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AFLP fingerprinting in *Medicago* spp.: Its development and application in linkage mapping

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Abstract

Cultivated alfalfa (*Medicago sativa* L., $2n = 4x = 32$) is one of the most important forage crops in temperate climates. The genus *Medicago* includes diploid species that are a valuable source of wild germplasm for studying the reproductive system of alfalfa and its abnormalities. A linkage map of an apomeiotic mutant of *Medicago falcata* (L.) Arcang. ($2n = 2x = 16$) that spanned 368.6 cM and included 29 amplified fragment length polymorphism (AFLP), 35 random amplified polymorphic DNA (RAPD) and three restriction fragment length polymorphism (RFLP) loci was constructed using a one-way pseudo-testcross mapping strategy. The success of such a strategy depends on the presence of sufficiently high levels of heterozygosity in the individual plant which is being mapped and on the informativeness of the marker system that is used. In general: (1) highly informative and reproducible RAPD and AFLP fingerprints were generated and several genome-specific primers selected; (2) of 67 marker loci mapped, 51 were arranged in 11 main linkage groups and eight additional couples of linked marker loci were detected; (3) mapping of an F_1 population theoretically allowed a better estimation of linkage distances since it avoided segregation distortion (χ^2 analyses revealed segregation distortion in only 5.2% of marker loci); (4) the high frequency of unlinked marker loci obtained suggests that, in this alfalfa genotype, DNA markers are distributed throughout the genome. This type of genetic map should find application and prove useful in marker-assisted selection and map-based breeding programmes in meiotic mutants of alfalfa for which there is a lack of suitable genetic markers.

Key words: *Medicago sativa* — genetic map — meiotic mutant — molecular markers

Cultivated alfalfa (*Medicago sativa* ssp. *sativa* L., $2n = 4x = 32$) is an open-pollinated species characterized by tetrasomic inheritance with multiple allelism and by pronounced inbreeding depression (Busbice and Wilsie 1966). Therefore, breeding methods used to develop cultivars usually focus on intermating a number of selected individuals in all combinations (i.e. synthetics), rather than creating partial inbred lines suitable for the production of hybrids. The selection of superior individuals is usually based on a visible phenotype, for example plant vigour, or on measurable traits such as forage yield. However, the efficiency of this selection may be reduced by environmental effects on the measured character and by the complex inheritance of multigenic traits (McCoy and Echt 1992).

Classical alfalfa breeding could be supplemented by direct selection at the genotypic level using molecular markers that allow the fingerprinting of plant germplasm and that cosegregate with the plant genes of interest. The objectives of alfalfa breeders are to improve selection methods and develop new strategies to enhance forage yield and quality by better exploitation of allelic and non-allelic interactions (Bingham et al. 1994). New biotechnological developments have expanded the range of plant DNA polymorphism assays for linkage mapping, gene targeting and assisted breeding, as well as for characterizing and investigating germplasm resources and genetic relatedness (reviewed by Powell et al. 1996). These techniques include not only restriction fragment length polymorphism (RFLP) markers, but also polymerase chain reaction (PCR)-based molecular markers, such as simple sequence repeats (SSRs or microsatellites), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers. In particular, the AFLP technique (Vos et al. 1995) provides a molecular assay that combines the reliability of the RFLP technique with the power of the PCR technique.

Tetraploid cultivated alfalfa belongs to the *M. sativa–coerulea–falcata* complex which includes both diploid (*M. sativa* ssp. *coerulea* and *M. sativa* ssp. *falcata*) and tetraploid (*M. sativa* ssp. *falcata* and *M. sativa* ssp. *sativa*) forms, which are interfertile and have the same karyotype. The use of diploid populations has given impetus to the development of genetic linkage maps based on molecular markers in alfalfa. However, it should be noted that the absence of homozygous inbred lines in alfalfa has made segregation analysis more complex than in other diploids (Helentjaris et al. 1986, Schumacher et al. 1997). Crosses realized between heterozygous parents and inbreeding depression could be a major cause of segregation distortion (Bailey 1961). Alfalfa genetic linkage maps have been obtained by using RFLP markers alone (Brummer et al. 1993, Tavoletti et al. 1996a) or in combination with RAPD markers (Kiss et al. 1993, Echt et al. 1994) and SSR markers (Diwan et al. 1995).

