *L. filicaulis*. The success of the crosses was checked in the F1. All plants should be wild type and have anthocyanin pigment and trichomes on the stem (Gifu type). The F_2 seedlings were grown in vermiculite immersed in nitrogen-free 1/2-HM nutrient solution (Imaizumi-Anraku et al. 1997) and infected with *M. loti* JRL501 at a density of 10^7 cells/ml. The 70–250 seedlings were grown on a 16 h/8 h day/night cycle at 25°C in a Biotron LH-300 in small pot arrays (10×20) and evaluated for setting nodules 1 month after inoculation.

Results

Collection sites of the accessions

Plants of *L. japonicus* from 15 localities were collected from throughout Japan on a sampling tour, and through

Fig. 1 Geographic origins of 15 accessions of *L. japonicus*. The region outlined by the square (Kanagawa Prefecture) is enlarged at the *top left*

the kind donation of seeds by other researchers (Table 1). Their geographic origins are shown in Fig. 1. The standard accession, Gifu B-129, was originally collected by Dr. Isawo Hirayoshi on a riverbank in Gifu Prefecture, in the central region of the Japanese mainland. Funakura B-581, used by Jiang and Gresshoff (1997) as a mating partner, was originally collected by Dr. N. Satomi on an isolated island in the Sea of Japan, Hegurajima. Taiki MG-2 and Miyakojima MG-20 were collected on a sampling tour to the Northern- and Southernmost parts of the habitat, respectively. The resulting 15 accessions can be considered as representing the major ecotypes of *L. japonicus* in Japan.

Morphological traits of Miyakojima MG-20

The Southernmost accession, Miyakojima, is easily distinguished from Gifu (Fig. 2). The former is more erect (plagiotropic), while the latter has a more crawling habit (ascending to decumbent) (Fig. 2A, D). Gifu accumulates anthocyanins on the mature stem and petiole (or lower part of the peduncle). Pigmentation of Gifu is also apparent as stripes on the basal part of the standard petal of the flower. Miyakojima does not accumulate anthocyanin on the stem (Fig. 2B, E) and only weakly in the petal (Fig. 2C, F). Leaflets and petals of Miyakojima are wider than those of Gifu and adjacent leaflets often overlap. Stipule-like leaflets of Miyakojima are also wider and have a tendency to embrace the stem (be amplexicaul), while those of Gifu show no such tendency (Fig. 2B, E). In Gifu, trichomes are numerous especially on the calyx tubes and at the bases of the pedicels, but sparse on the stems and leaflets in Gifu, while there are few trichomes in Miyakojima (Fig. 3A, C). Seeds of Miyakojima are darker in color and larger than those of Gifu (Fig. 3B, D). Hypocotyls and young shoots of Miyakojima are longer than those of Gifu – like those of *hy* mutants of *Arabidopsis* (data not shown). The red color of the stem and the presence of trichomes are each determined by single dominant genes (see below), and can be used as markers to check the success of the crosses when using Miyakojima as the female parent.

HEGS analysis of plants of 15 accessions of *L. japonicus* and *L. filicaulis*

In order to assess the degree of polymorphism in all combinations among the accessions including Gifu B-129, Funakura B-581 and *L. filicaulis* B-37, AFLP analyses were done for these 16 genomic DNA samples using the HEGS system (see Kawasaki and Murakami 2000, for review). As shown in Fig. 4, HEGS can handle 64 lanes of samples per gel, and 256 samples can easily be processed at a time. This made it possible to finish the electrophoretic analysis of the 16 accessions with 31 selective primer pairs in 3 days, verifying the high throughput capacity of the HEGS system. As the lanes were close to each other, direct comparison of the bands was easy. A total of 21,141 major bands were detected from 15 accessions of *L. japonicus* and *L. filicaulis*. Thus, the system could resolve approximately 43 bands, on average, per lane from an amplification with $+3/+3$ primer pairs. This is close to the expected value of 59, assuming that only the fragments with hetero enzyme-digested ends are amplified (Vos et al. 1995), and taking the genome size to be 494 Mb (Kawasaki and Murakami 2000). The polymorphic band numbers per primer pair between the accessions were assembled into a half matrix (Table 2). The data indicated clearly that Miyakojima is the most polymorphic with respect to Gifu, among Japanese accessions. The polymorphism rate of Miyakojima/Gifu (4.4%; 1.9 polymorphic bands per primer pair) was more than twice that of Funakura/Gifu (2.1%; 0.9 bands per primer pair). Miyakojima also gave the highest in polymorphism rate in comparisons with all the other Japanese accessions (1.8 to 2.2 bands per primer pair). The degree of DNA polymorphism between *L. japonicus* and the African species *L. filicaulis* was very high (36%; 15.5 polymorphic bands per primer

