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First RFLP linkage map of red clover (*Trifolium pratense* L.) based on cDNA probes and its transferability to other red clover germplasm

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Abstract We constructed a genetic linkage map of red clover (*Trifolium pratense* L., $2n=2x=14$) using RFLP markers from cDNA probes of a backcrossed mapping population, and investigated the transferability of the markers to other red clover germplasm. The map contains 157 RFLP markers and one morphological marker on seven linkage groups. The total map distance was 535.7 cM and the average distance between two markers was 3.4 cM. All of the cDNA probes of the map were hybridized to the fragments of genomic DNA from 12 plants derived from three varieties, and 87% of the cDNA probes detected polymorphic bands that corresponded to those of mapping parents. This result indicated that RFLP markers on the present map were transferable to the genome analysis of other red clover germplasm. This is the first report to construct a linkage map of *Trifolium* species; it should provide fundamental and useful genetic information relevant to the breeding of red clover and genus *Trifolium*.

Introduction

Red clover (*Trifolium pratense* L.) is one of the major forage legumes in moist temperate regions around the world. Most of the varieties in use today are diploid ($2n=14$), cross-pollinated with a gametophytic self-incompatibility system (Taylor and Smith 1979). Due to its high seedling vigor, ease of establishment, rapid growth and soil enhancing characteristics, it was first domesti-

cated in the temperate regions of Europe, then distributed to the United States and other regions worldwide (Sjodin 1996). However, its usefulness as forage is limited by its lack of perenniality, which is a common deficiency in red clover varieties throughout the world. Achieving perenniality is thus currently the primary breeding objective, though efforts are also being made to improve other agronomic characteristics. However, because many of these characteristics, including perenniality, are thought to be controlled by quantitative traits loci (QTLs), it is difficult to improve them efficiently using only conventional breeding systems.

Molecular marker technologies have led to the rapid development of detailed genetic maps of many crops. These linkage maps are used to isolate genes and identify quantitative trait loci linked to traits of agricultural importance, which may then be used for marker-assisted selection (Staub et al. 1996). Linkage maps have been constructed for several legume species, including soybean (Keim et al. 1990; Yamanaka et al. 2001), cowpea (Ubi et al. 2000), chickpea (Winter et al. 1999), *Lotus japonicus* (Hayashi et al. 2001) and *Medicago truncatula* (Thoquet et al. 2002). However, the only linkage map constructed for a forage legume is that for diploid alfalfa (Brunner et al. 1993; Kiss et al. 1993; Kaló et al. 2000), and no linkage maps for *Trifolium* species have been reported.

Various types of DNA-based markers have been developed in the past several years, including restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs). PCR-based markers (i.e., RAPD, AFLPs and SSRs) are less costly and require less time and DNA, but few are capable of cross-species amplification of homologous sequences (Westman and Kresovich 1998). RFLP markers, especially those derived from complementary DNA (cDNA) probes, have the ability to detect homologous sequences in distantly related genomes, and have been used to construct consensus maps (sugar beet, Schumacher et al. 1997; Loblolly pine, Sewell et al. 1999; Fuel wood, Butcher et al. 2000). Gale and Devos (1998)

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and Jones et al. (2002) demonstrated that the genetic map structure and gene order were conserved among many *Poaceae* species using heterologous RFLPs. These facts implied that RFLP markers from cDNA probes might be the most stably expressed markers over a wide range of diverse genomes.

Several studies have reported that genomic heterozygosity among intra-populations was higher than those of inter-populations in outcrossing forage species, such as red clover (Milligan 1991; Kongkiatngam et al. 1995; Campos-de-Quiroz et al. 2001), white clover (Gustine et al. 2002), alfalfa (Yu et al. 1993) and perennial ryegrass (Sweeney et al. 1994). Because these forages have self-incompatibility systems and hence are cross-pollinated breeding systems, their varieties are a heterogeneous population of individual genotypes. Thus the breeding materials of forage species allow a wide range of genomic diversity during breeding procedures.

