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Development of an RFLP map in diploid alfalfa

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Summary. We have developed a restriction fragment length polymorphism (RFLP) linkage map in diploid alfalfa (*Medicago sativa* L.) to be used as a tool in alfalfa improvement programs. An F_2 mapping population of 86 individuals was produced from a cross between a plant of the W2xiso population (*M. sativa* ssp. *sativa*) and a plant from USDA PI440501 (*M. sativa* ssp. *coerulea*). The current map contains 108 cDNA markers covering 467.5 centimorgans. The short length of the map is probably due to low recombination in this cross. Marker order may be maintained in other populations even though the distance between clones may change. About 50% of the mapped loci showed segregation distortion, mostly toward excess heterozygotes. This is circumstantial evidence supporting the maximum heterozygote theory which states that relative vigor is dependent on maximizing the number of loci with multiple alleles. The application of the map to tetraploid populations is discussed.

Key words: RFLP – Alfalfa – Genetic map – Segregation distortion – Plant breeding

Introduction

Despite the agricultural importance of alfalfa, no complete genetic map is available. Cultivated alfalfa is an autotetraploid (2n=4x=32), a feature which has undoubtedly slowed the development of a genetic map. However, tetraploid alfalfa, *M. sativa* ssp. *sativa* has a diploid form, ssp. *coerulea*. The closely related ssp. *falcata* also exists at both diploid and tetraploid levels (Quiros and Bauchan 1988). Additionally, a population, cultivated alfalfa at the diploid level (CADL), has been developed through the isolation of haploids (n=2x=16) from cultivated germplasm (Bingham and McCoy 1979). The availability of these alternative germplasm sources makes the construction of a molecular-marker-based map for alfalfa much easier than working with tetraploids.

The potential usefulness of a genetic map in alfalfa is far reaching. Some of the most intransigent problems may be amenable to solution using molecular linkage information. Complex traits, such as acid-soil tolerance and bloat resistance, may be more advantageously selected through indirect, map-based methods. Maximum heterozygosity, or the presence of multiple alleles at a locus, has been postulated to be important for traits such as yield and vigor (Dunbier and Bingham 1975). Map-based methods which assess the amount of allelic variation present throughout the genome may enable breeders to more effectively develop cultivars carrying multiple alleles at many loci. Lastly, long-term goals such as integrating genes from related, but incompatible, species of *Medicago* [such as weevil resistance (Sorenson et al. 1988)] could be facilitated by map-based cloning. We are developing an RFLP-based map in a diploid population of alfalfa. The probes can be easily shared among many labs.

Materials and methods

Methods for DNA extraction, gel electrophoresis and hybridization, and information about the probe library, are presented elsewhere (Brummer et al. 1991). Clones are from a cDNA library made from alfalfa seedlings and are identified by UGAc (*U*niversity of *G*eorgia *c*DNA) while the loci they identify are denoted as *Xugac* (*X* indicates a mapped locus). Previously,

