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Construction of an improved linkage map of diploid alfalfa (*Medicago sativa*)

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Abstract An improved genetic map of diploid (2n=2x=16) alfalfa has been developed by analyzing the inheritance of more than 800 genetic markers on the F_2 population of 137 plant individuals. The F_2 segregating population derived from a self-pollinated F_1 hybrid individual of the cross *Medicago sativa* ssp. *quasifalcata* × *Medicago sativa* ssp. *coerulea*. This mapping population was the same one which had been used for the construction of our previous alfalfa genetic map. The genetic analyses were performed by using maximum-likelihood equations and related computer programs. The improved genetic map of alfalfa in its present form contains 868 markers (four morphological, 12 isozyme, 26 seed protein, 216 RFLP, 608 RAPD and two specific PCR markers) in eight linkage groups. Of the markers 80 are known genes, including 2 previously cytologically localized genes, the rDNA and the β-tubulin loci. The genetic map covers 754 centimorgans (cM) with an average marker density of 0.8/cM. The correlation between the physical and genetic distances is about 1000–1300 kilobase pairs per centiMorgan. In this map, the linkage relationships of some markers on linkage groups 6, 7, and 8 are different from the previously published one. The cause of this discrepancy was that the genetic linkage of markers displaying distorted segregation (characterized by an overwhelming number of heterozygous individuals) had artificially linked genetic regions that turned out to be unlinked. To overcome the disadvantageous influence of the excess number of heterozygous genotypes on the recombination fractions, we used recently described maximum-likelihood formulas and colormapping, which allowed us to exclude the misleading linkages and to estimate the genetic distances more precisely.

Key words *Medicago sativa* · Linkage map · Distorted segregation

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Introduction

Alfalfa (*Medicago sativa* L.) is a very important forage crop species in many countries throughout the world. It is widely adaptable to diverse environmental conditions partly because of its deeply penetrable root system and its ability to fix atmospheric nitrogen in symbiosis with *Rhizobium meliloti*. These obvious soil-improving properties make it an important member of the crop rotation. The cultivated alfalfa is a tetraploid, cross-pollinated plant for which homozygous, inbred lines can not be established (Hanson et al. 1988). As a consequence, the understanding of its genetic system, which would be extremely beneficial for both basic and applied science, is in its infancy. Because of the complex segregation pattern of the tetraploid inheritance geneticists often study the diploid alfalfa species and subspecies belonging to the *Medicago sativa* complex (Quiros and Bauchan 1988; Echt et al. 1992; Kiss et al. 1993; Endre et al. 1996). As these diploid alfalfa plants are crossable with the cultivated, tetraploid counterpart, genetic systems developed for the diploids can be adaptable for the tetraploid ones as well.

Preliminary genetic mapping studies on diploid alfalfa species demonstrated that the genotypes of some markers in the F_2 segregating populations originating from self-pollinated F_1 hybrids had significant distorted segregation (Brummer et al. 1993; Kiss et al. 1993). In these mapping populations, the dominant (3:1) or the codominant (1:2:1) segregation ratios deviated significantly from the theoretical values in several regions in which heterozygous genotypes dominated. Heterozygote excesses have been observed for a few markers in the mapping populations of other plant species, such as lettuce (Landry et al. 1987;

Kesseli et al. 1994), tomato (Bernatzky and Tanksley 1986; Helentjaris et al. 1986); maize (Helentjaris et al. 1986), and pepper (Prince et al. 1993), but in none of these was the segregation distortion as severe as that seen in alfalfa. Deviations in the segregation ratio result from the reduced viability of the parental gametes (gametic selection) or the zygotes (zygotic selection). The type of the selection can be identified numerically from the segregation ratio by two succesive χ^2-tests (Pham et al. 1990; Lorieux et al. 1995). In extreme cases the homozygotes for one allele can be completely absent, therefore the presence of a lethal allele is suggested, such as that reported in sugar beet by Pillen et al. (1993). In many segregating populations, higher proportions of one or both homozygous allele configurations have also been found for some markers, e.g., tomato (Bernatzky and Tanksley 1986), lettuce (Landry et al. 1987), oilseed rape (Landry et al.1991), and pepper (Prince et al. 1993), which may be explained by the inbred nature of the parents.

Several investigations have been performed to reveal the effect of the distorted segregation ratio on the estimation of the recombination fractions (RFs). Bailey (1949) analyzed the linkage relationships between dominant markers in the case of zygotic selection, and Heun and Gregorius (1987) also investigated dominant markers under gametic and zygotic selections. Lorieux et al. (1995) developed "special" maximum-likelihood (ML) formulas to estimate the viability factors and recombination fractions for both codominant and dominant markers in the case of both gametic and zygotic selections. These investigations indicated that the distorted segregation ratio had a strong effect on the estimation of the recombination fractions, which linked marker(s) and, consequently, unlinked genetic regions together.

During our mapping work we have come to the conclusion that markers with extreme segregation distortion behave confusingly when linkage is calculated. The increased numbers of heterozygous genotypes influence the linkage values on such a way that genetically unlinked regions are artifically linked together. To overcome this obstacle, we calculated recombination fractions by the "special" maximum-likelihood formulas developed by Lorieux et al. (1995) by which false linkage values can be distinguished from genuine ones for those chromosomal regions where the number of individuals with a heterozygous allele configuration overwhelmingly exceed the homozygotes. The fidelity of this approach is sustained by an independent, non-mathematical method called colormapping, described by Kiss et al. (1998). By the combined use of these two methods an improved genetic map of alfalfa was constructed and is presented in this paper.

Materials and methods

Plant material

The 137 individual plants of the F_2 segregating population – used previously for genetic analysis of alfalfa by Kiss et al. (1993) – were the progeny of a self-mated F_1 hybrid plant deriving from a cross between a diploid (2n=2x=16), yellow-flowered *Medicago sativa* ssp. *quasifalcata* (Mqk93) as a female parent and a diploid (2n=2x=16), blue-flowered *Medicago sativa* ssp. *coerulea* (Mcw2) as a male parent. The conditions for growing the plants were the same as described by Kiss et al. (1993).

DNA isolation and hybridization

Total DNA was isolated from young leaves according to the slightly modified method described by Dellaporta et al. (1983). Aliquots of 10–15 µg of total DNA were digested with four different restriction enzymes (*Eco*RI, *Eco*RV, *Hin*dIII, and *Dra*I; Amersham and Promega) according to the suppliers' instructions. The DNA fragments were separated on 1.1% agarose gels and transferred by the capillarity method (Southern 1975) onto nylon membranes (Hybond-N, Amersham). The DNA fragments used as probes were isolated from agarose gels using the QIAEX Gel Extraction Kit (Qiagen GmbH, Germany) and were labelled with α-[^{32}P]dCTP by the random priming method (Feinberg and Vogelstein 1983). The hybridizations were performed at 55–60°C and the washings were carried out according to Kiss et al. (1993).

DNA amplification

The PCR amplifications with random amplified polymorphic DNA (RAPD) primers were based on the optimized method of Williams et al. (1990). The reaction mix consisted of 5 pmol 10-mer primer, 1 U *Taq* polymerase enzyme (Promega), 2.4 m*M* $MgCl_2$, 200 m*M* of each dNTP, and 25 ng total DNA of the individuals in 1× *Taq* polymerase buffer in a final volume of 25 µl. The reactions were overlayed with 20 µl mineral oil (Sigma). The polymerase chain reactions (PCRs) for RAPD markers were carried out in 40 cycles with the following steps: 5 s at 94°C; 1 min at 37°C, and 1 min at 72°C in a PREM III thermocycler (LEP Scientific) or in a Programmable Thermal Controller (MJ Research).

The specific PCR amplifications were carried out with 16- to 27-base-long oligonucleotides in the same mix as for RAPDs except that the $MgCl_2$ concentration was 1.5 m*M*. The reactions were carried out in 35 cycles of 30 s at 94°C; 1 min at 55°, 60° or 65°C depending on the primers, 1 or 2 min at 72°C, the reactions were terminated with a final extension at 72°C for 4 min.

Amplified products were separated on 2.0% agarose gel and photographed after staining with ethidium bromide.

Cloning and sequencing of PCR fragments

After separation, the PCR products were reisolated and purified by QIAEX Gel Extraction Kit (Qiagen GmbH, Germany). The PCR fragments were treated with the Klenow fragment of DNA polymerase I in order to create blunt ends. The fragments were ligated into *Hin*cII-digested M13mp19 phage vector, and the sequencing reactions were carried out by the chain termination method described originally by Sanger et al. (1977).

Other DNA manipulations (restriction digestion of DNA, ligation, transformation, isolation of plasmid DNA, etc.) were carried out according to Maniatis et al. (1982).

Isozyme analysis

The phosphoglucoseisomerase (PGI, E.C.5.3.1.9.) [=glucose-6-phosphate isomerase (GPI)] isoenzyme assay was performed on cellulose acetate plates following the method described by Hebert and Beaton (1989). Double-concentrated staining mixture – prepared according to the procedure described by Wendel and Weeden (1989) – was mixed with an equal volume of melted agar and poured over the plate to stain the gels for enzyme activity. Other isozyme analyses were performed with the method described by Kiss et al. (1993).

Source and nomenclature of the markers

Morphological markers

The sticky leaves (Stl, formerly marked as STLF), the dwarf phenotypes and the colors of the flowers [the presence of anthocyan (Ant) and xanthophyll (Xant) pigments] were the same as described before (Kiss et al. 1993).

Restriction fragment length polymorphism (RFLP) markers

To determine the genotype of RFLP markers, we hybridized cDNA and genomic probes to restriction enzyme-digested total DNA of the individuals of the segregation population. cDNA clones with prefixes U and L originated from libraries described by Brummer et al. (1991) and Györgyey et al. (1991), respectively. W prefixes indicate clones that originated from a genomic library described by Echt et al. (1994). Clones denoted by CG are genomic clones from a *PstI* library of *Medicago sativa* cv 'Nagyszénási' (Kiss et al. 1993). Several genes with known function were mapped by RFLP using specific amplified fragments as probes (Table 4).

The rDNA locus was mapped by dominant evaluation after *Hin*fI digestion of the amplified 858-bp length region containing the 5.8S sequence (Table 4) and with RFLP (also with dominant evaluation) using an isolated fragment containing the 17S sequence of the rDNA unit as a probe (G. Endre, PhD thesis, 1997).

Two previously mapped leghemoglobin loci, LbMs1 and LbMs3 (Kiss et al. 1993), were renamed LbMsI and LbMsII to be consistent with the nomenclature of leghemoglobin genes (Löbler and Hirsch 1992). Using a cDNA fragment as hybridization probe (Table 5), we identified a third leghemoglobin locus, LbMsIII.

Dominant RFLP markers inherited from either the maternal or paternal parent are labelled with 5 or 4, respectively, in superscript after the marker names in Fig. 3A–H.

Markers detected by PCR using specific primers

To identify specific DNA fragments 16- to 27-base oligonucleotide primers were synthesized (Biological Research Center, Szeged) for polymerase chain reactions. They are listed in Table 4.

RAPD markers

Random amplified polymorphic DNA (RAPD markers) fragments (Williams et al. 1990) were generated using 203 different 10-base-long oligonucleotide primers. The names of the markers which were detected by these primers (purchased from Operon Technologies, Alameda, Calif.) start with O. Other RAPD markers were produced with the AP8b (5'-TCGTCACT GA-3') and AP8e (5'-TGGTGACTGA-3') primers. Although in most cases the RAPD fragments were dominant, 17 (2.6% of the mapped RAPD markers) of them showed codominant segregation. These markers are indicated with asterisks in Fig. 3A–H. The AP8b12 RAPD fragment was sequenced and based on its sequence, 20-base-long primer-pairs were synthesized (Table 4) and used in specific PCR reactions to detect a sequence-characterized amplified (SCAR) marker (Paran and Michelmore 1993).