We have generally developed individual varieties by comparing 3,000 to 4,000 individuals and selecting several fundamental clones (Isobe et al. 2002). On the other hand, one linkage map was usually constructed by one mapping population which was derived from two parental clones. While thousands of individuals with high genomic variation were compared in breeding procedures, the one linkage map reflected genomic information from only four kinds of haplotype (if a mapping population was developed from a two-way pseudo-test cross). This suggested that the genomic information derived from one linkage map is a quite few, compared with numerous breeding materials. Therefore, we considered that the first linkage map should contain genetic information transferable to numerous red clover germplasm, like a consensus map. For this reason, it was important to choose a marker system with stable expression of polymorphisms in voluntary red clover individuals. The RFLP markers from cDNA probes were considered to offer the most stable expression in various germplasms; we decided to use this marker system to establish the first red clover linkage map.

To perform linkage mapping in outbreeding species, several research strategies have been proposed (Ritter et al. 1990; Grattapaglia and Sederoff 1994; Hemmat et al. 1994; Maliepaard et al. 1997). In these strategies, two-way pseudo-test crosses with heterozygous parents were used as the mapping populations. Because the genetic

segregations were the result of meiotic recombination from both parents, two parental linkage maps were developed from one mapping population; these were then integrated by the markers that were heterozygotic in both parents, known as “bi-markers” or “bridge markers”. The two-way pseudo-test cross can detect a maximum of four different alleles at one locus, while the use of first backcrossed progeny in outbreeding species detected only three. We considered that if the first backcrossed progeny was used as the mapping population, a much larger number of bi-markers could be obtained than by a two-way pseudo-test cross.

In this study, we constructed the first RFLP linkage map of red clover using cDNA probes with a backcrossed mapping population. We also discussed the transferability of the markers on the map to other red clover germplasm and application of the map to quantitative traits analysis and breeding programs.

Materials and methods

Plant materials

Two diploid red clover plants, ‘WF1680’ and ‘1588’, were crossed at the All-Russian Williams Fodder Crop Research Institute in 1997. ‘WF1680’, which was used as the paternal parent of the cross, was originated from a Russian variety cultivated in central Russia and was characterized by a very late flowering and white flowers (Table 1). ‘1588’, which was used as the maternal parent of the cross, originated from a wild accession collected in the Arhangelsk region (approximately N 65°, E 40°), and was characterized by early flowering and bright red flowers. From the F₁ progenies of the cross ‘1588’ × ‘WF1680’, a single F₁ plant (named ‘272’) with a phenotype similar to that of ‘1588’ was selected as the maternal parent of the mapping population. ‘WF1680’ was used as the paternal parent and backcrossed to ‘272’, and then 167 segregating progenies were used as the mapping population.

RFLP assays

Total DNA was isolated from young leaves using the CTAB extraction technique described by Doyle et al. (1990), with the exception that the extraction buffer contained 2% CTAB reagent with 100 mM of Tris-HCl pH 8.0, 20 mM of EDTA, 0.7 M of NaCl, 0.3 M of sorbitol, 0.5% polyvinylpyrrolidone, 2% polyethylene glycol and 0.3% 2-mercapethanol. Approximately 3 µg of total DNA were digested with the six restriction enzymes, that is, *Bam*HI, *Dra*I, *Eco*RI, *Hind*III, *Kpn*I and *Pst*I. The digested DNA fragments were separated on 0.6% agarose gels and blotted onto

Table 1 Phenotypes of parental plants evaluated in Russia in 1997

Phenotype	1588	272 ^{a)}	WF1680 ^{b)}
Plant type	Winter type ^{c)}	Winter type ^{c)}	Spring type ^{d)}
Flowering time	Very early	Early	Very late
Flower color	Bright pink	Bright pink	White
Plant height	Short	Short	Tall
Winter hardiness	Tolerant	Unknown	Medium
Size and shape of spots on leaflet	Small, round	Medium, triangular	Large, triangular

^{a)} ‘272’=‘1588’ × ‘WF1680’; maternal parent of mapping population

^{b)} Paternal parent of mapping population

^{c)} Spring regrowth is moderate and fall dormancy is low

^{d)} Spring regrowth is vigorous and fall dormancy is high

nylon membranes (Hybond-N⁺; Amersham Pharmacia Biotech, Buckinghamshire, UK).

