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Chromosomal Map of the Model Legume *Lotus japonicus*

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ABSTRACT

Lotus japonicus is a model plant for the legume family. To facilitate map-based cloning approaches and genome analysis, we performed an extensive characterization of the chromosome complement of the species. A detailed karyotype of *L. japonicus* Gifu was built and plasmid and BAC clones, corresponding to genetically mapped markers (see the accompanying article by Sandal *et al.* 2002, this issue), were used for FISH to correlate genetic and chromosomal maps. Hybridization of DNA clones from 32 different genomic regions enabled the assignment of linkage groups to chromosomes, the comparison between genetic and physical distances throughout the genome, and the partial characterization of different repetitive sequences, including telomeric and centromeric repeats. Additional analysis of *L. filicaulis* and its F_1 hybrid with *L. japonicus* demonstrated the occurrence of inversions between these closely related species, suggesting that these chromosome rearrangements are early events in speciation of this group.

LEGUMES are important worldwide for their use as food and forage. They are also remarkable for their ability to grow independently of externally added reduced nitrogen due to symbiosis with rhizobia. Although studies concerning the molecular basis of the plant role in nitrogen fixation and symbiosis have been reported (Scheres *et al.* 1990; Kouchi and Hata 1993), fast progress has been achieved only recently with the use of the model legume *Lotus japonicus* (Handberg and Stougaard 1992; Szczyglowski *et al.* 1997; Schauser *et al.* 1998; Wegel *et al.* 1998).

To facilitate the isolation of new genes by map-based cloning, genetic linkage maps have been established for *L. japonicus* (Hayashi *et al.* 2001; Sandal *et al.* 2002) and a bacterial artificial chromosome (BAC) library has been constructed (N. Sandal, N. Ellis and J. Stougaard, unpublished results). The map presented here was based on a cross between *L. japonicus* Gifu and the related species *L. filicaulis* (Sandal *et al.* 2002). Until recently, only conventional karyotypes were described for both species (Cheng and Grant 1973). With the increasing interest in Lotus, including a genome sequencing project (Cyranoski 2001; Sato *et al.* 2001), karyotypes based on the prophase condensation pattern and on fluorescence *in situ* hybridization (FISH) with rRNA genes were determined for two *L. japonicus* accessions (Ito *et al.* 2000).

FISH has been used, among other applications, for mapping repetitive sequences and multicopy genes and for correlating genetic linkage groups to chromosomes that could be followed in hybrid lines. FISH has also been useful to compare genetic and physical distances, since it is known that recombination is not uniform along chromosomes and, therefore, these distances correlate poorly (Schwarzacher 1994; Gill 1995). The use of FISH for assigning individual markers to plant chromosomes has been demonstrated (Jiang *et al.* 1995; Fuchs *et al.* 1998; Dong *et al.* 2000; Fransz *et al.* 2000; Cheng *et al.* 2001a,b; Kulikova *et al.* 2001).

We have used different cytogenetic approaches, including FISH, to develop an extensive chromosomal map of *L. japonicus* Gifu. Additionally, FISH was used to compare the parental lines *L. japonicus* Gifu and *L. filicaulis* and the F_1 hybrid from which the F_2 linkage mapping population was derived. The alignment of genetic and chromosomal maps in several positions along the chromosomes provided an understanding of the map in a genome perspective. Furthermore, chromosomal rearrangements between these two closely related species were demonstrated, which led to the resolution of difficulties with the linkage analysis.

MATERIALS AND METHODS

Plant material: *L. japonicus* Gifu B-129-S9 seeds (Stougaard and Beuselinck 1996) were obtained from the USDA-ARS Regional Plant Introduction Station (Pullman, WA). *L. filicaulis* B37 seeds were obtained from W. F. Grant's collection. The *L. filicaulis* × *L. japonicus* F_1 hybrid plant 4-6 is the parent of the F_2 mapping population and is maintained as cuttings.

Pretreatment and fixation: Root tips were obtained from seeds germinating on moist filter paper or, in the case of the F_1 hybrid, from plants growing in pots. Root tips were pretreated with 8-hydroxyquinoline (2 mm) for 4–5 hr at 16°,

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AY040715 and AF390569.

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fixed in methanol/acetic acid 3:1 (v/v), and stored in fixative at −20° for up to several weeks.

Feulgen staining and chromosome measurements: *L. japonicus* roots were hydrolyzed in 1 N HCl at 60° for 10 min, stained in Schiff's reagent (Merck, Darmstadt, Germany) for 2 hr in the dark at room temperature, and squashed in 45% acetic acid. After removal of the coverslip in a cold plate, slides were made permanent in Euparal.

C-banding: Root tips were macerated in an enzyme solution [3% (w/v) cellulase "Onuzuka R-10" (Serva Electrophoresis, Heidelberg, Germany) plus 10% (v/v) pectinase (Sigma-Aldrich, Vienna) in 0.01 M citric acid-sodium citrate buffer, pH 4.8] at 37° for 1–2 hr, transferred to a drop of 45% acetic acid, and flamed before squashing. C-banding was performed according to Schwarzacher *et al.* (1980), except for the final staining, which was done by mounting the slides with 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield (Vector, Burlingame, CA).

Nuclear DNA measurements: Nuclear DNA content was estimated by Feulgen densitometry and propidium iodide (PI) flow cytometry. Feulgen densitometry was performed according to Greilhuber and Ebert (1994), using *Pisum sativum* "Kleine Rheinländerin" as standard. Ten nuclei were measured per seedling/slide and three pairs of slides (*L. japonicus*/*P. sativum*) were analyzed in each of the four replications. PI flow cytometry was performed according to Baranyi and Greilhuber (1996) and Greilhuber and Obermayer (1997), using *Glycine max* "Polanka" and *Zea mays* line CE-777 as standards. A total of 5000 nuclei were analyzed in three joint measurements per replication, with two and three replications with *G. max* and *Z. mays*, respectively.

Cloning of 5S rRNA gene from *L. japonicus*: The 5S rRNA repeats were amplified from genomic DNA of *L. japonicus* by PCR using the primers 5′-GTGCGATCATACCAGC(AG)(CT)TAATGCACCGG-3′ and 5′-GAGGTGCAACACGAGGACTTCCCAGGAGG-3′. The PCR product, ~400 bp, corresponding to the dimer form, was purified using the Quantum Prep Freeze 'N Squeeze spin column (Bio-Rad, Vienna), ligated, and transformed using the TA cloning kit (Invitrogen, Groningen, The Netherlands). The insert of one positive clone (D2) was determined by cycle sequencing on an ABI 377 automated sequencer (PE Applied Biosystems, Vienna) and the sequence of one repeat unit was included in the GenBank database (accession no. AY040715).