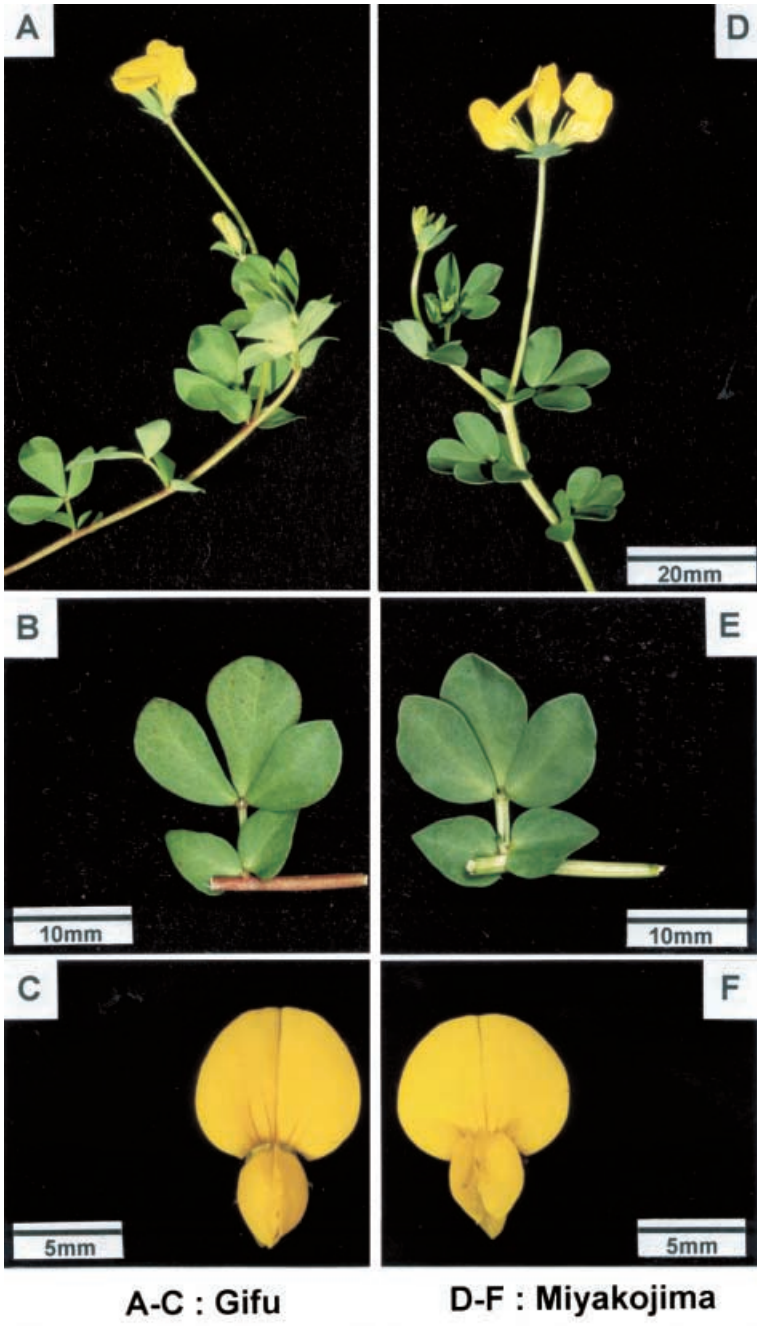


Fig. 2A–F Morphological traits of two accessions of *L. japonicus*; Gifu (**A–C**) and Miyakojima (**D–F**). Gifu plants are rich in anthocyan pigments in the mature stem (**A, B**), and have purple stripes around the basal part of the standard petal (**C**). Miyakojima does not deposit pigments in the mature stem. The leaflets, stipule-like leaflets, petioles and petals of Miyakojima are wider and thicker than those of Gifu (**B** and **E**; **C** and **F**)

pair), more than 10 times greater than those detected between accessions of *L. japonicus*. However, this high degree of polymorphism raises concerns regarding segregation distortion and the inhibition of crossing-over between homologous chromosomes of the parents.