Red clover cDNA fragments were used as probes for Southern hybridization. Total RNA was isolated from seedlings of red clover (cv 'Hokuseki') with TRIzol reagents (Life Technologies, Rockville, Md.). A cDNA library was constructed according to the manual from a cDNA library construction-kit (Stratagene, La Jolla, Calif.). A total of 2,213 cDNA clones of red clover were tested for their ability to hybridize clearly and the frequency of RFLP among eight randomly selected progenies of the mapping cross. Southern hybridization was performed with probes labeled by an ECL direct-labeling system (Amersham Pharmacia Biotech) and the hybridization signals were detected by chemiluminescence on X-ray film.

Linkage analysis

A combined genetic linkage map was established using JoinMap software (version 2.0; Stam and Van Ooijn 1995) with the segregation data from both parental genotypes. Segregation of the loci was tested by a chi-square goodness-of-fit test (the JMSLA module with 5% significance levels). Segregation data were analyzed using the cp population type. For grouping, a JMGRP module with a LOD threshold of 4.0 was used. Several markers that were not classifiable into any of the groups were excluded from the analysis in this step. Each recombinant value was calculated by JMREC with LOD and REC thresholds set at 0.01 and 0.49, respectively. The linkage groups were ordered by a JMMAP module with the following parameters: Kosambi's mapping function, LOD threshold 2.0, REC threshold 0.35, jump threshold 4.0, triplet threshold 7.0 and ripple value 3. In these steps, several markers that were not ordered in the groups were excluded from analysis.

Polymorphisms of the markers of other red clover plants

Three varieties bred in different countries were used for polymorphic analysis of the markers on the map: 'Hokuseki' (Japan), 'Altaswede' (Canada) and 'Runny-2' (Russia). Genomic DNA was extracted from four individuals of each of the three varieties (12 plants in total) and RFLP analysis was done using 90 cDNA probes of the map. The number and size of the polymorphic bands were investigated, and compared to those of the parents of the mapping population.

Results

Marker selection

Thirty six percent (808) of the tested 2,213 cDNA probes were found to have hybridized clearly, and 5.5% (121) of the cDNA probes detected polymorphic bands among eight mapping progenies; these 121 cDNA probes were used for RFLP analysis of all the mapping population. Sixty five-screened cDNA probes displayed multiple polymorphic bands and were separately scored. In cases where multiple polymorphic bands were mapped at the same position, the bands were taken for one marker. The size of probes ranged from approximately 800 bp to 3 kbp; the average size was 1.7 kbp.

Table 2 The number of cDNA probes which detected multiple polymorphic bands

Number of bands ^{a)}	Number of cDNA probes		
	Total	Within LG ^{b)}	Among LGs ^{c)}
Single bands	49	49	
Multiple bands			
2	22	17	5
3	14	4	10
4	4	0	4
6	1	0	1

^{a)} Number of polymorphic bands which were individually detected by one cDNA probe

^{b)} Number of cDNA probes which multiple polymorphic bands identified markers located on the same linkage group

Linkage map construction

Two hundred and forty nine genetic markers were grouped by JoinMap's JMGRP module with a LOD threshold of 4.0. Of these, 191 markers were distributed into seven linkage groups while 58 markers remained unlinked. The markers of each linkage group were ordered by a JMMAP module with a LOD threshold of 2.0 and a Rec threshold of 0.35. Thirty three of the 191 markers did not map at LOD>2.0 and were excluded from analysis. The 158 markers separated on seven linkage groups, and then the linkage groups were numbered in the order of their length (Fig. 1). Twenty one multiple polymorphic bands were mapped on overlapping positions and taken for ten markers. The 158 markers consisted of one morphological marker (white flower color; Flower-c) and 157 RFLP markers. The morphological marker was mapped at the genetic distance of 8.0 cM from either of the ends on LG1.