DNA probes: The probes used were R2, a 6.5-kb fragment of a 18S-5.8S-25S rDNA repeat unit from *Arabidopsis thaliana* (Wanzenböck *et al.* 1997); D2, a 5S rRNA clone from *L. japonicus*; an Arabidopsis-like telomeric probe, amplified by PCR according to Ijdo *et al.* (1991) using the oligomer primers $(5'\text{-TTTAGGG-}3')_5$ and $(5'\text{-CCCTAAA-}3')_5$; and large plasmid and BAC clones containing inserts with known map position (Table 1). The *L. japonicus* BAC library (N. Sandal, N. Ellis and J. Stougaard, unpublished results) was screened using genomic and cDNA clones, corresponding to DNA markers. Southern hybridization to some BAC clones with *L. japonicus* genomic DNA was performed to identify and exclude the BACs bearing highly repetitive sequences. BACs were confirmed by PCR using specific primers. BAC DNA was isolated using a Plasmid mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. All probes were labeled by nick translation (Roche Diagnostics, Vienna; Life Technologies, Vienna) with Cy3-dCTP or Cy3-dUTP (Amersham Pharmacia Biotech, Vienna). R2 and D2 were also labeled with biotin-14-dATP (Life Technologies).

FISH: Slides were prepared as described for C-banding and selected after staining with 2 μg/ml DAPI in McIlvaine's pH 7.0 buffer. Good preparations were destained in 3:1 (v/v) ethanol-acetic acid (30 min) and absolute ethanol (1 hr) and pretreated as described in Pedrosa *et al.* (2001). Chromosome and probe denaturation, post-hybridization washes, and detection were performed according to Heslop-Harrison *et al.* (1991), except for the stringent wash, which was performed, in subsequent experiments, with 0.1× SSC at 42°. Hybridization mixes consisted of 50% (v/v) formamide, 10% (w/v) dextran sulfate, 2× SSC, and 2–5 ng/μl probe. The slides were denatured for 5 min at 75° and hybridized for up to 2 days at 37°. A total of 10 μg of salmon sperm sheared DNA and 10- to 1000-fold excess *L. japonicus* C_0t 1 fraction (Zwick *et al.* 1997) were added to the hybridization mix in few experiments. In those cases, slides were denatured first in 50% formamide and 2× SSC and dehydrated in 96% ethanol for 5 min at −20°. Denatured probe and blocking DNA were reannealed in the hybridization mix for 1–5 hr at 37° before being added to the slides. Biotin-labeled probes were detected using ExtrAvidin-FITC conjugate (Sigma-Aldrich, Vienna) in 1% (w/v) BSA. All preparations were counterstained and mounted with 2 μg/ml DAPI in Vectashield. Reprobing of slides for localization of different DNA sequences on the same cell was performed according to Heslop-Harrison *et al.* (1992) up to three times.

Image analysis: Photographs were taken on a Zeiss Axioplan (Carl Zeiss, Vienna) equipped with a mono cool view CCD camera (Photometrics, Tucson, AZ). Images from the camera were combined and pseudocolored using spectrum software (IPLab, Fairfax, VA). For construction of the idiogram, 5 well-spread, complete, DAPI-stained metaphases, in which each chromosome arm could be recognized by hybridizing with specific probes, were used. For each chromosome, the total length and the length of its presumable short arm were measured in arbitrary units. Relative lengths and arm ratio were calculated for each chromosome and a mean value was calculated for each pair. The total length of the chromosome complement measured from 10 Feulgen-stained metaphases was used to calculate the length of each chromosome in micrometers. At least 10 metaphases bearing clear signals were photographed in order to calculate the position of a clone. Assignment of a clone to a specific chromosome arm was confirmed by reprobing the slides with a clone of known position. All measurements were performed using the "analyze/measure length" function of the IPLab software. Digital images were imported into Adobe Photoshop version 5.0 for final processing.

RESULTS

Karyotype of *L. japonicus*: To establish a chromosomal map of *L. japonicus*, an idiogram was constructed on the basis of the relative chromosome lengths and arm ratios obtained from DAPI-stained cells in which all chromosome arms could be recognized. Feulgen-stained metaphase plates [Figure 1(1)] were used to measure the total length of the chromosome complement in micrometers. The complement was divided into three groups: one large (1), three medium (2–4), and two small (5 and 6) metacentric pairs. After C-banding [Figure 1(2)], heterochromatin was observed predominantly at the pericentromeric region, varying in size among different chromosome pairs. Additional C-positive bands were observed in chromosome 2 in the region corresponding to the nucleolar organizer and at the intercalary position in the long arm of chromosome 4.

Nuclear DNA content of the species was estimated by Feulgen densitometry and propidium iodide flow