Only by estimating the linkage relationships among molecular markers, can one thoroughly investigate genetic diversity and identify chromosome regions carrying genes of interest. Saturated linkage maps are also required for the application of marker-assisted selection (MAS).

In the present study, AFLP markers, in combination with RAPD and RFLP markers, are used to investigate their potential applications in linkage mapping of *Medicago* spp. and this is, to our knowledge, the first time that the AFLP technique has been applied to alfalfa linkage mapping.

Table 1: List of 10-*mer*s, *Eco*-RI/*Mse*-I primer combinations and genomic probes used to detect RAPD, AFLP and RFLP markers

RAPD 10-*mer* primers		
OP-P03	OP-R15	OP-Q08
OP-P08	OP-R16	OP-Q10
OP-P10	OP-R17	OP-Q12
OP-R02	OP-B03	OP-Q18
OP-R05	OP-B07	OP-Q20
OP-R08	OP-B08	BAR-01
OP-R07	OP-B09	BAR-02
OP-R09	OP-B10	BAR-03
OP-R10	OP-B20	BAR-06
AFLP *Eco*RI + 3/*Mse*I + 3 primers		
CAC/AGC	CAC/AGA	CCA/ACA
CAC/CAA	CAC/AGG	CCA/CAA
CAC/CCA	CCA/AGA	CCA/AGG
RFLP genomic DNA probes		
Hg2 A7	Vg2C2	UWg170
Vg2B9	Vg2F8	UWg190

Materials and Methods

Plant materials: An apomeiotic mutant of *M. falcata* (L.) Arcang. ($2n = 2x = 16$), named PG-F9 that produces diplosporous eggs (Tavoletti 1994, Barcaccia et al. 1995a; Tavoletti et al. 1996b; Barcaccia et al. 1997a,b) was crossed with a wild type of *M. coerulea* (Less.) Schm. ($2n = 2x = 16$). The F_1 progeny was used as a mapping population where segregating maternal AFLP, RAPD and RFLP markers were scored.

Genomic DNA isolation: Approximately 25 mg of alfalfa leaflets were collected from healthy plants and frozen in liquid nitrogen. Total genomic DNA from tissue samples was isolated into 2-ml Eppendorf tubes according to a protocol similar to that described by Dellaporta et al. (1983). The DNA pellet was washed twice with 70% ethanol, dried and redissolved in 100 μl of 0.33 × TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The concentration of DNA samples was determined by optical density (OD) reading (DU650 spectrophotometer, Beckman Instruments Inc., Fullerton, CA) at 260 nm (1 OD = 50 ng/μl) and the purity calculated by the OD_{260}/OD_{280} ratio (Sambrook et al. 1989). An aliquot of genomic DNA was also assayed by electrophoresis on 1% agarose (Gibco-BRL) gels.

AFLP markers: AFLP fingerprinting was performed according to the procedure reported by Barcaccia et al. (1998a), slightly modified to adapt it to the alfalfa DNA template. The analysis of AFLP marker loci was based on the detection of *Eco*RI/*Mse*I genomic restriction fragments by PCR amplification with nine different primer combinations having three selective nucleotides (Table 1). Labelled restricted-selectively amplified DNA fragments separated on standard 4.75% polyacrylamide gels were visualized by autoradiogram (Biomax MR-1 film, Kodak) after 18 h exposure at –80°C using a hypercassette with intensifying screens (Amersham, Life Science, Uppsala, Sweden).

RAPD markers: PCR experiments for the detection of RAPD markers and gel electrophoresis were performed according to the procedures described by Barcaccia et al. (1995b). A total of 24 10-*mer* primers, selected by Barcaccia et al. (1997b) and purchased from Operon Technologies Inc. (Alameda, CA), were used to detect polymorphic markers that segregated in the mapping population (Table 1).

RFLP markers: Template restriction, gel blotting and membrane hybridizations were conducted according to the methods described by Echt et al. (1994) and Tavoletti et al. (1996b). Six genomic DNA single-copy probes (Table 1), kindly supplied by Prof. T. C. Osborn, University of Wisconsin, Madison, WI, USA, were employed for the detection of RFLP markers by the chemiluminescent system Gene Images kit (Amersham, Life Science).