All 157 RFLP markers on the map were detected by 90 cDNA probes. Forty one of the 90 cDNA probes detected multiple polymorphic bands, while the others detected single polymorphic bands (Table 2). The number of multiple polymorphic bands detected by one cDNA probe ranged from two to six. The numbers of cDNA probes detecting 2, 3, 4 and 6 multiple polymorphic bands were 22, 14, 4 and 1, respectively. The cDNA probes detecting 4 or 6 multiple polymorphic bands were available for simultaneously mapping their markers across several linkage groups. On the other hand, most of the cDNA probes that detected only two multiple polymorphic bands identified markers located on the same linkage group.

The total map distance was 535.3 cM and each of the linkage groups covered between 63.7 cM (LG7) and 104.6 cM (LG1) (Table 3). The average distance between two markers was 3.4 cM with a maximum distance of 23.2 cM between marker C1483a and C27 on LG1. The correlation between the physical distance and the distance on the map was estimated as 874 kb/cM, taking the physical DNA-length of the haplo-

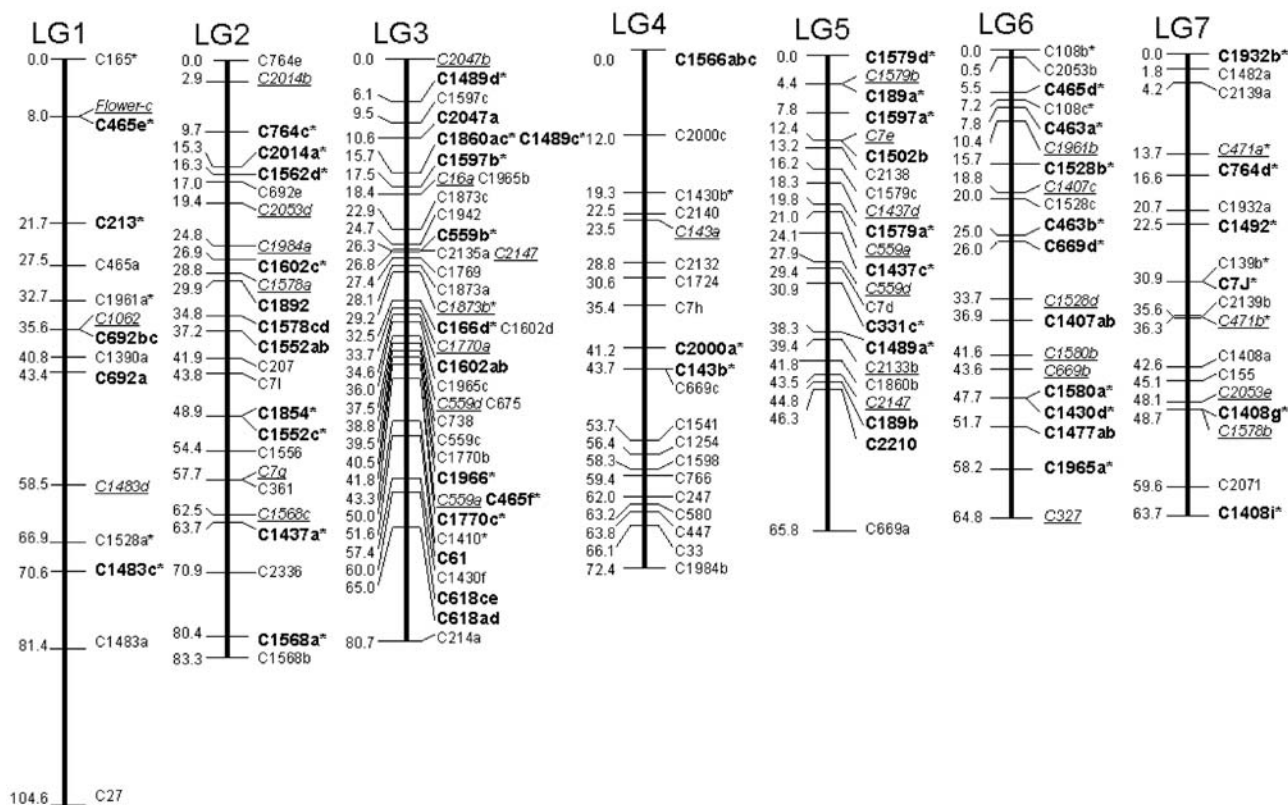


Fig. 1 The seven linkage groups of the red clover map constructed using 157 RFLP markers and one morphological marker (Flower-c = white flower color). The markers are indicated by three kinds of characters based on their segregation types: *large bold characters* indicate bi-parental markers (ab × ac, ao × ao and ab × ab), *small characters* indicate mono-donor parental markers (ab × aa), and

italicized characters indicate mono-recurrent parental markers (aa × ab). The markers derived from multiple polymorphic bands are indicated by xa, xb, etc. (e.g., C7a, C7b). If several multiple polymorphic bands were taken as one marker, they are indicated with *plural alphabets* (e.g., C1407ab). Distorted markers are indicated by *asterisks*

Table 3 Map distance and number of markers of seven red clover linkage groups