TABLE 1

List of plasmid and BAC clones used as probes for *in situ* hybridization

Clone	Gene/marker	Size (kb)	Localization			
			Chromosome	Arm	Position[a]	n^b
1. LS32	*Nin*	10–15	1	Short	2.43 ± 4.06[c]	25
2. BAC S1-1	*s1-1*		1	Short	7.96 ± 4.47	33
3. BAC 81J4	*Nlp1*	45	1	Long	31.91 ± 5.24	12
4. BAC 53C15	*Cyc2*		1	Long	23.14 ± 5.50	56
5. BAC 72G19	*Lnp*		1	Long	14.96 ± 3.80	37
6. pZF	*pZF*		1	Long	7.50 ± 4.12	33
7. BAC 35P5	*GAP2*		1	Long	6.50 ± 3.04	12
8. R2	45S rDNA (*A. thaliana*)	6.5	2	Short	22.77 ± 4.36[d]	34
			5	Short	20.73 ± 7.66	16
			6	Short	39.07 ± 6.69	19
9. D2	5S rRNA gene	0.4	2	Short	35.51 ± 4.92	38
10. 936-5.3	*sym13*	11[e]	2	Long	31.08 ± 5.46	35
11. BAC 58K7	*sym5*		2	Long	13.68 ± 7.07	39
BAC 92C16	*sym5* region		Dispersed			
12. BAC 61A22	*Cyc1*		2[f]	Long	11.61 ± 4.00	17
13. BAC 92J16	*Nlp2*		3	Short	8.00 ± 3.76	26
			5	Long		
BAC 3C16			3	Short	8.54 ± 4.73	21
			5	Long		
14. BAC 85D15	Related to *har1-3*		3	Long	31.47 ± 6.36	45
15. BAC 45N23			3	Long	23.78 ± 5.02	36
16. BAC 19J6	*sym8*	100	3	Long	21.14 ± 4.20	40
LS79/E3.1		16	3	Long	28.44 ± 5.88	48
17. BAC 82C22	Fructose biphosphate aldolase		3	Long	15.29 ± 6.38	14
BAC 12M21						
BAC 81G23						
18. pλ3.2	*har1-3*	15	3	Long	10.35 ± 3.83	25
BAC 35H14		95	Dispersed (pericentric)			
19. BAC 50I19	Related to *P9*		4[f]	Short	21.31 ± 4.55	12
20. BAC 94F4	*Enod40-2*		Dispersed			
94F4-*Bam*HI	Large *Bam*HI fragment	13	4[f]	Short	29.72 ± 6.15	14
21. BAC 60D1	*Myo2*		4	Long	16.21 ± 4.77	11
BAC 1K17			4[f]	Short	14.08 ± 6.84	10
22. BAC 1M10	*Lb*	50	Dispersed (pericentric)			
1M10-71	1M10 *Bam*HI subclone	9	5	Long		
Lb 112		0.62				
Lb 123		0.61				
23. 431	*Gln1*	15	6	Short	22.20 ± 6.00	22
24. BAC 47F13	*GTP*		6	Long	12.27 ± 4.16	32
25. BAC 10F21	Telomere-like	60	Centromeres			
BAC 41P18		100	Centromeres			
BAC 17N10		65	Centromeres			
26. BAC 32K9	*ENBP1*		Dispersed (pericentric)			
27. BAC 52M7	Related to *P9*		Dispersed (pericentric)			
28. BAC 10N16	*Aldo2,3*		Dispersed (pericentric)			
29. BAC 16L1	*Uni*		Dispersed (pericentric)			
30. BAC 90B16	*Ndx3*		Dispersed			
31. BAC 70O3	*Ndx2*		Dispersed			
32. BAC 36A4	*Goc8*		Dispersed			

[a] Position is given as a percentage of chromosome length in relation to the closest telomere.
[b] Number of chromatids used for measurements.
[c] Mean ± SD.
[d] The large 45S rDNA site is located terminally and this value represents the length of the cluster.
[e] The clone was digested with *Kpn*I and only the 11-kb band was purified and used as probe.
[f] Signals were present on the indicated chromosome plus overall dispersed signal.

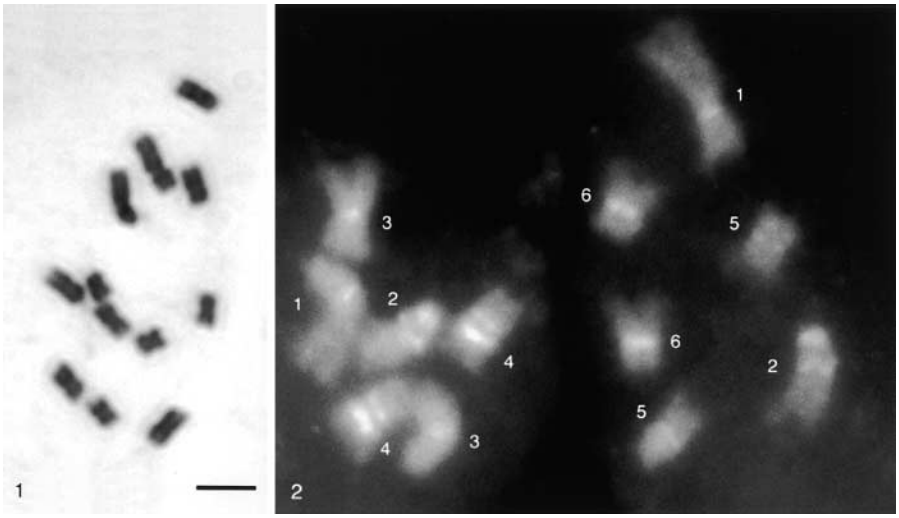


FIGURE 1.—Metaphase plates of *L. japonicus* Gifu after Feulgen staining (1) and C-banding-DAPI (2). Chromosomes in 2 are indicated by numbers. Bar, 2.5 μm.

cytometry (Table 2). The DNA content ratios obtained between *L. japonicus* and three standards were used to calculate the ratio between *L. japonicus* and *A. thaliana* (DOLEZEL *et al.* 1998). *L. japonicus* has a genome 3.46-fold larger than that of *A. thaliana* and, therefore, has a mean 1C value of 432 Mbp, if a 1C value of 125 Mbp (ARABIDOPSIS GENOME INITIATIVE 2000) is assumed for *A. thaliana*. This estimation is close to the already reported values for *L. japonicus* (1.0–1.1 pg/2C, GRANT 1995; 442.8 Mbp/1C, ITO *et al.* 2000) and was further used for calculating the size of each chromosome in megabase pairs.

***In situ* hybridization on *L. japonicus* mitotic chromosomes:** Altogether, DNA clones corresponding to 32 different genomic regions plus a centromeric repeat were used as probes for FISH to *L. japonicus* chromosomes. Clones were screened out of a BAC library (N. SANDAL, N. ELLIS and J. STOUGAARD, unpublished results) or from λ libraries using genetic markers distributed over the entire genetic map. Occasionally, subclones were used (Table 1). In addition, the Arabidopsis-like telomeric oligomer repeat was used to generate a telomeric probe by PCR. Eighteen genetically mapped genes plus one genetically mapped BAC end could be used for correlating the linkage and the chromosomal maps.

Some of the BACs, however, showed a strong, dispersed pattern along the chromosomes and the position of those clones could not be determined. Blocking with the C_0t 1 fraction of *L. japonicus* genomic DNA was not effective for most of the BACs tested. The patterns observed with those BACs were basically of three types: almost uniform along chromosomes, more intense labeling of proximal regions, or more intense labeling of pericentromeric region [Figure 2(3)]. Most likely, these different patterns reflected the distribution of different repetitive DNA families.