Inferring phylogenetic relationships between the accessions

A dendrogram was constructed from the above data matrix based on the degree of polymorphism between accession pairs (Table 2) using the NEIGHBOR program (which uses the UPGMA method) of PHYLIP 3.5c (Felsenstein 1993) (Fig. 5). The genetic distance (in arbitrary units) between *L. filicaulis* and *L. japonicus* was about four times larger than that between the accessions Gifu and Miyakojima, which, in turn, was about twice that between Gifu and Funakura. The fact that Miyakojima occupies a rather isolated position relative to other Japanese accessions in the dendrogram is consistent with the fact that Miyakojima is quite distinct from the others also in its morphological traits. The dendrogram for the accessions roughly correlates with geographical distribution (see Figs. 1 and 5), supporting the general form of the dendrogram. However, there

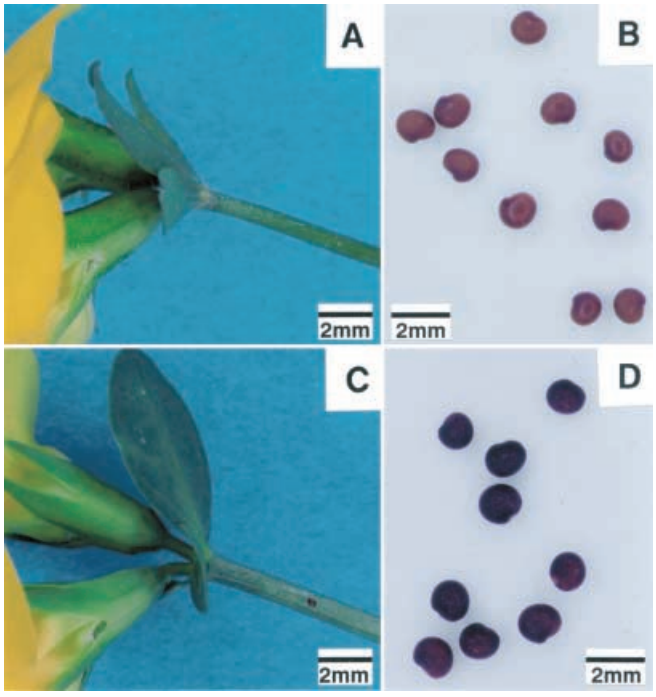


Fig. 3A–D Trichomes and seeds of Miyakojima and Gifu. There are trichomes on the stem, and around the calyx tube and the bases of pedicels in Gifu (**A**) but not in Miyakojima (C). Seeds of Miyakojima (**D**) are darker and larger than those of Gifu (**B**)

were some exceptions to this concordance. Plants from the Bishamon locality, on the Pacific coast in Kanagawa Prefecture (Fig. 1), were very closely related to those from Taiki, near the northern limit of the habitat, but also located near the Pacific coast. The accession Bishamon is also specific in its red petal color. Plants of the accession Kameoka are quite divergent from other Japanese mainland accessions and are found in a basin which is surrounded by low mountains. This region belongs to the Sohaya area, which is known to have specific characteristics, based on other distributional studies of the Japanese flora.

Comparison of segregation patterns in F_2 progeny

In order to confirm normal segregation, F_2 siblings derived from crosses of representative nodulation mutants of Gifu [*Ljsym70*, Nod$^-$; *Ljsym72*, Nod$^-$ Ami (abuscular mycorrhizal interaction)$^-$; *Ljsym74-1* [*alb1-1*], Hist$^-$; *Ljsym78*, Nod^{++}) with Miyakojima were analyzed. The cross of *Ljsym 74-1* with *L. filicaulis* was also analyzed for comparison. In the crosses with Miyakojima, all the mutant phenotypes segregated in a normal Mendelian manner with a good χ^2 fit (Table 3), while the cross with *L. filicaulis* showed a severely distorted segregation, with the assumption of a 3:1 ratio coming close to being rejected at the 5% level. The segregation rate for the cross with *L. filicaulis* makes mapping rather treacherous, although several markers may be obtained in the apparent neighborhood of the target. The wild-type traits of green stem [*viridicaulis* (*vic*) *6*] and *few trichomes* (*fet1*) in Miyakojima behaved as if each were controlled by a recessive single gene in the cross with Gifu (Table 4), and their segregation patterns strictly fit a Mendelian scheme.

