**AN ABSTRACT OF THE THESIS OF**

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Marker information in the new oilseed crop, meadowfoam, is limited. Molecular markers to facilitate meadowfoam breeding and cultivar improvement are not available. The knowledge of genetic relationships among recent germplasm is not known. The objectives of this study were (i) to gain an understanding of genetic diversity and relationship patterns among germplasm. (ii) to construct a genetic linkage map, and (iii) to map genes and QTLs (Quantitative Trait Loci) underlying erucic and dienoic acid concentrations in seed oils. We fingerprinted meadowfoam 41 accessions of section inflexae of family Limnanthaceae using 176 AFLP markers. Polymorphic information content (PIC) scores were high in 42.6% of the markers and ranged from 0.45 to 0.5. Genetic distance estimates ranged from 0.14 to 0.55 with an average of 0.44. The clustering phenogram showed concordance with taxonomic classification. The first three principal component analyses accounted for 37% of the total variation of genetic distance estimated. We concluded that the genetic diversity of elite and exotic germplasm in section Inflexae was high.

The AFLP genetic linkage map for meadowfoam was built using inter-subspecific backcross progeny between OMF40-11 (*Limnanthes. alba* spp. *alba*) and OMF64 (*L. alba* spp. *versicolor*). The map was comprised of 104 loci in five linkage groups, with 14 to 28 loci per linkage group. The map

covered 698.3 cM with a mean density of 6.7 cM. The lengths of the linkage groups varied from 110.3 to 168.0 cM. AFLP loci were randomly distributed throughout the genome with no centromeric clustering. Genetic maps of meadowfoam can be rapidly constructed using a small number of AFLP primer combinations.

We utilized the AFLP genetic linkage map to map genes and QTLs underlying erucic and dienoic acid concentrations in seed oils. The QTL analyses were performed using interval mapping. QTL affecting erucic and dienoic acids was mapped to linkage group four at the *E* locus, which controlled seed oil phenotypic differences between the two subspecies, *alba* and *versicolor*. The effect of *E* locus was pleiotropic. QTLs with significant effects on content of erucic and dienoic acid other than the effects of *E* locus were not found in this backcross population.

# DNA Fingerprinting and Genome Mapping in Meadowfoam

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Sureeporn Katengam, Author

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## CONTRIBUTION OF AUTHORS

Professor Steven J. Knapp proposed, advised, and supervised all aspects of the projects. He substantially assisted in the interpretation of data and made revisions and suggestions to the manuscripts. Jimmie M. Crane helped with fatty acid analysis, greenhouse and field work.

## TABLE OF CONTENTS

## TABLE OF CONTENTS (Continued)

## LIST OF FIGURES

## LIST OF FIGURES (Continued)

## LIST OF FIGURES (Continued)

**Figure** **Page**

## LIST OF TABLES

## LIST OF TABLES (Continued)

**Table** **Page**

# DNA Fingerprinting and Genome Mapping in Meadowfoam

## CHAPTER 1

## INTRODUCTION

Molecular breeding has dramatically changed the pace of genetic improvement of many plant species in the past few decades. It holds great potential for crop improvement, as it promises to expedite the time taken to produce crop varieties with desirable characters. A variety of molecular markers have been developed based on two main technological methods, hybridization-based and PCR-based methods (Rafalski et al. 1996). Hybridization-based marker is dominated by restriction fragment length polymorphism (RFLP) (Botstein et al. 1980). The advent of polymerase chain reaction (PCR) techniques (Mullis et al. 1986) accelerated and expanded the efficiency of new DNA marker systems. These include simple sequence repeat (SSR) (Morgante and Olivieri 1993), amplified fragment length polymorphism (AFLP) (Vos et al. 1995), and random amplified polymorphic DNA (RAPD) (Williams et al. 1990). The utility of these DNA markers depends on their unique properties such as abundance, reproducibility, multiplex ratio, information content, cost and convenience (Powell et al. 1996a, 1997; Russell et al. 1997a; Jones et al. 1997, Pejic et al. 1998). Molecular markers are powerful tools for constructing genetic linkage maps, DNA fingerprinting and germplasm identification, quantitative trait loci (QTL) analysis, genetic diversity analysis and germplasm organization, marker-assisted breeding, and map-based cloning (Lee 1995; Staub et al. 1996, Mohan et al. 1997).

Ideal DNA markers for plant breeding are simple, abundant, cost and time effective. Based on these criteria, amplified fragment length polymorphisms (AFLPs) are the prominent marker system for various applications in crop improvement. AFLP was developed by combining the

strength of RFLP marker and the efficiency of PCR-based method (Zabeau and Vos 1993). AFLP is a bi-allelic and multilocus PCR-based marker, which uses selective amplification of restriction fragments. Polymorphisms are usually revealed as the presence or absence of amplified restriction fragments, and are therefore dominant. Outstanding features of AFLPs as valuable markers for plant genome analysis are that they are highly abundant, reproducible, relatively high multiplex ratio, small amount of DNA required, and extensive genome coverage (Vos et al. 1995; Powell et al. 1996b, 1997; Bai et al. 1999; Hansen et al. 1999; Knapp et al. 1999). Moreover, unlike simple sequence repeat (SSR), AFLP markers require no prior sequence information and a larger number of markers can be produced in a short period of time (Maughan et al. 1996; Hill et al. 1997; Zhu et al. 1998).

Meadowfoam (*Limnanthes* spp.) is a new oil seed crop native to Southern Oregon and Northern California (Mason 1952; Kalin 1971; Jain 1986). Seed oil of meadowfoam contains unique unsaturated very long-chain fatty acids ($C_{20}$ and $C_{22}$) with outstanding oxidative stability (Smith et al. 1960; Bagby et al. 1961; Miller et al. 1964; Knapp and Crane 1995, 1998; Isbell 1997). The market for this seed oil was successfully expanded owing to the specialty of its fatty acids (Knapp and Crane 1999).

Marker information for meadowfoam is limited even though it is needed to assess the feasibility of conducting molecular breeding and genome mapping in this crop. The availability of markers in this crop was based on morphological traits and allozyme markers (Arroyo 1973, 1975; McNeill and Jain 1983; Kessili and Jain 1985). DNA markers are not yet developed in meadowfoam to speed up the efficiency of cultivar improvement. Our goals were to assess the genetic diversity and relationships pattern of elite and exotic germplasm accessions of section Inflexae, to construct a genetic linkage map of meadowfoam, and to increase our understanding of the genetics underlying economically important traits, particularly fatty acid profile differences in the very long-chain seeds oil. These can be accomplished more

efficiently by the use of molecular markers and AFLP was chosen as the marker of choice for this study.

Knowledge of germplasm diversity and relationships among breeding germplam has a significant impact on cultivar improvement (Hallauer et al. 1988). This information can be used for organizing germplasm, identification of cultivars, assisting the selection of parents for hybridization, identifying breeding bottlenecks and describing heterotic groups and patterns for crop species (Smith et al. 1990, 1992; Thormann et al. 1994; Mumm and Dudley 1994). AFLPs are successfully used to study genetic diversity and relationships in many crop species, for instance soybean (Powell et al. 1996; Maughan et al. 1996), barley (Ellis et al. 1997; Pakniyat et al. 1997), rice (Zhu et al. 1998, Aggarwal et al. 1999), lettuce (Hill et al. 1997); sunflower (Hongtrakul et al. 1997); and wheat (Barrett and Kidwell 1998). AFLP diversity and DNA fingerprinting are not described for meadowfoam. One of our aims was to gain an understanding of the pattern of genetic diversity and relationships among elite and exotic germplasm accessions of section Inflexae (chapter 2). AFLP markers were used to assess the genetic diversity of 41 accessions including nine inbred lines, eight open pollinated cultivars, and 24 wild populations of *Limnanthes* species. Polymorphic information content (PIC) was estimated for AFLP markers. Genetic distance between lines was estimated and subsequently used to construct a phenogram depicting the genetic relationships among meadowfoam germplam based on cluster analysis and principal component analysis.

The demand for meadowfoam (*Limnanthes alba* Benth.) oil is dramatically increasing along with the demand for new cultivars to boost seed yield and profits. One of our aims is to increase the supply of meadowfoam oil by increasing seed yield and oil content and developing new cultivars. This process can be facilitated using molecular breeding tools. A genetic linkage map has been useful for identifying and localizing gene controlling both simple and complex traits. AFLP linkage maps have been constructed for many crop

species for example barley; rice, peach, lentil, melon, and eucalyptus (Becker et al. 1995; Cho et al. 1996; Wang et al. 1997; Eujayl et al. 1998; Lu et al. 1998; Marques et al. 1998). Due to high multiplex ratio, AFLPs, therefore have been widely used for rapidly increasing the density of map in many crop species (Becker et al. 1995; Keim et al. 1997; Cho et al. 1998; Nandi et al.1997). A genetic linkage map for meadowfoam has not been developed. The second study (chapter 3) involves constructing genetic linkage map for meadowfoam (*L. alba*) using high throughput AFLP markers. A genetic linkage map was produced using inter-subspecific backcross progeny between *L. alba* spp. *alba* x *L. alba* spp. *versicolor*. The initial map described here promises to provide a framework for better understanding of the genome structure, to contribute a useful resource for genetic improvement, and to facilitate marker assisted selection in the meadowfoam breeding program.

The use of molecular markers has greatly simplified the genetic analysis of quantitative traits. Marker-based methods applied to segregating populations provide a means to locate quantitative trait loci (QTL) to chromosome regions and to estimate the effects of QTL allele substitution (Lander and Botstein 1989; Tanksley et al. 1989; Xiao et al. 1996). QTL mapping leads to an increased understanding of genes involved in the inheritance of quantitative traits, and may improve genetic gain in breeding programs through marker-assisted selection (Edwards et al. 1987; Tanksley 1993). The third study (chapter 4) involves mapping the *E* locus and QTL underlying fatty acid profile differences in meadowfoam seed oil. Since meadowfoam seed oil is of great industrial interest, knowledge of genes and QTLs underlying these fatty acids should facilitate cultivar improvement with desirable fatty acids fit to market need. *L. alba* subspecies have different wild type fatty acid profiles. *L. alba* spp. *versicolor* produces significantly more erucic acid (22:1Δ13) and significantly less dienoic acid (22:2 Δ5, Δ13) than *L. alba* spp. *alba*. The erucic and dienoic acid content differences between the subspecies are controlled by a dominant gene (*E*) (Knapp and Crane 1997).

Erucic and dienoic acid concentration differences are found among accessions within subspecies (Knapp and Crane 1995). Furthermore, erucic and dienoic acid concentrations vary continuously among *E_* and *ee* progeny in segregating populations. These differences could be caused by an allelic variant of the *E* locus or perhaps by quantitative trait loci (QTL). We used the genetic map of meadowfoam to map the *E* locus and search for quantitative loci (QTL) affecting erucic and dienoic acid content (chapter 4). The information about QTL detection in this study not only contributes to a better understanding of genetic control of very long-chain fatty acid profile differences in seed oils but also assists breeders in constructing allelic combinations for the development of superior genotypes for seed oil fatty acid in meadowfoam.

# CHAPTER 2

## DNA Fingerprinting in Meadowfoam: Analysis of the Genetic Diversity of Elite and Exotic Germplasm Accessions of Section Inflexae *Limnanthes*

Sureeporn Katengam, Jimmie M. Crane, and Steven J. Knapp

## ABSTRACT

DNA fingerprinting is widely used in crop plants and wild relatives for phylogenetic and biodiversity analysis. Such analysis has not been conducted in meadowfoam (*Limnanthes* spp.) We utilized amplified fragment length polymorphism (AFLP) markers to assess the genetic diversity of 41 meadowfoam accessions of Inflexae section of family Limnanthaceae including nine inbred lines, eight open-pollinated cultivars, and 24 wild populations and species. Our objectives were (1) to estimate polymorphic information content (PIC) for AFLP markers and genetic distance among germplasm, (2) to assess the pattern of genetic diversity of elite and exotic germplasm accessions using UPGMA cluster analysis and principal component analysis. Six AFLP primer pairs produced 176 polymorphic bands, with an average of 29.3 polymorphic bands per primer combination. The mean polymorphic information content (PIC) was 0.39. Forty-two percent of the markers showed high PIC scores between 0.45 and 0.5, indicating high diversity. Genetic distances estimated by Roger-W ranged from 0.14 to 0.55 with an average of 0.44. The UPGMA (unweighted pair group method on the basis of arithmetic averages) clustering phenogram based on the genetic distance matrix had a high cophenetic value indicating a good fit of the performed cluster analysis. The first three principal component analyses accounted for 37% of the total variation of the estimated genetic distance. Cluster analysis and principal component analysis separated meadowfoam germplasm into three diverse clusters. One was primarily comprised of *Limnanthes alba* spp. *alba*, another was comprised of *L. alba* spp. *versicolor*, and the other was primarily comprised of *L. floccosa* accessions. The patterns of diversity were concordant with species, subspecies, geographic, and breeding origin. Our results suggested that genetic diversity patterns of elite and exotic germplasm in Inflexae section is very diverse. This information

provides a valuable framework for meadowfoam improvement for enhancing the productivity and performance of cultivated meadowfoam.

## INTRODUCTION

Meadowfoam (*Limnanthes* spp.) is an annual oil seed crop, native to Southern Oregon and Northern California (Mason 1952; Kalin 1971; Jain 1986). Meadowfoam seed oil contains unique unsaturated very long-chain fatty acids ($C_{20}$ and $C_{22}$) with outstanding oxidative stability (Smith et al. 1960; Bagby et al. 1961; Miller et al. 1964; Knapp and Crane 1995, 1998; Isbell 1997). These unique characteristics of the seed oil are of industrial interest and have propelled the development of meadowfoam as an oilseed crop with successful marketing of a variety of specialty very long-chain fatty acids and triglycerides (Knapp and Crane 1999).

The genus *Limnanthes* comprises nine species belonging to the family Limnanthaceae (McNeill and Jain 1983; Jain 1986). Mason (1952) divided the genus into two sections, Inflexae and Reflexae, based on the morphological character of petals folding inward or outward during seed maturation. A variety of breeding systems are found in this genus ranging from almost complete self-pollination due to cleistogamy to highly outcrossed due to protandry (Mason 1952; Jain 1978; McNeill and Jain 1983, Kesseli and Jain 1985). All species are diploid (2x =2n = 10), hermaphroditic and self-compatible but protandry varies among species, which probably enhances outcrossing in many members of this genus (Kessili and Jain 1985).

Cultivated meadowfoam is based on *Limnanthes alba*, which belongs to section Inflexae. This section comprises of 4 species, namely *L. alba*, *L. floccosa*, *L. gracilis* and *L. montana*. The primary gene pool of *L. alba* is composed of *L. alba* spp. *alba* and *L. alba* spp. *versicolor,* whereas *L. floccosa*, *L. gracilis*, and *L. montana* are identified as a secondary gene pool of

*L. alba*. Based on the fertility of inter-subspecific and inter-specific hybridization with *L. alba*, the species belonging to section Reflexae including *L. bakeri*, *L. douglasii*, *L. macounii*, *L. striata and L. vinculans* are classified as the tertiary gene pool of *L. alba* (Knapp and Crane 1999).

Meadowfoam has been cultivated since 1973 (Jain 1986). *L. alba* was evaluated as the most promising species in this genus for its lower moisture requirements, adaptation to a wide range of environments, and high seed yield (Gentry and Miller 1965). The first non- shattering cultivar, Foamore, developed for commercial production was released in 1974 (Calhoun and Crane 1975). Breeding and cultivar development is underway at Oregon State University with the goals of increasing productivity of meadowfoam through superior cultivars, discovering and developing novel phenotypes, and advancing understanding of the genetics of economically important traits (Knapp and Crane 1999).

