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The development of a genetic map for meadowfoam comprised of amplified fragment length polymorphisms

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Abstract *Limnanthes alba* Benth. (meadowfoam), a diploid (*x*=5) winter annual, produces novel very long-chain seed oils (C_{20} and C_{22}) with less than 2% saturated fatty acids. The first genetic map of meadowfoam, a recently domesticated species, is described herein. Two phenotypically diverse inbred lines, OMF40–11 (*L. alba* ssp. *alba*) and OMF64 (*L. alba* ssp. *versicolor*), were screened for amplified fragment length polymorphisms (AFLPs) using 16 primer combinations. Twenty three percent of the AFLP bands (415 out of 1,801) were polymorphic between OMF40–11 and OMF64. One hundred (OMF40–11×OMF64)×OMF64 BC_1 progeny were genotyped for 107 polymorphic AFLP markers produced by nine AFLP primer combinations. One hundred and three AFLP loci amalgamated into five linkage groups with 14 to 28 loci per linkage group (four loci segregated independently). The map was 698.5-cM long with a mean interlocus spacing of 6.7 cM and no dense clustering of loci. The segregation ratios for 25 loci (23.2%) were significantly distorted. Twenty one of the distorted loci (84%) had an excess of *L. alba* ssp. *versicolor* (recurrent parent) alleles. The distorted loci, apart from one locus on linkage group 4, were distally clustered on both ends of linkage groups 1, 4 and 5. The development of the map was facilitated by the small chromosome number, an abundance of restriction site polymorphisms between the two subspecies (23%), and a high multiplex ratio of the AFLP markers (112 per primer combination).

Keywords Meadowfoam · *Limnanthes alba* · Amplified fragment length polymorphisms

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Introduction

Limnanthes alba Benth. (meadowfoam) is one of nine diploid (*x*=5) species belonging to the Limnanthaceae family (Mason 1952; Ornduff and Crovello 1968). The *Limnanthes* are native to temporally wet habitats (vernal pools) in California, Oregon and British Columbia. The seed oils of meadowfoam are a rich source of novel very long-chain fatty acids (C_{20} and C_{22}), and have low saturated fatty acid concentrations (typically less than 2%) (Smith et al. 1960; Bagby et al. 1961; Isbell 1997). The industrial utility and novelty of the oil (Isabell 1997) has been a catalyst for the development of meadowfoam as an oilseed crop (Knapp and Crane 1999).

Meadowfoam, a recently domesticated species, has been seriously cultivated for less than a decade, but has gained economic momentum with the release of new cultivars (Knapp and Crane 1999) and the development of specialty chemicals from meadowfoam fatty acids and triglycerides (Isabell 1997). Genetic maps have not been developed for meadowfoam, partly because restriction fragment length polymorphism (RFLP), simple sequence repeat (SSR), and other locus-specific DNA markers, apart from a limited number of biochemical markers (Arroyo 1975; Brown and Jain 1979; McNeill and Jain 1983; Kesseli and Jain 1985), have not been developed. The development of universal DNA marker systems, e.g., amplified fragment length polymorphisms (AFLPs) (Vos et al. 1995), has eliminated impediments to developing genetic maps in species lacking DNA markers.

The widespread popularity and utility of AFLP markers for molecular breeding and genomics research can be attributed to a high multiplex ratio, high assay reproducibility, universality and versatility – several hundred AFLPs can be assayed in most species using fewer than ten universal primer combinations with no prior sequence information (Vos et al. 1995). AFLP markers have been particularly useful for increasing genetic map densities in species with well-developed maps (Becker et al. 1995; Keim et al. 1997; Cho et al. 1998; Qi et al. 1998; Gedil et al. 2001) and constructing genetic maps

in species that lacked DNA markers, such as peach (*Prunus persica* L.) (Lu et al. 1998) and muskmelon (*Cucumis melo* L.) (Wang et al. 1997).

The development of inbred lines for *L. alba* (Knapp and Crane 1999), a self-compatible, allogamous species (Jain 1978), has facilitated the development of mapping populations with known linkage phases and simple pedigrees. We developed a backcross population from an intersubspecific cross between inbred lines (*L. alba* ssp. *alba*×*L. alba* ssp. *versicolor*) for the purpose of producing a genetic map. The screening of the inbred lines for AFLPs and the development of an AFLP map from the backcross are described herein.