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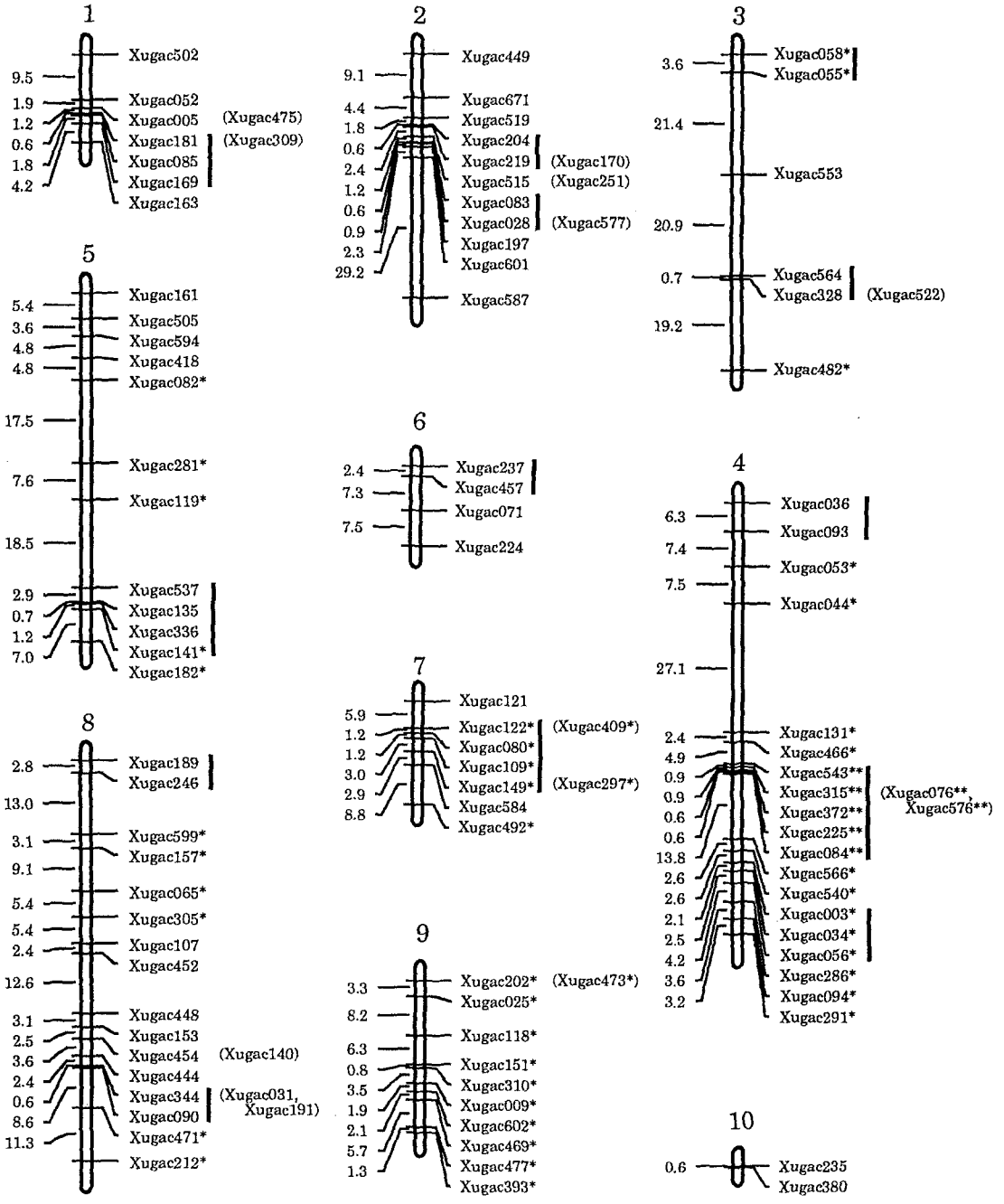

Fig. 1. RFLP map of diploid alfalfa. All markers are cDNA markers. Map distances in centimorgans (cM) are given to the left of each linkage group; marker names are given to the right. Markers *in parentheses* indicate loci which map to the same location as the marker to the left. *Dark bars* indicate markers whose order could not be confirmed with a LOD of 2.0. *Asterisks* indicate loci with segregation distortion ($P<0.05$); *two asterisks* indicate clones for which >90% of the F_2 progeny were heterozygous

these clones were denoted by "U" or "I" (Brummer et al. 1991). Two plants from the previous study with highly polymorphic RFLP patterns were chosen as parents for the mapping population. One parent (W2xiso #3) was derived from W2*xiso-1* germplasm (Bingham 1991); the other (440501 #2) was a plant from USDA PI 440501 (*M. sativa* ssp. *coerulea*). The parents were crossed to produce an F_1 hybrid which was selfed to produce an F_2 mapping population of 86 individuals. Clones were screened with chloroplast DNA and total DNA to eliminate any chloroplast or highly repetitive sequences. The remaining clones were hybridized to Southern blots containing DNA of the two parents and the F_1 hybrid digested with *Eco*RI, *Eco*RV, and *Hin*dIII. Loci which showed clear polymorphism between the parents and were heterozygous in the F_1 were scored in the F_2 population. The expected segregation of alleles at most loci was thus 1:2:1. Five loci (*Xugac056, 140, 189, 246,* and *584*) were also mapped which segregated in an expected 3:1 ratio. Only loci whose dominant alleles were in coupling were used in the analyses because those in repulsion provide little information in F_2 populations (Reiter et al. 1992).

Table 1. Segregation data for 51 loci which exhibited segregation distortion ($P<0.05$) from the expected ratio of 1:2:1