Linkage analysis: The linkage analysis was carried out following a pseudo-testcross strategy (Grattapaglia and Sederoff 1994) using molecular data of 48 plants of the F_1 population. Dominant (RAPD and AFLP) and codominant (RFLP) markers segregating 1 : 1 for PG-F9 alleles were selected. Linkage groups were established with MAPMAKER Exp 3.0 software (Lander et al. 1987, Lincoln et al. 1992) by treating segregation data of markers as an 'F_2 backcross' (e.g. BC_1) population. The function 'group' was applied by setting an LOD score of 5 and a maximum recombination frequency r = 0.25 to detect groups of linked markers. Subsequently, marker loci within each linkage group were

Table 2: Model, expected ratio, observed ratio of progeny segregation and χ^2 values for RAPD, AFLP and RFLP markers

Marker type	Parental genotypes[1] (PG-F × 2-DI)	Expected ratios[2] and progeny plant genotypes	No.	Average χ^2 for $P \leqslant 0.05$ and range	No.	Average χ^2 at $0.05 < P \leqslant 0.001$ and range	Total
RAPD	a/− × −/−	1 : 1 (a/− : −/−)	79	0.61 (0.00–3.84)	5	5.18 (3.91–6.55)	84
	−/− × a/−	1 : 1 (−/− : a/−)	30	0.87 (0.09–2.67)	2	6.76 (5.42–8.10)	32
	a/− × a/−	3 : 1 (a/a : a/− : −/−)	18	0.51 (0.01–1.99)	0	—	18
AFLP	a/− × −/−	1 : 1 (a/− : −/−)	97	0.93 (0.00–3.84)	6	5.02 (3.86–5.65)	103
	−/− × a/−	1 : 1 (−/− : a/−)	65	0.85 (0.00–3.52)	4	5.32 (4.34–6.54)	69
	a/− × a/−	3 : 1 (a/a : a/− : −/−)	14	0.22 (0.00–1.00)	0	—	14
RFLP	a/b × −/−	1 : 1 (a/− : b/−)	5	0.84 (0.07–1.79)	0	—	5
	−/− × a/−	1 : 1 (a/− : −/−)	2	0.42 (0.40–0.43)	0	—	2
	a/− × −/−	1 : 1 (a/− : −/−)	1	0.84	0	—	1

[1]a and b indicate the presence of distinct marker alleles, − the absence of markers.
[2]Presence vs. absence of the marker.

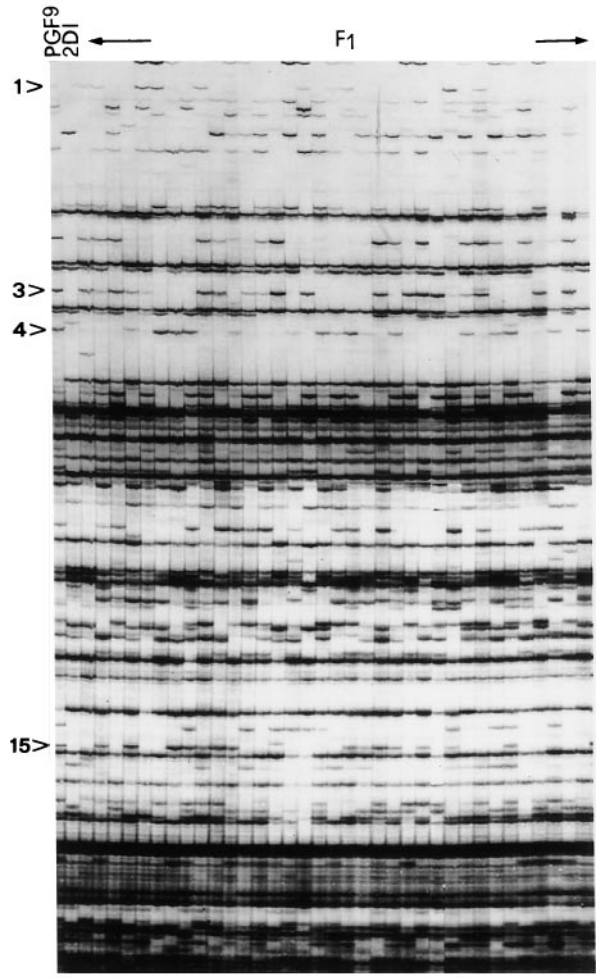

Fig. 1: AFLP fingerprints generated by primer combination *Eco*RI + CCA/*Mse*I + AGA in the parents PG-F9 and 2-DI and F_1 population. Arrows indicate markers segregating from mutant PG-F9 included in the genetic map

ordered using the 'Compare' and 'Try' functions. Markers which could be ordered with an LOD > 2 were adopted as a framework map and the remaining marker loci located at their most probable position. The 'Ripple' function was then used to confirm the final order of the marker loci. The map distances, expressed in centimorgans (cM), were calculated using the Kosambi function (Kosambi 1944).