Seed protein markers

Seed protein markers (indicated by 2D prefixes) were produced by separating seed proteins by means of two-dimensional polyacrylamide gel electrophoresis (K. Felföldi manuscript in preparation).

Data analysis

The genotypes of the loci were scored as numerical characters as described by Kiss et al. (1993). The female and male homozygous genotypes, the heterozygous, and maternal and paternal dominant genotypes were marked with 1, 3, 2, 5 and 4, respectively. Missing data were designated with 0.

Deviation of the segregation ratio of the core markers from the expected 1:2:1 ratio were tested by χ^2 analysis (Table 1.). The type of selection acting at a given locus was determined by two successive χ^2-tests (Pham et al. 1990); where the first tested the 1:1 ratio of the allelic frequencies (gametic selection), and the second tested the random assortment of the alleles according to the p^2:2pq:q^2 distributions (zygotic selection).

The recombination fractions were calculated by two methods: in one case RFs were calculated by the so-called "classical" maximum-likelihood method using MAPMAKER/EXP 3.0 (Lander et al. 1987; Lincoln et al. 1992). In the second case RFs were determined by the recently described "special" formulas by Lorieux et al. (1995) in which segregation distortion is taken into consideration. The "special" maximum-likelihood formulas were developed only for those loci which were affected by the same type of selection or for markers with a normal segregation ratio (Lorieux et al. 1995). Therefore, the RFs were calculated by the "special" formulas only for the marker pairs under normal-normal, zygotic-normal, zygotic-zygotic, normal-gametic, and gametic-gametic selection. In those cases when 2 markers were affected by a different type of selection (zygotic-gametic) no RFs could be determined using the "special" maximum-likelihood method.

For the determination of the RFs by the "special" maximum-likelihood equations of Lorieux et al. (1995), first the viability values for each marker were determined by comparing the actual segregation ratio with the expected ones with no selection. These viabilities were then used as parameters in the search for the RFs which maximized the corresponding log-likelihood functions. First an initial estimate for RF was obtained by a grid search over possible RF values. Using this initial estimate we computed the true maximum iteratively by the Nelder-Mead (simplex) algorithm. The programs were written in Matlab (The MathWorks, Natick, Mass.). The RFs were converted to map distances according to the Haldane function.

Results and discussion

Selection of the core markers and preliminary grouping

A genetic map with eight linkage groups (LGs) had been constructed earlier for diploid alfalfa using 89 molecular, isozyme, and morphological markers (Kiss et al. 1993). More genetic markers have been mapped during the last 5 years, and as the number of the mapped markers increased some showed spurious and ambiguous linkages to the existing eight linkage groups (LGs) using MAPMAKER/EXP 3.0 (Lander et al. 1987; Lincoln et al. 1992) as a mapping program. Because of these discrepancies, we have restarted the construction of the genetic map of alfalfa from the beginning. As a first step, 121 so-called core markers were selected to identify the number of genetic linkage groups and to establish a frame map. The core markers represented all those codominant markers which showed recombination to each other, that is, only one of those codominant markers was included which showed 100% linkage. Codominant markers were selected as core markers, since they reveal more recombination events than dominant markers; consequently, they ensure more reliable linkage data. The resulting "frame map" was used afterwards to map more than 700 additional, mostly dominant, markers.

The genetic analysis of the 121 core markers (see Table 1) was started with the help of the MAPMAKER/EXP

Table 1 The observed segregation ratio of the 121 core markers. The markers are aligned according to their established order in the linkage groups (see Results and discussion). The LG 1, LG 2, LG 3, LG 4, LG 5, LG 6, LG 7, and LG 8 correspond to groups A, B, G, H, F, C, D and E in Fig. 1, respectively. The genotypes are as follows: a_1a_1, maternal homozygotes; a_1a_2, heterozygotes; a_2a_2, paternal homozygotes

LG 1 (group A)

Serial number	Name of the locus	Segregation ratio a_1a_1:a_1a_2:a_2a_2	χ^2_{zyg}[a]	χ^2_{gam}[b]	$\chi2_{segr}$[c]
1	AAT2	24:74:39	1.20	3.28	4.17
2	LbMsII	23:65:37	0.35	3.14	3.34
3	U237	22:61:37	0.44	2.09	2.40
4	U80	24:61:33	0.19	1.37	1.51
5	W151	25:66:30	1.04	0.41	1.41
6	U779	28:82:27	5.32	0.01	5.34
7	CG5	28:79:25	5.15	0.14	5.26
8	CG8	28:81:23	6.92	0.38	7.20
9	CG1	32:74:20	4.33	2.29	6.13
10	U502	35:71:20	2.58	3.57	5.60
11	Med1	30:80:21	6.76	1.24	7.66
12	Enod2	28:82:21	8.55	0.75	9.06
13	OQ5B	29:74:22	4.41	0.78	5.02
14	L234	29:82:19	9.41	1.54	10.43
15	NOD14	32:78:20	5.75	2.22	7.42
16	L391	32:86:19	9.76	2.47	11.41*
17	CAD5A	31:79:15	10.16	4.10	12.81*
18	L56	32:89:16	13.79*	3.74	16.01*
19	Chi	32:86:17	11.35*	3.33	13.47*

LG 2 (group B)

Serial number	Name of the locus	Segregation ratio a_1a_1:a_1a_2:a_2a_2	χ^2_{zyg}[a]	χ^2_{gam}[b]	χ^2_{segr}[c]
20	U212D	28:47:42	4.00	3.35	7.87
21	EST3	23:50:43	1.44	6.90	9.10
22	AP8B12	23:68:46	0.06	7.72	7.73
23	U73	23:68:45	0.10	7.12	7.12
24	U68A	20:66:40	0.70	6.35	6.63
25	PRX	20:77:40	3.02	5.84	7.95
26	L591	15:81:32	10.64	4.52	13.55*
27	W170	12:58:35	2.70	10.08*	11.23
28	U107	10:75:35	11.29	10.42*	17.92*
29	U212A	7:87:34	22.87*	11.39*	27.92*
30	U305	4:87:26	34.33*	8.27	36.04*
31	L408BD	3:109:24	56.07*	6.49	55.93*
32	NOD22A	11:96:19	35.39*	1.02	35.59*
33	PEPC	11:99:21	35.46*	1.53	35.79*
34	U599	12:81:21	21.08*	1.42	21.63*
35	CG6	16:95:18	28.89*	0.06	28.91*
36	U189A	17:84:19	19.24*	0.07	19.27*
37	CAD5B	17:87:21	19.35*	0.26	19.46*
38	L295	18:94:19	24.81*	0.02	24.82*

LG 3 (group G)

Serial number	Name of the locus	Segregation ratio a_1a_1:a_1a_2:a_2a_2	χ^2_{zyg}[a]	χ^2_{gam}[b]	χ^2_{segr}[c]
39	U515A	29:45:44	6.13	2.6	9.51
40	L313	29:47:45	5.47	3.4	9.26
41	U28	30:66:44	0.35	2.6	2.51
42	LAP2	27:69:41	0.04	2.6	2.87
43	CG11	26:71:40	0.31	2.6	3.04
44	OD3E	25:78:34	2.83	1.8	3.82
45	U587	24:63:35	0.21	1.8	2.11
46	GSb	25:60:35	0.01	1.7	1.67
47	OR5I	26:77:31	3.05	0.7	3.36
48	NMs25	23:80:27	6.99	0.5	7.17
49	LAP1	23:89:25	12.29*	0.6	12.33*

LG 4 (group H)

Serial number	Name of the locus	Segregation ratio a_1a_1:a_1a_2:a_2a_2	χ^2_{zyg}[a]	χ^2_{gam}[b]	χ^2_{segr}[c]
50	NOD22B	40:63:24	0.01	4.03	4.04
51	L408AC	45:68:24	0.04	6.44	6.45
52	β–tubulin	48:58:23	0.56	9.69*	11.00
53	NOD1G	46:61:22	0.05	8.93	9.31
54	U466	47:68:18	0.72	12.65*	12.71*
55	U131	48:63:19	0.05	12.94*	13.06*
56	W157A	49:58:19	0.07	14.29*	15.08*
57	L23	54:70:13	2.05	24.54*	24.61*
58	OA17A	55:67:11	2.29	29.11*	29.12*
59	SKDH1	47:78:12	6.52	17.88*	20.52*
60	U84	45:68:14	2.44	15.13*	15.77*
61	OQ3B	45:73:14	3.84	14.56*	16.05*
62	U40A	44:78:15	5.06	12.28*	14.91*
63	U68B	44:73:14	4.07	13.74*	15.46*

3.0 computer program which was used previously. To try to establish the number of linkage groups, we performed a "classical" "two-point" or pair-wise linkage analysis of the 121 core markers which generated 7260 RFs and LOD scores. These data were then used to identify groups of the markers by the "group" command of the program by altering systematically the LOD scores from 3 to 17 and the maximum distances between 10 and 40 cM. The number of groups generated by this analysis varied from 3 to 20 as shown in the matrix of Table 2A.

Since the haploid chromosome number of diploid alfalfa is eight, our attention focussed on those elements of the matrix which contained eight groups. Such elements appeared in two blocks: the first block (Block 8/I) was in the column of LOD score 13 between 20 and 30 cM (light-gray area of Table 2A); the second block (Block 8/II) was in the row of 15 cM from LOD score 3 to 10 (dark-gray area of Table 2A). Block 8/I and Block 8/II are formed by three ($8^{\alpha-\gamma}$) and eight (8^{a-h}) elements of the matrix, respectively. The marker compositions of the groups in each element of Block 8/I were identical, that is, element 8^{α} was equal to element 8^{β} and 8^{γ}. Similarly, the markers in the groups of any element of Block 8/II were identical with the markers of the corresponding groups in any other element of Block 8/II; that is, element 8^{a} was equal to element 8^{b}, 8^{c} 8^{d}, 8^{e}, 8^{f}, 8^{g}, and 8^{h}, respectively. Comparing the eight groups of the elements in Block 8/I and Block 8/II showed that the marker constitution of some groups in the elements of Block 8/I and Block 8/II were identical, but some were different (see

Table 1 (continued)

LG 5 (group F)

Serial number	Name of the locus	Segregation ratio a_1a_1:a_1a_2:a_2a_2	χ^2_{zyg}[a]	χ^2_{gam}[b]	χ^2_{segr}[c]
64	U89A	27:80:25	5.95	0.06	6.00
65	U584B	23:88:24	12.46*	0.01	12.47*
66	F3OH	19:79:22	12.10*	0.15	12.18*
67	CycIIIMs	18:90:20	21.16*	0.06	21.19*
68	U224	13:93:18	31.30*	0.40	31.40*
69	CG13	9:98:24	34.89*	3.44	35.69*
70	Enod40	11:101:22	35.91*	1.81	36.31*
71	U202	6:90:32	27.89*	10.56*	31.69*
72	ALD	6:87:33	25.22*	11.57*	29.86*
73	U70	7:95:27	33.44*	6.20	35.05*
74	GSc	8:93:27	30.22*	5.64	31.92*
75	LbMsI	7:97:30	32.40*	7.90	34.76*
76	U151	8:87:29	24.52*	7.11	27.27*
77	U26	4:94:32	34.68*	12.06*	37.94*
78	CG9	4:92:33	32.54*	13.04*	36.49*
79	Calmod.	3:96:33	37.61*	13.64*	40.91*

LG 6 (group C)