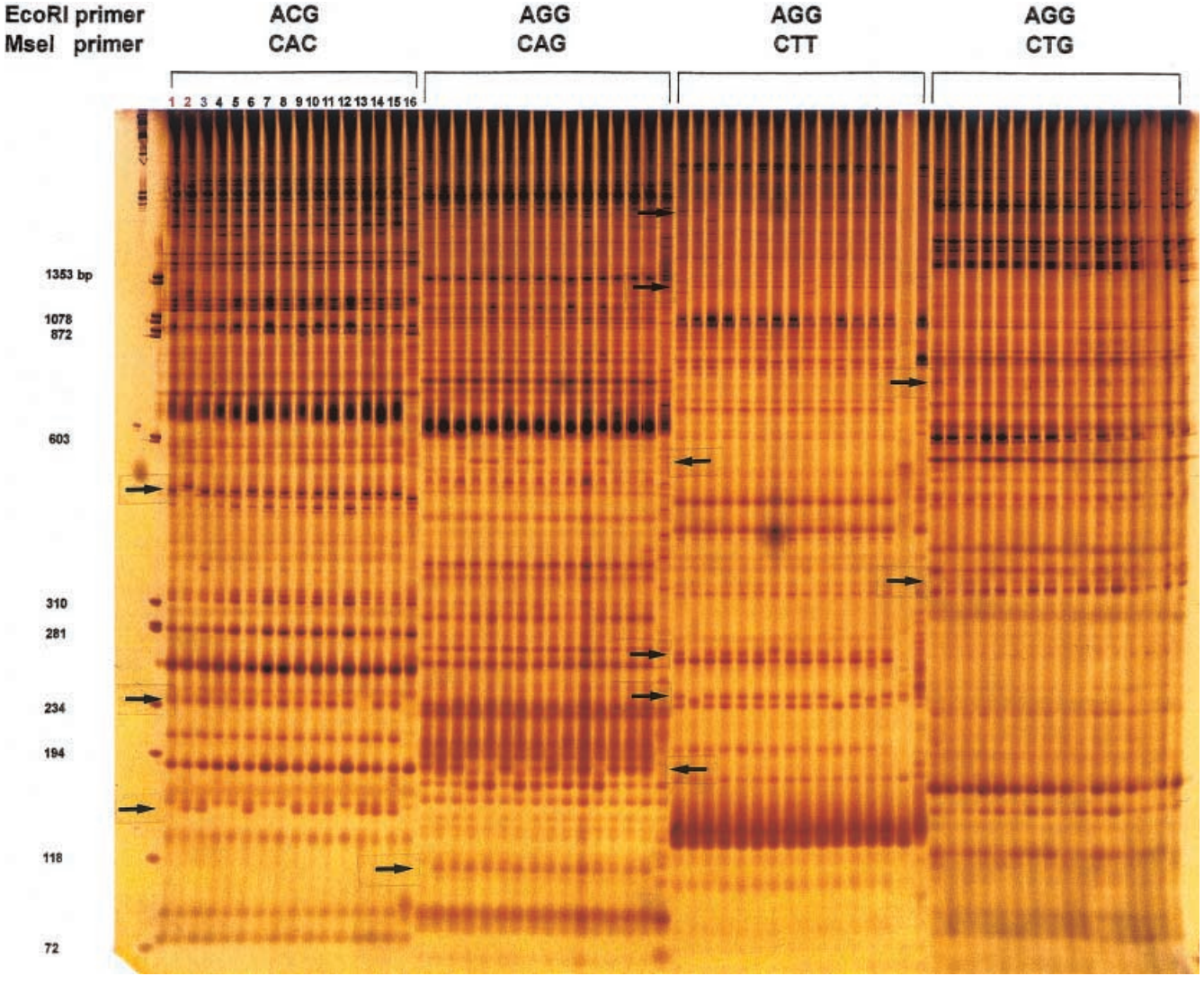
Discussion

Based on the AFLP and genetic analyses, the accession Miyakojima MG-20, collected near the Southern tip of the Japanese archipelago, was found to be the most useful crossing partner for the standard accession Gifu B-129. Miyakojima could easily be distinguished from other Japanese accessions by its morphology (Figs. 2 and 3). The genetic isolation of Miyakojima from other Japanese accessions was verified by their rates of DNA polymorphism and supported by a dendrogram derived from these data (Table 2 and Fig. 5). The high-throughput HEGS system proved its usefulness for this kind of analysis (Fig. 4), and it also was demonstrated to provide a powerful tool for F_2 and QTL analyses. This is especially important for *L. japonicus*, for which no ready-made map is available for use in positional cloning. Although a polymorphism rate of 4.5% is not high compared with examples in other crops, e.g. the 10–15% detected between the rice subspecies *japonica* and *indica* determined with HEGS (see A. Shimizu et al., manuscript submitted), it is sufficient for any genetic analysis with the HEGS system, which can process 256–512 samples a day. That means that 64–128 primer pairs can be scanned per day, by bulked segregant analysis with four lanes (Michelmore et al. 1991). With 1.9 polymorphic bands among 40 bands developed per lane (4.4% polymorphism; Table 2), one can scan through 120–250 polymorphic markers/day; that means that about 7700 polymorphic markers can be expected to be obtained from all of the 4096 combinations of the 64 (*Eco*RI) and 64 (*Mse*I) primers with $+3/+3$ selective nucleotides in 32–64 working days, at a very reasonable cost. Considering that total map sizes for most crop plants are converging around 1500 cM; irrespective of genome size (O'Brien 1993), and the genome size of *L. japonicus* is 494 Mb (Kawasaki and Murakami 2000), this system will provide about three markers/cM or one marker/$\sim$120 kb on average – smaller than the average insert size in the BAC library (140 kb; Y. Murakami and S. Kawasaki, manuscript submitted). Although only markers in repulsion phase, about half of the total, can be used for the recessive mutant F_2 analyses, due to the dominant nature of the AFLP makers, this marker density will be sufficient to proceed directly from genetic to physical map analysis using the BAC library.