Genetic variation is the basis for crop improvement (Allard 1960). Knowledge of the genetic diversity and relationships among germplasm is essential to the improvement of crop plants (Hallauer et al. 1988). Genetic diversity of germplasm collections can be established from pedigree records, morphological traits, isozyme and DNA markers (Smith et al. 1990; Melchinger et al. 1994; Mumm and Dudley 1994). But the small number of polymorphic isozyme markers and unfavorable phenotypic expression of morphological traits due to environmental effects limit the utility of these markers (Smith et al. 1990; Stuber 1992; Dudley 1993; Melchinger et al. 1994; Staub et al. 1996). The isozyme markers often fail in classification or identification of breeding genotypes because of poor genome coverage (Smith et al. 1990; Dudley 1993; Melchinger et al. 1994; Bai et al. 1999). Pedigree records and DNA markers are successfully and widely used to classify germplasm and describe heterotic groups in many crop species (Smith and Smith 1991; Smith et al. 1992; Mumm and Dudley 1994; Hongtrakul et al. 1997; Cheres and Knapp 1998). The resulting information is useful in planning crosses for hybrids and

line development and in plant variety protection (Smith et al. 1990; Smith and Smith 1991; Graner et al. 1994; Kisha et al. 1998; Pejic et al. 1998). Nonetheless, pedigree data are usually biased since they do not account for the effects of selection, mutation and random genetic drift. Moreover, they are sometimes unavailable or erroneous (Smith and Smith 1991; Melchinger et al. 1994). In contrast to pedigree data, molecular markers can provide accurate evaluation of genetic diversity since they not only allow direct comparison of genotypes at the DNA level but also provide a more complete sampling of genome coverage (Smith et al. 1990; Dudley 1993). DNA markers can reveal tremendous numbers of genetic loci which are phenotypically neutral and not subject to environmental effects. Polymorphic DNA markers are highly informative and superior to traditional estimation, such as morphological traits, in resolving genetic differences (Tanksley et al. 1989 and Stuber 1992).

A variety of molecular markers are applied to cultivar improvement and germplasm management. Because a large number of marker loci can be developed in a short period of time, AFLP (Amplified fragment length polymorphism) is the leading DNA based-marker system (Vos et al. 1995; Hill et al 1997; Powell et al. 1996). AFLP is a bi-allelic and multilocus PCR-based marker that uses selective amplification of restriction fragments. Polymorphisms are visualized as the presence or absence of amplified restriction fragments and are therefore dominant. Useful features of AFLP markers for plant genome analysis are (1) high marker abundance, (2) efficient genome coverage, (3) relatively high multiplex ratio (described as number of loci simultaneously analyzed per assay), (4) high reproducibility, (5) no requirement for prior sequence information, and (6) small amount of DNA required (Vos et al. 1995; Maughan et al. 1996; Powell et al. 1996; Hill et al. 1997; Zhu et al. 1998; Bai et al. 1999; Knapp et al. 1999; Hansen et al. 1999). AFLPs are successfully utilized for DNA and RNA fingerprinting, genetic mapping, and marker assisted plant breeding (Cervera et al. 1996; Cho et al. 1996,1998; Cnops et al. 1996; Mackill et al. 1996; Hill et al. 1997; Hongtrakul

et al. 1997; Nandi et al. 1997; Wang et al. 1997; Lu et al. 1998; Qi et al. 1998). Moreover, AFLPs are powerful tools for evaluating genetic diversity and determining the relationships within and among species in many plants such as soybean (Powell et al. 1996; Maughan et al. 1996), barley (Ellis et al. 1997; Pakniyat et al. 1997), rice (Zhu et al. 1998, Aggarwal et al. 1999), lettuce (Hill et al. 1997); sunflower (Hongtrakul et al. 1997), cassava (Roa et al. 1997), tea (Paul et al. 1997), yam (Mignouna et al. 1998), wheat (Barrett and Kidwell 1998), tef (Bai et al. 1999), and olive (Angiolillo et al. 1999). Due to the large number of polymorphisms that can be screened per assay, AFLPs are the markers of choice for saturation of particular genomic regions necessary for map-based cloning of economically important genes (Thomas et al. 1995; Cnops et al. 1996; Cho et al. 1996).

Biosystematic surveys of genetic resources and agronomic evaluation for domestication of meadowfoam were initiated in 1973. Genetic variability and differentiation among natural populations of meadowfoam including phylogenetic studies in the genus *Limnanthes* were determined using electrophoretic (allozyme), morphological, and hybrid fertility data (Ornduff and Crovello 1968; Ornduff 1971; Arroyo 1973,1975; Parker 1976; McNeill and Jain 1983). Nonetheless, the genetic diversity among elite and exotic germplasm accessions of section Inflexae has not been described. In the present study, we present the first report of DNA fingerprinting of elite and exotic germplasm in Inflexae section and evaluation of their genetic relationships using AFLP markers. Our objectives were (1) to estimate the polymorphic information content (PIC) of AFLP markers and estimate genetic distance among inbred lines, open-pollinated cultivars, wild populations, and all genotypes, and (2) to assess the patterns of genetic diversity and relationships of elite and exotic germplasm using UPGMA cluster analysis and principal component analysis.

# MATERIALS AND METHODS

## Plant Materials

One hundred and three samples representing 41 accessions including nine inbred lines, eight open-pollinated cultivars, and 24 wild populations of meadowfoam germplasm in Inflexae section were included in this diversity study (Table 2.1). Sampling strategies were employed differently among inbred lines and open-pollinated cultivars and wild populations. For each inbred line, ten plants were grown and approximately equal amounts of leaf tissue were bulked for DNA extraction. For open-pollinated cultivars and wild meadowfoam populations, several individuals were planted and three individuals were randomly chosen from each population. Leaf tissues from these three individuals were collected separately for DNA extraction. There were two accessions, PI 420137 and Mermaid, that had two samples. PI 420137 had only two viable seeds. For Mermaid, out of three DNA samples, one was excluded since it was poorly amplified by AFLP primer combinations.

**Table 2.1** Meadowfoam germplasm (41 accessions) for AFLP fingerprinting

| Accession | Description | |
|---|---|---|
| | Type | Source |
| OMF63 $S_5$ | Self-pollinated inbred line | Selected from OMF159 |
| OMF64 $S_5$ | Self-pollinated inbred line | Selected from OMF160 |
| OMF66 $S_5$ | Self-pollinated inbred line | Selected from OMF66 |
| OMF109-1 | Self-pollinated inbred line | Selected from Mermaid x OMF62/ OMF64) |
| OMF109-2 | Self-pollinated inbred line | Selected from Mermaid x OMF62/ OMF64 |
| OMF109-3 | Self-pollinated inbred line | Selected from Mermaid x OMF62/ OMF64) |
| OMF40-11 (Mermaid $S_5$) | Insect-pollinated *L. alba* spp. *alba* inbred line | Selected from PI 283703 |

**Table 2.1** Continued

| Accession | Description | |
|---|---|---|
| | Type | Source |
| LAG109 $F_4$ | Self-pollinated inbred line | Mermaid x *L. gracilis* spp. *parishii* |
| LAG111 $F_4$ | Self-pollinated inbred line | Mermaid x *L. gracilis* spp. *parishii* |
| OMF66 (Redding) | Wild population | *L. alba* spp. *versicolor* |
| OMF 158 | Wild population | *L. alba* spp. *versicolor* (Recollected PI 283705) |
| OMF159 | Wild population | *L. alba* spp. *versicolor* (Recollected PI 374791) |
| OMF160 | Wild population | *L. alba* spp. *versicolor* (Recollected PI 374801) |
| OMF161 | Wild population | *L. alba* spp. *versicolor* (Recollected PI 374802) |
| OMF57 | Wild population | *L. alba* spp. *versicolor* (UC328 or UC457) |
| OMF52 | Wild population | *L. alba* spp. *alba* (UC- Calaveras) |
| OMF53 | Wild population | *L. alba* spp. *alba* (UC-Sonoma) |
| PI 374792 | Wild population | *L. alba* spp. *alba* (Shasta county) |
| PI 374793 | Wild population | *L. alba* spp. *alba* (Placer county) |
| PI 374794 | Wild population | *L. alba* spp. *alba* (Placer county) |
| PI 374795 | Wild population | *L. alba* spp. *alba* (Placer county) |
| PI 374796 | Wild population | *L. alba* spp. *alba* (Butte county) |
| PI 374797 | Wild population | *L. alba* spp. *alba* (Butte county) |
| PI 374798 | Wild population | *L. alba* spp. *alba* (Butte county) |
| PI 367900 | Wild population | *L. alba* spp. *alba* (Sacremento county) |
| Foamore | Open-pollinated cultivar | Selected from PI 283704 |
| Mermaid | Open-pollinated cultivar | Selected from PI 283703 |

**Table 2.1** Continued

| Accession | Description | |
|---|---|---|
| | Type | Source |
| Floral | Open-pollinated cultivar | Mermaid x *L. floccosa* spp. *grandiflora* |
| Knowles (OMF69) | Open-pollinated cultivar | Selected from bulk of *L. alba* spp. *alba* |
| OMF86 | Open-pollinated cultivar | Selected from Knowles |
| OMF78 | Open-pollinated cultivar | Selected from intermating between *L. alba* spp. *alba* and spp. *versicolor*) |
| OMF87 | High oil open-pollinated population | Selected from OMF62 |
| OMF62-29 | High oil open-pollinated population | *L. alba* spp. *alba* |
| PI 283724 | Wild species | *L. gracilis* spp. *parishii* |
| PI 420137 | Wild species | *L. gracilis* spp. *gracilis* |
| PI 283720 | Wild species | *L. floccosa* spp. *bellingeriana* |
| PI 420133 | Wild species | *L. floccosa* spp. *grandiflora* |
| PI 283719 | Wild species | *L. floccosa* spp. *floccosa* |
| OSU-LF-4 | Wild species | *L. floccosa* spp. *californica* |
| PI 283721 | Wild species | *L. floccosa* spp. *pumila* |
| PI 283725 | Wild species | *L. montana* |

Meadowfoam seeds were germinated at 4°C on moist blotter paper in covered 11- by 11- by 3- cm plastic boxes as described in Knapp and Crane (1998). Seedlings were transplanted to potting soil (pumice: peat moss: sandy loam) in 7.5 x 7.5 cM plastic pot and grown in a growth chamber (Model CEL 37-14, Sherer-Gillett Co., Marshall, MI) for 25 to 28 days at 15°C with 8 h of fluorescent light. These plants were subsequently grown in a greenhouse at 22°C with 16 h fluorescent light for two weeks and then young leaves were harvested and frozen at -80°C prior to DNA extraction.

## DNA Extraction

Genomic DNA was extracted from frozen tissue according to Lodhi et al. (1994) with minor modification. One to two grams of frozen tissues were ground in the presence of liquid nitrogen and incubated with 2% CTAB (cityltrimetrylammonium bromide) extraction buffer for 1 h at 65°C. Chloroform extraction was carried out once and the aqueous phase was transferred and mixed with 0.5 volume of 5M NaCl, precipitated with 2 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. After dissolving, DNA was treated with Rnase (10 μg /μl) for 1 h at 37°C.

## AFLP Analysis

AFLP analysis was carried out essentially as developed by Keygene (Waeningen, NL) (Zabeau and Vos 1993) with minor modification that the selection of a subset of fragments on steptavidin beads was omitted (Vos et al. 1995). Genomic DNA samples (500 ng) were digested with 5 units of *EcoR*I (rare 6-base cutter) and 5 units of *Mse*I (frequent 4-base cutter) (New England Biolab, Schwalbach, Germany) in a reaction volume of 50 ml in 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 *EcoR*I adapter (5 pmol/ml), 1 μl *Mse*I adapter (50 pmol/ml), 5U *EcoR*I, 5U *Mse*I, 1.2 ml 10 mM ATP, 1 μl 10x RL buffer, and 1U T4 DNA ligase (New England Biolabs) was added to the solution and incubated for 3 h at 37°C. Twelve μl of restriction ligation products were electrophoresed on a 1% agarose gel to ensure that the DNA had been completely digested.

Two consecutive amplifications (preamplification and selective amplification) were performed in a PE-9600 DNA thermocycler (Perkin Elmer Corp., San Francisco, CA., USA). Preamplification utilized primers complementary to the adaptor-ligated DNA fragments with a single selective nucleotide added at the 3' end of the PCR primers, *EcoR*I+A and *Mse*I+C (Table 2.2). Thirty cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s were used. Twelve microliters of the total 50 µl PCR products were electrophoresed on 1% agarose gel to determine that DNA preamplification was successful. The preamplification products were then diluted ten-fold in 0.1% TE buffer to be used as template for the selective amplification with radioactive labeling.

Selective PCR amplification was carried out with primers complementary to the adaptor-ligated DNA fragments and having three selective nucleotides added at the 3' end of the PCR primers (Table 2.2). *EcoR*I primers were end-labeled with $\gamma$-$^{33}$P using T4 polynucleotide kinase (New England Biolabs, Inc., Bervely, Mass.). A touch down cycle profile was performed with the first cycle at 94°C for 30 s, 65°C for 30 s and 72°C for 60 s, followed by 11cycles in which the annealing temperature decreased 0.7°C per cycle, and finally followed by 24 cycles with the annealing temperature at 56°C. A total of six primer combinations were used in this study (Table 2.2).

The selective radioactive PCR products were mixed with an equal volume of formamide dye (98% formamide, 10 mM EDTA pH 8.0, 0.025% bromo phenol blue and 0.025% xylene cyanol as tracking dye), denatured at 94°C for 3 min and then quickly cooled on ice. Eight microliters of each sample were loaded into pre-warmed 6% denaturing polyacrylamide gel and 1X TBE running buffer. The gel was run at 60 Watts of constant power for 2 h, transferred to chromatography paper (3MM) (Fisher Scientific, Pittsburgh, PA, USA), dried on a gel dryer (Fisher Biotech, Fisher Scientific, Pittsburgh, PA) under vacuum for 2 h at 80°C, and then exposed to standard X-ray film for 3 to 7 days. AFLP bands were visually scored from autoradiographs.

**Table 2.2** Oligonucleotide adapters and AFLP primers used for meadowfoam DNA fingerprinting.

| Adaptors or Primers | Name | Sequence |
|---|---|---|
| *EcoR*I Adaptors | 91M35 | 5'-CTCGTAGACTGCGTACC-3' |
| | 91M36 | 3'-CTGACGCATGGTTAA-5' |
| *Mse*I Adaptors | 92A18 | 5'-GACGATGAGTCCTGAG-3' |
| | 92A19 | 3'-TACTCAGGACTCAT-5' |
| *EcoR*I +1 Primer | 92R11 | 5'-AGACTGCGTACCAATTC/A-3' |
| *Mse*I +1 Primer | 92H20 | 5'-GACGATGAGTCCTGAGTAA/C-3' |
| *EcoR*I +3 Primers | 92SO5 | 5'-GACTGCGTACCAATTC/ACA-3' |
| *Mse*I +3 Primers | 92G23 | 5'-GATGAGTCCTGAGTAA/CAG-3' |
| | 92G24 | 5'-GATGAGTCCTGAGTAA/CAT-3' |
| | 92G29 | 5'-GATGAGTCCTGAGTAA/CTG-3' |
| | 92G30 | 5'-GATGAGTCCTGAGTAA/CTC-3' |
| | 92F10 | 5'-GATGAGTCCTGAGTAA/CAC-3' |
| | 92F41 | 5'-GATGAGTCCTGAGTAA/CAA-3' |

**Data Analysis**

Heterozygosities were used to refer to the relative value of each marker with respect to the degree of polymorphism exhibited for each polymorphic locus. Heterozygosities were estimated as:

$$H = 1 - \sum_{i=1}^{k} p_i^2$$

where $p_i$ is the frequency of $i^{th}$ allele and $k$ is the number of alleles (Ott 1991). This heterozygosity value is essentially the same as polymorphic information content (PIC) which was described by Botstein et al. (1980). Due to a bi-allelic

feature, the PIC value for AFLP markers therefore ranges from 0.0 (monomorphic) to 0.5 (polymorphic).