Serial number	Name of the locus	Segregation ratio a_1a_1:a_1a_2:a_2a_2	χ^2_{zyg}[a]	χ^2_{gam}[b]	χ^2_{segr}[c]
80	W284	21:86:17	18.72*	0.26	18.84*
81	OY16E	20:94:16	26.04*	0.25	26.12*
82	U58	6:109:12	65.91*	0.57	65.77*
83	GSa	6:121:10	80.79*	0.23	80.71*
84	OW13B	6:115:8	79.16*	0.06	79.14*
85	OAD16A	3:124:4	104.52*	0.02	104.51*
86	U553	4:107:5	82.82*	0.02	82.81*
87	OF20A	9:114:9	69.82*	0.00	69.82*
88	U328A	21:90:14	24.70*	0.78	24.98*
89	U1294	24:80:18	12.08*	0.59	12.43*
90	L27A	23:96:18	22.29*	0.36	22.45*
91	U1208	28:84:18	11.70*	1.54	12.65*
92	U63	23:99:15	27.77*	0.93	28.09*
93	U784A	21:89:15	22.82*	0.58	23.05*

LG 7 (group D)

Serial number	Name of the locus	Segregation ratio a_1a_1:a_1a_2:a_2a_2	χ^2_{zyg}[a]	χ^2_{gam}[b]	χ^2_{segr}[c]
94	U829	22:72:30	3.42	1.3	4.26
95	U235	19:75:30	5.96	1.5	7.40
96	L500	15:73:31	7.41	4.0	10.43
97	L59	14:86:31	14.74*	4.1	17.24*
98	U291	7:107:17	54.19*	1.3	54.11*
99	U62	1:112:6	93.35*	0.2	93.07*
100	U286	2:121:6	99.39*	0.5	99.23*
101	U745B	4:102:7	73.52*	0.6	73.44*
102	U56	5:106:7	74.98*	0.7	74.95*
103	OC13B	6:113:9	75.22*	0.4	75.17*
104	OQ3M	5:115:9	79.42 *	0.5	79.33*
105	L589	6:120:10	79.85 *	0.4	79.76*
106	U88	9:100:13	50.14 *	0.6	50.13*
107	L285	10:110:16	52.41*	0.3	52.41*
108	Med2	16:89:18	24.64*	0.7	24.66*
109	U69	18:93:18	25.19*	0.0	25.19*
110	L83	29:90:14	18.29*	3.8	19.99*

LG 8 (group E)

Serial number	Name of the locus	Segregation ratio a_1a_1:a_1a_2:a_2a_2	χ^2_{zyg}[a]	χ^2_{gam}[b]	χ^2_{segr}[c]
111	L590	26:83:22	9.43*	0.24	9.60
112	U161	27:80:21	8.18	0.56	8.56
113	CG19	29:86:11	19.51*	5.14	21.94*
114	U594	30:83:10	18.3*	6.50	21.54*
115	L482	21:103:2	57.06*	5.73	56.52*
116	U281	20:107:4	56.77*	3.91	56.50*
117	U119	22:108:4	55.15*	4.84	55.01*
118	OG8A	25:93:18	18.75*	0.72	19.10*
119	U336	26:81:20	9.85*	0.57	10.21
120	U515B	28:70:24	2.70	0.26	2.92
121	U86A	28:80:29	3.86	0.01	3.88

* Significant at the 1% level

[a] χ^2_{zyg}, Value of the χ^2-test for zygotic selection

[b] χ^2_{gam}, Value of the χ^2-test for gametic selection

[c] χ^2_{seg}, Value of the χ^2-test for distorted segregation

Fig. 1). Consequently, at this point it was impossible to resolve whether the groups in Block 8/I or the groups in Block 8/II, or neither, represented the genuine marker composition of the eight LGs of alfalfa.

To see the fate of the markers as they were assorted into groups by tightening the parameters of the "group" command of MAPMAKER/EXP 3.0, we followed the formation of new groups and their marker compositions. The result of this analysis is shown in Fig. 1 and in Table 2B. By increasing the LOD scores and decreasing the maximum genetic distance the markers were assorted into more and more groups, as was expected. The number of the groups in the elements remained the same or increased only by one from Block 3 to Block 7 as the conditions of the "group" command were tightened (see Table 2). From Block 3 to Block 7, the groups in the elements of each Block contained identical markers, and the new groups of the consecutive element were always formed from a group of the previous element (Fig. 1). However, elements in Block 8/I and 8/II containing eight groups could be deduced from elements with seven and six groups, respectively, as shown by arrows in Table 2, and in Fig. 1. In the case of set 8/I, group CD (in the set of seven groups) was split into two parts, C_1D, and C_2 (route I in Fig. 1). In the case of set 8/II, groups CDE and H (in the set of six groups) resulted in groups C_1DE, C_2 and H_1, H_2 (route II on Fig. 1). In both cases the other linkage groups remained unchanged (see groups A, B, F, G, H, and groups A, B, F, G). During the formation of eight groups, one common, similar event could be observed in route I and II.

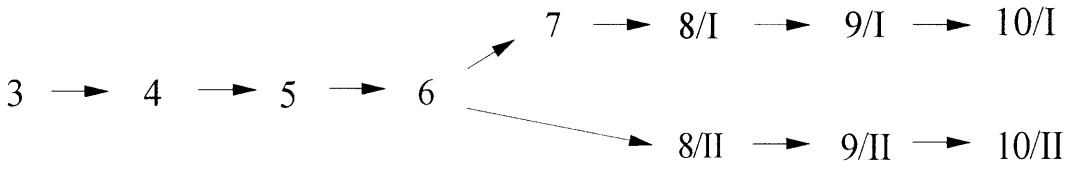
Table 2. A Formation of groups established by the "group" command of the MAPMAKER/EXP 3.0 program. To establish linkage groups of the markers we altered the LOD scores and maximum distances [in centiMorgans (cM) by Haldane function) step by step. The eight groups which formed at different parameters are shaded in light and dark gray (Block 8/I and Block 8/II). The small arrows indicate the development of the groups from the previous ones. **B** The simplified schema of the formation of the groups shown in part **A**

A.

LOD / cM	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
40	3	4	4	4	5	5	6	6	7	7	7	9	9	10	13
30	3	4	4	4	5	5	6	6	7	7	8^{α}	9	9	10	13
25	4	4	4	4	5	5	6	6	7	7	8^{β}	9	9	10	13
20	4	4	4	4	5	5	6	6	7	7	8^{γ}	9	9	10	13
15	8^{a}	8^{b}	8^{c}	8^{d}	8^{e}	8^{f}	8^{g}	8^{h}	9	9	9	10	10	10	14
10	19	19	19	19	19	19	19	19	19	19	19	19	19	19	20

Block 8/II Block 8/I

B.



In both cases group C_2 was separated from group CD or CDE, respectively, without the disjunction of group C_1D or C_1DE (the group C_1DE was split into group C_1D and group E at a LOD score of 11, 12, and 13 at 15 cM, but group C_1D remained in its entirety).

Subsequent genetic mapping revealed that unambiguous marker order could be established for groups A, B, E, F, G, and H, but under no circumstances could the marker order for groups C_1DE or C_1D be determined with MAPMAKER/EXP 3.0 or with any other mapping programs. This is surprising since markers in group C_1D behaved as they would have belonged to one group because they remained together even at a very high threshold using the "group" command (LOD 15, maximum distance 20 cM). The inconsistency between the failure to set the gene order and the apparent one-group attribute must reside in the nature of the markers in group C_1D.

The influence of segregation distortion on linkage

As the core markers were sorted into groups by the "group" command, we realized that most of the markers with severe distorted segregation ratios accumulated in groups C_1DE or C_1D (see black regions in Fig. 1). We therefore suspected that markers displaying extreme distorted segregation may be responsible for the failure of proper grouping and setting the gene order in groups C_1D or C_1DE.

As it can be seen in Fig. 1 and is documented by numbers in Table 1, many of the core markers showed significant deviations from the expected 1:2:1 segregation ratio. Most of these distortions were the consequences of zygotic selections (individuals with heterozygous configuration were dominating in the mapping population). Only some of the markers in group H were of gametic selection. The most extreme zygotic selection was detected for the markers in group CDE (Fig. 1), where nearly all of the markers displayed distorted segregation with an overwhelming number of heterozygous genotypes.

It was demonstrated earlier (Lorieux et al. 1995) that in the case of zygotic selection the calculation of RF values by the "classical" maximum likelihood (ML) estimation (Allard 1956) resulted in significantly lower RFs if the viability (number) of the individuals with heterozygous configuration exceeded by tenfold the viability of the individuals with homozygous configuration. It is demonstrated in Fig. 1 that such an extreme distortion occurred only in group CDE. The lower RF values calculated by the "classical" maximum likelihood estimation were supposed to

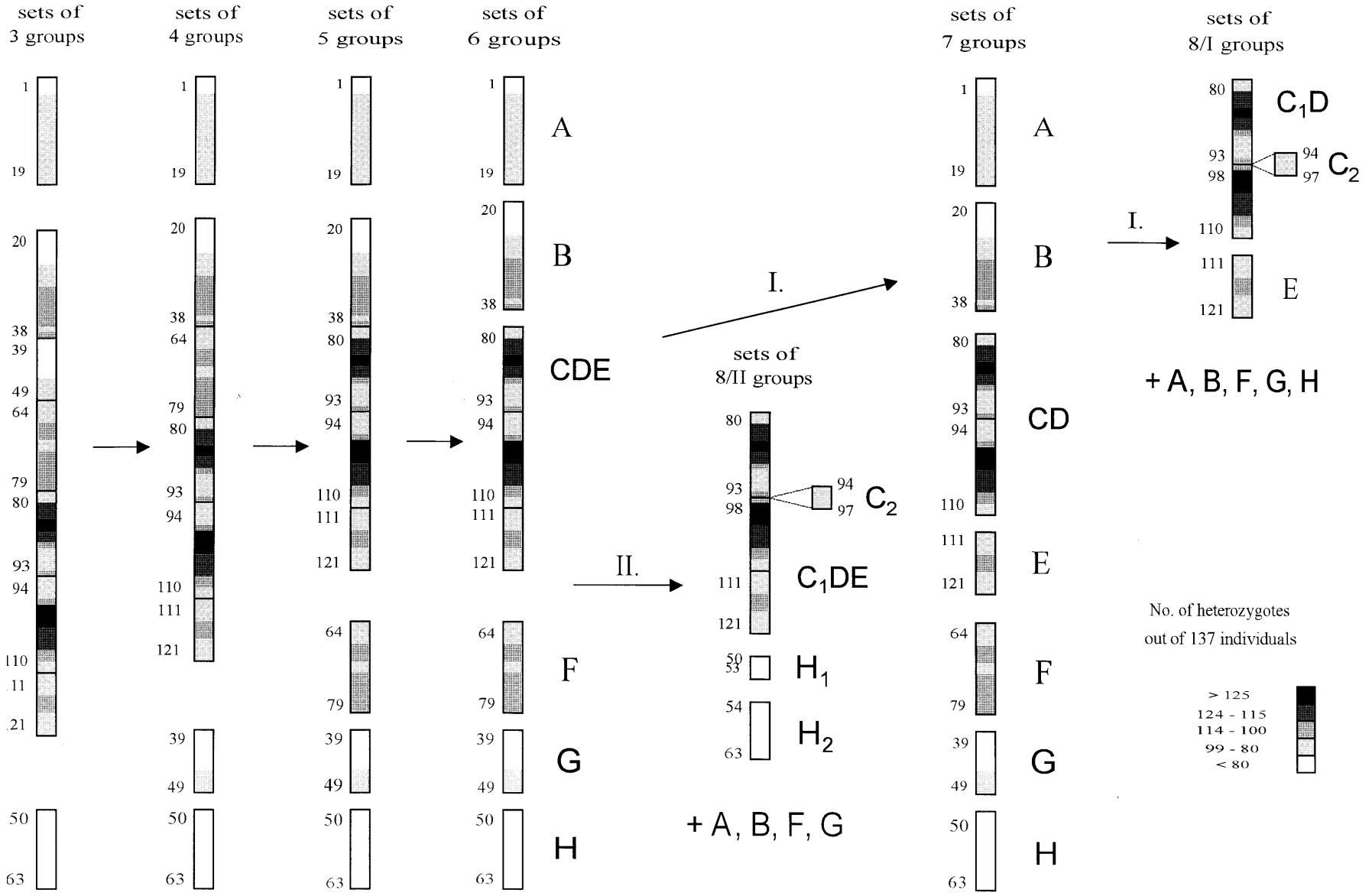


Fig. 1 The marker composition of the groups identified by the "group" command of MAPMAKER/EXP 3.0. Each *bar* represents a group of markers established at a certain LOD score and maximum distance. The parameters for creating the groups are shown in Table 2A. The designation of the groups (*A, B, C, D, E, F, G, H*) corresponds to the eight linkage groups determined by the "special" maximum-likelihood equations (see Results and discussion). The two ways that resulted in eight groups with altering of the parameters of the "group" command are designated with I and II above the *arrows*. The *intensity* of *shading* in the *bars* reflects the degree of distorted segregation (see also in Table 1). The *numbering* of the markers corresponds to the serial numbers in Table 1