Individual RAPD loci were named using the 10-*mer* primer nomenclature from Operon Technologies, Inc., and the relative marker number ordered by decreasing molecular weight, AFLP loci were named using the *Eco*RI/*Mse*I selective base combination and the relative marker number ordered by decreasing molecular weight, while the RFLP loci were named using the original probe nomenclature (Table 1). Linkage groups were numbered as in Tavoletti et al. (1996a).

Results

A total of 186 polymorphisms were detected by nine AFLP primer combinations, with an average of 20.7 polymorphisms per primer combination; the minimum was 11 with the primer combination *Eco* + CAC/*Mse* + AGA and the maximum 29 with *Eco* + CAC/*Mse* + CAA. A total of 134 RAPD polymorphisms were obtained using 27 10-*mer* primers with an average of 5.0 polymorphisms per primer; the minimum was three with primer OP-R02 and the maximum nine with OP-P10. A total of eight RFLP polymorphisms were scored by using six genomic DNA probes; of the maternal RFLP markers, one segregated as a dominant (UWg190) and five were codominant.

In particular, 103 AFLP markers (55.4% of polymorphisms), 97 of which segregated in the F_1 mapping population without significant deviation from the expected values (Table 2), proved to be maternal. Sixty-nine AFLP markers (37.1% of polymorphisms) showed a paternal origin and 14 (7.5% of polymorphisms) were shared between parental plants PG-F9 and 2-DI. The best primer combinations were *Eco* + CCA/*Mse* + CAA and *Eco* + CAC/*Mse* + CAA, which originated DNA fingerprints with 79 and 84 restricted-amplified products and detected 16 and 14 markers segregating from PG-F9 and 10 and 15 segregating from 2-DI, respectively. Figure 1 gives an example of the AFLP marker segregation patterns.

Eighty-four of 134 RAPD markers (62.7% of polymorphisms), 79 of which segregated 1 : 1 in the F_1 mapping population without segregation distortion (Table 2), were of maternal origin. Furthermore, 32 RAPD markers (23.8% of polymorphisms) were found to be paternal and 18 (13.5% of polymorphisms) were shared between PG-F9 and 2-DI. The six genomic DNA probes detected six maternal and two paternal (dominant) RFLP loci, all of which segregated 1 : 1 in the F_1

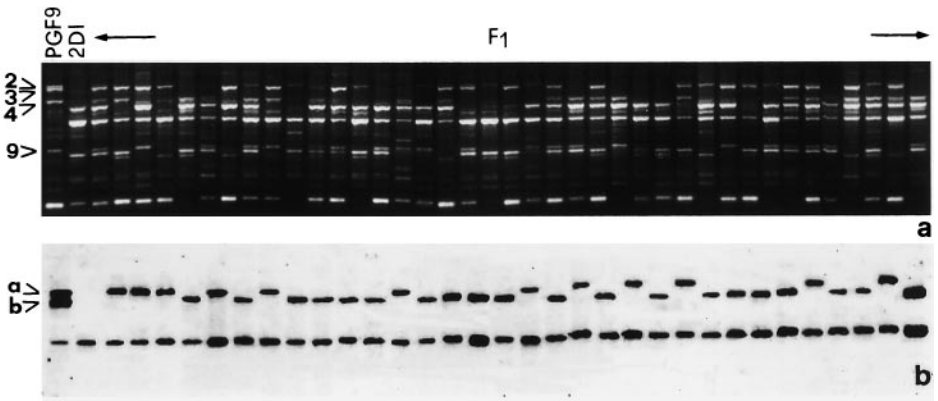

Fig. 2: Examples of dominant RAPD (a) and codominant RFLP (b) markers segregating from mutant PG-F9. Arrows indicate polymorphisms generated by primer OP-Q10 (RAPD polymorphisms 2, 3, 4 and 9) and probe UWg170 on *Eco*R5-digested genomic DNA (RFLP alleles a and b) that were mapped in the linkage groups