Telomeres and centromeres of *L. japonicus* chromosomes: An Arabidopsis-like telomeric probe was generated by PCR and proved to be highly specific and sensitive to detect telomeres of *Phaseolus vulgaris*, another legume species (A. PEDROSA, unpublished data). However, in the case of *L. japonicus*, signals were always weak

TABLE 2

Estimates of 1C nuclear DNA content in *L. japonicus* using Feulgen densitometry and PI flow cytometry

	Ratios of relative DNA content			*L. japonicus/A. thaliana* ratios (indirect)[d]
	Mean	SD	SE	
L. japonicus/P. sativum[a]	0.1388	0.0081	0.0023	3.28
L. japonicus/G. max[b]	0.5215	0.004	0.0016	3.61
L. japonicus/Z. mays[b]	0.2356	0.003	0.001	3.48
L. japonicus/A. thaliana mean ratio				3.46
L. japonicus 1C (Mbp)[c]				432.5

[a] Feulgen densitometry measurement.

[b] PI flow cytometry measurement.

[c] 1C of *A. thaliana* = 125 Mbp (ARABIDOPSIS GENOME INITIATIVE 2000). If a 1C DNA content of 147 Mbp is assumed for *A. thaliana* (ARUMUGANATHAN and EARLE 1991), correcting the ratio of 1 pg = 0.98×10^6 kb (CAVALIER-SMITH 1985), *L. japonicus* 1C DNA content would be 508.6 Mbp.

[d] Genome size ratios between each standard and *A. thaliana* were obtained from DOLEZEL *et al.* (1998).

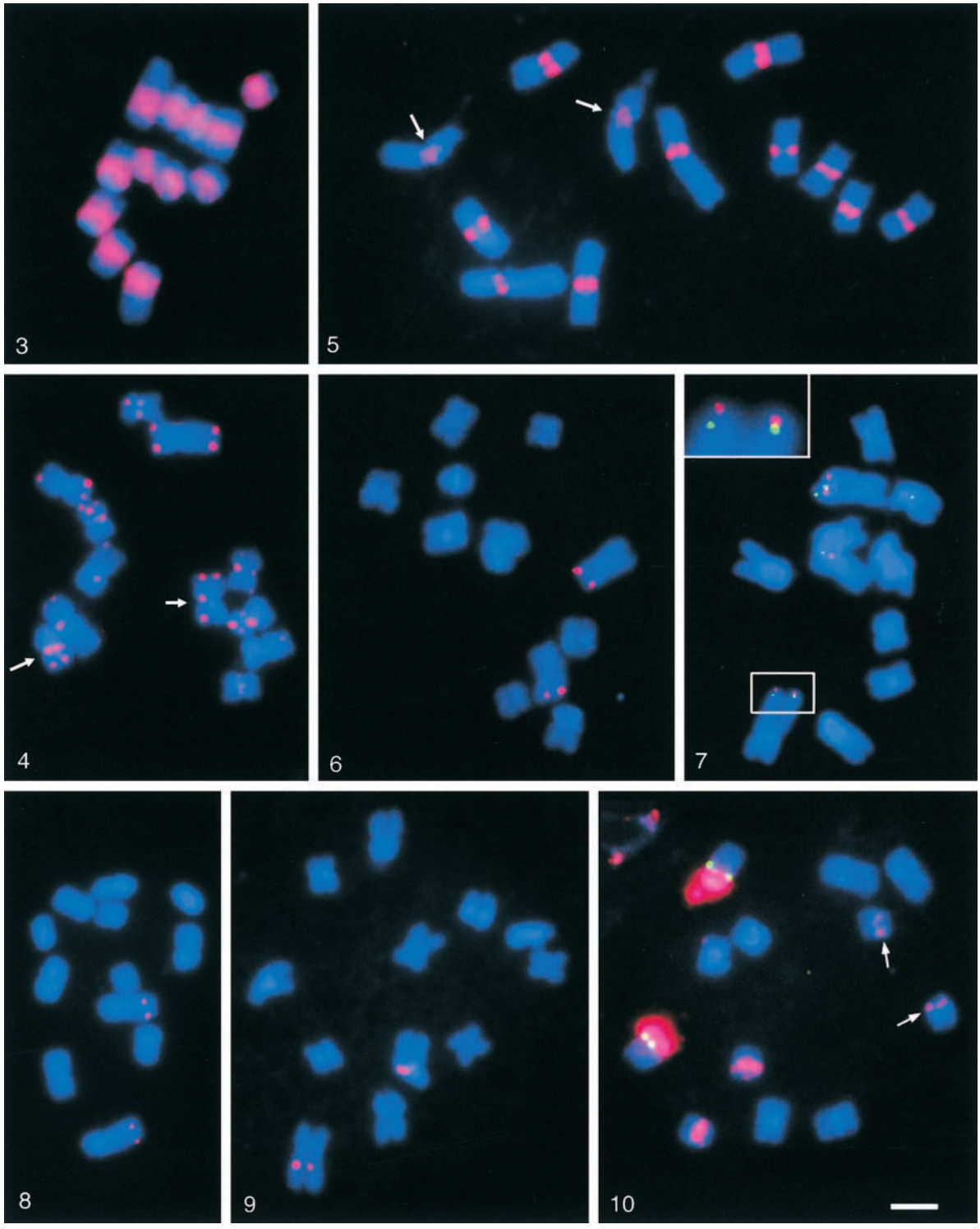


FIGURE 2.—*In situ* hybridization to *L. japonicus* Gifu chromosomes. (3) BAC 1M10 (*Lb*) showing pericentromeric distribution. (4) Arabidopsis-like telomeric repeat, with arrows showing some centromeric signals. (5) BAC 41P18 showing centromeric localization (arrows indicate centromeres of chromosome 2). (6–9) Chromosome 1 specific clones: (6) BAC S1-1; (7) *GAP2* (BAC 35P5, red) and *pZF* (green; insertion shows enlargement of a representative chromosome 1; the second chromosome 1 is partly folded in this cell). (8) *Lnp* (BAC 72G19). (9) *Nlp1* (BAC 81J4). (10) 45S rDNA (red) on chromosomes 2, 5, and 6 and 5S (green) rRNA genes on chromosome 2 (minor sites on chromosome 5 are indicated by arrows). Bar, 2.5 μm.

and never detected in all chromosome ends. Furthermore, additional centromeric signals were observed with the same intensity and frequency as the telomeric ones [Figure 2(4)]. When the BAC library was screened with the telomeric oligonucleotides and three of those clones were hybridized to *L. japonicus* chromosomes,

none showed hybridization to the telomeres. However, all showed labeling of centromeres, which was less intense for chromosome 2 than for the others [Figure 2(5)].