Materials and methods

Plant materials

The genetic map was constructed using 100 (OMF40–11× OMF64)×OMF64 backcross progeny, where OMF40–11 and OMF64 are S_5 lines. OMF40–11 was developed by single-seed descent (without artificial selection) from the open-pollinated *L. alba* ssp. *alba* cultivar Mermaid by randomly selecting and manually selfing individuals. OMF64, a self-pollinated inbred line, was developed from the wild *L. alba* ssp. *versicolor* population PI374801 (Knapp and Crane 1997; Crane and Knapp 2000). Hybrid and backcross progeny were produced by emasculating and hand-pollinating OMF40–11 and OMF40–11×OMF64, respectively.

Seeds of the parents, hybrid, and backcross progeny were produced on greenhouse-grown plants. Whole seeds of the inbred lines and hybrid and half-seeds of 100 BC_1 progeny were germinated at 4°C in the dark on moistened blotter paper in 11×11×3 cm clear plastic boxes. Half-seed samples were produced by latitudinally slicing seeds in half, germinating the intact- embryo halves, and saving the non-embryo halves for chemical analysis as described by Knapp and Crane (1998). Germinants were transplanted to potting soil (pumice: peat moss: sandy loam) in 7.5 cm^2 plastic pots. The plants were grown at 15°C for 25 to 28 days in a growth chamber (Model CEL 37–14, Sherer-Gillett Co., Marshall, Mich.) with 8 h of fluorescent light per day. Four-week-old plants were transferred to a greenhouse and grown to maturity at 18°C (night) to 25°C (day) with 16 h of light per day. The bases of the pots and protruding roots were continuously submerged in 1–3 cm of water in the growth chamber and greenhouse. Leaves from 50 to 55 day old plants were harvested, immediately frozen, and stored at –80°C.

AFLP marker analyses

Genomic DNA was extracted from frozen leaves using a protocol similar to that of Lodhi et al. (1994). One to two grams of leaf tissue were ground in liquid nitrogen and incubated with 2% CTAB (cetyltrimetylammonium bromide) extraction buffer for 1 h at 65°C. The DNA was chloroform-extracted once. The aqueous phase was mixed with a half volume of 5 M NaCl, precipitated with two volumes of cold 95% ethanol, and refrigerated at 4°C overnight. The DNA pellets were dissolved in TE (10 mM Tris HCl and 0.1 mM EDTA, pH 8.0) buffer. The dissolved DNA samples were treated with RNase (100 µg/ml) for 1 h at 37°C.

AFLP marker assays were performed using the protocols described by Zabeau and Vos (1993) without streptavidin beads (Vos et al. 1995). Genomic DNA samples (0.5 µg) were digested with 5 U of *Eco*RI and 5 U of *Mse*I (New England Biolab, Schwalbach, Germany) in a reaction volume of 50-µl restriction-ligation (RL) buffer (10 mM Tris-acetate, 10 mM Mg acetate 50 mM K acetate, and 5 mM DTT, pH 7.5) (Pharmacia, Upsala, Sweden) for 3 h at 37°C. Ten microliters of ligation solution containing 1 µl of *Eco*RI adapter (5 pmol/µl), 1 µl of *Mse*I adapter (50 pmol/µl), 5 U of *Eco*RI, 5 U of *Mse*I, 1.2 µl of 10-mM ATP, 1 µl of 10×RL buffer and 1 U of T4 DNA ligase (New England Biolabs) was added to the solution and incubated for 3 h at 37°C. Twelve microliters of restriction ligation products were electrophoresed on a 1% agarose gel to assess whether or not the DNA had been completely digested.

Restriction fragments were selectively amplified from adaptor-ligated DNA samples in two steps. First, fragments were PCR-amplified using one selective nucleotide (+1) on each oligonucleotide primer (*Eco*RI +A and *Mse*I +C) (Table 1). The PCRs were performed for 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s using a Perkin Elmer 9600 DNA thermal cycler (San Francisco, Calif.). Twelve microliters of each PCR product were electrophoresed on 1% agarose to check for the presence of amplicons. The amplicons were diluted ten-fold in 0.1% TE buffer and stored at –20°C.