Binary data representing the presence (1) and absence (0) of specific AFLP marker was generated. Only unambiguous polymorphic bands were scored and entered into a binary matrix as input for the genetic distance analysis. The genetic distance of Roger as modified by Wright (1978) was estimated among all genotypes using NTSYS-pc (Numerical taxonomy and Multivariate Analysis System), version 1.8 (Rohlf 1993). A phenogram was subsequently generated by cluster analysis based on the unweighted pair group method on the basis of arithmetic averages (UPGMA) using a genetic distance matrix (Sneath and Sokal 1973). Goodness of fit of a cluster analysis was tested using the cophenetic correlation (r) value (Mantel 1967) from MXCOMP program in NTSYS, which allows direct comparison between the original dissimilarity matrix that was clustered and the cophenetic value matrix. Principal component analysis based on genetic distance matrix was carried out using the PROC PRINCOMP procedure of SAS (1996) (SAS Institute, Inc., Cary, NC) to visualize the dispersion of individuals in relation to the first three principal axes of variation.

## RESULTS

### AFLP Fingerprinting

The AFLP fingerprinting was performed using 6 primer combinations (Table 2.2) on the 103 meadowfoam samples of 41 accessions of Inflexae section including nine inbred lines, eight open-pollinated cultivars, and 24 wild population of four species (10 taxa) (Table 2.1). These primer combinations were chosen based on previous information of polymorphic rate from screening parents for an AFLP meadowfoam mapping study (Katengam et al.

1999). Each pair of primers produced a large number of polymorphic bands but only clear polymorphic markers across all accessions were included in this study. The six primer combinations revealed 176 AFLP markers, which were polymorphic in at least two or more accessions across the 41 meadowfoam accessions (Table 2.3). The number of polymorphic markers varied from 18 to 40 markers per primer pair with an average of 29.33 markers (Table 2.3). The size of markers ranged from 50 to 250 bp. Out of 176 AFLP markers, 142 were polymorphic in at least two inbred lines, 138 in two open-pollinated cultivars, and 175 AFLP markers were polymorphic in at least two wild populations.

**Table 2.3** Total number of informative AFLP markers detected with six primer combinations (one *EcoR*I primer and six *Mse*I primers) used in DNA fingerprinting of 41 meadowfoam accessions of Inflexae section.

| Primer combinations | | Total polymorphic AFLP markers |
|---|---|---|
| *EcoR*I | *Mse*I | |
| ACA | CTC | 28 |
| ACA | CAG | 38 |
| ACA | CTG | 40 |
| ACA | CAC | 32 |
| ACA | CAA | 18 |
| ACA | CAT | 20 |
| Total | | 176 |
| Average | | 29.33 |

## Genetic Diversity

Estimation of genetic diversity in meadowfoam germplasm was represented by polymorphic information content (PIC) value. One individual from each accession of wild and cultivated meadowfoam populations was sampled, and PIC scores were calculated across 41 accessions. The PIC scores for 176 AFLP markers ranged from 0.0 to 0.5 (Fig. 2.1). Mean PIC scores were 0.31 for inbred lines (142 AFLP markers), 0.30 for open-pollinated cultivars (138 AFLP markers), 0.40 for wild populations (175 AFLP markers), and 0.39 for all genotypes (176 AFLP markers). Forty-two percent of the markers showed maximum PIC scores ranging from 0.45 to 0.50.

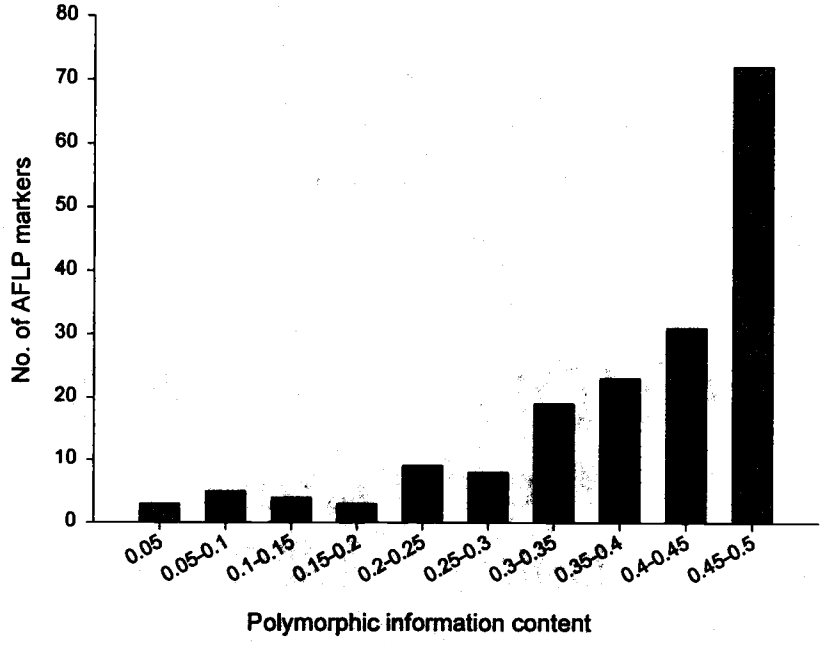


**Figure 2.1** Distribution of polymorphic information content for 176 AFLP markers among 41 meadowfoam accessions including nine inbred lines, eight open-pollinated cultivars, and 24 wild populations and species.

## Distance Analysis

Genetic distance among 41accessions based on 176 AFLP markers was estimated using Rogers genetic distance as modified by Wright (1978), and ranged from 0.14 to 0.55, with an average of 0.44 (Table 2.4). PI 283719 (*L. floccosa* spp. *floccosa*) and OMF159 (*L. alba* spp. *versicolor*) were most distantly related while OMF109-1 and OMF109-3 were closely related. The distance estimated among nine inbred lines varied from 0.14 (between OMF109-1 and OMF109-3) to 0.47 (between LAG109$F_4$ and OMF109-1, OMF109-2, and OMF109-3) with an average of 0.39 (Table 2.4). Among the eight open-pollinated cultivars the distance estimated varied from 0.34 to 0.46 with an average of 0.40 (Table 2.4). The greatest distance was found between Foamore and OMF62-29 (0.46), whereas the least distance (close relationship) was found between OMF86 and Knowles (0.34) and OMF86 and OMF78 (0.34).

Among 24 wild meadowfoam populations including four species (*L. alba*, *L. floccosa*, *L gracilis*, *and L. motana*) and 10 taxa, the greatest distance (0.55) was found between OMF159 (*L. alba* spp. *versicolor*) and PI 283719 (*L. floccosa* spp. *floccosa*). The least distance (0.32) was found between two wild populations of *L. alba* spp.*versicolor* (OMF66 and OMF158) (Table 2.4). The average genetic distance among wild populations was 0.45, indicating high genetic diversity in these wild populations.

## Cluster Analysis

Cluster analysis using UPGMA (Unweighted pair group method based on arithmetic mean) was performed to estimate the genetic relationships among meadowfoam germplasm. A phenogram was produced from the UPGMA cluster analysis of genetic distance matrix for 41 accessions (Fig. 2.2) based

**Table 2.4** Genetic distance matrix estimated by Roger-W from AFLP fingerprints of 41 meadowfoam accessions

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. OMF63$S_5$ | 0 | | | | | | | | | | | | | | | | | | | |
| 2..OMF64$S_5$ | 0.31 | 0 | | | | | | | | | | | | | | | | | | |
| 3. OMF66$S_5$ | 0.33 | 0.44 | 0 | | | | | | | | | | | | | | | | | |
| 4 OMF109-1 | 0.37 | 0.39 | 0.46 | 0 | | | | | | | | | | | | | | | | |
| 5 OMF109-2 | 0.37 | 0.38 | 0.46 | 0.25 | 0 | | | | | | | | | | | | | | | |
| 6 OMF109-3 | 0.36 | 0.39 | 0.45 | 0.14 | 0.27 | 0 | | | | | | | | | | | | | | |
| 7 OMF40-11 | 0.35 | 0.43 | 0.42 | 0.42 | 0.43 | 0.43 | 0 | | | | | | | | | | | | | |
| 8 LAG109$F_4$ | 0.41 | 0.44 | 0.45 | 0.47 | 0.47 | 0.47 | 0.38 | 0 | | | | | | | | | | | | |
| 9 LAG111$F_4$ | 0.35 | 0.42 | 0.40 | 0.44 | 0.44 | 0.44 | 0.42 | 0.30 | 0 | | | | | | | | | | | |
| 10 OMF66 | 0.31 | 0.41 | 0.32 | 0.42 | 0.43 | 0.43 | 0.41 | 0.45 | 0.42 | 0 | | | | | | | | | | |
| 11 OMF158 | 0.30 | 0.38 | 0.35 | 0.42 | 0.42 | 0.42 | 0.41 | 0.43 | 0.38 | 0.32 | 0 | | | | | | | | | |
| 12 OMF159 | 0.33 | 0.41 | 0.39 | 0.42 | 0.42 | 0.42 | 0.40 | 0.47 | 0.41 | 0.37 | 0.35 | 0 | | | | | | | | |
| 13 OMF160 | 0.32 | 0.38 | 0.38 | 0.41 | 0.42 | 0.41 | 0.41 | 0.46 | 0.42 | 0.35 | 0.37 | 0.34 | 0 | | | | | | | |
| 14 OMF161 | 0.32 | 0.38 | 0.38 | 0.37 | 0.37 | 0.36 | 0.44 | 0.47 | 0.41 | 0.34 | 0.34 | 0.38 | 0.38 | 0 | | | | | | |
| 15 OMF57 | 0.43 | 0.49 | 0.47 | 0.47 | 0.47 | 0.49 | 0.43 | 0.44 | 0.44 | 0.47 | 0.45 | 0.46 | 0.47 | 0.46 | 0 | | | | | |
| 16 OMF52 | 0.43 | 0.49 | 0.45 | 0.49 | 0.48 | 0.50 | 0.42 | 0.47 | 0.44 | 0.47 | 0.47 | 0.48 | 0.48 | 0.48 | 0.42 | 0 | | | | |
| 17 OMF53 | 0.38 | 0.44 | 0.43 | 0.46 | 0.46 | 0.46 | 0.40 | 0.47 | 0.44 | 0.42 | 0.43 | 0.44 | 0.42 | 0.43 | 0.47 | 0.44 | 0 | | | |
| 18 PI 374793 | 0.38 | 0.46 | 0.39 | 0.45 | 0.46 | 0.45 | 0.33 | 0.41 | 0.38 | 0.41 | 0.41 | 0.43 | 0.38 | 0.41 | 0.44 | 0.44 | 0.41 | 0 | | |
| 19 PI 374794 | 0.39 | 0.49 | 0.39 | 0.45 | 0.44 | 0.46 | 0.42 | 0.47 | 0.43 | 0.41 | 0.43 | 0.45 | 0.43 | 0.43 | 0.47 | 0.44 | 0.44 | 0.36 | 0 | |
| 20 PI 374795 | 0.40 | 0.47 | 0.45 | 0.50 | 0.46 | 0.49 | 0.41 | 0.45 | 0.45 | 0.44 | 0.47 | 0.46 | 0.43 | 0.44 | 0.49 | 0.47 | 0.46 | 0.42 | 0.40 | 0 |
| 21 PI 374796 | 0.38 | 0.46 | 0.41 | 0.47 | 0.45 | 0.48 | 0.41 | 0.46 | 0.42 | 0.40 | 0.41 | 0.43 | 0.41 | 0.43 | 0.45 | 0.48 | 0.46 | 0.34 | 0.38 | 0.39 |
| 22 PI 374797 | 0.37 | 0.43 | 0.40 | 0.46 | 0.44 | 0.47 | 0.40 | 0.45 | 0.41 | 0.38 | 0.40 | 0.42 | 0.39 | 0.43 | 0.45 | 0.46 | 0.40 | 0.38 | 0.38 | 0.43 |
| 23 PI 374798 | 0.37 | 0.40 | 0.42 | 0.46 | 0.45 | 0.46 | 0.42 | 0.47 | 0.44 | 0.40 | 0.40 | 0.40 | 0.39 | 0.43 | 0.49 | 0.46 | 0.43 | 0.40 | 0.42 | 0.42 |
| 24 PI 367900 | 0.38 | 0.47 | 0.43 | 0.46 | 0.45 | 0.47 | 0.43 | 0.48 | 0.45 | 0.44 | 0.44 | 0.46 | 0.44 | 0.44 | 0.47 | 0.43 | 0.40 | 0.40 | 0.41 | 0.45 |
| 25 PI 374792 | 0.38 | 0.42 | 0.40 | 0.42 | 0.43 | 0.42 | 0.41 | 0.46 | 0.42 | 0.40 | 0.38 | 0.36 | 0.37 | 0.39 | 0.46 | 0.43 | 0.43 | 0.41 | 0.41 | 0.47 |
| 26 Foamore | 0.37 | 0.45 | 0.40 | 0.46 | 0.46 | 0.45 | 0.43 | 0.49 | 0.43 | 0.44 | 0.41 | 0.41 | 0.38 | 0.45 | 0.49 | 0.44 | 0.44 | 0.38 | 0.40 | 0.42 |
| 27 Mermaid | 0.40 | 0.46 | 0.43 | 0.49 | 0.48 | 0.49 | 0.37 | 0.48 | 0.42 | 0.46 | 0.43 | 0.44 | 0.42 | 0.46 | 0.46 | 0.45 | 0.43 | 0.40 | 0.41 | 0.44 |
| 28 Floral | 0.38 | 0.43 | 0.44 | 0.45 | 0.43 | 0.46 | 0.37 | 0.41 | 0.38 | 0.42 | 0.42 | 0.40 | 0.39 | 0.45 | 0.46 | 0.42 | 0.42 | 0.40 | 0.45 | 0.44 |
| 29 Knowles | 0.40 | 0.39 | 0.44 | 0.44 | 0.43 | 0.45 | 0.40 | 0.47 | 0.43 | 0.44 | 0.43 | 0.44 | 0.40 | 0.43 | 0.46 | 0.43 | 0.43 | 0.40 | 0.44 | 0.42 |
| 30 OMF86 | 0.37 | 0.39 | 0.42 | 0.41 | 0.43 | 0.42 | 0.38 | 0.46 | 0.45 | 0.41 | 0.41 | 0.42 | 0.37 | 0.42 | 0.47 | 0.47 | 0.40 | 0.40 | 0.46 | 0.44 |
| 31 OMF78 | 0.35 | 0.40 | 0.43 | 0.39 | 0.41 | 0.40 | 0.40 | 0.49 | 0.45 | 0.41 | 0.40 | 0.40 | 0.41 | 0.40 | 0.46 | 0.46 | 0.42 | 0.39 | 0.43 | 0.47 |
| 32 OMF87 | 0.36 | 0.41 | 0.43 | 0.42 | 0.42 | 0.42 | 0.39 | 0.47 | 0.43 | 0.40 | 0.41 | 0.41 | 0.41 | 0.43 | 0.49 | 0.46 | 0.43 | 0.41 | 0.44 | 0.44 |
| 33 PI 283724 | 0.47 | 0.51 | 0.49 | 0.49 | 0.48 | 0.50 | 0.47 | 0.46 | 0.44 | 0.52 | 0.48 | 0.49 | 0.50 | 0.49 | 0.45 | 0.48 | 0.45 | 0.47 | 0.47 | 0.51 |
| 34 PI 420137 | 0.38 | 0.47 | 0.44 | 0.46 | 0.47 | 0.46 | 0.41 | 0.45 | 0.41 | 0.46 | 0.43 | 0.42 | 0.43 | 0.43 | 0.49 | 0.47 | 0.44 | 0.38 | 0.46 | 0.44 |
| 35 PI 283720 | 0.47 | 0.52 | 0.49 | 0.49 | 0.50 | 0.50 | 0.48 | 0.48 | 0.46 | 0.53 | 0.51 | 0.54 | 0.52 | 0.52 | 0.47 | 0.46 | 0.47 | 0.49 | 0.45 | 0.49 |
| 36 PI 420133 | 0.46 | 0.52 | 0.51 | 0.50 | 0.52 | 0.51 | 0.47 | 0.50 | 0.46 | 0.51 | 0.49 | 0.52 | 0.50 | 0.53 | 0.48 | 0.48 | 0.49 | 0.51 | 0.47 | 0.49 |
| 37 PI 283719 | 0.46 | 0.52 | 0.51 | 0.50 | 0.51 | 0.49 | 0.49 | 0.46 | 0.45 | 0.49 | 0.49 | 0.55 | 0.51 | 0.52 | 0.48 | 0.48 | 0.50 | 0.49 | 0.47 | 0.49 |
| 38 OSU-LF$_4$ | 0.47 | 0.52 | 0.50 | 0.48 | 0.49 | 0.48 | 0.48 | 0.51 | 0.48 | 0.52 | 0.52 | 0.52 | 0.51 | 0.52 | 0.48 | 0.49 | 0.51 | 0.49 | 0.46 | 0.51 |
| 39 PI 283721 | 0.45 | 0.49 | 0.46 | 0.46 | 0.46 | 0.46 | 0.46 | 0.49 | 0.45 | 0.47 | 0.46 | 0.49 | 0.47 | 0.47 | 0.47 | 0.45 | 0.51 | 0.48 | 0.45 | 0.49 |
| 40 PI 283725 | 0.38 | 0.41 | 0.43 | 0.46 | 0.45 | 0.45 | 0.43 | 0.45 | 0.40 | 0.46 | 0.39 | 0.38 | 0.44 | 0.40 | 0.46 | 0.47 | 0.41 | 0.42 | 0.46 | 0.44 |
| 41 OMF6229 | 0.39 | 0.42 | 0.44 | 0.43 | 0.42 | 0.44 | 0.39 | 0.45 | 0.42 | 0.46 | 0.47 | 0.45 | 0.45 | 0.46 | 0.47 | 0.44 | 0.44 | 0.45 | 0.45 | 0.47 |