Linkage groups	Length (cM)	Mean distance (cM)	Number of markers					Total
			ab × aa ^{a)}	aa × ab	ab × ac	ao × ao	ab × ab	
LG1	104.6	7.0	7	3	1	4	0	15
LG2	83.3	3.3	8	6	3	8	0	25
LG3	80.7	2.1	16	8	3	10	1	38
LG4	72.4	3.6	16	1	1	2	0	20
LG5	65.8	3.0	5	7	1	9	0	22
LG6	64.8	3.2	4	6	2	8	0	20
LG7	63.7	3.5	8	4	0	6	0	18
Total	535.3	3.4	64	35	11	47	1	158
(%)			40.5	22.2	7.0	29.7	0.6	

^{a)} Segregation type of the markers. ab × aa and aa × ab were derived from donor and recurrent parents, respectively (mono-parental markers). ab × ac, ao × ao and ab × ab were derived from both parents (bi-parental markers)

type genome as 468 Mbp (Arumuganathan and Earle 1991).

Markers segregating from the donor (maternal) and recurrent (paternal) parents, or both, were spread across the seven linkage groups (Table 3). Forty percent (64) of the markers on the total map were donor parental markers and 22% (35) of them were recurrent markers. Thirty seven percent (59) of the markers on the total map were

bi-parental markers. The bi-parental markers were classified into three segregation types, ab × ac, ao × ao and ab × ab, for which there were 11, 47 and 1 markers, respectively.

Distorted segregation was observed for 37% of all the markers on the map (Table 4). The skewed markers were randomly distributed throughout the linkage groups (Fig. 1). The distortion ratios differed depend-

Table 4 The number of distorted segregation markers and distortion ratio

Segregation types	Number of markers		Distortion ratio (%)
	Normal ^{a)}	Distorted ^{a)}	
ab × aa	58	6	9.4
aa × ab	33	2	5.7
ab × ac	7	4	36.4
ao × ao	1	46	97.9
ab × ab	0	1	100.0
Total	99	59	37.3

^{a)} Significant at $P > 0.05$

ing on the types of marker segregation (Table 4). The distortion ratios of markers of segregation type ab × ac and ao × ao were 36% and 98%, respectively. The distortion ratios of the mono-parental markers were lower than those of the bi-parental markers; those of the donor and recurrent parental markers were 9.4% and 5.7%, respectively.