Second, fragments were PCR-amplified from the diluted +1 PCR products using three selective nucleotides (+3) on each oligonucleotide primer (Table 1). *Mse*I +3 primers were unlabelled, while *Eco*RI +3 primers were end-labeled with [γ-^{33}P]-ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.). Touchdown PCRs were performed with the first cycle at 94°C for 3 s, 65°C for 30 s and 72°C for 60 s, 11 cycles with annealing temperatures reduced 0.7°C per cycle, and 24 cycles with an annealing temperature of 56°C (Zabeau and Vos 1993; Vos et al. 1995).

The amplicons were mixed with an equal volume of loading buffer [98% formamide, 10 mM EDTA (pH 8.0), 0.025% xylene cyanol, and 0.025% bromophenol blue as a tracking dye], heated at 94°C for 3 min, and rapidly cooled on ice. Eight microliters of

Table 1 Amplified fragment length polymorphism oligonucleotide primers and adaptors

Adaptor or Primer	Name	Sequence
*Eco*RI adaptors	91M35	5′-CTCGTAGACTGCGTACC-3′
	91M36	3′-CTGACGCATGGTTAA-5′
*Mse*I adaptors	92A18	5′-GACGATGAGTCCTGAG-3′
	92A19	3′-TACTCAGGACTCAT-5′
*Eco*RI+1 primer	92R11	5′-AGACTGCGTACCAATTC/A-3′
*Mse*I+1 primer	92H20	5′-GACGATGAGTCCTGAGTAA/C-3′
*Eco*RI+3 primers	92SO5	5′-GACTGCGTACCAATTC/ACA-3′
		5′-GACTGCGTACCAATTC/ACG-3′
*Mse*I+3 primers	92G23	5′-GATGAGTCCTGAGTAA/CAG-3′
	92G24	5′-GATGAGTCCTGAGTAA/CAT-3′
	92G28	5′-GATGAGTCCTGAGTAA/CTA-3′
	92G29	5′-GATGAGTCCTGAGTAA/CTG-3′
	92G30	5′-GATGAGTCCTGAGTAA/CTC-3′
	92G31	5′-GATGAGTCCTGAGTAA/CTT-3′
	92F10	5′-GATGAGTCCTGAGTAA/CAC-3′
	92F41	5′-GATGAGTCCTGAGTAA/CAA-3′

each sample were loaded per lane into pre-warmed 6% denaturing polyacrylamide gels. Gels were run in 1×TBE running buffer (0.045 M Tris borate and 0.001 M EDTA, pH 8.0) at 60 W for 2 h, transferred to chromatographic paper (3 MM) (Fisher Scientific, Pittsburgh, Pa., USA), dried on a gel-dryer under vacuum at 80°C for 2 h, and exposed to X-ray film (Bioworld, Dublin, Ohio, USA) at room temperature for 3 to 5 days.

AFLP fingerprints were produced for OMF40–11 and OMF64 using 16 AFLP primer combinations (two *Eco*RI +3 and eight *Mse*I +3 oligonucleotide primers) (Table 1) and for 100 backcross progeny using nine of the 16 primer combinations. The autoradiographs were manually scored for the presence or absence of bands. Loci were named by using the selective nucleotide sequences of the *Eco*RI and *Mse*I +3 oligonucleotide primers and estimated fragment lengths, e.g., the locus name for a 250-bp fragment produced by the *Eco*RI-ACG and *Mse*I-CAA primers is ACG-CAA-250. Fragment lengths were visually estimated using the Sequenase DNA sequencing ladder from Amersham Life Science (Arlington Heights, Ill).

Genetic mapping

Genetic maps were constructed using MAPMAKER (Lander et al. 1987) and G-MENDEL (Holloway and Knapp 1993). Chi-square tests for segregation distortion were performed for each locus. Loci were grouped using a likelihood odds (LOD) threshold of 7.0 and a recombination frequency threshold of 0.25. Loci were ordered using the MAP function of MAPMAKER (Lander et al. 1987) and the ORDER function of G-MENDEL (Holloway and Knapp 1993). MAP estimates locus orders by comparing mutipoint likelihoods, whereas ORDER estimates locus orders by comparing map lengths (sums of adjacent recombination frequencies). Multipoint likelihood was used to select the final locus-order estimate for each linkage group. If the likelihood for the locus order produced by MAPMAKER was greater than the likelihood of the locus order produced by G-MENDEL, then the order produced by MAPMAKER was selected. Similarly, if the likelihood for a locus order produced by G-MENDEL was greater than the likelihood of the locus order produced by MAPMAKER, then the order produced by G-MENDEL was selected.