**Table 2.4** Continued

| | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 20 PI 374795 | 0 | | | | | | | | | | | | | | | | | | | | | |
| 21 PI 374796 | 0.39 | 0 | | | | | | | | | | | | | | | | | | | | |
| 22 PI 374797 | 0.43 | 0.35 | 0 | | | | | | | | | | | | | | | | | | | |
| 23 PI 374798 | 0.42 | 0.37 | 0.39 | 0 | | | | | | | | | | | | | | | | | | |
| 24 PI 367900 | 0.45 | 0.43 | 0.41 | 0.39 | 0 | | | | | | | | | | | | | | | | | |
| 25 PI 374792 | 0.47 | 0.42 | 0.41 | 0.40 | 0.40 | 0 | | | | | | | | | | | | | | | | |
| 26 Foamore | 0.42 | 0.40 | 0.40 | 0.35 | 0.40 | 0.40 | 0 | | | | | | | | | | | | | | | |
| 27 Mermaid | 0.44 | 0.43 | 0.42 | 0.43 | 0.44 | 0.42 | 0.41 | 0 | | | | | | | | | | | | | | |
| 28 Floral | 0.44 | 0.43 | 0.42 | 0.41 | 0.42 | 0.42 | 0.41 | 0.41 | 0 | | | | | | | | | | | | | |
| 29 Knowles | 0.42 | 0.43 | 0.42 | 0.39 | 0.43 | 0.38 | 0.41 | 0.39 | 0.38 | 0 | | | | | | | | | | | | |
| 30 OMF86 | 0.44 | 0.44 | 0.41 | 0.38 | 0.42 | 0.40 | 0.44 | 0.38 | 0.39 | 0.34 | 0 | | | | | | | | | | | |
| 31 OMF78 | 0.47 | 0.43 | 0.43 | 0.39 | 0.43 | 0.41 | 0.40 | 0.41 | 0.36 | 0.40 | 0.34 | 0 | | | | | | | | | | |
| 32 OMF87 | 0.44 | 0.43 | 0.40 | 0.35 | 0.42 | 0.40 | 0.42 | 0.40 | 0.41 | 0.40 | 0.38 | 0.37 | 0 | | | | | | | | | |
| 33 PI 283724 | 0.51 | 0.47 | 0.49 | 0.48 | 0.45 | 0.46 | 0.46 | 0.46 | 0.47 | 0.48 | 0.47 | 0.49 | 0.49 | 0 | | | | | | | | |
| 34 PI 420137 | 0.44 | 0.43 | 0.43 | 0.39 | 0.42 | 0.42 | 0.38 | 0.41 | 0.42 | 0.40 | 0.43 | 0.41 | 0.40 | 0.47 | 0 | | | | | | | |
| 35 PI 283720 | 0.49 | 0.51 | 0.49 | 0.49 | 0.46 | 0.49 | 0.52 | 0.49 | 0.51 | 0.50 | 0.48 | 0.52 | 0.50 | 0.44 | 0.49 | 0 | | | | | | |
| 36 PI 420133 | 0.49 | 0.49 | 0.49 | 0.49 | 0.49 | 0.49 | 0.49 | 0.45 | 0.50 | 0.51 | 0.50 | 0.51 | 0.48 | 0.48 | 0.50 | 0.40 | 0 | | | | | |
| 37 PI 283719 | 0.49 | 0.50 | 0.48 | 0.51 | 0.51 | 0.50 | 0.51 | 0.48 | 0.52 | 0.51 | 0.50 | 0.51 | 0.49 | 0.48 | 0.51 | 0.32 | 0.41 | 0 | | | | |
| 38 OSU-LF$_4$ | 0.51 | 0.52 | 0.52 | 0.50 | 0.49 | 0.48 | 0.51 | 0.48 | 0.53 | 0.49 | 0.50 | 0.52 | 0.49 | 0.49 | 0.48 | 0.39 | 0.34 | 0.41 | 0 | | | |
| 39 PI 283721 | 0.49 | 0.47 | 0.46 | 0.48 | 0.48 | 0.44 | 0.47 | 0.46 | 0.48 | 0.47 | 0.49 | 0.49 | 0.45 | 0.49 | 0.49 | 0.44 | 0.33 | 0.43 | 0.33 | 0 | | |
| 40 PI 283725 | 0.44 | 0.44 | 0.42 | 0.41 | 0.44 | 0.39 | 0.41 | 0.40 | 0.43 | 0.40 | 0.40 | 0.42 | 0.38 | 0.46 | 0.39 | 0.52 | 0.51 | 0.52 | 0.49 | 0.48 | 0 | |
| 41 OMF6229 | 0.47 | 0.49 | 0.46 | 0.45 | 0.46 | 0.44 | 0.46 | 0.44 | 0.41 | 0.42 | 0.43 | 0.42 | 0.40 | 0.47 | 0.47 | 0.49 | 0.49 | 0.49 | 0.47 | 0.45 | 0.43 | 0 |

on mean AFLP data from each accession and from genetic distance matrix of 103 samples from 41 accessions (Fig. 2.3). There were three major diverse clusters. The largest cluster was primarily comprised of *L. alba* spp. *alba*, with two distinct subclusters. The first subcluster included wild populations of *L. alba* spp. *alba* and one inbred line of OMF40-11 (Mermaid $S_5$), which was derived from *L. alba* spp. *alba* (PI 283703). The wild populations included PI 374793, PI 374794, PI 374795, PI 374796, and PI 374797. They were collected from a geographically continuous area (Table 2.1). The second subcluster included all elite germplasm (open-pollinated cultivars) and three wild populations of *L. alba* spp. *alba* (PI 374798), *L. gracilis* spp. *gracilis* (PI 420137), and *L. montana* (PI 283725). Even though these elite germplasm were grouped together, genetic distances between accessions was high, ranging from 0.34 to 0.42. All open-pollinated cultivars were derived from an *L. alba* spp. *alba*, except Floral which was derived from intersubspecific cross between *L. alba* spp. *alba* (Mermaid) and *L. floccosa* spp. *grandiflora* (Joliff 1994).

The second cluster was comprised of *L. alba* spp. *versicolor*. Wild populations of *L. alba* spp. *versicolor* as well as inbred lines derived from them tended to group together in this cluster. There were two distinct subgroups. Wild populations of *L. alba* spp. *versicolor* formed one subgroup, composed of OMF158, OMF66, OMF159, OMF160, and OMF161, in addition to two inbred lines, which were derived from *L. alba* spp. *versicolor* including OMF63 $S_5$ and OMF66 $S_5$. PI 374792, identified as *L. alba* spp. *alba*, was included in this subgroup. The other subgroup consisted of four inbred lines. One was derived from *L. alba* spp. *versicolor*, OMF64 $S_5$, and the remainder were derived from inter- subspecific crosses between *L. alba* and *L. versicolor* including OMF109-1, OMF109-2, and OMF109-3.

The third cluster was composed of five taxa of *L. floccosa* including subspecies *bellingeriana*, *floccosa*, *grandiflora*, *californica* and *pumila* (PI

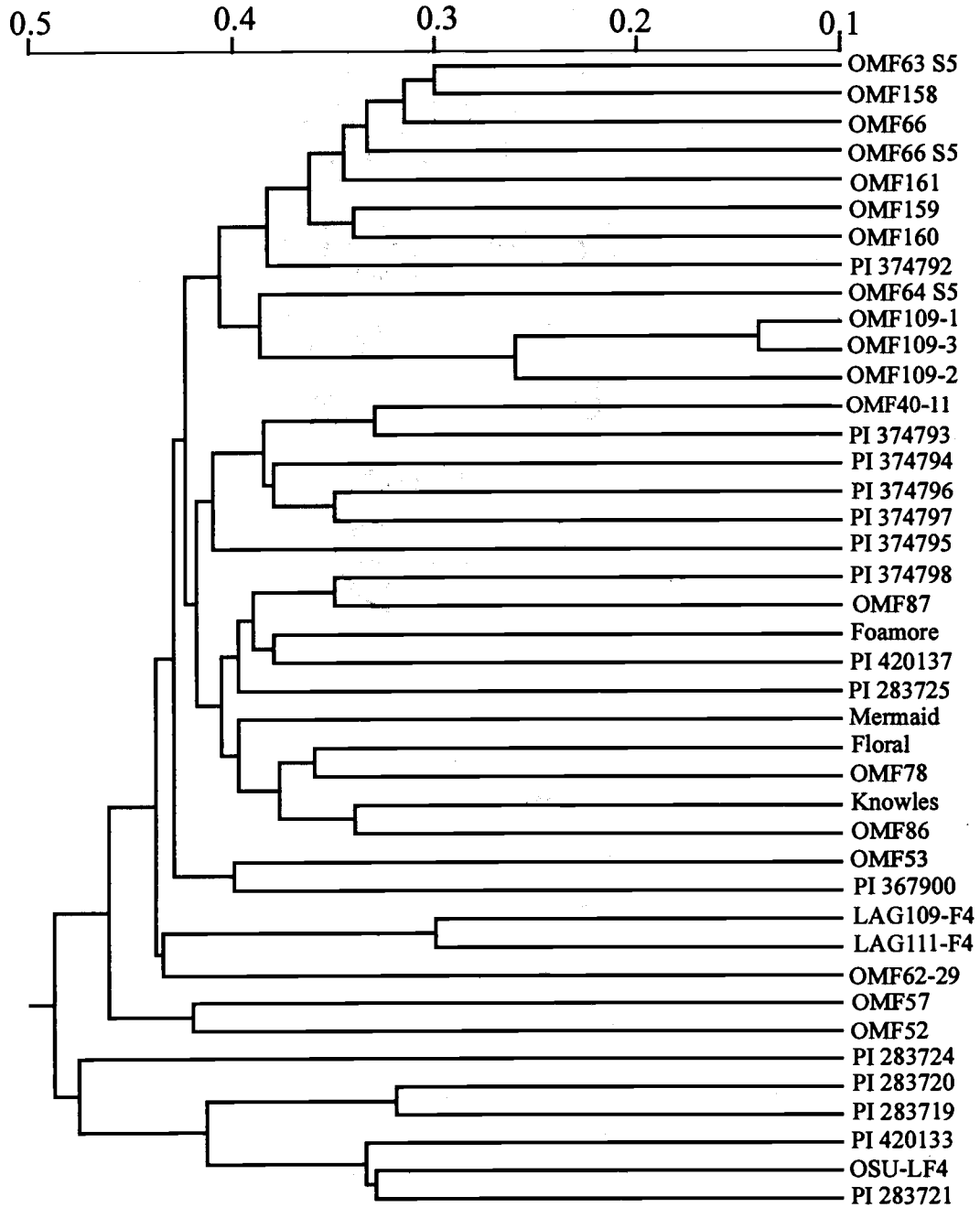


**Figure 2.2** A phenogram produced by UPGMA clustering of Roger-W genetic distance matrix estimated from AFLP fingerprints (176 markers) among 41 meadowfoam accessions of Inflexae section.

**Figure 2.3** A phenogram produced by UPGMA clustering of Roger-W genetic distance matrix estimated from AFLP fingerprints (176 markers) among 103 samples from 41 meadowfoam accessions of Inflexae section.

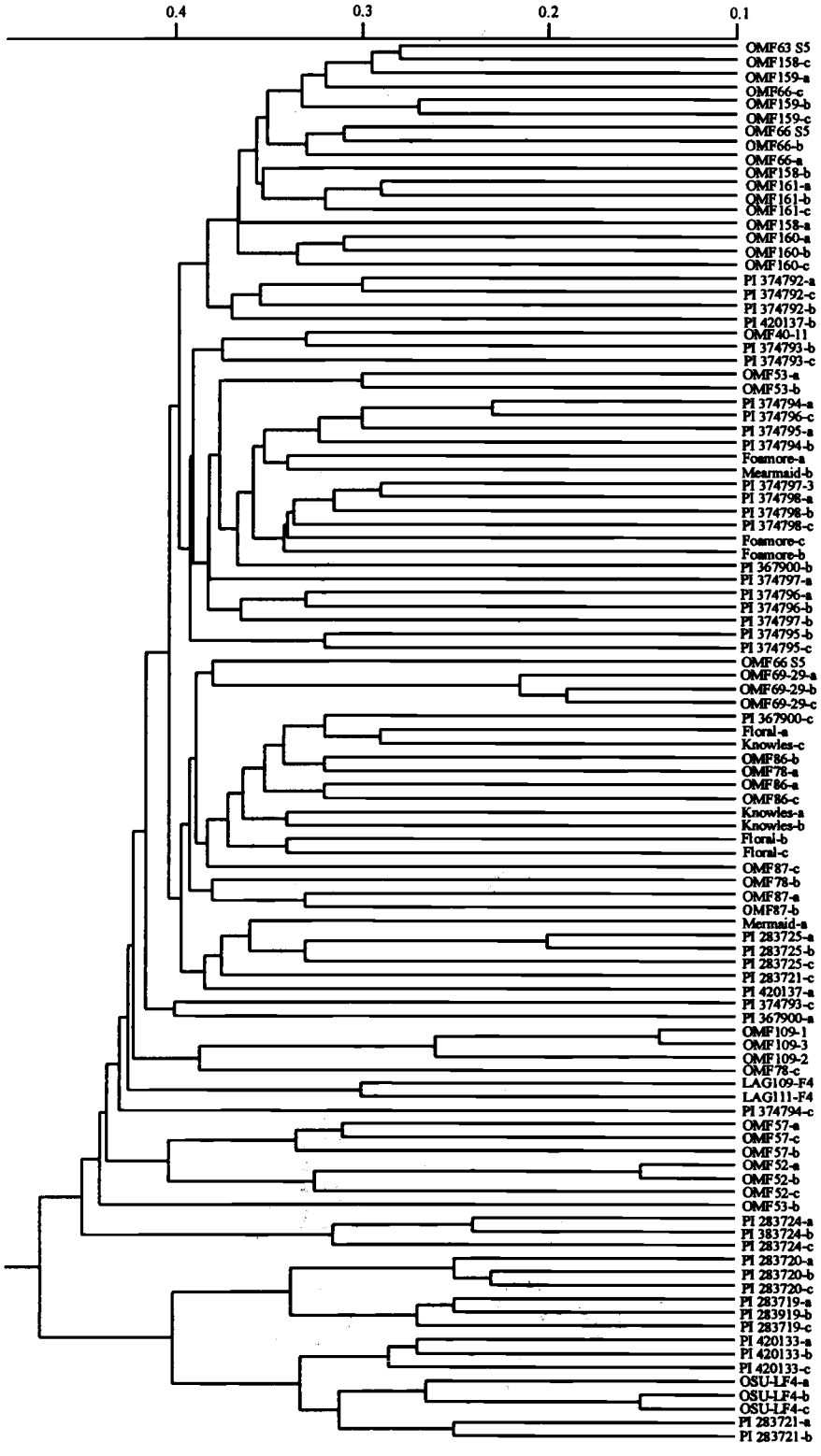
0.4
0.3
0.2
0.1
OMF63 S5
OMF158-c
OMF159-a
OMF66-c
OMF159-b
OMF159-c
OMF66 S5
OMF66-b
OMF66-a
OMF158-b
OMF161-a
OMF161-b
OMF161-c
OMF158-a
OMF160-a
OMF160-b
OMF160-c
PI 374792-a
PI 374792-c
PI 374792-b
PI 420137-b
OMF40-11
PI 374793-b
PI 374793-c
OMF53-a
OMF53-b
PI 374794-a
PI 374796-c
PI 374795-a
PI 374794-b
Foamore-a
Mearmaid-b
PI 374797-3
PI 374798-a
PI 374798-b
PI 374798-c
Foamore-c
Foamore-b
PI 367900-b
PI 374797-a
PI 374796-a
PI 374796-b
PI 374797-b
PI 374795-b
PI 374795-c
OMF66 S5
OMF69-29-a
OMF69-29-b
OMF69-29-c
PI 367900-c
Floral-a
Knowles-c
OMF86-b
OMF78-a
OMF86-a
OMF86-c
Knowles-a
Knowles-b
Floral-b
Floral-c
OMF87-c
OMF78-b
OMF87-a
OMF87-b
Mermaid-a
PI 283725-a
PI 283725-b
PI 283725-c
PI 283721-c
PI 420137-a
PI 374793-c
PI 367900-a
OMF109-1
OMF109-3
OMF109-2
OMF78-c
LAG109-F4
LAG111-F4
PI 374794-c
OMF57-a
OMF57-c
OMF57-b
OMF52-a
OMF52-b
OMF52-c
OMF53-b
PI 283724-a
PI 383724-b
PI 283724-c
PI 283720-a
PI 283720-b
PI 283720-c
PI 283719-a
PI 283919-b
PI 283719-c
PI 420133-a
PI 420133-b
PI 420133-c
OSU-LF4-a
OSU-LF4-b
OSU-LF4-c
PI 283721-a
PI 283721-b