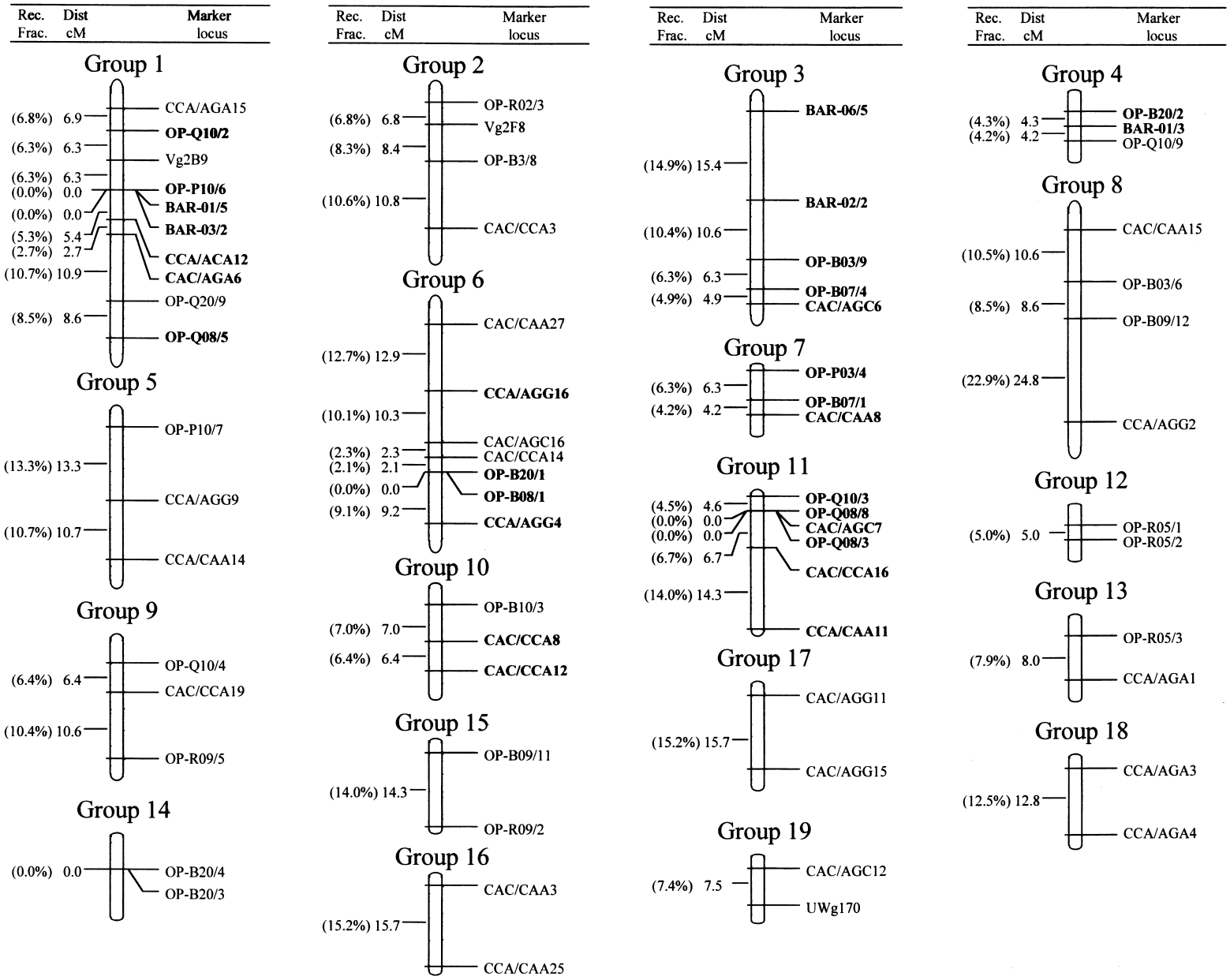

Fig. 3. Integrated AFLP, RAPD and RFLP map of the *M. falcata* mutant PG-F9. Recombination frequencies (%) and map distances in cM are given to the left of each group, while marker names are on the right. Markers loci that could be ordered with an LOD > 2 are in bold type

mapping population (Table 2). Figure 2 shows examples of RAPD and RFLP marker segregation in the F_1 progeny.