Sequencing of both ends of two of those BACs revealed the presence of a repeated DNA family, named *Ljcen1* (GenBank accession no. AF390569). A 440-bp fragment of *Ljcen1* showed the same centromeric distribution. Variation among the four sequenced segments was restricted to 1 or 2 bp out of ~600 bp. The repeats do not show significant homology to the telomeric DNA or to any sequence in the EMBL/GenBank database, but the telomeric oligonucleotide hybridized to large *Bam*HI fragments that also contained *Ljcen1*. Furthermore, one centromeric BAC labeled the telomeres of *P. vulgaris* (A. Pedrosa, unpublished data).

Mapping of *L. japonicus* chromosomes: Clones bearing unique sequences enabled the alignment of genetic markers onto chromosomes. Examples of hybridization of unique sequences to each chromosome are shown in Figures 2(6–10) and 3(11–15). The identification of the chromosome arm bearing the signals was based on the relative chromosome length, centromeric position, and heterochromatin pattern and confirmed by rehybridization with previously assigned clones. The 45S rRNA probe was particularly useful for identification of chromosomes [Figure 2(10)]. Chromosome 2 bears the major, active rDNA site. Chromosome 6 possesses the second largest site and can be easily distinguished from chromosome 5, which bears a minor site, difficult to detect and not reported before (Ito *et al.* 2000). Although neither 45S nor 5S rRNA gene clusters could be mapped genetically, they could be assigned to linkage groups after both maps were correlated.

Positioning of the clones along chromosomes was based on means of several measurements (Table 1). All clones were assigned to a unique chromosome position, except for clone *Nlp2*, which, in a subset of cells, was detected on chromosome 5 instead of 3, suggesting the presence of a translocation in some individuals. With the exception of chromosome 5, a minimum of two mapped markers per chromosome were assigned to confirm the orientation of genetic *vs.* chromosome map.

Marker alignment was particularly useful for chromosome 1, because segregation distortion provided two linkage groups instead of one (IA and IB) and additional unlinked markers. We mapped at least two markers per linkage group, plus two unlinked markers, and demonstrated that all are derived from chromosome 1, with unlinked markers residing in the region that connects linkage groups IA and IB [Figure 2(6–9)].

Physical distances: A summary with the karyotype data, the position of all mapped clones, and the correlation to the genetic map (Sandal *et al.* 2002) is given in Figure 4 and in Table 3. The relative distances between the clones in relation to the chromosome length could be compared with their distances on the genetic map. It is clear that these distances often did not correlate.

Chromosomes 1, 5, and 6 seemed to be well covered in the genetic map, with mean ratios of 0.77, 1.03, and 1.05 Mbp/cM, respectively. Chromosomes 2, 3, and 4, on the other hand, showed ratios of 1.42, 1.81, and 2.07 Mbp/cM, respectively, suggesting either suppression of recombination or lack of certain chromosome segments in the genetic map.

When subchromosomal regions were analyzed in more detail, major differences in recombination frequencies along chromosomes were detected (Table 3). In subtelomeric regions of chromosome 1, comprising around 23% of the chromosome length, the ratio between physical and genetic distances was 0.36 Mbp/cM in the short arm (region comprising from the top of the chromosome to the *s1-1* gene) and 0.31 Mbp/cM in the long arm (from *Lnp* to the bottom). Along the central part of chromosome 1, however, the ratio increases to 1.3 Mbp/cM. The major part of linkage group II comprises ~30% of the distal part of the long arm of chromosome 2 (from *sym13* to the bottom). In this region, the physical *vs.* genetic ratio is 0.52 Mbp/cM, in contrast to 6.64 Mbp/cM along the rest of the chromosome. In chromosome 3, a lower ratio was again observed for the terminal region of the long arm (0.83 Mbp/cM, from *sym8* to the bottom), in contrast to a ratio of 3.04 Mbp/cM for the remaining 75% of the chromosome. Chromosome 4 showed a particularly strong suppression of recombination on its middle part, comprising the centromeric region and an intercalary heterochromatic block. Altogether, except for the rRNA gene clusters on the short arm of chromosome 2, all linkage groups seem to cover the chromosome ends, and lower levels of recombination were observed mainly on proximal chromosome regions.

To check whether chromosome rearrangements between the two parental species were involved in segregation distortions, repression of recombination, and shortening of the genetic map length, the chromosome complement of *L. filicaulis* and of the F_1 hybrid that served as a source for the mapping population were also analyzed.

Chromosomal analysis in *L. filicaulis* and in the F_1 hybrid: After DAPI staining, the chromosome complement of *L. filicaulis* proved to be similar to the *L. japonicus* complement. However, the intercalary heterochromatic band on chromosome 4 was not observed and one of the two largest midsized chromosome pairs was acrocentric, as was confirmed by hybridization with a centromeric BAC. Using this clone, the same centromeric pattern was observed, only slightly more dispersed toward the arms. This correlates to the higher content of pericentromeric heterochromatin in *L. filicaulis*, as inferred from its C-banding-like pattern observed after FISH [Figure 3(16)].