**Figure 2.3**

283720, PI 283719, PI 420133, OSU-LF$_4$, and PI 283721, respectively). The simplified phenogram from 41 accessions showed concordance with the phenogram from 103 samples (Fig. 2.3). The three individuals within genotypes were grouped together, even though some were dispersed. This may be because meadowfoam populations are heterogeneous and variation within populations might account for this dispersion. *L. gracilis* spp. *parishii* (PI 283724) was grouped to *L. floccosa* species in the simplified phenogram (Fig. 2.2). Our studies showed that subspecies *floccosa* and *bellingeriana* of *L. floccosa* have a close genetic relationship (0.32) (Table 2.4.), and they were subgrouped together.

Three inbred lines (OMF62-29, LAG109-F$_4$ and LAG111-F$_4$) separately formed a small cluster far from the others. OMF62-29 is high-oil germplasm derived from *L. alba* spp. *alba*, while LAG109-F$_4$ and LAG111-F$_4$ were inbred lines derived from an interspecific cross between *L. alba* spp. *alba* (Mermaid) and *L. gracilis* spp. *parishii*. These two inbred lines fell between Mermaid and *L. gracilis* spp. *parishii* in this phenogram, indicating that they were almost equally related to their parents. The remaining two small clusters consisted of two wild populations of *L. alba* spp. *alba*, OMF53 and PI 367900 and two wild populations of *L. alba alba* (OMF52) and *L. alba versicolor* (OMF57). The latter were distantly related to their groups (Fig. 2.2).

The goodness of fit of this UPGMA cluster analysis was performed based on the cophenetic correlation (r) value between the cophenetic value matrix and the original distance matrix. The cophenetic correlation was high (r=0.85), indicating a good fit of the UPGMA cluster analysis performed.

**Principal Component Analysis (PCA)**

A two dimensional presentation of genetic distance produced by principal component analysis is shown in Fig. 2.4. The first three principal coordinates

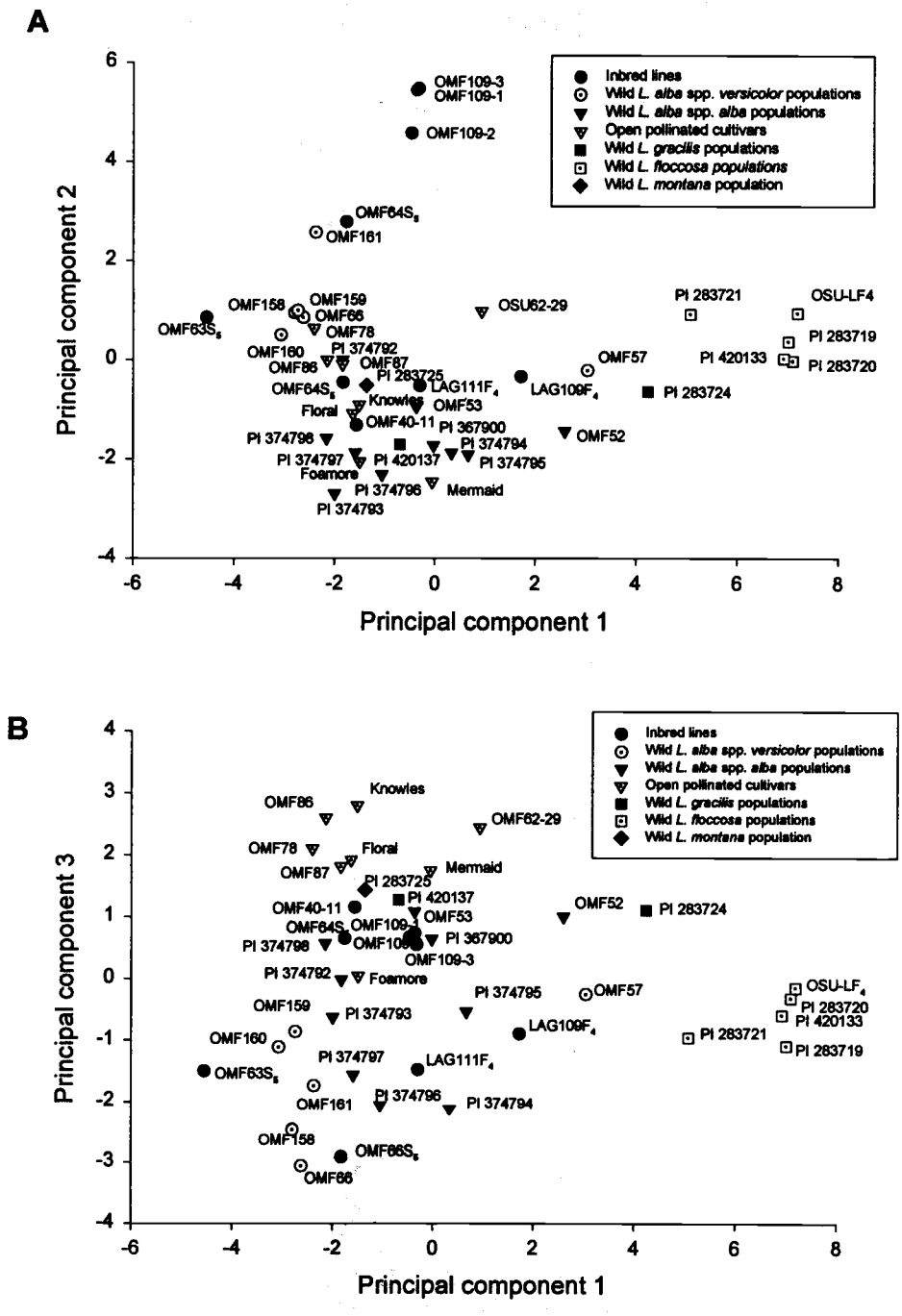


**Figure 2.4** Plot of the principal scores for the first and second (panel A), first and third (panel B) principal components estimated from a genetic distance matrix estimated from AFLP fingerprints (176 markers) of 41 meadowfoam accessions.

accounted for 37% of the total variation in AFLP-based genetic distance (the first, second, and the third eigenvalues were 0.22, 0.09, and 0.06, respectively). The first and the second as well as the first and the third coordinate clearly separated the wild populations of *L. floccosa* from the other populations. Within wild populations of *L. alba*, *L. alba* spp. *versicolor* populations were clustered and separated from the *L. alba* spp. *alba* populations. OMF57 (*L. alba* spp.*versicolor*) was separated from its group. Both of the coordinates 1 and 2 and coordinates 1 and 3 placed *L. gracilis* spp. *gracilis* and *L. montana* in *L. alba* spp. *alba* cluster, however, *L. gracilis* spp. *parishii* was placed close to *L. floccosa.*

## DISCUSSION

Abundance of AFLP markers provides an efficient means to evaluate the pattern of genetic diversity and relationships of meadowfoam germplasm. We fingerprinted 41 meadowfoam accessions of Inflexae section gene pool using AFLP markers. Genetic variation in *Limnanthes* spp. was reported using allozyme markers, however, only 11 to 18 loci were found to be polymorphic and employed to establish phylogenetic analysis (Brown and Jain 1979; McNeill and Jain 1983; Kessili and Jain 1985; Ritland and Jain 1984). AFLP is a bi-allelic marker and it was shown to have less polymorphic information content than SSRs (Simple Sequence Repeats) and RFLPs (Restriction Fragment Length Polymorphisms). The maximum PIC score for either SSR or RFLP marker is 1.0. Several studies have reported that SSRs revealed the highest polymorphic information content (Powell et al. 1996, Russell et al. 1997; Smith et al. 1997; Pejic et al. 1998). The maximum PIC scores for an AFLP marker is 0.5, however, AFLP has the highest multiplex ratio as compared to RFLPs or SSRs. This outstanding feature of AFLP is useful for many applications in genome analysis, for instance genome mapping, genetic

diversity and marker assisted selection (Powell et al. 1996; Russell et al.1997; Pejic et al. 1998).

The section Inflexae gene pool was diverse. The phenogram showed that there was only one cluster with a genetic distance below 0.3, comprised of three self-pollinated inbred lines (the OMF109 series) developed from the same *L. alba* spp. *alba* x *L. alba* spp. *versicolor* cross (Mermaid x OMF62/ OMF64). OMF109-2 was selected for *L. alba* spp.*versicolor* fatty acid profile with a high dienoic (22:2 Δ5, Δ13) and low erucic acid (22:1Δ13) content while OMF 109-3 was selected based on *L. alba* spp. *alba* fatty acid profile with a high erucic acid but low dienoic acid content. OMF109-1 was selected based on heterozygous progeny, with fatty acid profiles between these two subspecies.

The pattern of diversity as illustrated in the phenogram resulting from cluster analysis was concordant with species, subspecies, geographic and breeding origin. The principal component analysis provided a three-dimensional presentation of estimated genetic distance and supported the results of the UPGMA cluster analysis. *L. floccosa* subspecies were distinctly separated from the other species. Two subgroups were clearly distinguished within *L. floccosa* in which *L. floccosa* subspecies *floccosa* and *bellingeriana* were closely related, while the other members of this species, *grandiflora*, *californica*, and *pumila* formed more distantly related groups. Our result was in agreement with morphological and taxonomical classification of this species as described by Mason (1952) and Arroyo (1973). The subspecies *floccosa* and *bellingeriana* were grouped and classified as fully autogamous, producing cleistogamous flowers, while the remaining three subspecies, *grandiflora*, *californica*, and *pumila*, were grouped together and assigned as semi-autogamous due to relatively more chasmogamous flowers and the presence of a small degree of protandry.

*L. gracilis* appeared closely related to *L. alba* (Ornduff and Crovello 1968; Ornduff 1971; McNeill and Jain 1983). Two subspecies, *gracilis* and *parishii*

(PI 420137 and PI 283724), were separated far from each other in our study (Fig. 2.2 and 2.4). Two members of *L. gracilis* are found in different geographical areas (Mason 1952). *L. gracilis* spp. *gracilis* was found in the Klamath Mountain region of southwestern Oregon while *L. gracilis* spp. *parishii* is found only in a few sites in San Diego County, California. *L. montana* (PI 283725) was closer to *L. gracilis* spp. *gracilis* than to *L. gracilis* spp. *parishii* (Table 2.4). *L. montana* is distributed intermediate in the range of these two subspecies of *L. gracilis,* from Mariposa County, Sierra Nevada southward to Tulare County California. Our results showed that two subspecies of *L. gracilis* were distantly related (D=0.47, Table 2.4) from each other, but were closely related to *L. montana*. Only *L. gracilis* spp. *gracilis* was grouped together with *L. alba* spp. *alba* and *L. montana* was also included in this cluster. This agrees with a numerical taxonomic study using morphological traits (Ornduff and Crovello 1968) and an artificial hybridization study (Ornduff 1971). Hybrids between *L. alba* and *L. gracilis* spp. *parishii* have relatively sterile pollen whereas those of *L. gracilis* spp. *gracilis* and *L. alba* spp. *alba* have highly viable pollen. *L. montana* and *L. gracilis* are so morphologically similar that some populations of the two species are barely separable, but they are consistently separated by sterility barriers (Ornduff 1971). Moreover, our results support the hypothesis described by Mason (1952) that *L. montana* might be the remnant of these two populations of *L. gracilis*. Once these two populations were continuously distributed, and climatic and geographical changes along with the extinction of many *Limnanthes* populations caused subdivision and subsequent isolation of these two subspecies. However, this disagrees with the conclusion of McNeill and Jain (1983) that the two subspecies of *L. gracilis* are closely related to each other but distantly related to *L. montana*. The inconsistency may have occurred because of the heterogeneous nature of meadowfoam populations.

*Limnanthes* species in Inflexae section contain a wide range of mating systems from cleistogamy involving full autogamy in *L. floccosa* through

intermediate stages in *L. gracilis* spp. *parishii*, *L. graclilis* spp. *gracilis*, and *L. montana*, to *L. alba* with dominantly protandous, showy, insect-pollinated flower and with the lowest autofertility (Mason 1952; Arroyo 1973; McNeill 1983). *L. alba* was cultivated in 1971, and several open-pollinated cultivars were developed for commercial production. Our results revealed two distinct clusters in *L. alba.* One was primarily comprised of *L. alba* spp. *alba* and the other was primarily comprised of *L. alba* spp *versicolor.* Each cluster included wild populations and inbred lines derived from their wild populations. Commercial open-pollinated cultivars formed a subgroup within *L.alba* spp. *alba* cluster. Foamore was the first meadowfoam cultivar developed (Calhoun and Crane, 1975), followed by Mermaid and Floral (Calhoun and Crane 1984; Joliff 1986, 1994). All of these cultivars were developed by mass selection. Knowles and OMF86 were closely related since they were derived from OMF58 by one and two cycles of recurrent half-sib family selection (Knapp and Crane 1999). OMF78 was developed by one cycle of recurrent half-sib family selection in OMF59 (Knapp and Crane 1999). Even though these three cultivars showed close relationships the genetic distance among them was more than 0.3.

*L. alba* was addressed as an outcrossing species and primarily consisted of two subspecies, *alba* and *versicolor* (Mason 1952; Arroyo 1973; Brown et al. 1979). The mating systems *L. alba* and the other species in section Inflexae are widely investigated (Arroyo 1975; Brown 1977; Brown and Jain 1979; McNeill 1983; McNeill and Jain 1983). Several studies report the presence of self-pollinated progeny in wild populations of *L. alba* (Arroyo 1975; Brown 1977; McNeill 1983). Knapp and Crane (1997) screened 26 accessions of *L. alba* for self-pollinated phenotypes and found that six populations of *L. alba* spp. *versicolor* produced seed in a high percentage of flowers, indicating allelic diversity for self-pollination among these geographically isolated populations. *L. alba* spp. *versicolor* is distributed from ~37° to 41°N and ~ 120° to 123 °W in central and Northern California (Mason 1952; Brown et al.