Table 3 The calculated linkage values of the core markers in group CDE (see Fig. 1). The recombination fractions (RFs; upper figures) with the pertaining LOD scores (bottom figures) calculated by the MAPMAKER/EXP 3.0 program are presented in the upper triangle of the LOD tables. RFs more than 0.3 and LOD scores less than 3 are not shown (double dashes). RFs generated by the "special" maximum-likelihood formula developed by Lorieux et al. (1995) for zygotic selection are listed in the lower left triangle, RFs more than 0.3 are also not shown. The markers are listed according to the determined order of the markers (see Results and discussion)

LG6, 7 & 8	W284	OY16E	U69	GSa	OW13B	OAD16A	U553	OF20A	U328A	U1294	L27A	U1208	U63	U784A	U829	U236	L500	L59	U291	U62	U286	U745B	VR	OC13B	OQ3M	L589	U98	L295	Med2	U69	L83	L590	U161	CG19	U594	L482	U281	U119	OG8A	U336	U515B	U86A
W284		0.052 28.22	0.163 6.05	0.160 6.26	0.167 7.10	0.193 4.57	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.209 3.52	0.214 3.59	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OY16E	0.071		0.101 15.51	0.102 15.76	0.103 14.83	0.151 7.73	0.190 4.54	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.210 3.34	0.192 4.47	0.189 4.90	0.204 4.02	0.210 3.86	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U69	0.262	0.173		0.008 39.58	0.017 32.72	0.061 19.09	0.096 12.10	0.134 8.68	-	-	-	-	-	-	-	-	-	-	-	0.106 9.74	0.121 8.76	0.125 7.93	0.123 8.54	0.134 7.88	0.126 9.01	0.131 8.89	0.164 6.82	0.164 6.33	0.213 3.44	-	-	-	-	-	-	0.170 4.99	-	-	-	-	-	-
GSa	0.260	0.184	0.026		0.008 39.57	0.062 22.63	0.079 15.12	0.113 11.56	-	-	-	-	-	-	-	-	-	-	0.184 5.56	0.093 13.24	0.106 11.20	0.109 10.12	0.104 11.17	0.111 10.96	0.113 10.97	0.116 11.20	0.149 7.27	0.146 8.59	0.166 5.11	0.207 4.33	-	-	-	-	-	0.149 6.92	0.189 3.87	0.171 5.64	-	-	-	-
OW13B	0.274	0.187	0.067	0.030		0.046 22.70	0.074 15.04	0.110 11.34	0.207 3.56	-	-	-	0.210 3.44	0.216 3.12	-	-	-	-	0.159 5.56	0.078 13.24	0.098 12.83	0.107 9.40	0.100 10.68	0.116 9.64	0.116 9.86	0.120 9.89	0.154 6.24	0.163 6.21	0.211 3.30	-	-	-	-	-	0.162 5.29	0.192 3.45	0.172 5.19	-	-	-	-
OAD16A	-	-	0.231 -	0.227 -	0.214 -		0.019 27.46	0.066 20.80	0.150 7.79	0.209 3.84	0.174 6.30	0.211 3.93	0.157 7.56	0.164 6.45	-	-	-	-	0.134 7.64	0.066 15.58	0.060 16.58	0.079 13.00	0.087 12.43	0.097 11.66	0.089 13.24	0.089 14.17	0.123 8.96	0.122 10.01	0.174 5.03	0.184 4.71	-	-	-	-	-	0.134 8.00	0.140 7.42	0.141 7.76	-	-	-	-
U553	-	-	-	0.296 -	0.287 -	0.116 -		0.043 22.29	0.140 8.58	0.182 5.63	0.150 8.14	0.197 5.38	0.144 8.61	0.149 7.83	-	-	-	-	0.152 5.21	0.061 11.68	0.076 13.08	0.101 9.71	0.117 7.47	0.117 8.31	0.105 9.74	0.107 10.24	0.157 5.10	0.149 6.70	-	-	-	-	-	-	0.155 5.50	0.159 5.45	0.157 5.90	-	-	-	-
OF20A	-	-	-	-	-	0.211 -	0.148 -		0.108 14.46	0.174 7.80	0.157 9.51	0.190 6.84	0.141 10.92	0.147 9.75	-	-	-	-	0.171 4.72	0.104 10.45	0.108 10.45	0.125 7.34	0.137 6.70	0.154 5.89	0.145 6.71	0.141 7.76	0.189 3.79	0.187 4.64	-	-	-	-	-	-	0.164 6.04	0.173 5.60	0.164 6.51	-	-	-	-
U328A	-	-	-	-	-	0.299 -	0.261 -	0.181 -		0.046 29.72	0.054 28.80	0.066 25.81	0.067 25.23	0.084 20.45	-	-	-	-	-	-	0.209 3.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U1294	-	-	-	-	-	-	0.278 -	0.240 -	0.060 -		0.012 42.66	0.018 39.41	0.025 37.46	0.036 31.99	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L27A	-	-	-	-	-	-	0.266 -	0.245 -	0.075 -	0.016 -		0.019 42.27	0.026 40.07	0.037 33.79	-	-	-	-	-	0.199 3.45	0.201 3.81	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U1208	-	-	-	-	-	-	0.279 -	0.257 -	0.084 -	0.021 -	0.026 -		0.040 34.93	0.053 29.16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U63	-	-	-	-	-	-	0.273 -	0.235 -	0.096 -	0.033 -	0.037 -	0.061 -		0.008 44.01	-	-	-	-	-	0.186 4.14	0.182 4.86	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U784A	-	-	-	-	-	-	0.271 -	0.236 -	0.116 -	0.047 -	0.061 -	0.066 -	0.012 -		-	-	-	-	-	-	0.191 3.99	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U829	-	-	-	-	-	-	-	-	-	-	-	-	-	-		0.014 42.17	0.057 29.27	0.075 26.55	0.197 7.74	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U236	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.015 -		0.043 31.70	0.061 29.07	0.177 9.11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L500	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.062 -	0.048 -		0.022 39.34	0.148 11.24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L59	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.083 -	0.070 -	0.026 -		0.139 12.90	-	0.232 3.03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U291	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.231 -	0.219 -	0.190 -	0.194 -		0.080 15.78	0.080 14.66	0.152 6.24	0.144 7.12	0.152 6.92	0.146 7.82	0.148 7.73	0.184 4.63	0.208 3.57	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U62	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.235 -		0.013 29.81	0.060 15.20	0.067 16.45	0.067 16.04	0.062 17.15	0.063 17.75	0.098 11.37	0.109 11.13	0.145 7.00	0.159 6.37	0.190 4.27	-	-	-	-	0.137 6.70	0.130 7.92	0.138 7.15	-	-	-	-
U286	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.258 -	0.093 -		0.068 16.57	0.068 16.80	0.079 15.06	0.072 16.56	0.077 16.39	0.114 10.10	0.127 9.92	0.176 5.22	0.178 5.43	-	-	-	-	0.134 7.59	0.111 10.84	0.124 9.40	0.197 4.01	-	-	-
U745B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.277 -	0.296 -		0.006 32.17	0.015 26.71	0.019 27.81	0.023 28.13	0.061 19.49	0.066 20.40	0.108 11.43	0.130 9.94	0.157 7.86	-	-	-	-	0.125 7.93	0.136 7.24	0.161 5.28	-	-	-	-
VR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.263 -	0.298 -	0.021 -		0.014 30.17	0.018 29.25	0.026 29.53	0.054 20.12	0.068 19.29	0.125 10.59	0.136 9.93	0.147 9.17	-	-	-	-	0.154 5.79	0.152 6.18	0.165 5.25	-	-	-	-
OC13B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.279 -	-	0.066 -	0.064 -		0.004 37.83	0.012 35.70	0.041 24.58	0.060 24.66	0.106 14.08	0.113 13.88	0.133 11.63	-	-	-	-	0.168 6.26	0.158 6.19	0.166 5.53	-	-	-	-
OQ3M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.274 -	-	0.076 -	0.072 -	0.016 -		0.008 39.27	0.036 26.48	0.045 26.44	0.098 15.15	0.110 14.60	0.131 12.01	-	-	-	-	0.147 7.22	0.159 6.25	0.179 4.80	-	-	-	-
L589	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.268 -	-	0.086 -	0.096 -	0.044 -	0.030 -		0.025 31.15	0.038 30.23	0.089 17.54	0.100 16.79	0.131 12.75	-	-	-	-	0.160 7.32	0.160 6.53	0.173 5.48	-	-	-	-
U98	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.297 -	-	0.139 -	0.145 -	0.109 -	0.097 -	0.069 -		0.025 32.66	0.086 18.14	0.096 17.58	0.132 12.57	-	-	-	-	0.184 4.28	0.199 3.38	-	-	-	-	-
L295	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.144 -	0.171 -	0.124 -	0.125 -	0.097 -	0.066 -		0.060 24.73	0.082 21.67	0.121 15.60	-	-	-	-	0.179 5.50	-	-	-	-	-	-
Med2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.299 -	-	0.199 -	0.228 -	0.186 -	0.188 -	0.162 -	0.140 -	0.100 -		0.025 35.98	0.088 21.18	-	-	-	-	0.203 3.80	-	-	-	-	-	-
U69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.216 -	0.230 -	0.193 -	0.193 -	0.168 -	0.142 -	0.121 -	0.025 -		0.074 26.20	-	-	-	-	-	-	-	-	-	-	-
L83	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.246 -	0.233 -	0.204 -	0.211 -	0.204 -	0.188 -	0.171 -	0.115 -	0.086 -		-	-	-	-	-	-	-	-	-	-	-
L590	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		0.017 42.92	0.096 21.49	0.110 18.85	0.167 10.61	-	-	-	-	-	-	-
U161	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.019 -		0.080 24.06	0.090 22.29	0.154 11.01	-	-	-	-	-	-
CG19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.114 -	0.095 -		0.009 42.27	0.076 21.48	0.211 5.45	0.231 4.42	-	-	-	-
U594	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.130 -	0.106 -	0.012 -		0.079 20.55	0.217 5.09	0.240 3.99	-	-	-	-
L482	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.206 -	0.200 -	0.119 -	0.121 -		0.114 13.09	0.134 10.76	0.224 4.27	-	-	-	-
U281	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.240 -		0.032 32.12	0.129 13.88	0.195 7.01	0.259 3.51	0.257 3.75	-
U119	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.274 -	0.070 -		0.093 20.26	0.156 10.79	0.214 6.39	0.221 6.36	-
OG8A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.191 -	0.139 -		0.060 31.81	0.091 23.07	0.103 22.75	-
U336	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.261 -	0.211 -	0.065 -		0.031 36.06	0.067 29.35	-
U515B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.298 -	0.246 -	0.100 -	0.038 -		0.038 37.79	-
U86A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.297 -	0.265 -	0.114 -	0.076 -	0.039 -		-