Hybridization with the 45S and 5S rRNA probes re-

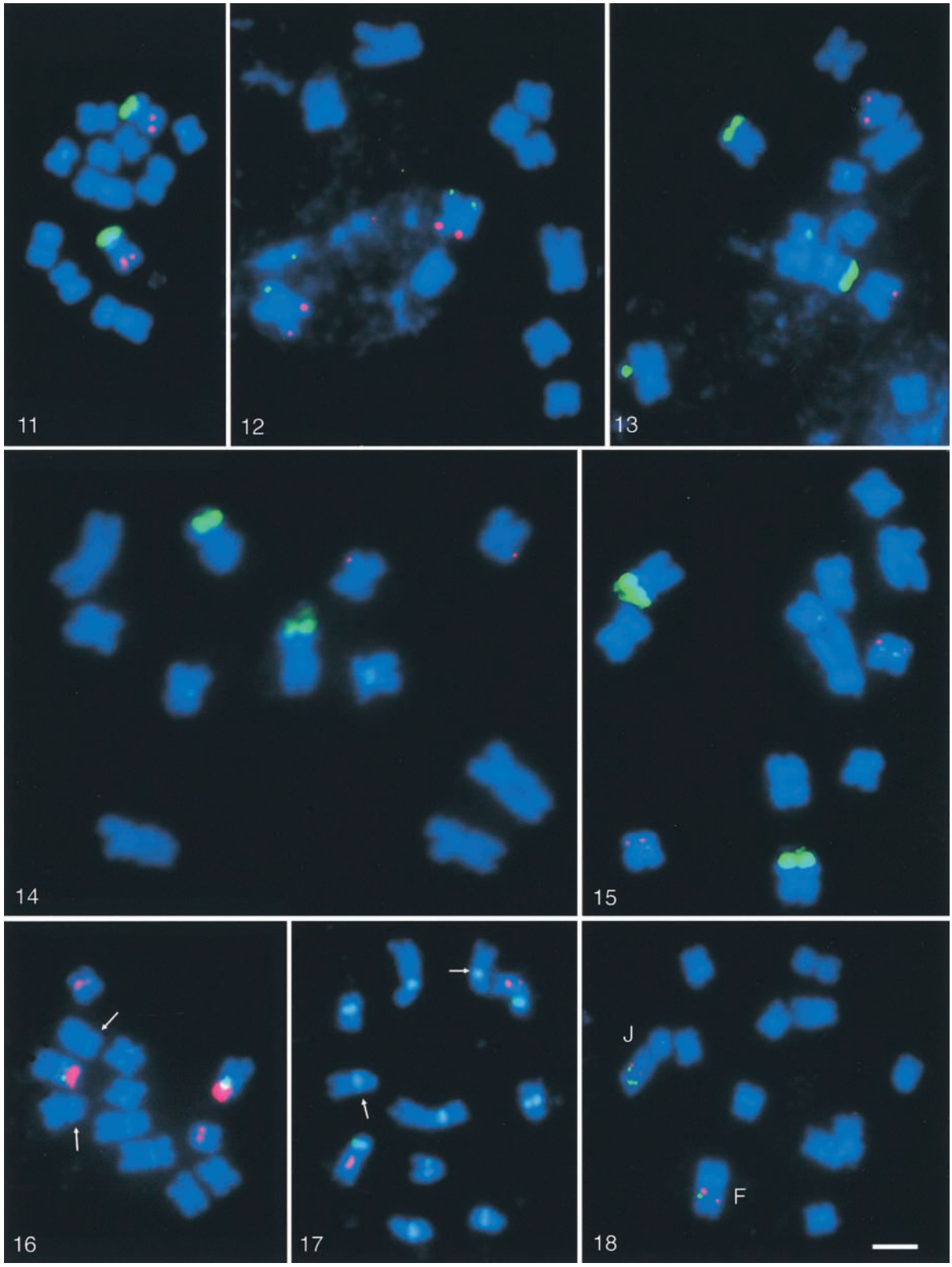
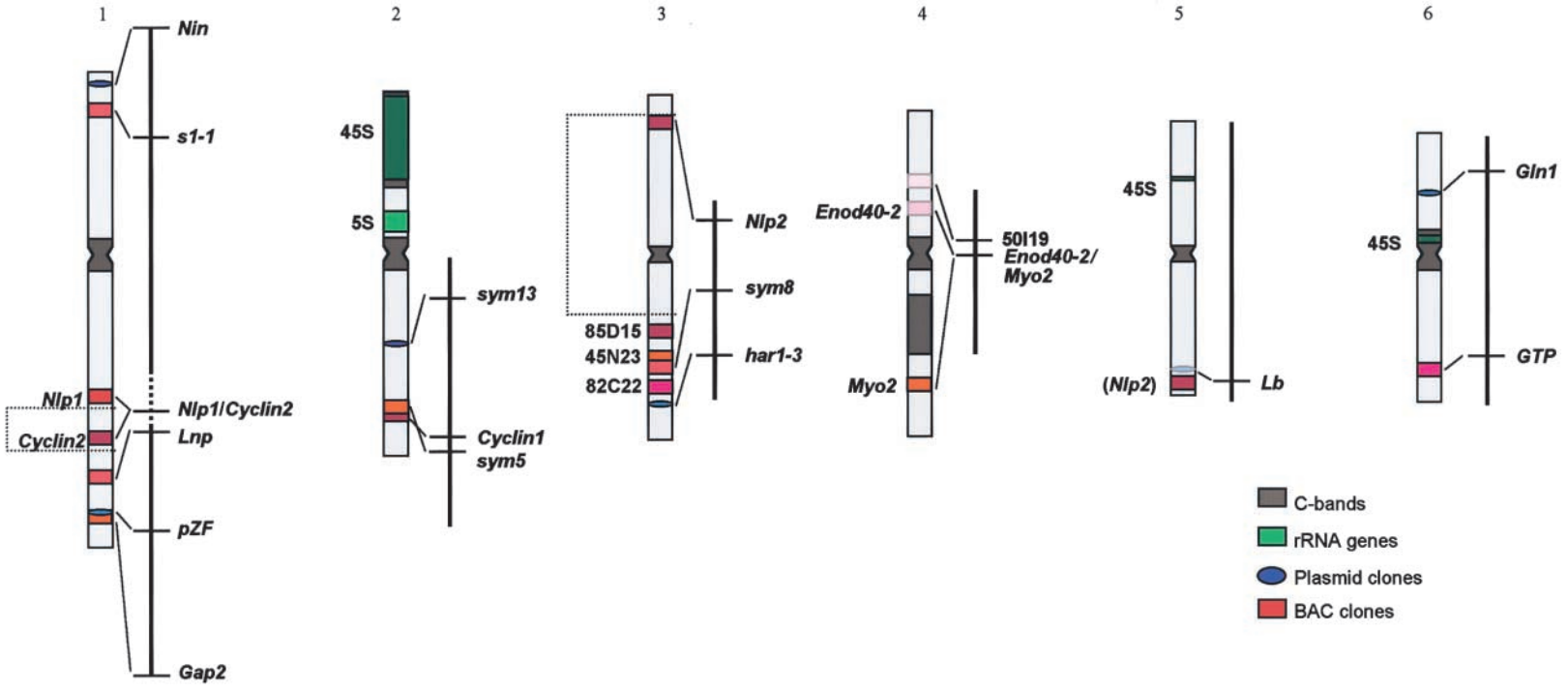