1979, McNeill and Jain 1985). Self pollination seems to be concentrated in populations originating near Redding California (40.5 °N, 122.4 °W), and OMF66 (Redding) is a source of self-pollinated phenotypes (Knapp and Crane 1997). Self-pollinated inbred lines were developed from OMF66 and two other wild populations of *L. alba* spp. *versicolor* (OMF159 and OMF160) (Table 2.1). The self-pollinated inbred lines (Table 2.1) developed from these species provided useful resources for developing elite meadowfoam cultivars.

In conclusion, AFLP fingerprinting was useful technique for evaluating genetic diversity in addition to constructing a genetic linkage map in meadowfoam (Katengam et al. 1999). AFLPs revealed great diversity among elite and exotic meadowfoam germplasm in the Inflexae section. *L. alba* spp. *alba* gene pool seems to be more diverse than the other gene pools in the section. The history of meadowfoam is so short that the breeding bottlenecks have not yet arisen in this elite gene pool.

## ACKNOWLEDGMENTS

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# CHAPTER 3

## A Genetic Map for the Oilseed Meadowfoam Comprised of Amplified Fragment Length Polymorphisms

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## ABSTRACT

Meadowfoam (*Limnanthes alba* Benth.) is a diploid (x = 5), winter annual native to vernal pools in California and the Pacific Northwestern US. This oilseed crop produces novel long-chain seed oils ($C_{20}$ and $C_{22}$) with low concentrations of saturated fatty acids (typically less than 2%) and outstanding oxidative stability. DNA markers and genetic maps have not been developed for meadowfoam. Our aims were to (i) screen two genetically diverse inbred lines (OMF40-11 and OMF64) for amplified fragment length polymorphisms (AFLPs) and (ii) develop a genetic map comprised of AFLPs. OMF40-11 and OMF64 were screened for AFLPs using 16 primer combinations with three selective nucleotides each. These primers produced 1,376 monomorphic and 425 polymorphic bands between the two lines. One hundred [(OMF40-11 x OMF64) x OMF64] $BC_1$ progeny were screened for AFLPs using nine primer combinations. One hundred and eight segregating AFLPs were scored and mapped. The genetic map was comprised of 104 loci in five linkage groups, one per haploid chromosome ($x$ = 5), with 14 to 28 loci per linkage group, a total length of 698.3 cM, and a mean density of 6.7 cM. The lengths of the linkage groups varied from 110.3 to 168.0 cM. AFLP loci were randomly distributed throughout the genome with no centromeric clustering, a finding contrary to that in several other plant species. There was an excess of OMF64 or OMF40-11 alleles in three telomeric regions and one non-telomeric region, but recombinants were recovered throughout the genome. Genetic maps of meadowfoam can be rapidly constructed using a small number of AFLP primer combinations. AFLPs should have tremendous utility for molecular breeding, especially marker-assisted backcross breeding, in meadowfoam.

# INTRODUCTION

Meadowfoam (*Limnanthes alba* Benth.) is a diploid, (2n=2x=10) winter annual native to vernal pools in California and the Pacific Northwestern US. It belongs to the Inflexae section of the family Limnanthaceae (Mason 1952) and is grown mainly for seed oil and to some extent for seed meal. This oilseed crop produces novel long-chain seed oils ($C_{20}$ and $C_{22}$) with low concentrations of saturated fatty acids (typically less than 2%) and outstanding oxidative stability owing to its $\Delta 5$ bond, long chain fatty acid in nature, and the lack of polyenoic fatty acids (Smith et al. 1960; Bagby et al. 1961, Isbell 1997). The unique characteristics of this seed oil promoted the development of meadowfoam as a special oilseed crop with a great opportunity of industrial markets.

The use of genetic markers in meadowfoam is documented in several studies. Morphological characters such as floral and nutlet morphology and allozyme markers are utilized to study biosystematic and genetic relationships, to examine basic genetic variation underlying breeding systems, and to investigate an outcrossing rate among *Limnanthes* populations (Ornduff and Crovello 1968; Arroyo 1975; Jain 1978; Brown and Jain 1979; Brown et al. 1979; McNeill and Jain 1983; Kesseli and Jain 1985). The small number of polymorphic markers obtained from allozyme markers (~11- 18 polymorphic marker loci) and unfavorable phenotype expressions of morphological traits due to environmental effects have limited the utility of these markers. DNA markers overcome these limitations since polymorphisms revealed at the DNA level are much more abundant than those identified at the protein and morphological levels. Moreover, they are phenotypically neutral, independent of environmental influences, lack of deleterious effects, and are able to detect DNA from all living tissues at all stages of development (Tanksley et al. 1989; Stuber 1992). Polymorphisms based on DNA markers are highly informative

and superior to markers revealed by traditional methods in resolving genetic differences.

Genetic maps are potentially important in plant breeding and are a powerful tool for localizing and isolating genes underlying both simple and complex traits. The DNA markers greatly facilitate and enhance the efficiency for constructing genetic maps in several plant species. Ideal genetic markers for plant breeding are simple and abundant as well as cost and time effective. Amplified fragment length polymorphism (AFLP) has been developed (Zabeau and Vos 1993; Vos et al. 1995) by combining the strength of restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR). Polymorphisms are usually revealed as presence or absence of an amplified restriction fragment, and are therefore dominant. AFLPs provide efficient high throughput markers for various applications in crop improvement since a large number of markers can be generated per assay in a short period of time. AFLP markers have several useful features including high abundance, reproducibility, relatively high effective multiplex ratio, and extensive genome coverage (Powell et al. 1996a, 1996b; Keim et al. 1997; Qi et al. 1998; Knapp et al. 1999: Ridout and Donini 1999). AFLPs are widely used for constructing framework genetic maps (Wang et al. 1997; Lu et al. 1998) as well as high density linkage maps (Becker et al. 1995; Keim et al. 1997; Cho et al. 1998; Qi et al.1998), identification of crop variety (Ellis et al. 1997), evaluation of genetic diversity and relationships (Maughan et al. 1996; Hill et al. 1997; Hongtrakul et al. 1997; Zhu et al. 1998) and position cloning of genes of interest (Thomas et al. 1995; Cho et al. 1996).

DNA markers and genetic maps are not available for meadowfoam. We employed AFLP marker technology to develop a framework genetic map for meadowfoam using inter- subspecies backcross progeny from a cross between a self-pollinated inbred line, OMF64 (*Limnanthes alba* ssp. *versicolor*) and a cross pollinated inbred line, OMF40-11 (*L. alba* ssp. *alba*). As no genetic linkage map of meadowfoam exist at this point, the initial map will

provide a framework to understanding the genome structure, contribute a useful resource for genetic improvement, and facilitate marker assisted selection in meadowfoam breeding program.

## MATERIALS AND METHODS

### Plant Materials

Seeds of inbred lines and crosses were produced on greenhouse grown plants. Seeds were germinated at 4°C in the dark on moistened blotter paper in 11 x 11 x 3 cm clear plastic boxes. Seedlings were transplanted to potting soil (pumice: peat moss: sandy loam) in 7.5 x 7.5 cm plastic pots and grown in growth chamber (Model CEL 37-14, Sherer-Gillett Co., Marshall, MI) at 15°C for 25 to 28 days with 8 h of fluorescent light per day. The plants were transferred to a greenhouse and grown to maturity with 16 h of light per day. Daily temperatures ranged from 18°C (night) to 25°C (day) in the greenhouse.

The inbred line OMF40-11 was developed from the open-pollinated cultivar Mermaid by randomly selecting and manually selfing one individual per generation. Several OMF40-11 plants were emasculated and crossed to OMF64, a self pollinated inbred line (Knapp and Crane, 1997). OMF40-11 originated from *L. alba* ssp. *alba* germplasm, while OMF64 originated from *L. alba* ssp. *versicolor* germplasm.

Several OMF40-11 x OMF64 plants were emasculated and backcrossed to OMF64. One hundred $BC_1$ seeds were latitudinally dissected to produce half seed samples. The embryonic halves were germinated on blotter paper and grown in the greenhouse as described earlier. Leaves were harvested from 50 to 55 day-old plants and immediately stored at -80°C.

**AFLP Marker Analyses**

Genomic DNA was extracted from frozen leaves using the protocol described by Lodhi et al. (1994) with a few minor changes. 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 5M 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 (10 mg/ml) for 1 h at 37°C.

AFLP marker assays were performed using the original protocols described by Zabeau and Vos (1993) without using streptavidin beads to eliminate a subset of fragments (Vos et al. 1995). Genomic DNA samples (0.5 μg) were digested with 5 units of *EcoR*I and 5 units of *Mse*I (New England Biolab, Schwalbach, Germany) in a reaction volume of 50 ml in 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 μl of ligation solution containing 1 μl *EcoR*I adapter (5 pmol/ml), 1 μl *Mse*I adapter (50 pmol/ml), 5U *EcoR*I, 5U *Mse*I, 1.2 ml 10 mM ATP, 1 μl 10x RL buffer, and 1U T4 DNA ligase (New England Biolabs) was added to the solution and incubated for 3 h at 37°C. Twelve μl of restriction ligation products were electrophoresed on a 1% agarose gel to verify that 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 (*EcoR*I +A and *Mse*I +C) (Table 3.1). The PCRs were performed for 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s on a Perkin Elmer 9600 DNA thermal cycler (San Francisco, CA). Twelve μl of each PCR product were electrophoresed

on 1% agarose to check for PCR products. The PCR products 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 3.1). *Mse*I +3 primers were unlabelled, while *EcoR*I +3 primers were end-labeled with [$\gamma$-$^{33}$P]-ATP using a T4 polynucleotide kinase (New England Biolabs, Beverly, MA). 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 the annealing temperature reduced 0.7°C per cycle, and 24 cycles with an annealing temperature of 56°C (Zabeau and Vos 1993; Vos et al. 1995).

**Table 3.1** Oligonucleotide adapters and primers used for analyses of amplified fragment length polymorphisms (AFLPs) in meadowfoam (*Limnanthes alba*).

| Adaptors or Primers | Name | Sequence |
|---|---|---|
| *EcoR*I Adaptors | 91M35 | 5'-CTCGTAGACTGCGTACC-3' |
| | 91M36 | 3'-CTGACGCATGGTTAA-5' |
| *Mse*I Adaptors | 92A18 | 5'-GACGATGAGTCCTGAG-3' |
| | 92A19 | 3'-TACTCAGGACTCAT-5' |
| *EcoR*I +1 Primer | 92R11 | 5'-AGACTGCGTACCAATTC/A-3' |
| *Mse*I +1 Primer | 92H20 | 5'-GACGATGAGTCCTGAGTAA/C-3' |
| *EcoR*I +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' |

The PCR products 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 tracking dye). These solutions were heated at 94°C for 3 m and rapidly cooled on ice. Eight μl were loaded into pre-warmed 6% denaturing polyacrylamide gels and 1x TBE running buffer (0.045 M Tris borate and 0.001 M EDTA, pH 8.0). Gels were run at constant 60 watts 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, OH, USA) at room temperature for 3 to 5 days.

AFLP assays were performed on OMF40-11 and OMF64 using 16 primer combinations (two *EcoR*I +3 and eight *Mse*I +3 oligonucleotide primers) (Table 3.1) and on 100 backcross progeny using nine primer combinations. The autoradiographs were manually scored for the presence or absence of bands. Locus names were developed using the selective nucleotide sequences of the *EcoR*I and *Mse*I +3 oligonucleotide primers in order, and estimated length of the fragment, e.g., the locus name for a 250 bp fragment amplified with *EcoR*I-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, IL).

**Genetic Mapping**

Genetic maps were constructed using MAPMAKER (Lander et al. 1987) and G-MENDEL (Holloway and Knapp 1993). Log-likelihood ratio (G) tests for segregation distortion were performed for each locus. The observed ratio was significantly different from the expected ratio (1:1) when $G > \chi^2_{1,0.01}$, where G is a log-likelihood ratio test statistic and $\chi^2_{1,0.01}$ is a random variable from the $\chi^2$ distribution with one degree of freedom. Tests for linkage between loci were performed using likelihood odds ratios. 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 orders by comparing multipoint likelihoods, whereas ORDER estimates orders by comparing map lengths (sums of adjacent recombination frequencies, SAR). 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 estimated by G-MENDEL was selected.

Monte Carlo analyses of locus orders were performed using the MONTE function of G- MENDEL (Holloway and Knapp 1993). This function produces n locus order estimates from n repeat runs of the locus ordering algorithm. We assessed the consistency or similarity of 100 locus order estimates per linkage group using the Kendall coefficient of concordance (Kendall and Gibbons, 1990). Concordance was estimated by

$$W = \frac{12\sum_{i=1}\left[S_i - \frac{n(k+1)}{2}\right]^2}{n^2(k^3-k)}$$

where $S_i$ is the sum of the ranks (rank sum) for the i$^{th}$ locus, $n$ is the number of rankings (locus order estimates), and $k$ is the number of loci. $W$ varies from 0 to 1. 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 conceptually analogous to a mean correlation among the $n$ orders. There are $(n!/2)$ possible correlations among n orders. $W$ estimates the sum of squared deviations of rank sums from the rank mean (Kendall and Gibbons 1990).

The statistical significance of W was tested using the $\chi^2$ -distribution (Kendall and Gibbons 1990). The null hypothesis was that the $n$ locus orders were independent. There are $(k!/2)^n$ possible sets of ranks for $k$ loci, rather than $(k!)^n$, because each locus order has a mirror image. The test statistic $C = n(k-1)W$ is approximately distributed as a $\chi^2$ -variable with $v = k-1$ degrees of freedom when $k > 7$ (Kendall and Gibbons 1990). The null hypothesis was rejected with a probability of $\alpha$ when $C > \chi^2_{v;\alpha}$

## RESULTS

Sixteen AFLP primer combinations produced 1,376 monomorphic and 425 polymorphic bands between OMF40-11 and OMF64 (Table 3.2). The number of polymorphic bands varied from 19 for the *Eco*RI-ACG/*Mse*I-CAG primer pair to 34 each for the *Eco*RI-ACG/*Mse*I-CAA and *Eco*RI-ACG/*Mse*I-CAT primer pairs. The percentage of polymorphic bands ranged from ~ 6 to 19 % with a mean of 12%.

The number of polymorphic bands and distribution of null alleles between OMF40-11 and OMF64 were used to select nine primer pairs (*Eco*RI-ACG/*Mse*I-CAA, CAC, CAT, CTA, and CAG and *Eco*RI-ACA/*Mse*I-CAC, CTG, CTC, and CAG) for analysis in the backcross population. Only AFLPs with null alleles in OMF64 segregated in the backcross, so roughly half of the polymorphisms between OMF40-11 and OMF64 could not be mapped (Fig.3.1). The selected primer pairs produced a total of 971 bands (98 to 120 bands per primer pair). Of these, 128 were polymorphic among the backcross

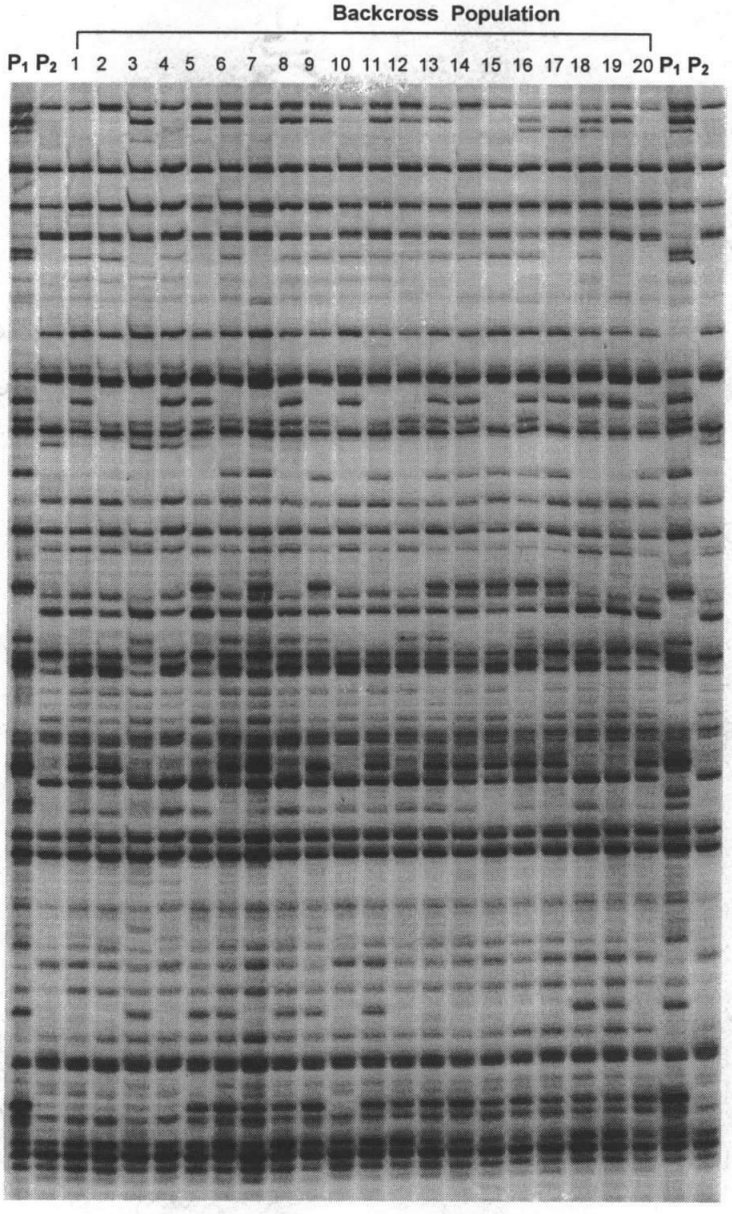


**Figure 3.1** Autoradiograph showing AFLP markers derived from selective amplification of restriction fragments by *EcoR*I+ACA and *Mse*I+CTC. The first two lanes from the left and the right are parents OMF40-11 ($P_1$) and OMF64 ($P_2$), respectively, the remaining lanes are [(OMF40-11 x OMF64) x OMF64] $BC_1$ progeny.