Polymorphisms of the markers of other red clover germplasm

RFLP analysis was performed on 12 plants from three varieties using 90 cDNA probes on the map (Table 5). All cDNA probes hybridized to fragments digested of genomic DNA from the 12 plants. Eighty five cDNA probes detected polymorphic bands among the 12 plants, while five cDNA probes did not. The average number of polymorphic bands detected by one cDNA probe was 3.3, with a range from 1 to 10. Seventy eight cDNA probes detected polymorphic bands of the same size at least of either mapping parents. On the other hand, seven cDNA probes detected polymorphic bands that strictly differed from the mapping parents. The number of cDNA probes that detected polymorphic bands within 'Hokuseki', 'Altaswede' and 'Runny-2' were 79, 83 and 83, respectively, while the number detecting polymorphic bands among all three varieties was 33.

Table 5 Number of cDNA probes detecting polymorphic bands among 12 plants from three red clover varieties.

Type	Monomorphic	Polymorphic		
		Same ^{a)}	Different ^{b)}	Total
Among 12 plants	5	78	7	85
Within varieties				
'Hokuseki'	11	66	13	79
'Altaswede'	7	72	11	83
'Runny-2'	7	75	8	83
Among varieties	57	15	18	33

^{a)} Number of cDNA probes those detected the same size of polymorphic bands of at least either mapping parent

^{b)} Number of cDNA probes those detected polymorphic bands were strictly different from both mapping parents

Discussion

Linkage map

We have constructed a genetic linkage map of red clover (*T.pratense*) containing 157 RFLP markers identified by cDNA probes and one morphological marker on seven linkage groups. Because the haplotype chromosome number of red clover is seven, these linkage groups were considered to correspond to each of the chromosomes of red clover. The total map distance was 535.3 cM, and the correlation between the physical and genetic distance was 874 kb/cM. These values were comparable to the total distance of 487.3–481.6 cM and the correlation between physical and genetic distance was 1,070–1,083 kb/cM in *Lotus japonicus* (Hayashi et al. 2001), the genome of which is approximately the same size as that of red clover.

One hundred and fifty eight markers were mapped on linkage groups, while 91 markers were unlinked. Because the map was constructed by combining two parental maps, the number of linked markers greatly depended on the existence of bi-parental markers. For example, when LG3 was re-ordered using the same module and without marker C1770c (ao × ao type, located on 43.3 cM in LG3), the length and marker number of LG3 was reduced to a size of 67.9 cM with 23 markers from a size of 80.7 cM with 38 markers. This fact suggested that the 91 un-linked markers might be mapped if additional bi-parental markers were screened.

Among the segregation type markers, ab × aa (mono and donor parental) markers were the most prevalent. The one haplotype genome of the donor parent (272) was originated from the wild accession collected in the Arhangelsk region (1588), which is located at a very high altitude (N 63°) in Russia. On the other hand, the other haplotype genome of the donor parent and both haplotype genomes of the recurrent parent (WF1680) were originated from the Russian cultivar. This might be the reason that there was a higher frequency of polymorphism between the haplotype genomes of the donor parent than between those of the recurrent parent.

The percentage of bi-parental markers described here (37%) was higher than the 22% observed in a pseudo-test cross of *Salix* (Tsarouhas et al. 2002) and lower than the

40% in a pseudo-test cross of *Eucalyptus globules* (Thamarus et al. 2002). The percentage of bi-markers in the trial using backcrossed mapping populations in this study was not higher than that of the pseudo-test cross mapping populations. One reason for this result might be the high polymorphism between the haplotype genomes of the donor parent.

Distorted segregation of markers has been reported in numerous species. Several reasons for marker distortion have been considered, i.e., statistical bias, genotyping and scoring errors (Plomin et al. 1995), in addition to biological mechanisms such as chromosome loss, viability, or lethal genes, genetic isolating mechanisms and genetic load (Bradshaw and Stettler 1994). In the present study, segregation distortion was randomly distributed among the linkage groups and the distortion ratios of markers differed depending on segregation type. Almost all $ao \times ao$ markers were skewed with heterozygote excess. Such a severe heterozygote excess of outcrossing species has been reported for diploid alfalfa in the F_2 populations from self-pollinated F_1 hybrids, and is considered to result from the reduced viability of the parental gametes or zygotes (Brummer et al. 1993; Kiss et al. 1993; Kaló et al. 2000). While it was difficult to decide the exact cause of marker distortion in the present study, several complex factors might have been involved. It would be indenting hypothetical reasons and strategies to prove them, and more detailed analysis is required to solve this problem.