LG *C*

LG *D*

LG *E*

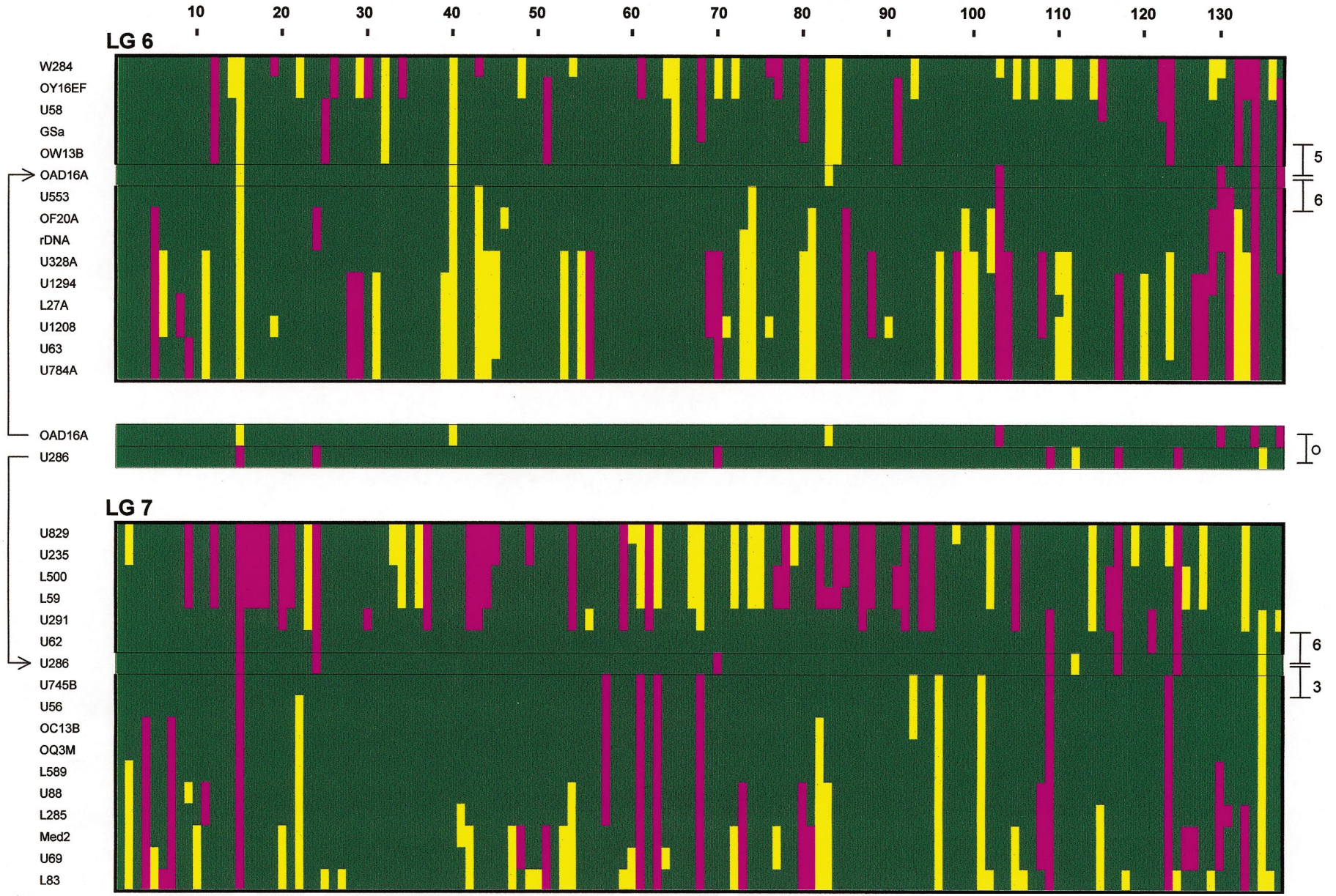


Fig. 2. Colormapping of markers (Kiss et al. 1998) showing the most extreme distorted segregation ratio. The colors in the matrix displays the genotypes of the core markers in LG 6 and LG 7 of the mapping population consisting of 137 individuals. The *yellow*, *green*, and *purple* colors represent the maternal homozygous, heterozygous, and paternal homozygous genotypes, respectively. The numbers at the *top* of the matrix are the serial numbers of the individuals in the population. The values to the *right* of the colormap represent the number of the individuals with the same homozygous genotypes between the markers ("homozygous links"). The interpretation of this figure is described in the Results and discussion section

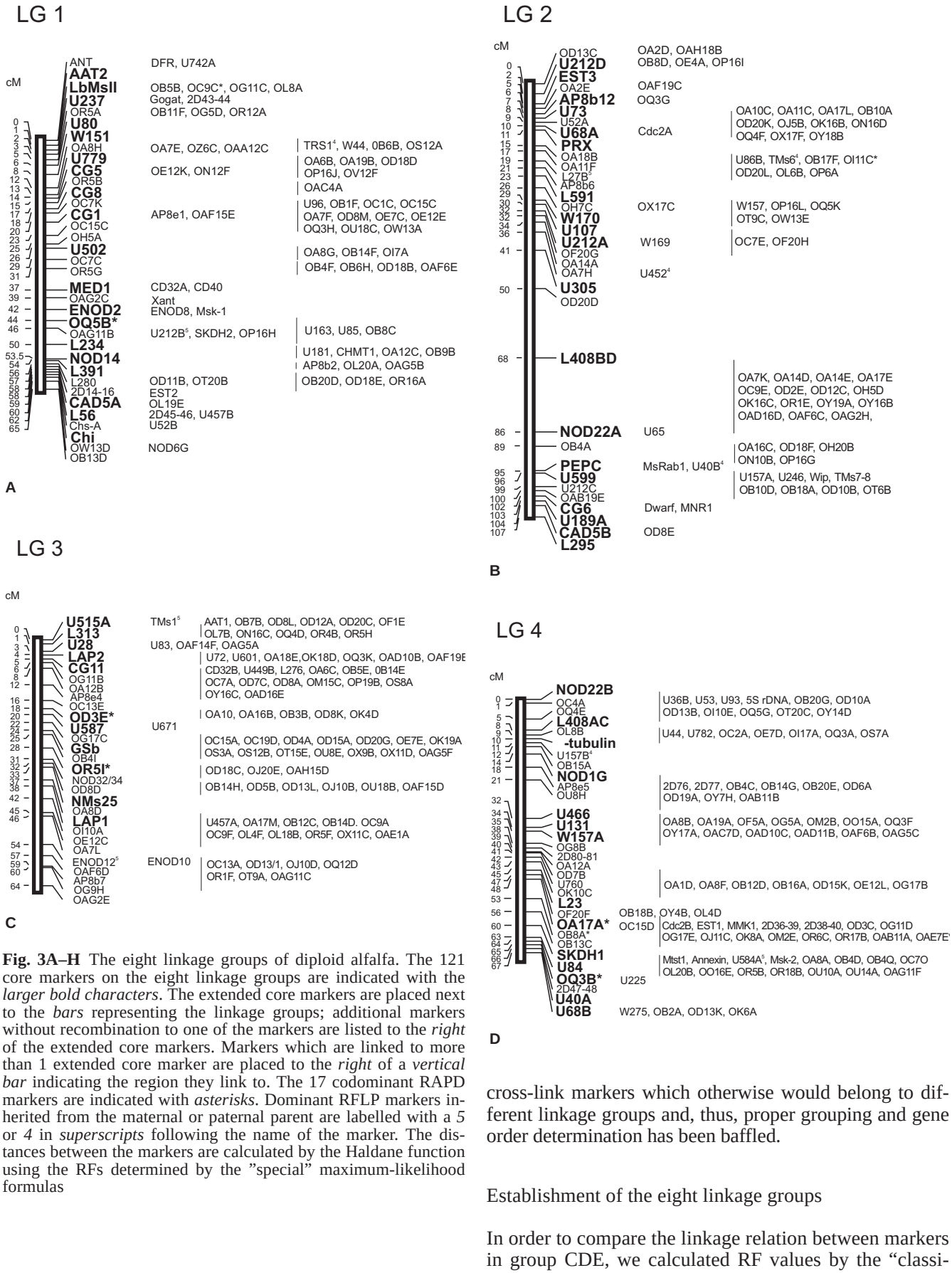


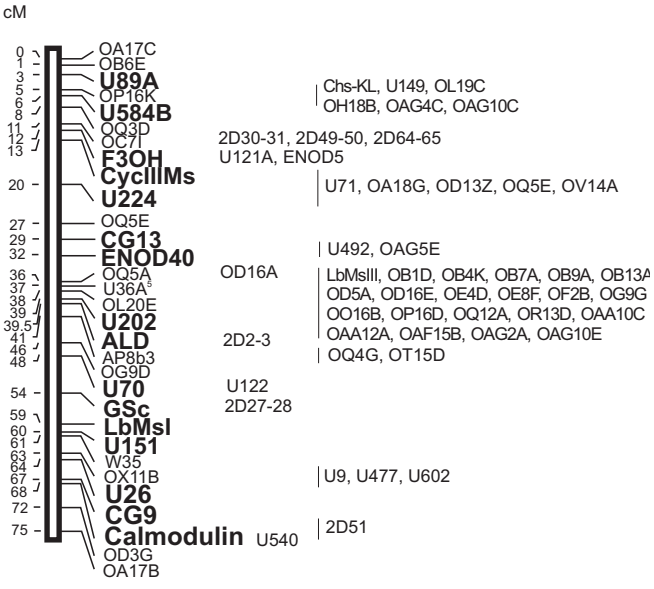
Fig. 3A–H The eight linkage groups of diploid alfalfa. The 121 core markers on the eight linkage groups are indicated with the *larger bold characters*. The extended core markers are placed next to the *bars* representing the linkage groups; additional markers without recombination to one of the markers are listed to the *right* of the extended core markers. Markers which are linked to more than 1 extended core marker are placed to the *right* of a *vertical bar* indicating the region they link to. The 17 codominant RAPD markers are indicated with *asterisks*. Dominant RFLP markers inherited from the maternal or paternal parent are labelled with a *5* or *4* in *superscripts* following the name of the marker. The distances between the markers are calculated by the Haldane function using the RFs determined by the ”special” maximum-likelihood formulas

cross-link markers which otherwise would belong to different linkage groups and, thus, proper grouping and gene order determination has been baffled.