FIGURE 3.—*In situ* hybridization to the chromosome complement of *L. japonicus* Gifu (11–15), of *L. filicaulis* (17–18), and of the F_1 hybrid (19). (11) *sym13* (936-5.3, red) together with the major rDNA site (green) on chromosome 2. (12) *Nlp2* (BAC 92J16, red) and *har1-3* (pλ3.2, green) on chromosome 3 (one chromosome 2 is missing). (13) *Myo2* (BAC 60D1, red) on chromosome 4, while chromosomes 2 and 6 are identified by rDNA sites (green). (14) Subclone 71 from BAC 1M10 (*Lb*) together with clones Lb 112 and 123 (red) on chromosome 5 and rDNA (green) indicating chromosomes 2 and 6 (one chromosome 4 is missing). (15) *Gln1* (no. 431, red) together with the rDNA (green) on chromosome 6. (16) 45S rDNA (red) on chromosomes 2 and 6 and 5S (green) rRNA genes on chromosome 2 of *L. filicaulis*. Centromeres are easily distinguishable by the presence of brighter heterochromatin. Arrows indicate centromeres of acrocentric chromosome. (17) *Nlp2* (BAC 92J16, red) on acrocentric chromosome 3 and BAC 10F21 (green) at centromeres of all chromosomes. Arrows indicate centromeres of chromosome 2. (18) *Nlp1* (BAC 81J4, red) and *Cyc2* (BAC 53C15, green) on chromosome 1 of *L. japonicus* (J) and *L. filicaulis* (F). Bar, 2.5 μm.



Size in μm	2.67	2.04	1.93	1.82	1.54	1.51
Arm ratio	1.60	1.24	1.16	1.28	1.06	1.23
Size in Mbp	100	77	73	68	58	57
Size in cM	130	54	40	33	56	54
Mbp/cM ratio	0.77	1.42	1.81	2.07	1.03	1.05

FIGURE 4.—Idiogram of *L. japonicus* Gifu in comparison to its genetic linkage map established by SANDAL *et al.* (2002). Idiogram shows relative chromosome length, position of centromeres, approximate distribution of C-banding heterochromatin, and mapping of plasmid and BAC clones. Clones are either named directly (left) or indicated by the name of the corresponding gene, when genetically mapped (right). Clones in light shading were tentatively localized. Parentheses represent the alternative position of clone *Nlp2* in some cells (see RESULTS). Chromosome 6 was used for normalizing chromosome and linkage group lengths. Dotted line in the genetic map indicates a region of uniparental inheritance (see SANDAL *et al.* 2002). Segments bordered by dotted brackets in chromosomes 1 and 3 indicate putative regions inverted between *L. japonicus* and *L. filicaulis*.

TABLE 3

Comparison of genetic and physical locations of genes and markers

Gene/marker	Chromosome number	Total cM	Genetic cM[a]	Physical FL[b]	Genetic %[c]	Physical %[d]
Nin	1	130	0	3.1	0	2.4
s1-1			22	10.4	16.9	8.0
Nlp1			77	88.5	59.2	68.1
Cyc2			77	100.0	59.2	76.9
Lnp			81	110.5	62.3	85.0
pZF			101	120.3	77.7	92.5
GAP2			130	121.6	100.0	93.5
sym13	2	54	8	37.2	14.8	68.9
sym5			39	46.6	72.2	86.3
Cyc1			36	49.8	66.7	92.3
Nlp2	3	40	4	3.2	10.0	8.0
sym8			18	31.6	45.0	78.9
har1-3			31	35.8	77.5	89.6
50I19	4	33	10	7.0	30.3	21.3
Enod40-2			13	9.8	39.4	29.7
Myo2			13	27.7	39.4	83.8
Gln1	6	54	7	12.0	13.0	22.2
GTP			44	47.4	81.5	87.7

[a] Position of markers and genes in the genetic map was determined by Sandal *et al.* (2002).
[b] Fractional length (FL) = (size of the linkage group in centimorgans) × (physical position of clones in %) × 100 (Cheng *et al.* 2001b).
[c] Position of clones as a percentage of the total linkage group size.
[d] Positions are derived from Table 1 and indicated in relation to the telomere of the short arm.

vealed that *L. filicaulis* chromosome 2 was metacentric [Figure 3(16)]. On the other hand, two genes that hybridized to the long arm of *L. japonicus* metacentric chromosome 3 hybridized, in the same orientation, to the end of the long arm of *L. filicaulis* acrocentric chromosome 3 (data not shown). Furthermore, *Nlp2*, which in *L. japonicus* hybridized to the short arm of chromosome 3 [see Figure 3(12)], hybridized to the middle of the long arm of the same chromosome in *L. filicaulis* [Figure 3(17)]. In the F_1 hybrid cells, both locations of *Nlp2* were observed. This indicates that a large pericentric inversion involving ~50% of chromosome 3 distinguishes *L. filicaulis* from *L. japonicus*.

Although no morphological alteration was observed in chromosome 1, the high segregation distortion that led to the separation of linkage group I prompted us to analyze the localization of *Nlp1* and *Cyc2*, which were mapped between IA and IB, in the F_1 hybrid [Figure 3(18)]. Due to the higher amount of pericentromeric heterochromatin in *L. filicaulis*, both chromosomes 1 could be distinguished.

The distance between the two clones was highly significant in the *L. japonicus* chromosome, but not significant in *L. filicaulis* (Table 4). *Nlp1* seems to have maintained the same relative position in the two species, whereas *Cyc2* was significantly more distal on the *L. japonicus* than on the *L. filicaulis* chromosome. Since the difference in total length of chromosome 1 was not highly significant, we favor the explanation that a paracentric inversion, not an insertion/deletion, is responsible for this deviation. Moreover, preliminary meiotic analysis of the F_1 hybrid showed the occasional formation of an anaphase I bridge between two large chromosomes (data not shown), as is typical for paracentric inversions.

DISCUSSION

We have constructed a detailed chromosome map of *L. japonicus* Gifu, integrating the position of BAC and plasmid clones from 32 genomic regions. Nineteen of these clones were also mapped genetically. This makes the *L. japonicus* map reported here one of the most extensive correlations of genetic and chromosomal maps in plants, enabling the determination of physical and genetic distance ratios along the whole chromosome complement. Furthermore, comparison to the closely related species *L. filicaulis* revealed at least two inversions between the genomes, giving insights into the karyotype evolution of the group.