The reproducibilities of locus orders were assessed using the MONTE function of G- MENDEL (Holloway and Knapp 1993). MONTE was used to produce n=100 locus-order estimates from 100 repeat runs of the locus-ordering algorithm. Kendall's coefficient of concordance (W) was estimated for each linkage group (Kendall and Gibbons 1990). The concordance between locus-order estimates is perfect when W=1 and random when W=0. W varies from 0 to 1 (rather than from –1 to 1 as for a rank correlation) because the agreement and disagreement between ranks are not "symmetrical opposites" when the number of ranks is greater than two (n>2) – a set of ranks can completely agree, but they cannot completely disagree (Kendall and Gibbons 1990). W is analogous to a mean correlation among the n orders with $\binom{n}{2}$ possible correlations among n orders. The statistical significance of W was tested using the χ^2 distribution (Kendall and Gibbons 1990). The null hypothesis was that the n locus orders estimated for a linkage group were independent. The test statistic $C=n(k-1)W$ is approximately distributed as a χ^2 variable with $v=k-1$ degrees of freedom when k>7, where k is the number of loci in a linkage group (Kendall and Gibbons 1990). The null hypothesis was rejected with a probability of α when $C>\chi^2_{v:\alpha}$.

Results and discussion

Four hundred and fifteen polymorphic and 1,386 monomorphic bands were produced between OMF40–11 and OMF64 by 16 AFLP primer combinations (Table 2). The number of polymorphic bands ranged from 19 for the *Eco*RI-ACG/*Mse*I-CAG primer combination to 34 each for the *Eco*RI-ACG/*Mse*I-CAA and *Eco*RI-ACG/*Mse*I-CAT primer combinations. The nine most polymorphic AFLP primer combinations (*Eco*RI-ACG/*Mse*I-CAA, CAC, CAT, CTA, and CAG and *Eco*RI-ACA/*Mse*I-CAC, CTG, CTC, and CAG) were used to genotype the mapping population. The selected primer pairs produced 971 bands (98 to 120 bands per primer pair). Of these, 246 were polymorphic between OMF40–11 and OMF64, and 128 segregated in the backcross population – loci with null alleles (–) transmitted by the donor parent (OMF40–11) were not be mapped, whereas loci with null alleles transmitted by the recurrent parent (OMF64) were mapped. The signal intensities between + allele homozygotes (+/+) and heterozygotes (+/–) varied for several loci; however, these loci were not scored because +/+ individuals could not be unequivocally distinguished from +/– individuals. Twenty-one of the polymorphic loci had faint bands or inconsistent band intensities, and were not genotyped.

Table 2 Number of monomorphic and polymorphic (total) and number of polymorphic amplified fragment length polymorphism (AFLP) bands produced by 16 AFLP primer combinations between two inbred lines (OMF40–11 and OMF64) of meadowfoam

Selective nucleotides		Number of bands	
*Eco*RI	*Mse*I	Total	Polymorphic
ACG	CAA	100	34
	CAC	98	22
	CAT	113	34
	CTA	106	23
	CAG	99	19
	CTG	77	22
	CTC	85	20
	CTT	98	26
ACA	CAC	114	31
	CTG	120	26
	CTC	107	25
	CAG	114	32
	CAT	133	28
	CTA	134	26
	CTT	160	21
	CAA	143	26
Total		1,801	415

One hundred and three out of 107 AFLP loci amalgamated into five linkage groups (Fig. 1). Four AFLP loci (ACG-CAC-205, ACA-CTC-184, ACA-CAC-150 and ACA-CTG-173) segregated independently. The five linkage groups (Fig. 1) presumably correspond to the five haploid chromosomes of this species. Concordance estimates for locus orders were 0.99 for linkage groups 1–4 and 0.98 for linkage group 5; thus, the locus orders were nearly 100% reproducible.