Establishment of the eight linkage groups

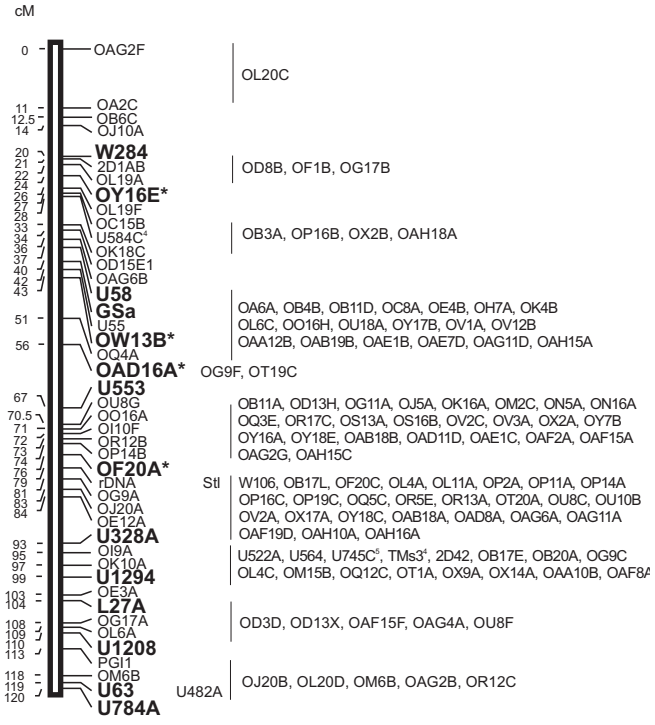
In order to compare the linkage relation between markers in group CDE, we calculated RF values by the “classi-

LG 5



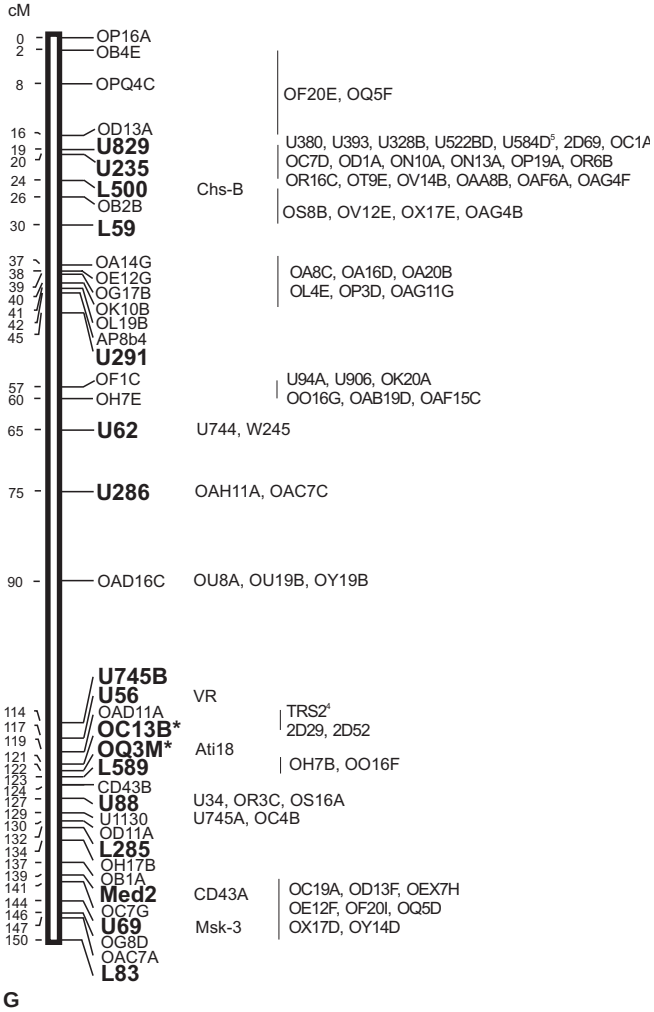
E

LG 6



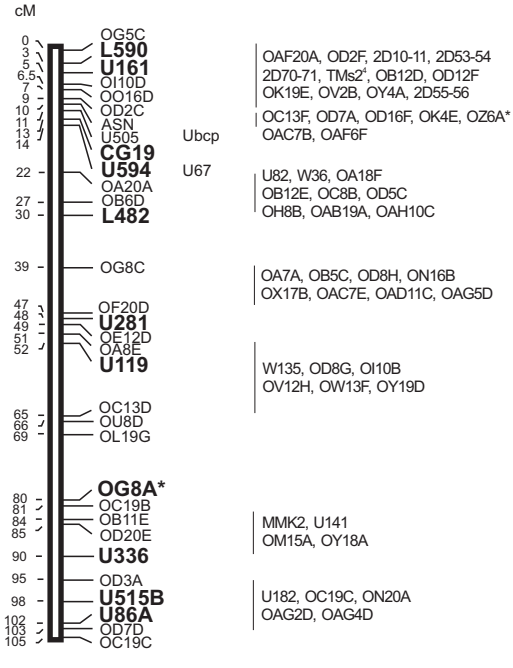
F

LG 7



G

LG 8



H

cal" (MAPMAKER/EXP 3.0) as well as by the "special" maximum-likelihood formula (Lorieux et al. 1995), respectively. The "special" maximum-likelihood formula was supposed to eliminate the disturbing effect of the distorted segregation between the marker pairs in group CDE, therefore we expected proper linkage group formation. In the upper right triangle of Table 3 the RF values calculated by the "classical" maximum-likelihood formula (MAPMAKER/EXP 3.0) are shown for all marker pairs in group CDE. Data were not given if the LOD score was less than 3 and/or the RF was more than 0.3. From these data one can see that only one group is formed because at least 4 markers (U286, U62, U553, OF20 A) linked to many markers in group CDE.

In the lower left triangle of Table 3, those RF values are shown which were calculated by the "special" maximum-likelihood formula developed for codominant markers under zygotic selection by Lorieux et al. (1995). With the exclusion threshold of 0.3, the markers of group CDE could be split unambiguously into three distinct and clear-cut groups (groups C, D, and E). We argued that the high linkage values calculated by the "classical" maximum-likelihood formula artificially linked the genetically unlinked markers in group CDE and if the disturbing effect of zygotic selection was compensated for, the genuine linkage groups could be revealed.

As a control, RF values for markers belonging to independent groups (inter-group relations) were determined, if possible, by the adequate "special" ML formulas (Lorieux et al. 1995) selected according to the selection type determined by the χ^2 tests (see Table 1). (No linkage could be calculated between the markers that displayed distorted segregation under zygotic selection and those markers in LG4 which displayed gametic selection, see Materials and methods). Based on the RFs calculated by this manner there was no inter-group linkage between the markers, that is markers belonging to a given linkage group did not show linkage to any markers in any other linkage groups (data not shown). On the other hand intra-group marker linkage could always be demonstrated, and there was no marker which remained unlinked (data not shown).

Comparing the marker composition of the linkage groups of the previous alfalfa linkage map (Kiss et al. 1993) to the present groups, groups A, B, F, G, and H corresponded to LG 1, LG 2, LG 5, LG 3, and LG 4. Based on the common marker sharing between the recent genetic groups and our previous map (Kiss et al. 1993), groups C, D, and E were designated to LGs 6, 7, and 8, respectively. The present genetic analysis demonstrated that some markers were linked to different markers in the previous map (Kiss et al. 1993). For example 6 markers (L590, L482, CG19, TMs2, ASN, and U82) from the previous LG VI is now part of LG 8, and 3 markers (L500, L59, and AP8b4) from the previous LG VIII now belong to LG 7. The reason for these rearrangements was the lack of sufficient marker saturation in the previous map as well as the false linkage values calculated by the "classical" maximum-likelihood formula for markers with extreme distorted segregation.

Genetic linkage can also be demonstrated and/or established visually by colormapping (Kiss et al. 1998). In Fig. 2 we show an example by which the mapping power of colormapping can be demonstrated. In the middle of Fig. 2, the color genotypes of markers U286 and OAD16A are highlighted. These markers of extreme distorted segregation are tightly linked to each other (RF=0.122, LOD=10.01) if the "classical" maximum-likelihood formula was used for calculation (see Table 3). On the other hand, if the RF between these 2 markers was calculated using the "special" maximum-likelihood formula, markers U286 and OAD16A turned out to be unlinked. According to the colormap these markers proved also to be unlinked, since no individual with the same homozygous genotype for this 2 markers could be observed, that is there is no "homozygous link" between markers U286 and OAD16A. On the other hand, several "homozygous links" were present between markers U286-U62, U286-U745B, and OAD16A-OW13B and OAD16A-U553, respectively, that is markers U286 and OAD16A belonged to different linkage groups (LG 7 and LG 6). This demonstrates that colormapping is a suitable and rapid method for determining linkage relationships for markers with distorted segregation ratios by overshadowing the linking effect of the heterozygous links and by giving more emphasis to homozygous links and detectable recombinations (for more detailed description of the method see Kiss et al. 1998).

Construction of the genetic map of alfalfa

The order of the core markers in the eight linkage groups was determined by calculating the RFs by the "special" maximum-likelihood formula followed by determining the genetic distances in centiMorgans using the Haldane function. This map generated the so-called frame map (Fig. 3A–H).

Further mapping was performed as follows: the additional 742 markers together with the core markers were first grouped by the "group" command of MAPMAKER/EXP 3.0 using LOD score 9 and maximum distances of 20 cM. Under these circumstances, all markers clustered in conjunction with the core markers into six groups, A, B, F, G, H, and CDE. None of the markers remained unlinked (data not shown). This grouping resulted in linkage groups containing either dominant or codominant markers which exhibited either distorted segregation of the same type as the core markers in the groups or showed normal distribution (no selection occurred). RFs were then calculated within the groups A, B, F, G, H, and CDE with the appropriate "special" maximum-likelihood formula corresponding to the type of selection which occurred in the given region (Table 1). Based on the calculated RFs, the order of the markers in the groups A, B, F, G, or H were established. The markers in group CDE fell apart into three LGs, C, D, and E (see above), and the order of the markers could be easily established within the groups. The fact that only eight linkage groups were generated after additional markers were included in the

Table 4 The nucleotide sequences of the specific primers used to amplify known sequences. The amplified fragments were used as hybridization probes in DNA-DNA hybridization (RFLP), or polymorphisms were detected by the electrophoresis of PCR products on agarose gels with or without *Hin*fI digestion [cleavage of amplified products (CAPs; Konieczny and Ausubel 1993)]

Gene		Primer sequences	References	Polymorphism detected by	
				PCR	RFLP
GSa		5′ GCACAAGGAGCACATTG 3′ 5′ ACCACTTGGACAATTACTAC 3′	Stanford et al. 1993	+	+
GSb		5′ GCACAAGGAGCACATTG 3′ 5′ GAAACCTATAACAAGGCCTC 3′	Stanford et al. 1993	–	+
GSc		5′ GCACAAGGAGCACATTG 3′ 5′ GCCCATAATTAAACATCATG 3′	Stanford et al. 1993	+	–
Calmodulin		5′ GATTTCCCTGAATTCCTTAACCTGATG 3′ 5′ GAAGTACAAATGCATGGTGGGAC 3′	Barnett and Long 1990	–	+
Chi		5′ GAGCACGCTGTTTCCCC 3′ 5′ GAAATAGTACAAGAGTAGAAAGC 3′	McKhann and Hirsch 1994	–	+
Chs		5′ CAAAGGGCAGAAGGCCC 3′ 5′ TGGTAAGACCCGGTCC 3′	McKhann and Hirsch 1994	–	+
Enod40		5′ CAAAACAGTTTGCTTTG 3′ 5′ CTAGAATACTCTTCAATTTC 3′	Crespi et al. 1994	+ *Hin*fI digestion	+
F3OH		5′ GCT/ATGC/TGAG/AGAATGGGG 3′ 5′ GGTTTG/ATCT/AGGCCAC/TCT 3′	Charrier et al. 1995	–	+
rDNA	18S–25S region	5′ ATGGTCCGGTGAAGTGTTCG 3′ 5′ CCCGGTTCGCTCGCCGTTAC 3′	Sun and Skinner 1994	+ *Hin*fI digestion	+
rDNA	5S region	5′ CTGCGGAGTTCTGATGGG 3′ 5′ GTTAAGCGTTCTTGGGCG 3′	Barcziszewska et al. 1987	–	+
β-tubulin		5′ GAAAACTGTGATTGCTTG 3′ 5′ TCCTCTCCATACTCATCC 3′	Liaud et al. 1992	+	+
AP8b12		5′ GTCCCTGTAAGTTGTGATGA 3′ 5′ TTAGAAACTTGGTGTCCGGC 3′	This study	+	–

"grouping" analysis confirmed that the eight groups represented the eight genuine genetic LGs of alfalfa.