Locus	A[a]	H	B	χ^2
Xugac003	3	67	11	36.26
Xugac009	23	53	8	11.12
Xugac025	22	54	8	11.52
Xugac034	2	70	12	39.71
Xugac044	12	43	29	6.93
Xugac053	11	47	27	6.98
Xugac055	17	54	14	6.44
Xugac056[b]	–	74	10	7.68
Xugac058	14	56	16	7.95
Xugac065	7	47	26	11.48
Xugac076	2	77	6	56.39
Xugac080	10	58	17	12.46
Xugac082	11	52	22	7.09
Xugac084	1	78	6	62.10
Xugac094	6	60	18	18.86
Xugac109	11	57	16	11.31
Xugac118	24	53	6	14.18
Xugac119	14	55	16	7.45
Xugac122	11	59	16	12.49
Xugac131	4	68	13	32.51
Xugac141	9	48	24	8.33
Xugac149	12	55	18	8.20
Xugac151	22	45	9	7.03
Xugac157	3	60	22	22.91
Xugac182	13	54	16	7.75
Xugac202	20	58	5	18.54
Xugac212	4	55	24	18.42
Xugac225	1	78	6	59.89
Xugac281	15	53	14	7.05
Xugac286	4	63	14	27.47
Xugac291	9	53	18	10.48
Xugac297	12	54	18	7.71
Xugac305	9	50	25	9.14
Xugac310	23	53	7	12.54
Xugac315	1	74	6	56.04
Xugac372	0	75	6	59.67
Xugac393	27	47	6	13.48
Xugac409	11	58	16	11.89
Xugac466	4	69	11	35.88
Xugac469	22	52	7	12.09
Xugac471	6	53	26	14.60
Xugac473	20	57	6	16.30
Xugac477	26	53	6	14.60
Xugac482	16	60	5	21.77
Xugac492	11	59	15	13.19
Xugac540	3	68	9	40.10
Xugac543	2	77	4	60.83
Xugac566	3	69	11	37.99
Xugac576	2	78	6	57.35
Xugac599	4	57	22	19.39
Xugac602	22	52	6	13.60

[a] F_2 progeny genotype: A, homozygous for the W2xiso #3 parental allele; H, heterozygous; B, homozygous for the PI440501 #2 parental allele

[b] Expected segregation ratio 3:1

Linkage was determined using MAPMAKER (Lander et al. 1987) on an Apple Macintosh computer. Markers were initially assigned to groups using the 'group' command. Next, a 'first order' approximation was made of the likely order, and selected loci were mapped with the 'three-point' command. Other loci were added to this framework using the 'try' command. All linkage orders were verified using either 'three-point' or 'compare'. A minimum LOD of 3.0 and a maximum recombination frequency of 0.25 was used. Linkage of markers separated by more than 20 cM were confirmed using the 'linked' command. A dark line is placed to the right of markers which could not be ordered conclusively using a LOD of 2.0 (see Fig. 1). Map distances were determined using the Kosambi mapping function.

Results and discussion

The current map (Fig. 1) consists of 108 markers and covers 467.5 centimorgans (cM). The map has ten linkage groups, but alfalfa has a basic chromosome number of eight. With the addition of more markers, the number of linkage groups should converge on eight.

An interesting feature of the map is its short length. Most likely, recombination was repressed in this cross. Alfalfa chromosomes are small (Lesins and Gillies 1972) which may make the frequency of chiasmata low. Whether this is a common feature of alfalfa crosses is not currently known. The only major problem of decreased recombination is uncertainty of locus ordering. Because of the proximity of some of the markers, their relative positions may change in another population. Nevertheless, the approximate position on the chromosome should be unchanged so that the map should be useful despite this problem.

An alternative explanation for the short map distance is poor chromosomal affinities between the parents. One parent was derived from a diploid population developed from tetraploid *M. sativa* ssp. *sativa* and the other parent originated from ssp. *coerulea*. These two subspecies are only differentiated by ploidy and are otherwise identical (Quiros and Bauchan 1988). Because of their similarities, it seems unlikely that poor chromosomal pairing would occur.

We have also found that 48% (52 of 108) of the mapped loci showed segregation distortion calculated by chi-square analysis as a deviation from the expected progeny ratios of 1:2:1 or 3:1 ($P<0.05$, Table 1; asterisks in Fig. 1). The majority of the distorted loci have excess heterozygotes although some revealed excess numbers of progeny homozygous for one or the other parental allele. Loci that display segregation distortion tend to be grouped together on the chromosomes (Fig. 1). Ninety percent or more of the progeny were heterozygous at seven loci in group 4 (two asterisks, Fig. 1). The surrounding markers also showed considerable skew away from both parental genotypes, particularly that of the W2xiso parent. Conversely, all markers on group 9 were distorted toward excess heterozygotes, and all had few progeny homozygous for PI440501 parental alleles.