Another great merit of Miyakojima as a crossing partner for Gifu is that its moderate genetic distance from the latter facilitates genetic analyses, with normal Mendelian segregation in the F_2 generation (Tables 3 and 4), and smooth crossing over between the

homologous chromosomes of the parents. The present AFLP map based on the Miyakojima × Gifu cross and assembled by our group using the HEGS system (Miyahara et al., manuscript in preparation; see Kawasaki and Murakami 2000) now spans 800 cM with 200 markers. Although *L. filicaulis* has also been proposed as a partner for Gifu (Sandal and Stougaard 1998), the genetic distance between these two species seems inconveniently large, judging from the polymorphism rate of 38% (Table 2), and the segregation distortion revealed in the cross with *Ljsym74-1* (*alb1-1*) in Table 3.

Fig. 4 HEGS-AFLP patterns of the 15 Japanese accessions of *L. japonicus* and *L. filicaulis*. The primer pairs used for the second amplification are indicated *above* the lane groups. HEGS makes it possible to scan four primer pairs/gel and 16 primer pairs/electrophoresis for the 16 samples. Some of the representative polymorphic bands are indicated by the *arrows*. Lanes: 1, Gifu; 2, Miyakojima; 3, Funakura; 4, Taiki; 5, Aomori; 6, Oga; 7, Towa; 8,Tono; 9, Totsuka; 10, Arasaki; 11, Ninomiya; 12, Bishamon; 13, Kameoka; 14, Ashizuri; 15, Kaseda; 16, *L. filicaulis*. The original dried gels measure 13×16.5 cm and can be kept in pocket photo albums



An optimal compromise between high polymorphism rate and low segregation distortion may be found around values of 10–15% in AFLP analysis. Too great a genetic distance between species often makes crossing-over between chromosomes difficult, as seen in the example of *Xa21* of rice, which had several markers at 0 cM, based on the cross of *O. sativa* and *O. longistaminata* (Ronald et al. 1992). Actually, the total length of a map derived from a *L. filicaulis* × Gifu seems to be small (378 cM) compared with the number of markers used (450; Sandal 2000).