Loci were well distributed within and among linkage groups with no dense clustering of loci in any linkage group (Fig. 1). The map was 698.3-cM long with 19 to 28 loci per linkage group and a mean interlocus spacing of 5.8, 7.2, 6.0, 9.1 and 6.8 cM among linkage groups in order. The longest gap between markers was 36.3 cM on linkage group 4 (Fig. 1). The longest gaps on the other four linkage groups were 25.9, 18.9, 16.9 and 26.1. Although the distances between the telomeres and distal markers on each

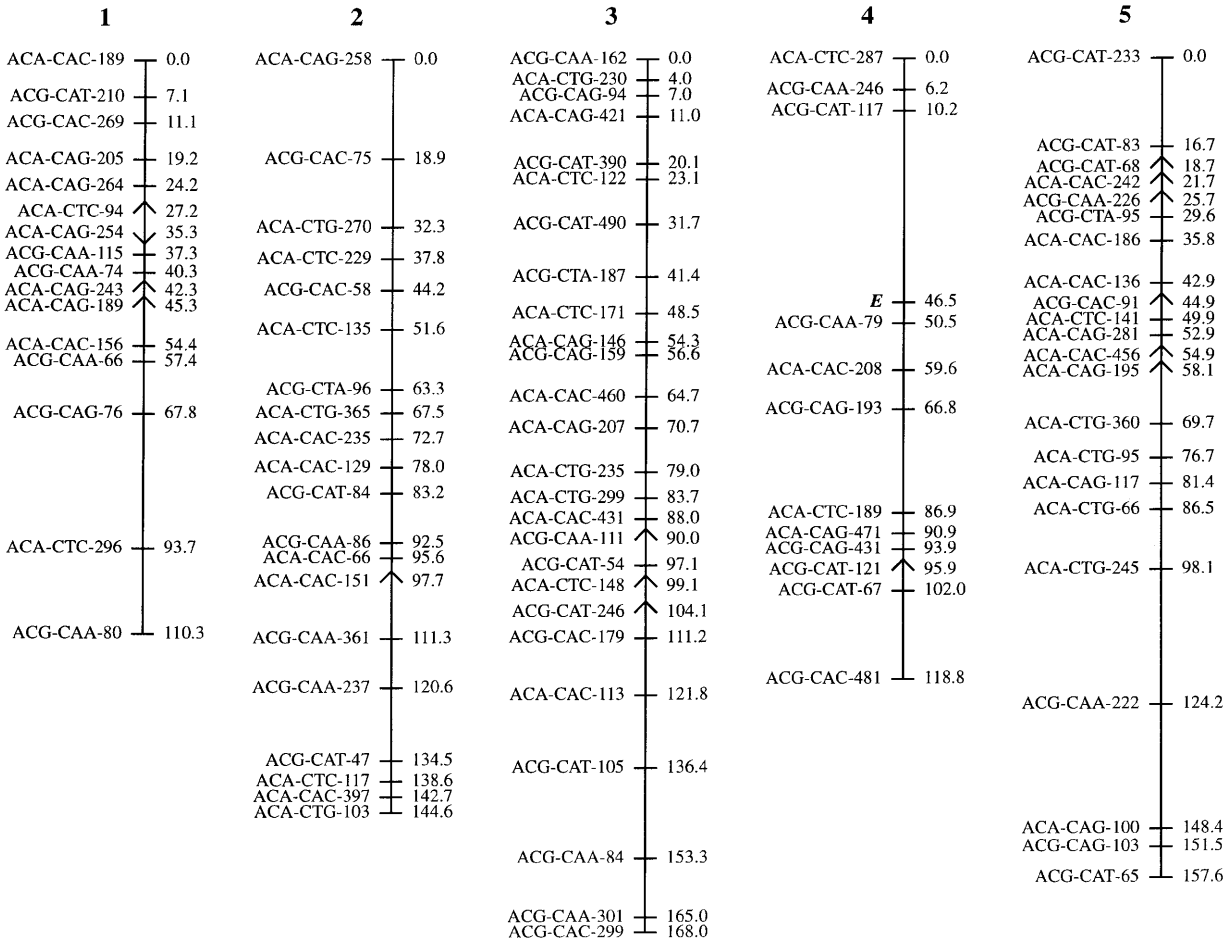

Fig. 1 Genetic linkage map of meadowfoam (*L. alba*) comprised of 103 amplified fragment length polymorphism markers