**Table 3.2.** The number of polymorphic bands and percentage of polymorphic bands produced between two inbred lines (OMF40-11 and OMF64) of meadowfoam (*Limnanthes alba*) by 16 amplified fragment length polymorphism primer pairs with three selective nucleotides each.

| Selective Nucleotides | | Number of Bands | | |
|---|---|---|---|---|
| *EcoR*I | *Mse*I | Total | Polymorphic | Polymorphic Bands (%) |
| ACG | CAA | 100 | 34 | 19.0 |
| | CAC | 98 | 22 | 10.2 |
| | CAT | 113 | 34 | 16.8 |
| | CTA | 106 | 23 | 11.3 |
| | CAG | 99 | 19 | 12.1 |
| | CTG | 77 | 22 | 13.0 |
| | CTC | 85 | 20 | 10.6 |
| | CTT | 98 | 26 | 9.2 |
| ACA | CAC | 114 | 31 | 14.9 |
| | CTG | 120 | 26 | 13.3 |
| | CTC | 107 | 25 | 12.1 |
| | CAG | 114 | 32 | 14.9 |
| | CAT | 133 | 28 | 9.8 |
| | CTA | 134 | 26 | 6.7 |
| | CTT | 160 | 21 | 8.1 |
| | CAA | 143 | 26 | 9.8 |
| Total | | 1801 | 425 | 23.6 |

progeny. The polymorphic fragments ranged in length from 47 to 490 base pairs (bp); however, 60% of the polymorphic fragments ranged in length from 100 to 250 bp. Twenty-one AFLPs were difficult to score and were not genotyped.

Segregation ratios for 25 loci (23.2 %) were distorted ($p \leq 0.01$). Twenty-one loci had an excess of OMF64 alleles, while only four had an excess of OMF40-11 alleles. Most of the allele frequency changes were systematic and were undoubtedly caused by gametic selection. Despite this, recombinants were recovered throughout the genome (Fig. 3.2). Clusters of telomeric or

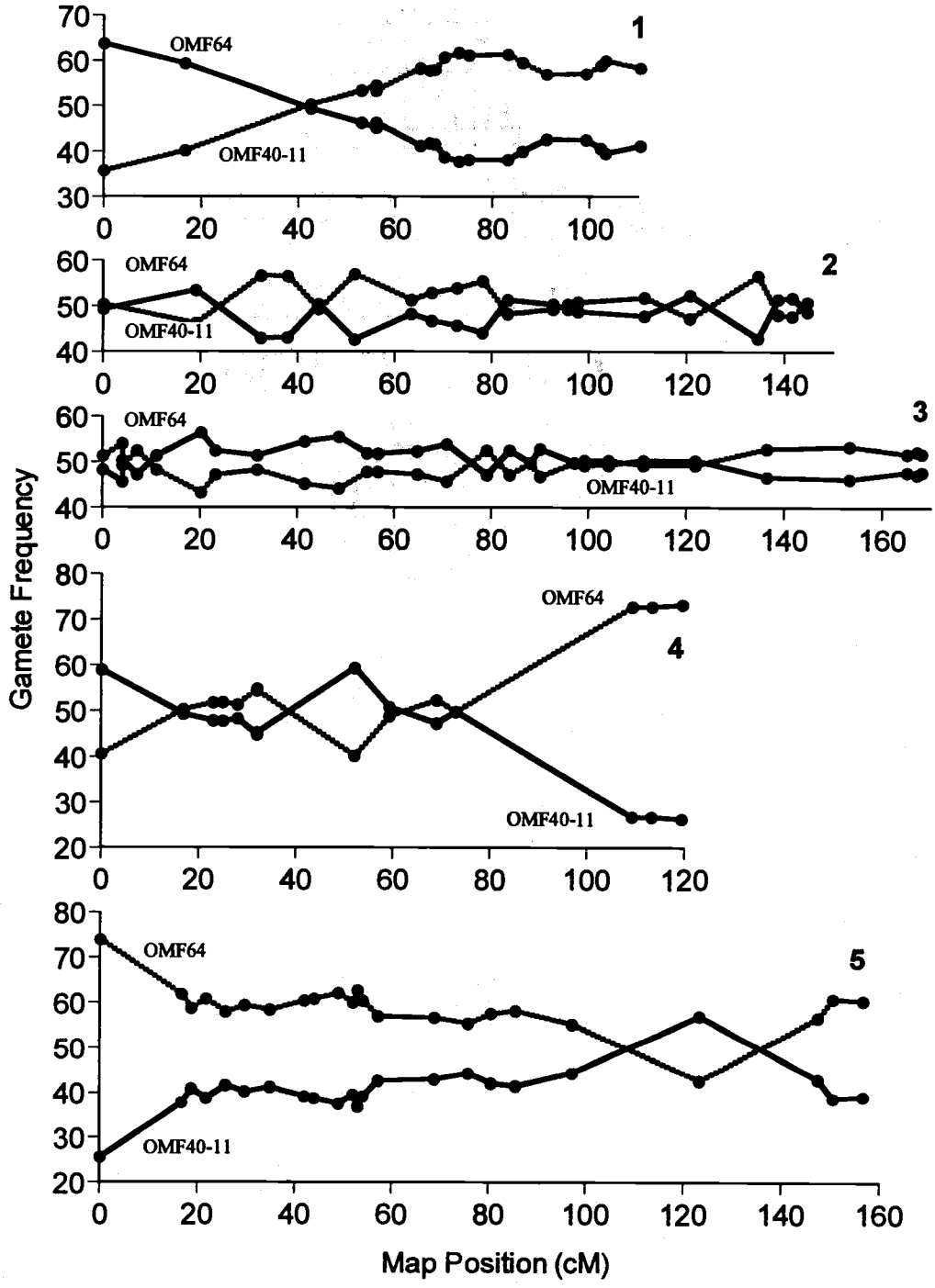


**Figure 3.2.** Amplified fragment length polymorphism allele percentages among [(OMF40-11 x OMF64) x OMF64] $BC_1$ progeny of meadowfoam (*Limnanthes alba*) across linkage groups 1, 2, 3, 4, and 5.

near-telomeric loci on linkage groups 1, 4, and 5 had distorted segregation ratios (Fig. 3.2). One cluster of non-telomeric loci on linkage group 1 had distorted segregation ratios (Fig. 3.2).

The genetic map was comprised of five linkage groups, one per haploid chromosome (Fig. 3.3). The map was comprised of 104 loci with 14 to 28 loci per linkage group (Table 3.3). Four loci (ACGCAC205, ACACTC184, ACACAC150 and ACACTG173) segregated independently. Locus orders estimated from multipoint likelihoods and map lengths were nearly identical and only varied locally. Concordance estimates for the five linkage groups (in order) were 0.99, 0.99, 0.99, 0.99, and 0.98; thus, the locus orders estimated from these data were highly reproducible. Lower concordance estimates are often indicative of genotyping or random sampling errors.

**Table 3.3** Number of markers and map distance in each linkage group, and average distance per marker interval of an AFLP genetic linkage map for meadowfoam (*Limnanthes alba*)

| Linkage group | Number of markers | Total distance (cM)* | Average distance per marker interval (cM) |
|---|---|---|---|
| 1 | 19 | 110.3 | 5.8 |
| 2 | 20 | 144.6 | 7.2 |
| 3 | 28 | 168.0 | 6.0 |
| 4 | 14 | 118.8 | 8.5 |
| 5 | 23 | 156.6 | 6.8 |
| Total | 104 | 698.3 | 6.7 |

*cM = centi Morgan

The longest gap between markers was 36.3 cM on linkage group 4. The longest gaps on the other four linkage groups were 25.9, 18.9, 16.9, and 26.1. The map was 698.3 cM long with a mean density of 6.7 cM. The lengths of the linkage groups varied from 110.3 to 168.0 cM (Table 3.3). Although the

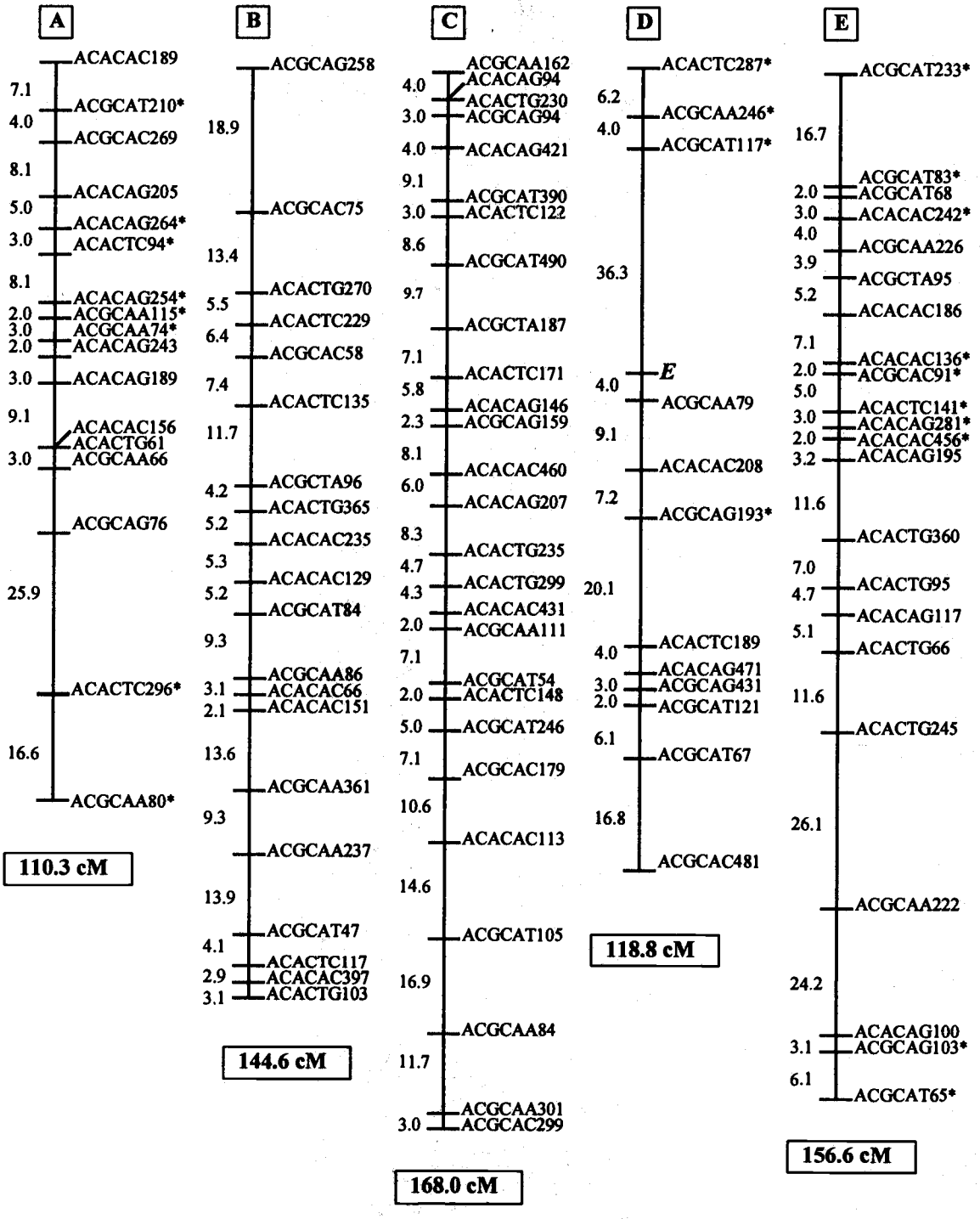


**Figure 3.3** A genetic map of meadowfoam (*Limnanthes alba*) comprised of 103 AFLP loci and one phenotypic marker (*E*). The map was built using 100 [(OMF40-11 x OMF64) x OMF64] $BC_1$ progeny.

distances between the most distal markers and the telomeres are not known the telomeres have not been mapped) and four markers did not map, the number of linkage groups and length of the map led us to conclude that a significant fraction of the genome was covered.

## DISCUSSION

AFLP analysis provides efficient, high throughput markers for building genetic linkage maps. The utility of AFLPs is greatest where scoring as a dominant marker is sufficient, for instance mapping in backcross progeny (Knapp et al. 1999). Even though only half of the polymorphisms between the two inbred parents, OMF40-11 and OMF64, were mapped in this study; nine combinations of AFLP primers provided sufficient AFLP markers for building a meadowfoam map. As compared to several plant species such as sunflower and barley, a large number of AFLP primer combinations are required for constructing those genetic maps. Sunflower genetic linkage map requires approximately 25 AFLP primer combinations (Gedil et al. 1999), similar to that in barley (Qi et al. 1998). This may be attributed the large genome sizes of sunflower (1C = 3.0 x $10^9$ bp) and barley (1C = 5.1 x $10^9$ bp, Bennett and Leitch 1995) which contain relatively high proportions of repetitive DNA sequences. We conclude that meadowfoam has a small genome size with relatively few repetitive DNA sequences.

The excess of OMF64 or OMF40-11 alleles in three telomeric regions and one non-telomeric region indicated occurrence of gametic selection between this intra-subspecific cross resulting in skewed markers. These skewed markers did not affect recombination frequency estimates, locus grouping or locus ordering statistics. A stringent LOD score (7.0) was used to construct this framework meadowfoam genetic map which increased the efficiency of detecting true linkage and minimizing false positives in assigning markers to

linkage groups (Type I errors). The concordance estimated from repeat runs of the G-MENDEL locus ordering algorithm were close to 1.0 for all five linkage groups, confirming true locus order estimation. The locus orders produced by MAPMAKER and G-MENDEL were nearly identical and only varied locally.

Skewed markers are commonly found in inter and intraspecific crosses, for example in rice (McCouch et al. 1988, Causse et al. 1994), barley (Graner et al. 1991), and *Medicago* (Jenczewski et al. 1997); and tend to increase with the level of divergence among the parents (Zamir and Tadmor 1986). The distortion of markers could be due to several factors such as linkage to either incompatible loci or a lethal allele in gametes (Wricke and Wehling 1985; Pillen et al. 1992), chromosome rearrangement (Faure et al. 1993; Jenczewski et al. 1997), competition among gametes, or abortion of the gamete or zygote (Lyttle 1991; Harushima et al. 1996).