Coupled with the AFLP data, geographic distribution and several morphological traits, the clearly different genome size of Miyakojima suggests that it may constitute a subspecies of *L. japonicus*, with respect to the mainland group. Besides its morphological features, among the Japanese accessions Miyakojima is the earliest to flower after seeding, and can easily produce flowers indoors, which is another useful trait that is important for an experimental plant.

It is interesting to note that the genetic distances between the accessions roughly reflect their geographical

Table 2 Average numbers of polymorphic bands detected per primer pair between pairs of accessions

Accession[a] Gifu	Gifu	Miya.	Funa.	Taiki	Aomori	Oga	Towa	Tono	Totsu.	Arasa.	Ninomi.	Nishiki	Kame.	Ashizu.	Kaseda	*L. filicaulis*
Miyakojima	1.9															
Funakura	0.9	1.7														
Taiki	0.9	2.0	1.2													
Aomori	0.9	2.2	1.2	0.3												
Oga	1.2	2.0	0.9	1.3	1.3											
Towa	0.9	2.1	1.2	0.5	0.6	1.2										
Tono	0.8	2.0	1.2	0.4	0.6	1.3	0.4									
Totsuka	0.7	1.7	0.9	1.1	1.2	1.1	1.2	1.2								
Arasaki	1.0	1.8	1.1	1.1	1.3	1.1	1.2	1.2	0.9							
Ninomiya	1.0	1.9	1.2	1.1	1.2	1.2	1.1	1.1	0.9	0.7						
Nishiki	0.8	2.0	1.1	0.2	0.3	1.2	0.5	0.5	1.0	1.0	1.1					
Kameoka	1.2	2.2	1.2	1.5	1.5	1.4	1.6	1.4	1.3	1.4	1.3	1.4				
Ashizuri	1.2	2.0	1.0	1.2	1.4	1.2	1.4	1.3	1.0	1.2	1.1	1.3	1.1			
Kaseda	0.8	1.8	0.8	1.0	1.1	0.8	1.0	0.9	0.8	0.9	0.9	0.9	0.8	0.7		
L. filicaulis	15.6	15.9	15.8	15.9	15.9	15.9	16.0	15.9	15.9	15.8	15.5	15.7	16.0	15.7	15.7	–
Total	42.5	42.9	42.6	42.9	42.7	42.7	42.9	43.0	42.6	42.6	42.4	42.7	42.9	42.9	42.7	40.9

[a]The polymorphic AFLP bands were detected and counted by the HEGS as in Fig. 4. Numbers are the average for the 31 primer pairs, electrophoresed on eight gels over a period of 3 days, including repetition. The high degree of polymorphism between Miyakojima and Gifu among the Japanese accessions and the large genetic distance between *L. japonicus* and *L. filicaulis* are apparent