The current map consists of 67 markers (29 AFLP, 35 RAPD and three RFLP) and covers 368.6 cM. Fifty-one of the 193 maternal markers analysed were arranged in 11 main linkage groups, with more than three marker loci each (on average, 4.6 markers per group), and also eight pairs of linked markers were detected (Fig. 3). The genetic map constructed is shown in Fig. 3. Linkage groups 1 and 2 included the RFLP loci Vg2B9 and Vg2F8, respectively. Since these loci were previously mapped for PG-F9, the RAPD and AFLP markers linked to these RFLP loci should prove useful for saturating these two linkage groups. Moreover, the RFLP locus UWg170 was linked to one AFLP locus (group 8). The RAPD markers OP-B20/3 and OP-B20/4 of group 14, which were linked at 0 cM probably identified a single codominant RAPD locus (Fig. 3). Moreover, it is worth noting that in a recent study the AFLP locus CAC/AGA6 (group 1) proved to be closely associated with one of the genes that potentially controls unreduced egg formation in PG-F9 (Barcaccia et al. 1998b).

A χ^2 analyses of single-marker segregation ratios (Table 2) demonstrated that, on the whole, only 17 out of 328 of the loci examined deviated from Mendelian expectations.

Discussion

The success of a pseudo-testcross strategy depends on the presence of sufficiently high levels of heterozygosity in the individual plant which is being mapped and on the informativeness of the marker system is used. An F_1 population obtained by crossing two highly heterozygous parents belonging to different interfertile subspecies (*M. sativa* ssp. *falcata* × *M. sativa* ssp. *coerulea*) was studied because it was believed that a non-inbred population would not only provide an effective strategy for avoiding segregation distortion, but also give better estimates of linkage distances. It was found that 311 out of 328 markers segregated according to a Mendelian 1 : 1 ratio and segregation distortion was seen in only 5.2% of loci.

Diploid alfalfa is extremely sensitive to inbreeding depression caused by the manifestation of deleterious recessives, which may be a major cause of segregation distortion in partially inbred generations, such as F_2 populations produced by selfing (Brummer et al. 1993, Kiss et al. 1993, Diwan et al. 1995) or backcross populations (Echt et al. 1994).

Previously reported maps were based on various segregating populations. Kiss et al. (1993) obtained a linkage map using an F_2 population and found that 50% of loci deviated significantly from the expected segregation ratio, as well as 48% of the Brummer et al. (1993) linkage map loci. Diwan et al. (1995), who also used tetraploid and diploid F_2 populations, documented the same level of segregation distortion. Mapping of a BC_1 population by Echt et al. (1994) revealed significant distortion in 34% of loci. Yu and Pauls (1993a) studied strategies for molecular mapping in an F_1 population of alfalfa with RAPD markers and obtained 6% segregation distortion. Tavoletti et al. (1996a), who also studied an F_1 population, reported that only 8.8% of loci exhibited segregation distortion.

The map obtained in this study spans about 369 cM, covering only part of the alfalfa genome. Fifty-one out of 67 marker loci mapped were arranged in 11 main linkage groups. The high frequency of unlinked marker loci suggests that the AFLP and RAPD markers used in our study are widely distributed across the genome of the alfalfa mutant. On the basis of these findings, it is thought that with a larger segregating population of mutant PG-F9 the number of groups should reduce to eight, that is the basic chromosome number of alfalfa.

The AFLP and RAPD data complemented the RFLP, while each of these markers alone would have been insufficient to assemble a representative genomic map. This shows that when AFLP markers are added to RAPD and RFLP markers, they effectively saturate the alfalfa linkage map and, regardless of their molecular nature, their Mendelian inheritance should make them valuable tools for genetically analysing reproductive mutants. In this study, the combination of markers proved efficacious in gathering data for linkage mapping. Such a map should find application in marker-assisted selection and map-based breeding programmes involving reproductive mutants of diploid alfalfa, such as those affecting female meiosis and egg cell formation, for which there is a scarcity of suitable genetic markers.

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