The distorted linkage groups most likely represent real associations among the loci. A chromosomal seg-

Table 2. The number and percent of cDNA clones producing the given number of restriction fragments per plant (averaged over three enzymes) as determined after hybridization of the clones to DNA from the mapping population parents digested with *Eco*RI, *Hin*dIII, and *Eco*RV

No. of restriction fragments per plant	Number (percent) of total cDNA clones tested
1–2	129 (41%)
3–4	86 (27%)
5–8	74 (24%)
>8	24 (8%)
Total clones tested	313 (100%)

ment which shows distorted segregation will cause skewed ratios in neighboring segments as well. The large linkage groups (e.g., 4 and 9) may in fact be combinations of several true linkage blocks which map to different areas in the genome. This will become more evident when the map is applied to another population, but should not pose a significant problem because entire groups of clones will be moved.

The maintenance of heterozygosity provides circumstantial evidence for the validity of the maximum heterozygosis hypothesis [Demarly (cited in Busbice et al. 1972); Dunbier and Bingham 1975]. This theory holds that multiple alleles at a locus are a prerequisite for high yield, vigor, and other traits relating to persistence. We have seen examples of four alleles per locus among the parents, the maximum possible to observe in diploids. The correlation between survival and the proportion and location of maximally heterozygous RFLP loci is unknown. Three large clusters of loci with excess heterozygotes have been identified (groups 4, 7, and 9). These might be interesting to further evaluate for their effect on traits thought to be associated with maximum heterozygosity. We did not perform any selection on the F_2 mapping population other than that resulting from choosing plants which were able to produce enough tissue for DNA extraction. Natural selection against homozygotes may have occurred at any developmental stage from the zygote to the mature plant.

Based on the number of bands seen on autoradiograms, many of the cDNA clones we tested probably represent repeated sequences (Table 2). We assumed that three or four bands per plant seen in three different restriction digestions represented two (or more) loci. Although it is possible that some of these are actually not repeated sequences, the assumption should be correct in the majority of cases. Because alfalfa is extremely heterozygous, many different alleles exist at a single locus, and attempting to correlate alleles with loci in different populations will be problematic.

The functionality of the map at the tetraploid level is the major question confronting breeders as they attempt to use it in their programs. Studying segregation in tetraploid progeny is much more complex than in diploids. If a maximally heterozygous individual is selfed, the maximum number of possible genotypes at a single locus is three diploids and 19 in tetraploids without double reduction or 35 with double reduction. Additionally, distinguishing among genotypes which only differ in allele frequency is difficult. A possible method to estimate linkage in polyploids has been proposed by Wu et al. (1992) and may need to be adopted by alfalfa geneticists for mapping at the tetraploid level. Further research needs to be conducted to better understand the potential for mapping directly in the tetraploid populations.

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References

Bingham ET (1991) Registration of isogenic populations of diploid and tetraploid alfalfa, W2xiso-1 and W4xiso-1. Crop Sci 31:496

Bingham ET, McCoy TJ (1979) Cultivated alfalfa at the diploid level: origin, reproductive stability, and yield of seed and forage. Crop Sci 19:97–100

Brummer EC, Kochert G, Bouton JH (1991) RFLP variation in diploid and tetraploid alfalfa. Theor Appl Genet 83:89–96

Busbice TH, Hill RR Jr, Carnahan HL (1972) Genetics and breeding procedures. In: Hanson CH (ed) Alfalfa science and technology. ASA, Madison, Wisconsin, pp 263–318

Dunbier MW, Bingham ET (1975) Maximum heterozygosity in alfalfa: results using haploid-derived autotetraploids. Crop Sci 15:527–531

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Lesins K, Gillies CB (1972) Taxonomy and cytogenetics of *Medicago*. In: Hanson CH (ed) Alfalfa science and technology. ASA, Madison, Wisconsin, pp 53–86

Quiros CF, Bauchan GR (1988) The genus *Medicago* and the origin of the *Medicago sativa* complex. In: Hanson AA, Barnes DK, Hill RR (eds) Alfalfa and alfalfa improvement. ASA, Madison, Wisconsin, pp 93–124

Reiter RS, Williams JGK, Feldmann KA, Rafalski JA, Tingey SV, Scolnik PA (1992) Global and local genome mapping in *Arabidopsis thaliana* by using recombinant inbred lines and random amplified polymorphic DNAs. Proc Natl Acad Sci USA 89:1477–1481

Sorensen EL, Byers RA, Horber EK (1988) Breeding for insect resistance. In: Hanson AA, Barnes DK, Hill RR (eds) Alfalfa and alfalfa improvement. ASA, Madison, Wisconsin, pp 859–902

Wu KK, Burnquist W, Sorrells ME, Tew TL, Moore PH, Tanksley SD (1992) The detection and estimation of linkage in polyploids using single-dose restriction fragments. Theor Appl Genet 83:294–300