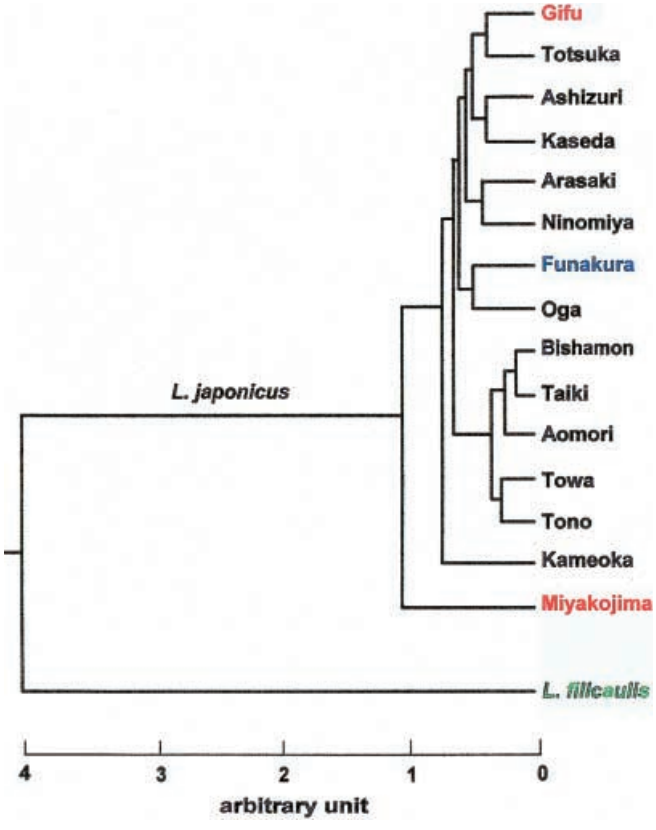


Fig. 5 Dendrogram representing the phylogenetic relationships between the accessions of *L. japonicus*. The data in Table 2 were processed with the NEIGHBOR program (UPGM method) in PHYLIP 3.5c. Compare the genetic distance between *L. filicaulis* and *L. japonicus* and the distance between Gifu and Miyakojima

distribution (Figs. 1 and 5). Oga and Funakura face the Sea of Japan and the accessions collected there are closely related;, the accessions Aomori, Tono; Towa – found in the Tohoku region of Japan – are also closely related. However, there is one striking exception, in that the accession Bishamon is very closely related to Taiki; both sites lie near the Pacific coast, but are very far apart. Such a correlation between geographic origin and the form of a dendrogram is not known for *Arabidopsis* ecotypes (Miyashita et al. 1999). However, this may depend, to some extent, on the appropriate selection of the programs applied in the analysis. In relation to the inter-species relationships of *Lotus*, although there has been a study of *Lotus* species relationships with the RAPD technique (Campos et al. 1994), very few polymorphic bands and species were used. Therefore, further examination using AFLP/HEGS analysis of *Lotus* species will provide a very rich source of data on the phylogenetic structure of the genus.

We (Y. Murakami and S. Kawasaki, submitted) also recently established another base for genome analysis of *Lotus*, a BAC library containing long inserts (average 140 kb) for *L. japonicus* (Gifu). With this size of inserts, the above mentioned marker density (120 kb/marker), expected from the polymorphism rate between Gifu and Miyakojima with the HEGS system, will be sufficient for positional cloning of various genes including the genes that are defective in the nodulation-related mutants. As mutations induced with chemical mutagens or radiation mainly involve single base changes or very localized alterations, they are much more likely to survive as homozygotes than mutants derived from insertion mutagenesis by transposons, T-DNA or promoter tagging, and their phenotypes should provide insight into the function of the genes involved. Therefore, positional cloning guided by the HEGS screening system will provide a powerful tool for a functional genomics of nodulation and symbiotic nitrogen fixation in *L. japonicus*. EST data for Gifu have also been accumulated in several laboratories [at Michigan State University (Szczyglowski

Table 3 F_2 segregation of nodulation mutants of Gifu crossed to Miyakojima or *L. filicaulis*

Locus	Crossing partner	Mutant phenotype[a]	F_2 segregation	χ^2 value for fit to 3:1 ratio	*p*
sym 70	Miyakojima	Nod–	199:67	0.005	0.95
sym 72	Miyakojima	Nod–, Ami–	130:38	0.507	0.5
sym 74-1 (*alb-1*)	Miyakojima	Hist–	147:48	0.051	0.8
sym 78	Miyakojima	Nod + +	181:53	0.689	0.4
sym 74-1 (*alb-1*)	*L. filicaulis*	Hist–	59:11	3.220	0.06

[a]Nod, nodulation; Ami, arbuscular mycorrhiza interaction; Hist, histogenesis

Table 4 Segregation of natural traits in F_2 progeny of the cross Miyakojima × Gifu

Locus	Phenotype (Miyakojima)	F1 plant	F_2 segregation	χ^2 value for fit to 3:1 ratio
vic 6	Green stem	Red stem	125:42	0.002
fet 1	Few trichomes	Many trichomes	93:31	0

et al. 1997), the Max Planck Institute at Golm, and at Aarhus University). Recently the Kazusa DNA Institute (Chiba, Japan) reported the large-scale generation of ESTs from Miyakojima (Asamizu et al. 2000). The three components introduced here – the crossing partner, HEGS, and the large-insert BAC library, can now be combined with these database resources. These new tools for molecular genetics are expected greatly to accelerate the advance of genome research on *L. japonicus*.

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