chromosome arm are not known (the telomeres have not been mapped), and four markers segregated independently, the length of the map led us to speculate that the genetic map covers 70% or more of the meadowfoam genome. Otherwise the 'complete' map, from telomere to telomere, would have to be longer than 1,000 cM, which seems improbable. The genetic map of meadowfoam described herein (Fig. 1) is 40% longer than the genetic map of *Arabidopsis thaliana* L. (approximately 500 cM) (Meinke et al. 1998), another species with five chromosomes in the haploid genome. The other prominent feature of the map was the absence of dense clusters of loci. AFLP markers characteristically cluster in heterochromatin-rich centromeric regions in plant species with large genomes (Becker et al. 1995; Keim et al. 1997; Cho et al. 1998; Qi et al. 1998; Gedil et al. 2001). The lack of clustering in meadowfoam might be a consequence of higher recombination rates in meadowfoam compared to other plant species.

The segregation ratios for 25 loci (23.2%) were significantly distorted ($p \leq 0.01$). Twenty one loci had an excess of recurrent parent (OMF64) alleles, whereas only four loci had an excess of donor parent (OMF40–11) alleles (Fig. 2). The distorted loci, apart from one locus (ACG- CAG-193) near the middle of linkage group 4, were distally clustered on the ends of linkage groups 1, 4 and 5 (Fig. 2). Selection operated against OMF40–11 alleles on four of six ends, and against OMF64 alleles (one or two loci only) on two of six ends, of the three linkage groups. The genomes of *L. alba* ssp. *alba* and *L. alba* ssp. *versicolor* seem to have diverged enough for selection to operate against loci in specific genomic regions, as is common in interspecific crosses (Zamir and Tadmor 1986; Harushima et al. 1996; Jenczewski et al. 1997; Lyttle 1991; Pillen et al. 1992). The two subspecies, although interfertile and sympartrically distributed throughout part of the natural range, are morphologically distinct (Mason 1952). Both subspecies are predominantly allogamous (Jain 1978); however, non-insect vectored selfing has only been observed in *L. alba* ssp. *versicolor* (Knapp and Crane 1997), a subspecies with reduced flower parts.

The rapid and efficient development of the genetic map of meadowfoam was facilitated by the small chromosome number, an abundance of restiction-site polymorphisms between the two subspecies (23% of the AFLP bands were polymorphic), and a high multiplex ratio of AFLP markers (112 bands per primer combination). This map builds the foundation for molecular breeding and genomics research in meadowfoam, and creates a framework for anchoring SSR and other sequence-based markers.

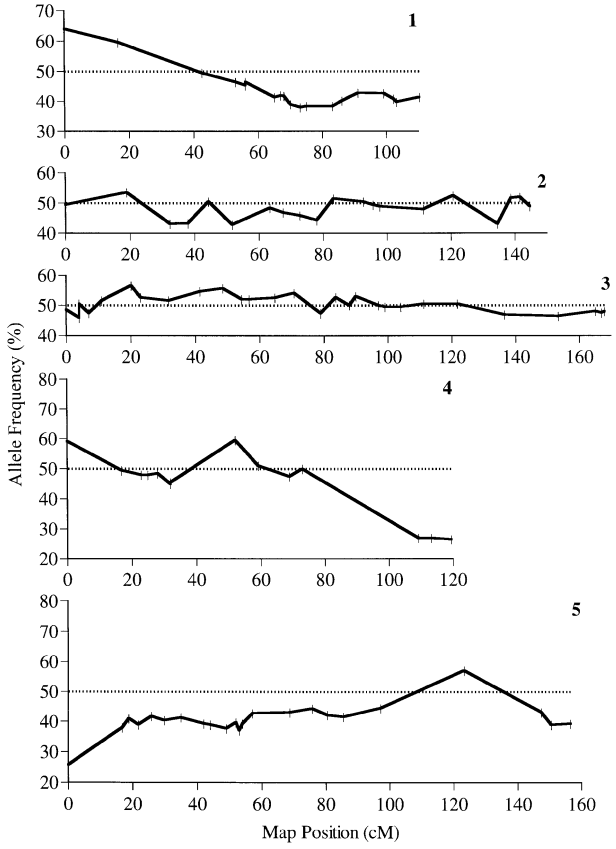

Fig. 2 OMF40–11 (*L. alba* ssp. *alba*) amplified fragment length polymorphism marker allele percentages among (OMF40–11× OMF64)×OMF64 BC_1 progeny for ordered loci on the genetic map of meadowfoam

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