To visualize the genetic map of alfalfa we converted the RFs – calculated by the "special" ML formulas between the neighboring markers – into genetic distances according to the Haldane function. These genetic distances were used to draw the genetic map of alfalfa shown in Fig. 3A–H. The genetic map was in complete agreement with the map constructed by colormapping (Kiss et al. 1998). In Fig. 3A–H the 121 core markers are highlighted by larger, bold characters. Additional markers could be sorted out into two classes: (1) those markers which had recombination with the core markers (RF >0) were inserted into the core markers (these enlarged sets of markers were called "extended core markers" and they are placed next to the bar representing the chromosomes (see Fig. 3A-H); (2) those markers which did not show recombination (RF=0) to the core or extended core markers were called "additional markers". Additional markers fell into two categories: (1) some markers were tightly linked (RF=0) to 1 of the core or extended core markers but exhibited recombination to the adjacent markers (these markers are shown to the right of the appropriate core or extended core markers); (2) some markers were tightly linked (RF=0) to more than 1 core or extended core markers, therefore their map location could not be determined precisely (these markers are placed to the right side of a vertical bar spanning the region in question). This ambiguity can be explained either by a lack of recombination which resides in the dominant character of the markers or by missing genotypes. The complete genetic map displaying the core, the extended core, and the additional markers is shown in Fig. 3A–H.

Characterization of the linkage map of alfalfa

The constructed linkage map of alfalfa contains 868 markers of which four were morphological, 216 RFLP (186 cDNA and 30 genomic markers), and 608 RAPD markers; 2 loci were detected exclusively by specific PCR amplification (see Table 4), and 12 isozyme and 26 seed protein markers were mapped by the analysis of zymograms and two-dimensional polyacrylamide gels, respectively.

The 868 genetic markers distributed into eight linkage groups span a genetic distance of 754 cM. The average marker density is 0.8 markers/cM with a maximum distance of 23.74 cM between markers OAD16C and U745B in LG 7. The correlation between the physical and genetic distances is 1000–1300 kb per centiMorgan, on average, taking the DNA content of the haploid genome as 750–1000 Mbp (Winicov et al. 1988; Arumuganathan and Earle 1991).

Eighty markers representing genes with known function or sequences are listed in Table 5. As it is shown in

Table 5 The list of the markers with known function or sequences which have been mapped on the linkage map of diploid alfalfa

Gene	Marker type	Gene product/phenotype	Number of mapped loci	LG	Reference
AAT1,-2	Isozyme, RFLP	Aspartate aminotransferase	2	3, 1	Kiss et al. 1993
ALD	RFLP	Aldolase	1	5	Kiss et al. 1993
Annexin	RFLP	Annexin	1	4	Pirck et al. 1994
Ant	Morphological	Anthocyanin pigment	1	1	Kiss et al. 1993
ASN	RFLP	Asparaginase	1	8	Lough et al. 1992
Ati18	RFLP	Wound-inducible trypsin inhibitor	1	7	McGurl et al. 1995
Cad5A, B	RFLP	Cinnamyl alcohol dehydrogenase	2	1, 2	Doorsselaere et al. 1995
Calmodulin	RFLP	Calmodulin	1	5	Barnett and Long 1990
Cdc2A, B	RFLP	Serine/threonine kinase	2	2, 4	Hirt et al.1993
CD32A, B	RFLP	B-type legumin (storage protein)	2	1, 3	Domoney and Casey 1985
CD40	RFLP	B-type legumin (storage protein)	1	1	Domoney and Casey 1985
CD43A, B	RFLP	A-type legumin (storage protein)	1	7	Domoney and Casey 1985
CHMT1	RFLP	Isoliquiritigenin 2'-O-Methyltransferase	1	1	Maxwell et al. 1993
Chi	RFLP	Chalcone isomerase	1	1	McKhann and Hirsch 1994
Chs-a,-b,-kl	RFLP	Chalcone synthase	3	1, 7, 5	McKhann and Hirsch 1994
CycIIIMs	RFLP	Mitotic cyclin	1	5	Savouré et al. 1995
DFR	RFLP	Dihydroflavonol-4-reductase	1	1	Charrier et al.1995
Dwarf	Morphological	Dwarf phenotype	1	2	Kiss et al. 1993
Enod2	RFLP	Early nodulin	1	1	Dickstein et al. 1988
Enod5	RFLP	Early nodulin	1	5	Scheres et al. 1990
Enod8	RFLP	Early nodulin	1	1	Dickstein et al. 1993
Enod10	RFLP	Early nodulin	1	3	Löbler and Hirsch 1993
Enod12	RFLP, PCR	Early nodulin	1	3	Kiss et al. 1993 and Csanádi et al. 1994
Enod40	RFLP, PCR	Early nodulin	1	5	Crespi et al. 1994
EST1,-2,-3	Isozyme	Esterase	3	4, 1, 2	Kiss et al. 1993
F3OH	RFLP	Flavanone-3-hydroxylase	1	5	Charrier et al.1995
Gogat	RFLP	Glutamate synthase	1	1	Gregerson et al. 1993
GSa,-b,-c	RFLP, PCR	Glutamine synthetase	3	6, 3, 5	Kiss et al.1993 & this study
LAP1,-2	Isozyme	Leucine aminopeptidase	2	3, 3	Kiss et al. 1993
LbMsI, II, III	RFLP, PCR	Leghemoglobin	3	5, 1, 5	Davidowitz et al. 1991 and Kiss et al.1993
Med1,-2	2D-PAGE	63–65 and 60 kD medicagin	2	1, 7	Felföldi unpublished
MMK1,-2	RFLP	Mitogen-activated protein (MAP) Kinase	2	4, 8	Jonak et al. 1993
MNR1	Isozyme	Menadion reductase	1	2	This study
MsK-1,-2,-3	RFLP	Serine/threonine kinase	3	1, 4, 7	Páy et al. 1993
MsRab1	RFLP	Small GTP-binding protein	1	2	Jonak et al. 1995
Mtst1	RFLP	Hexose transporter	1	4	Harrison 1996
NMs25	RFLP, PCR	Late nodulin	1	3	Kiss et al. 1993
Nod1G	RFLP	Nodulin	1	4	Dickstein et al. 1991
Nod6G	RFLP	Nodulin	1	1	Dickstein et al. 1991
Nod14	RFLP	Nodulin	1	1	Dunn et al. 1988
Nod22A,-B	RFLP	Nodulin	2	2, 4	Dunn et al. 1988
Nod32/34	RFLP	Nodulin	1	3	Dunn et al. 1988
PEPC	RFLP	Phosphoenolpyruvate carboxylase	1	2	Kiss et al. 1993
PGI1	Isozyme	Phosphoglucose isomerase	1	6	This study
PRX	Isozyme	Peroxidase	1	2	Kiss et al. 1993
rDNA	RFLP	Ribosomal RNA	1	6	This study
5S rDNA	RFLP	5S RNA	1	4	This study
SKDH1, 2	Isozyme	Shikimate dehydrogenase	2	4, 1	Kiss et al. 1993
Stl	Morphological	Sticky leaf phenotype	1	6	Kiss et al. 1993
TMs1,-2,-3,-6,-7–8	RFLP	Retrotransposon	5	3, 8, 6, 2, 2	Kiss et al. 1993
TRS1,-2	RFLP	Telomer related sequence	2	1, 7	Kiss et al. 1981
β-Tubulin	RFLP, PCR	β-Tubulin	1	4	This study
Ubcp	RFLP	Ubiquitin carrier protein	1	8	Pramanik and Bewley 1993
VR	RFLP	Vestitone reductase	1	7	Gou and Paiva 1995
Wip	RFLP	Wound induced protein	1	2	Bögre unpublished
Xant	Morphological	Xanthophyll pigment	1	1	Kiss et al. 1993

the fourth column of Table 5 some genes were detected to be present in one, two, three, or more loci in the genome. Considering only the RFLP markers, isozyme markers and 2 loci detected by specific PCR amplification, 63 out of the 230 loci (27%) were duplicated or multiplied. Thirteen cDNA clones (CAD5, CD32, L27, L408, NOD22, U36, U40, U52, U68, U157, U328, U457, U515), 1 genomic clone (W157) and 3 isozyme markers (AAT, LAP, SKDH) were mapped to 2 loci, while 7 genes (Chs, GS, EST, leghemoglobin, Msk, U522 and U745) are present at least in three copies. Two cDNA clones could be located at 4 loci (U584 and U212).

Two multicopy genes received special interest since both had been localized by cytological experiments earlier (Schaff et al. 1990; McCoy and Bingham 1988; Bauchan and Campbell 1994). These are: (1) the ribosomal RNA genes (rDNA) at one genomic region in LG 6 and (2) the β-tubulin genes in LG 4. The genetic mapping of these genes allowed us to correlate these two LGs with the appropriate chromosomes detected by cytology. Accordingly, LG 6 on which the ribosomal genes were mapped could be correlated with chromosome no. 8 carrying the nucleolus organizing region (McCoy and Bingham 1988; Bauchan and Campbell 1994; Falistocco et al. 1995). LG 4 carrying the β-tubulin genes could be correlated with the small metacentric or another submetacentric chromosome to which these genes hybridized in situ (Schaff et al. 1990).

Perspectives

The improved genetic map of alfalfa presented in this study is suitable for comparative mapping studies. The determination of the degree of synteny between the genetic maps of *Medicago sativa* and *Medicago truncatula*, *Pisum sativum*, and *Arabidopsis thaliana* is in progress. Since the diploid and the cultivated tetraploid alfalfa are crossable and belong to the *Medicago sativa* complex (Quiros and Bauchan 1988; Endre et al. 1996), the detailed genetic map of diploid *Medicago sativa* facilitates mapping and tagging agronomically important traits in different alfalfa populations. In addition, this map can be used in map-based cloning approaches for isolating genes conditioning important agronomic traits like symbiotic nitrogen fixation.

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References

Allard RW (1956) Formulas and tables to facilitate the calculation of recombination values in heredity. Hilgardia 24:235–278

Arumuganathan K, Earle ED (1991) Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9:208–219

Bailey NTJ (1949) The estimation of linkage with differential viability, II and III. Heredity 3:220–228

Barcziszewska MZ, Nalaskowska M, Mashkova TD, Kisselev LL, Barcziszewski J (1987) Heterogenity in the 3'-portion of Papilionaceae 5S rRNAs. The primary structure of alfalfa 5S rRNA. Nucleic Acids Res 15:362

Barnett MJ, Long SR (1990) Nucleotide sequence of an alfalfa calmodulin cDNA. Nucleic Acids Res 18:3395

Bauchan GR, Campbell TA (1994) Use of an image analysis system to karyotype diploid alfalfa (*Medicago sativa* L.). J Hered 85:18–22

Bentolila S, Hardy T, Guitton C, Freyssinet G (1992) Comparative genetic analyses of F_2 plants and anther culture derived plants of maize. Genome 35:575–582

Bernatzky R, Tanksley SD (1986) Majority of random cDNA clones correspond to single loci in the tomato genome. Mol Gen Genet 203:8–14

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

Brummer EC, Bouton JH, Kochert G (1993) Development of an RFLP map in alfalfa. Theor Appl Genet 86:329–332

Charrier B, Coronado C, Kondorosi Á, Ratet P (1995) Molecular characterization and expression of alfalfa (*Medicago sativa* L.) flavanone-3-hydroxylase and dihydroflavonol-4-reductase encoding genes. Plant Mol Biol 29:773–786

Crespi MD, Jurkevitch E, Poiret M, d'Aubenton Carafa Y, Petrovics G, Kondorosi É, Kondorosi Á (1994) *enod40*, a gene expressed during nodule organogenesis, codes for a non-translatable RNA involved in plant growth. EMBO J 13:5099–5112

Csanádi G, Szécsi J, Kaló P, Kiss P, Endre G, Kondorosi Á, Kondorosi É, Kiss GB (1994) *ENOD12*, an early nodulin gene, is not required for nodule formation and efficient nitrogen fixation in alfalfa. Plant Cell 6:201–213

Davidowitz EJ, Creissen G, Vincze É, Kiss GB, Lang-Unnasch N (1991) Sequence analysis of alfalfa (*Medicago sativa*) leghemoglobin cDNA and genomic clones. Plant Mol Biol 16:161–165

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: Version II. Plant Mol Biol Rep 1:19–21