Numerous plant species are considered to have genes that undergo several cycles of polyploidy formation and duplication (Stebbins 1966; Masterson 1994; Blanc et al. 2000). In the present study, 46% of the cDNA probes detected multiple polymorphic bands, and markers derived from these multiple polymorphic bands were located throughout all the linkage groups. Because the RFLP markers were detected by cDNA fragments, these multiple markers would seem to have been due to the duplication of genes. The finding that a high percentage of cDNA probes detected multiple polymorphic bands suggested that the duplicated genes had a greater number of polymorphisms than the non-duplicated genes.

Transferability of the markers to other red clover germplasm and application to breeding programs

Ninety four percent of cDNA probes on the present map detected polymorphic bands in all 12 plants and 87% of them detected polymorphic bands identical to those of the mapping parents. We therefore concluded that the RFLP markers on the present map are transferable to other red clover germplasm. The number of cDNA probes that detected polymorphic bands within each varieties, i.e., 'Hokuseki', 'Altaswede' and 'Runny-2', were almost same. This result suggested that the three varieties, although derived from different materials and regions, have roughly equal levels of genomic diversity.

The markers of the present map were distributed quite uniformly among the seven linkage groups, except for both ends of LG1, LG3, LG4 and LG5. Darvasi et al. (1993) reported that the power to detect QTLs does not significantly increase for marker densities greater than one every 10–20 cM. The maximum interval of the present map was 23.2 cM between C1483a and C27 on LG1, and no other regions existed with a distance of over 20 cM between two markers. These facts indicate that the present map should provide sufficient density for QTL analysis.

Among the agronomic characteristics of the mapping parents, flowering time and plant height were found to differ significantly, and these two characteristics have been considered to play a role in plant perenniality. These traits also segregated in the mapping population at field tests (data not shown); for example, the flowering time of the mapping population ranged from 24th June to the 15th August in Japan. The mapping population was considered suitable for QTL analysis of flowering time and plant height. However, there are many other important agronomic traits linked to plant perenniality in addition to flowering time and plant height. Additionally, the kind of genes linked to perenniality in the mapping population was fewer than that in the whole red clover germplasm. Therefore, in order to develop DNA markers from QTL analysis, it may be necessary to integrate the results of analyses from several different populations. The high transferability of the present map was sufficient to reconstruct linkage maps in other mapping populations and should play a large role in the development of DNA markers in the future.

The conventional breeding system namely means a series of work, i.e., comparing and screening functional genes by phenotypes. Because the markers on the present map were derived from cDNA clones, the polymorphism of the marker might reflect the diversity of functional genes, that is the most interesting parts for breeders. Chen et al. (2001) constructed a potato molecular-functional map by EST data and sequencing of RFLP markers, and analyzed the relationship between functional genes and QTLs. The present map could be used to analyze the functional genes by sequencing the cDNA probes. The sequencing of the cDNA probes is already underway in our laboratories. In this analysis, several cDNA clones showed high homology to genes with known functions (data not shown). If some QTLs were closely related to the markers on the map, we could analyze the relation of QTLs and the function of the markers, and develop them as DNA markers for marker-assisted selection.

In recent years, the genomes of two model legume plants, *Medicago truncatula* and *Lotus japonicus*, have been widely analyzed across the world. The sequence data of the present cDNA probes could be used for synteny analysis among these legumes. The results could then provide further information for the genome analysis of red clover and other *Trifolium* species, like the relationship between rice and Poaceae species.

The red clover linkage map constructed in the present study provided a fundamental basis for QTL analysis and was shown to have high transferability to red clover germplasm. This map should provide useful genetic information relevant to the breeding agronomic characters of red clover and the *Trifolium* species.

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