FISH to mitotic and pachytene chromosomes has been previously demonstrated to be very useful in plants for assigning linkage groups to chromosomes (Fuchs *et al.* 1998; Dong *et al.* 2000; Cheng *et al.* 2001a; Kulikova *et al.* 2001) and for establishing cytogenetic-based physical maps for single chromosomes and chromo-

TABLE 4

Comparison of the position of *Nlp1* (BAC 81J4) and *Cyc2* (BAC 53C15) on chromosome 1 of *L. japonicus* and *L. filicaulis* in the F_1 hybrid

	Position of clones on chromosome 1[a] (%)		Difference in position of clones (%)		
	Nlp1	*Cyc2*	*Nlp1 vs. Cyc2*	*Cyc2 L. japonicus vs. L. filicaulis*	Difference in length of chromosome 1 (%)
L. japonicus	36.53 ± 5.32[b]	24.42 ± 4.27	12.06 ± 2.43***	9.59 ± 4.64***	5.64 ± 6.88*
L. filicaulis	36.56 ± 2.84	34.01 ± 3.99	2.54 ± 5.15		

***Significant at $P < 0.001$; *significant at $P < 0.05$ only.
[a] Position is given as a percentage of chromosome length in relation to the closest telomere.
[b] Mean ± SD (n = 8–12).

some regions (Zwick *et al.* 1998; Zhong *et al.* 1999; Cheng *et al.* 2001b). Pachytene chromosomes have been used in many studies to obtain a higher mapping resolution, due to the more decondensed state when compared to mitotic metaphase chromosomes (Fransz *et al.* 1998). On the other hand, a mitotic idiogram, in contrast to a pachytene one, allows the estimation of the size of each chromosome in megabase pairs due to the uniform condensation of chromosomes at the mitotic metaphase stage (Lapitan *et al.* 1989) and is therefore useful for constructing first generation physical maps.

Telomeres, centromeres, and other repetitive sequences of *L. japonicus*: The weak hybridization signals obtained at terminal chromosomal locations suggest that telomeric tracts are relatively short in *L. japonicus*. However, hybridization of the telomeric repeat to its centromeres was observed, indicating some similarity between these two chromosome domains. Occurrence of telomere-like repeats in centromeric heterochromatin has been demonstrated previously for animals and plants (Southern 1970; Simoens *et al.* 1988; Meyne *et al.* 1990; Maluszynska and Heslop-Harrison 1991; Richards *et al.* 1991). Although the distribution of telomere-like motifs at the centromeric DNA in Lotus is still not clear, the isolation of *Ljcen1*, a centromeric repeat, will help to elucidate centromere structure in legumes.

From the dispersed hybridization patterns observed with some BACs, at least three types of repetitive sequences were evident: repeats clustered at centromeres, dispersed repeats enhanced at pericentromeric regions, and uniformly dispersed repeats. Similar distribution patterns were reported for repetitive sequences in other plant species. In Arabidopsis, for instance, transposon elements show mainly a centromeric or pericentromeric distribution (Arabidopsis Genome Initiative 2000).

Terminal regions of *L. japonicus* chromosomes were apparently enriched for single copy sequences, consistent with the findings that BAC clones with unique hybridization patterns mapped predominantly toward the chromosome ends (see also Zwick *et al.* 1997). Furthermore, when a pool of 500 cDNA clones was used as probe for FISH in *L. japonicus*, signals were predominantly located at terminal regions (Ito *et al.* 2000).

Correlation of chromosomal and genetic maps: Distorted correlation between chromosome and linkage group sizes was observed in *L. japonicus*. Furthermore, evaluation of the ratios between physical and genetic distances along chromosomes revealed remarkable differences and provided a clear indication for regions particularly suited for map-based cloning in this particular cross (see Sandal *et al.* 2002).

In general, higher recombination rates were observed toward the chromosome ends. This phenomenon had previously been demonstrated in wheat, barley, and tomato (Tanksley *et al.* 1992; Gill *et al.* 1996; Künzel *et al.* 2000). In Arabidopsis and rice, however, recombination hotspots seemed to be more randomly distributed and only centromeres are not subject to recombination (Schmidt *et al.* 1995; Harushima *et al.* 1998; Arabidopsis Genome Initiative 2000; Cheng *et al.* 2001b). A possible reason for this difference is the additional accumulation of repetitive sequences at proximal chromosome regions with the increase in genome size in most plant species. This is consistent with the idea of a typical plant chromosome, with genes predominantly clustered near chromosome ends (Schmidt and Heslop-Harrison 1998). Because *L. japonicus* has a small genome size, comparable to rice, it is possible that the higher recombination frequencies at distal chromosome regions observed in the species results mainly from the influence of chromosome rearrangements on recombination in the Lotus interspecific cross. More studies on legumes are necessary to clarify this issue.

Chromosome rearrangements and their influence on the *L. japonicus* genetic map: In chromosomes 1 and 3, the main distortions of the genetic map may be at least partially explained by the occurrence of a paracentric and a pericentric inversion, respectively. Comparison of genetic maps of related species such as tomato and potato, or among cereals, also suggested a large number of inversions in those genomes (Tanksley *et al.* 1992; Moore 1995). Furthermore, a translocation was recently demonstrated in *L. japonicus* when two ecotypes

were compared (Hayashi *et al.* 2001). Although clustering of markers was observed in chromosome 2, no morphological change was detected when *L. filicaulis* and *L. japonicus* chromosomes 2 were compared. It is likely that the lack of both 45S and 5S rRNA genes on the genetic map contributes to the short length of this linkage group (see Schweizer *et al.* 1987).

The paracentric inversion on chromosome 1 alone cannot explain the gap originally observed between linkage groups IA and IB. From the genetic data, it seems that strong selection for *L. filicaulis* alleles, or against *L. japonicus* alleles, is taking place in this region. It is likely, however, that the inversion extended the selection from one specific gene to a much larger area, influencing the segregation of markers on a chromosomal level.

The pericentric inversion on chromosome 3, on the other hand, resulted in the typical suppression of recombination in the inverted region and consequent reduction of the linkage group length. Indeed, a cluster of markers, including *Nlp2*, is observed in linkage group III, the total length of which is shorter than expected (Figure 4; Sandal *et al.* 2002). Such an inversion, which brought a subtelomeric, gene-rich region to the middle of the chromosome, significantly alters chromosome organization. More detailed comparison of single copy *vs.* repetitive sequences in *L. filicaulis* would offer the opportunity to investigate whether this presumably recent inversion triggered changes in repetitive or single copy sequences to reestablish maximal gene density on the chromosome end.

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