Dickstein R, Bisseling T, Reinhold VN, Ausubel FM (1988) Expression of nodule-specific genes in alfalfa root nodules blocked at an early stage of development. Genes Dev 2:677–687

Dickstein R, Scheirer DC, Fowle WH, Ausubel FM (1991) Nodules elicited by *Rhizobium meliloti* heme mutants are arrested at an early stage of development. Mol Gen Genet 230:423–432

Dickstein R, Prusty R, Peng T, Ngo W, Smith ME (1993) *Enod8*, a novel early nodule-specific gene, is expressed in empty alfalfa nodules. Mol Plant-Microbe Interaction 6:715–721

Domoney C, Casey R (1985) Measurement of gene number for seed storage proteins in *Pisum*. Nucleic Acids Res 13:687–699

Doorsselaere JV, Baucher M, Feuillet C, Boudet AM, Van Montagu M, Inzé D (1995) Isolation of cinnamyl alcohol dehydrogenase cDNAs from two important economic species:alfalfa and poplar. Demonstration of a high homology of the gene within angiosperms. Plant Physiol Biochem 33:105–109

Dunn K, Dickstein R, Feinbaum R, Burnett BK, Petermann TK, Thoidis G, Goodman HM, Ausubel FM (1988) Developmental regulation of nodule-specific genes in alfalfa root nodules. Mol Plant-Microbe Interaction 1:66–74

Echt CS, Erdahl LA, McCoy TJ (1992) Genetic segregation of random amplified polymorphic DNA in diploid cultivated alfalfa. Genome 35:84–87

Echt CS, Kidwell KK, Knapp SJ, Osborn TC, McCoy TJ (1994) Linkage mapping in diploid alfalfa (*Medicago sativa*). Genome 37:61–71

Endre G, Kaló P, Hangyel Tárczy M, Csanádi G, Kiss GB (1996) Reducing the tetraploid non-nodulating alfalfa (*Medicago sativa*) MnNC-1008(NN) germ plasm to the diploid level. Theor Appl Genet 93:1061–1065

Falistocco E, Falcinelli M, Veronesi F (1995) Karyotype and C-banding pattern of mitotic chromosomes in alfalfa, *Medicago sativa* L. Plant Breed 114:451–453

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Gou L, Paiva NL (1995) Molecular cloning and expression of alfalfa (*Medicago sativa* L.) vestitone reductase, the penultimate enzyme on medicarpin biosynthesis. Arch Biochem Biophys 320:353–360

Gregerson RG, Miller SS, Twary SN, Gantt JS, Vance C (1993) Molecular characterization of NADH-dependent glutamate synthase from alfalfa nodules. Plant Cell 5:215–226

Györgyey J, Gartner A, Németh K, Magyar Z, Hirt H, Heberle-Bors E. Dudits D (1991) Alfalfa heat shock genes are differentially expressed during somatic embryogenesis. Plant Mol Biol 16:999–1007

Hanson AA, Barnes DK, Hill RR (eds) (1988) Alfalfa and alfalfa improvement. American Society of Agronomy, Crop Science Society of America, Soil Science of America, Madison, Wis

Harrison MJ (1996) A sugar transporter from *Medicago truncatula*: altered expression in roots during vesicular-arbuscular (VA) mycorrhizal associations. Plant J 9:491–503

Hebert PDN, Beaton MJ (1989) Methodologies for allozyme analysis using cellulose acetate electrophoresis. Helena Laboratories, Beaumont, Tex

Helentjaris T, Slocum M, Wright S, Schaefer A, Nienhuis J (1986) Construction of genetic linkage maps in maize and tomato using resctriction fragment length polymorphisms. Theor Appl Genet 72:761–769

Heun M, Gregorius HR (1987) A theoretical model for estimating linkage in F_2 populations with distorted single gene segregation. Biom J 4:397–406

Hirt H, Páy A, Bögre L, Meskiene I, Heberle-Bors E (1993) *cdc2MsB*, a cognate *cdc2* gene from alfalfa, complements the G1/S but not the G2/M transition of budding yeast *cdc28* mutants. Plant J 4:61–69

Jonak C, Páy A, Bögre L, Hirt H, Heberle-Bors E (1993) The plant homologue of MAP kinase is expressed in a cell cycle-dependent and organ specific manner. Plant J 3:611–617

Jonak C, Heberle-Bors E, Hirt H (1995) A cDNA from *Medicago sativa* encodes a protein homologous to small GTP-binding proteins. Plant Physiol 107:263–264

Kesseli RV, Paran I, Michelmore RW (1994) Analysis of a detailed genetic linkage map of *Lactuca sativa* (Lettuce) constructed from RFLP and RAPD markers. Genetics 136: 1435–1446

Kiss GB, Amin AA, Pearlman RE (1981) Two separate regions of the extrachromosomal ribosomal deoxyribonucleic acid of *Tetrahymena thermophila* enable autonomous replication of plasmids in *Saccharomyces cerevisiae*. Mol Cell Biol 1:535–543

Kiss GB, Csanádi G, Kálmán K, Kaló P, Ökrész L (1993) Construction of a basic genetic map for alfalfa using RFLP, RAPD, isozyme and morphological markers. Mol Gen Genet 238:129–137

Kiss GB, Kereszt A, Kiss P, Endre G (1998) Colormapping: a non-mathematical procedure for genetic mapping. Acta Biol Hun 49:125–142

Konieczny A, Ausubel FM (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J 4:403–410

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

Landry BS, Kesseli RV, Farrara B, Michelmore RW (1987) A genetic map of lettuce (*Lactuca sativa* L.) with restriction fragment length polymorphism, isozyme, disease resistance and morphological markers. Genetics 116:331–337

Landry BS, Hubert N, Etoh T, Harada JJ, Lincoln SE (1991) A genetic map for *Brassica napus* based on restriction fragment length polymorphisms detected with expressed DNA sequences. Genome 34:543–552

Liaud MF, Brinkmann H, Cerff R (1992) The β-tubulin gene family of pea: primary structures, genomic organization and intron dependant evolution of genes. Plant Mol Biol 18:639–651

Lincoln S, Daly M, Lander E (1992) Constructing genetic maps with MAPMAKER/EXP 3.0 Whitehead Institute Technical Report, 3rd edn.

Lorieux M, Perrier X, Goffinet B, Lanaud C, González de Leon D (1995) Maximum-likelihood models for mapping genetic markers showing segregation distortion. 2. F_2 populations. Theor Appl Genet 90:81–89

Lough TJ, Reddington BD, Grant MR, Hill DF, Reynolds PHS, Farnden KJF (1992) The isolation and characterization of a cDNA clone encoding L-asparaginase from developing seeds of lupin (*Lupinus arboreus*). Plant Mol Biol 19:391–399

Löbler M, Hirsch AM (1992) An alfalfa (*Medicago sativa* L.) cDNA encoding an acidic leghemoglobin (MsLb3). Plant Mol Biol 20:733–736

Löbler M, Hirsch AM (1993) A gene that encodes a proline-rich nodulin with limited homology to PsENOD12 is expressed in the invasion zone of *Rhizobium meliloti*-induced alfalfa root nodules. Plant Physiol 103:21–30

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Laboratory Press, New York

Maxwell CA, Harrison MJ, Dixon RA (1993) Molecular characterization and expression of alfalfa isoliquiritigenin 2'-*O*-methyltransferase, an enzyme specifically involved in the biosynthesis of an inducer of *Rhizobium meliloti* nodulation genes. Plant J 4:971–981

McCoy TJ, Bingham ET (1988) Cytology and cytogenetics of alfalfa. In: Hanson AA, Barnes DK, Hill RR (eds) Alfalfa and alfalfa improvement. American Society of Agronomy, Crop Science Society of America, Soil Science of America, Madison, Wis., pp 737–776

McGurl B, Swarup M, Kahn M, Ryan CA (1995) Characterization of two proteinase inhibitor (ATI) cDNAs from alfalfa leaves (*Medicago sativa* var. Vernema): the expression of ATI genes in response to wounding and soil microorganisms. Plant Mol Biol 27:995–1001

McKhann HI, Hirsch AM (1994) Isolation of chalcone synthase and chalcone isomerase cDNAs from alfalfa (*Medicago sativa* L.): highest transcript levels occur in young roots and root tips. Plant Mol Biol 24:767–777

Paran I, Michelmore RW (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor Appl Genet 85:985–993

Páy A, Jonak C, Bögre L, Meskiene I, Mairinger T, Szalay A, Heberle-Bors E, Hirt H (1993) The MsK family of alfalfa protein kinase genes encodes homologues of *shaggy/glycogen synthase kinase-3* and shows differential expression pattrens in plant organs and development. Plant J 3:847–856

Pham JL, Glaszmann JC, Sano R, Barbier P, Ghesquiére A, Second G (1990) Isozyme markers in rice: genetic analysis and linkage relationships. Genome 33:348–359

Pillen K, Steinrücken G, Herrmann RG, Jung C (1993) An extended linkage map of sugar beet (*Beta vulgaris* L.) including nine putative lethal genes and the restorter gene. X. Plant Breed 111:265–272

Pirck M, Hirt H, Heberle-Bors E (1994) The cDNA sequence encoding an annexin from *Medicago sativa*. Plant Physiol 104:1463–1464

Pramanik SK, Bewley JD (1993) A ubiquitin carrier preotein cDNA from developing alfalfa embryos. Plant Physiol 102:1049–1050

Prince JP, Pochard E, Tanksley SD (1993) Construction of a molecular linkage map of pepper and a comparison of synteny with tomato. Genome 36:404–417

Quiros FC, 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. American

Society of Agronomy, Crop Science Society of America, Soil Science of America, Madison, Wis., pp 93–124
Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with the chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467
Savouré A, Fehér A, Kaló P, Petrovics G, Csanádi G, Szécsi J, Kiss G, Brown S, Kondorosi Á, Kondorosi É (1995) Isolation of a full-length mitotic cyclin cDNA clone CycIIIMs from *Medicago sativa*: Chromosomal mapping and expression. Plant Mol Biol 27:1059–1070
Schaff DA, Koehler SM, Matthews BF, Bauchan GR (1990) *In situ* hybridization of β-tubulin to alfalfa chromosomes. J Hered 81:479–483
Scheres DA, Van Engelen F, Van Der Knaap E, Van De Wiel C, Van Kammen A, Bisseling T (1990) Sequential induction of nodulin gene expression in the developing pea nodule. Plant Cell 2:687–700
Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Note added in Proof An agreement was made on the 2nd *Medicago truncatula* Workshop (Amsterdam, July 22–23, 1999) about the uniform designation of the linkage groups as well as the corresponding chromosomes of all *Medicago* species. According to the agreement, the nomenclature of the linkage groups described in this paper will be used based on the common genetic markers mapped in both *Medicago sativa* and *Medicago truncatula*. The corresponding chromosomes will be identified by *in situ* hybridization using the common genetic markers or the corresponding *Medicago truncatula* BAC clones.

Stanford AC, Larsen K, Barker DG, Cullimore JV (1993) Differential expression within the glutamine synthetase gene family of the model legume *Medicago truncatula*. Plant Physiol 103:73–81
Sun Y, Skinner DZ (1994) A phylogeny of *Medicago* species based on ribosomal DNA sequences. In: Michaud R, Elgin JH (eds), Rep 34th N Am Alfalfa Improve Conf. Guelph, Ont, Canada, p 65
Wendel JF, Weeden NF (1989) Visualization and interpretation of plant isozymes. In: Soltis DE, Soltis PS (eds) Isozymes in plant biology. Dioscorides Press, Portland, Oreg, pp 5–45
Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535
Winicov I, Maki DH, Waterborg JH, Riehm MR, Harrington RE (1988) Characterization of the alfalfa (*Medicago sativa*) genome by DNA reassociation. Plant Mol Biol 10:369–371