The markers were fairly evenly distributed among the five linkage groups, in contrast to AFLP maps in several crops such as tomato, rice and sunflower, in which AFLP markers tend to cluster in some regions, particularly around centromeric areas (Tanksley et al. 1992; Becker et al. 1995; Qi et al. 1997, 1998; Gedil et al. 1999). The suppression of recombination in centromeric areas may result from both a centromere effect and (or) lower levels of recombination in heterochromatin around the centromeres (Tanksley et al. 1992). The level of recombination in heterochromatin is less than that in euchromatin presumably due to the more condensed state of heterochromatin during meiosis at the time of crossing over (Roberts 1965).

The meadowfoam genetic map covers 698.3 cM, assigned to five linkage groups corresponding to the number of haploid chromosomes (x = 5). The linkage groups themselves indicated the degree of genome coverage. The total map distance was around one and a half fold longer than that of *Arabidopsis* (500 cM, Meinke et al. 1998). Our results showed that this map had excellent genome coverage. Knowing the position of telomeric sequences

on genetic map provides the confirmation that genome is covered. Telomeres are terminal sequences of linear chromosomes that play a role both in the stability and replication of chromosome ends (Burr et al. 1992; Wu and Tanksley 1993). The first plant telomere sequences were isolated from *Arabidopsis thaliana* (Richards and Ausubel 1988) and these sequences were used as heterologous probes for identifying telomere sequences in several plant species such as tomato (Ganal et al. 1991, 1992), barley (Kilian and Kleinhofs, 1992; Roder et al. 1993), maize (Burr et al. 1992), and rice (Wu and Tanksley 1993). Mapping telomeres would help us to define the boundaries of this genetic map and estimate the total genetic distance and potential genetic distance expansion for each chromosome. Telomere sequences are investigated by means of pulse field gel electrophoresis (PFGE) and *in situ* hybridization. Mapping the telomere sequences is performed by monitoring their segregation using PFGE (Ganal et al. 1991, 1992; Roder et al. 1993; Wu and Tanksley 1993).

Although most loci were evenly distributed throughout the meadowfoam genome there were some gaps between pairs of markers (Fig. 3.3). The gaps were probably caused by a lack of polymorphic markers in the genome regions, representing areas of high recombination (Becker et al. 1995; Berry et al. 1995; Keim et al. 1997). These gaps could be filled as more polymorphic markers are identified and added to the map. Saturating this meadowfoam map with a variety of codominant markers such as simple sequence repeat (SSR) and intron fragment length polymorphism (IFLP) markers will provide the opportunity and flexibility for utilization of this genetic map for different purposes particularly marker-assisted selection (MAS) and map-based cloning. The development of SSR and IFLP markers for meadowfoam is underway in our laboratory. This will provide opportunities not only to anchor the locus specific markers to each linkage group and also to saturate the existing meadowfoam map with multi-allelic, highly polymorphic SSR and gene-specific IFLP markers. SSR markers are highly polymorphic and can be

multiplexed and automated for high throughput genotyping. Moreover, primer information is convenient to exchange between laboratories (Powell et al. 1996a, 1996b; Senior et al. 1996). IFLPs are gene-specific markers, which are developed from unique sequences flanking the introns of specific genes (Slabaugh et al. 1997; Hongtrakul et al. 1998). Polymorphisms are caused by intron fragment length differences. IFLP markers for fatty acid gene families, for example FatB (thioesterases) and SAD (Δ9-stearoyl ACP desaturase) genes are developed for meadowfoam (unpublished data). These PCR-based markers are useful for genome mapping as well as synteny mapping and candidate gene analysis.

We report here the framework of a genetic linkage map for meadowfoam. This map will be an important tool in meadowfoam breeding programs for identifying the genomic regions controlling economically important traits as well as for marker-assisted selection.

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# CHAPTER 4

## Quantitative Trait Loci Underlying Fatty Acid Profile Differences in the Very Long-Chain Oilseed Meadowfoam

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## ABSTRACT

Meadowfoam (*Limnanthes alba* Benth.) produces very long-chain seed oils with unique chemical and physical properties. The principal fatty acids of meadowfoam oil are 20:1Δ5, 22:1Δ5, 22:1Δ13 (erucic acid), and 22:2Δ5, Δ13 (dienoic acid). *L. alba* ssp. *versicolor* germplasm accessions typically produce more erucic acid and less dienoic acid than *L. alba* ssp. *alba* germplasm accessions. The fatty acid profile differences between the subspecies are primarily caused by allelic variants at the *E* locus. The dominant allele, which seems to be fixed in *L. alba* ssp. *alba*, decreases erucic and increases dienoic acid. Erucic and dienoic acid concentrations vary continuously within *E_* and *ee* phenotypic classes in segregating populations. Within class differences could be caused by quantitative trait loci (QTL). The aim of this study was to map the *E* locus and QTL affecting erucic and dienoic acid concentrations. Backcross progeny were produced using parent inbred lines (OMF40-11 and OMF64) with fatty acid profiles characteristic of the two subspecies. The fatty acid profiles of the backcross progeny were assayed using gas chromatography. The *E* locus segregated 94:86, a ratio that was not significantly different from 1:1 ($p = 0.55$). This locus was associated with 94 and 77% of the phenotypic variance for erucic and dienoic acid concentration, respectively. Erucic acid varied from 42 to 151 g $kg^{-1}$ among *E_* and 185 to 269 g $kg^{-1}$ among *ee* progeny, while dienoic acid varied from 151 to 318 g $kg^{-1}$ among *E_* and 66 to 209 g $kg^{-1}$ among *ee* progeny. The genome was searched for QTL using a genetic map constructed from 100 backcross progeny. The map was comprised of 66 loci spaced ~10 cM apart across five linkage groups (x = 5). The *E* locus mapped to linkage group four and pleiotropically affected the concentration of nearly every fatty acid. QTL affecting erucic, and dienoic acid concentrations mapped to linkage group four and were centered or near the *E* locus, but were not found elsewhere in the genome. Despite this the phenotypic distribution of the *E_* class was

reproduced by crossing extreme phenotypes, thus substantiating that *E_* class phenotypes were almost certainly produced by genetic and non-genetic factors.

## INTRODUCTION

The fatty acid profile and chemical and physical properties of meadowfoam (*Limnanthes alba* Benth.) seed oil are unique (Miller et al., 1964; Gentry and Miller, 1965; Higgins et al., 1971; Chang and Rothfus, 1977; Isbell, 1997). With saturated fatty acid concentrations of 20 g $kg^{-1}$ or less, meadowfoam oil has significantly less saturated fat (Smith et al. 1960; Miller et al. 1964; Moreau et al. 1981, Lardans and Tremolieres, 1991, 1992) than soybean (*Glycine max* L.), sunflower (*Helianthus annuus* L.), and other common seed oils (Hammond, 1994). One unique characteristic of the oil is the concentration of very long-chain fatty acids (VLCFs), specifically acids with $C_{20}$ and $C_{22}$ chain lengths. Although rapeseed (*Brassica napus* L.) and many other Cruciferae (Pollard and Stumpf, 1980a; Agrawal and Stumpf, 1985; Fehling et al., 1990; Taylor et al., 1992; James et al., 1995; Miller and Kunst 1997, Barret et al., 1998) produce VLCFs (primarily erucic acid), meadowfoam produces VLCFs in higher concentrations than the Cruciferae and is the richest known source of VLCFs in the plant kingdom (Princen, 1979; Purdy and Craig, 1987; Kleiman, 1990). VLCF concentrations typically range from 940 to 960 g $kg^{-1}$ in meadowfoam (Knapp and Crane, 1995).

The principal fatty acids found in meadowfoam are cis-5-eicosenoic (20:1Δ5), cis-13- docosenoic (22:1Δ13, or erucic acid), cis-5,cis-13-eicosenoic (22:2Δ5, Δ13, or dienoic acid), and cis-5-docosenoic (22:1Δ5) acid (Earle et al., 1959; Smith et al., 1960; Bagby et at., 1961). The presence and concentration of fatty acids with Δ5 double bonds is one of the unique characteristics of the oil. Fatty acids with Δ5 double bonds are found in low

concentrations in pines and other species (Hitchcock and Nichols, 1971; Harwood, 1989, 1996), but meadowfoam is presently the only commercial rich source of Δ5 fatty acids. The position of the double bond and high concentration of Δ5 fatty acids has led to the development of novel chemical derivatives often with high yields or processing efficiencies (Chang and Rothfus, 1977; Kaneniwa et al., 1988; Isbell, 1997). The triglyceride (crude oil), fatty acids, and chemical derivatives have been proposed for use as feedstocks for producing waxes, lubricants, surfactants, detergents, plastics, and other products (Miwa et al., 1962; Higgins et al., 1971; Chang and Rothfus, 1977; Pierce and Jain, 1977, Battey et al., 1989; Harwood, 1989, 1996; Klieman, 1990; Topfer et al., 1995; Isbell, 1997). The oil is presently widely used in cosmetics (Isbell, 1997).

The biosynthesis of certain VLCFs is fairly well understood in plants. De novo fatty acid biosynthesis is carried out in the chloroplast and other plastids where fatty acid synthase sequentially condenses two-carbon units into fatty acyl chains with $C_{16}$ and $C_{18}$ chain lengths in common oils (Browse and Somerville, 1991; Ohlrogge and Browse, 1995). The most common fatty acids produced through this process are palmitic (16:0), stearic (18:0), and oleic (18:1Δ9) acids. These fatty acids are released from acyl carrier protein by the activities of thioesterases. The acyl residues are exported to the cytoplasm and converted to acyl-CoA esters by acyl-CoA synthetase, thereby producing substrates that are further elongated in species that produce VLCFs and further desaturated in species that produce polyunsaturated fatty acids. These processes and the assembly of triacylglycerols (seed storage lipids) are carried out in the endoplasmic reticulum (Browse and Somerville, 1991).

Whereas the end products of de novo fatty acid synthesis are 16- or 18-carbon fatty acids in common seed oils, several species, as noted earlier, produce longer chains. Elongation of the fatty acid carbon chain from $C_{18}$ to $C_{20}$ and $C_{20}$ to $C_{22}$ occurs by sequential addition of one and two $C_2$ units from malonyl coenzyme A (CoA) to the $C_{18}$ carbon skeleton (James et al. 1995).

Four enzymatic reactions are involved in the elongation systems of plants: (1) condensation of malonyl-CoA with a long chain acyl CoA to form a β-ketoacyl CoA in which the acyl moiety has been elongated by two carbons, (2) reduction to β-hydroxyacyl-CoA, (3) dehydration to enoyl CoA, and (4) reduction of enoyl CoA to yield elongated acyl-CoA by two carbons (Fehling and Mukherjee 1991; Cassagne et al., 1994). These four activities of fatty acid elongation (FAE) are named elongase (von Wettstein-Knowles, 1982). A key component of this elongase complex is the β-ketoacyl-CoA synthase, a condensing enzyme which in *Arabidopsis* is encoded by *FAE1* (Kunst et al. 1992), which catalyzes the condensation of malonyl CoA with long-chain acyl-CoA.

The pathway for producing VLCFs in *Arabidopsis* and rapeseed have been well characterized. Cis-11-eicosenoic (20:1Δ11) and erucic acid are produced by elongating 18:1 to 20:1 and 20:1 to 22:1, respectively (Agrawal and Stumpf, 1985; Harwood, 1989, 1996; Kunst et al., 1992; Taylor et al., 1992; Ecker and Yaniv, 1993). The substrates are elongated by fatty acid elongases (*FAE1*) complex (Havey and Downey, 1964; Pollard and Stumpf, 1980a; Agrawal and Stumpf, 1985; Kunst et al., 1992; Taylor et al., 1992). This involves four different enzyme reactions (Fehling and Mukherjee, 1991). The concentration of erucic acid in rapeseed oil is primarily affected by two loci, (E1 and E2), with multiple alleles (Downey and Craig, 1964; Harvey and Downey, 1964; Siebel and Pauls, 1989; Ecke et al., 1995; Thormann et al., 1996). Most of the alleles additively affect 20:1Δ11 and erucic acid concentrations and are simply inherited (Kondra and Stefansson, 1965; Johnson, 1977; Ecke et al., 1995). These gene copies contribute about 9-10% erucic acid to the seed oil (Harvey and Downey, 1964). In *Arabidopsis* mutation of *FAE1* locus reduces the level of VLCF in seed oil (James and Dooner, 1990; Lemieux et al., 1990) and result in a deficiency in acyl chain elongation from $C_{18}$ to $C_{20}$ and $C_{20}$ to $C_{22}$ (Kunst et al., 1992). This work supports the notion that *FAE1* gene encodes a condensing enzyme, β-ketoacyl-CoA synthase (KCS). In rapeseed, the two

loci (E1 and E2) were found to co-segregate with the *FAE1* gene (Fourmann et al., 1998) which encodes a condensing enzyme β-ketoacyl-CoA-synthase (Kunst et al., 1992; Lassner et al., 1996; Todd et al., 1999). Two sequences homologous to the *FAE1* gene were isolated from a *Brassica napus* immature embryo cDNA library (Barret et al., 1998). Southern hybridisation showed that the rapeseed β-ketoacyl-CoA-synthase was encoded by a small multigene family. One of the *FAE1* genes is tightly linked to the E1 locus (Barret et al., 1998). The β-ketoacyl-CoA synthase (KCS) is proposed as a candidate gene for underlying erucic acid concentration in rapeseed (Fourmann et al. 1998).

Meadowfoam has evolved a variant of the pathway found in *Arabidopsis* and rapeseed. Pollard and Stumpf (1980b) propose two branches in the pathway underlying the synthesis of VLCFs in meadowfoam. They propose one branch whereby 18:0, 20:0 and 22:0 are produced by elongating 16:0. The elongated fatty acids are desaturated by Δ5 desaturase(s), thereby yielding 18:1Δ5, 20:1Δ5 and 22:1Δ5. The other branch yields 20:1Δ11, 22:1Δ13 (erucic acid), and 22:2Δ5, Δ13 (dienoic acid). The substrates in this branch are produced by elongating oleic acid (18:1Δ9). The diene (22:2Δ5, Δ13) is produced from 22:1Δ13 substrate by a Δ5 desaturase. Knapp and Crane (1998) showed that 22:1Δ13 and 22:2Δ5, Δ13 concentrations were strongly negatively correlated in meadowfoam populations segregating for a dominant gene (*E*) that decreases erucic and increases dienoic acid. Although this was not sufficient to show cause and effect between Δ5 desaturase allelic variants and phenotypic differences in fatty acid concentration, Δ5 desaturase is a logical candidate for the *E* locus. Allelic variants that encode Δ5 desaturases with less activity on erucic acid substrate, for example, should produce more erucic acid than allelic variants that encode Δ5 desaturases with greater activity on erucic acid substrate. Allelic variants of the *E* locus produce such phenotypic differences; thus we speculate that the *E* locus encodes a Δ5 desaturase.

Two wild-type fatty acid profiles have been described for *L. alba* (Knapp and Crane, 1995). *L. alba* ssp. *versicolor* germplasm accessions typically produce more erucic acid and less dienoic acid than those of *L. alba* ssp. *alba* germplasm accessions. The fatty acid profile differences between the subspecies are primarily caused by allelic variants at the *E* locus (Knapp and Crane, 1998). *L. alba* ssp. *alba* germplasm seems to be homozygous for dominant alleles, whereas *L. alba* ssp. *versicolor* seems to be homozygous for recessive alleles (Knapp and Crane, 1995, 1998). Nevertheless, erucic and dienoic acid concentration differences are found among accessions within the subspecies (Knapp and Crane, 1995). These differences could be caused by allelic variants of the *E* locus or perhaps by quantitative trait loci (QTL). Furthermore, erucic and dienoic acid concentrations vary continuously among *E_* and *ee* progeny in segregating populations. These differences could be caused by quantitative trait loci (QTL). The aim of this study was to map the *E* locus and QTL affecting erucic and dienoic acid concentrations among intersubspecific backcross progeny.

## MATERIALS AND METHODS

This study was performed using 180 progeny from the backcross population [(OMF40-11 x OMF64) x OMF64] described by Katengam et al. (1999). The donor parent (OMF 40-11) is an inbred line developed from the *L. alba* ssp. *alba* cultivar Mermaid. The recurrent parent (OMF64) is an inbred line developed from the *L. alba* ssp. *versicolor* accession PI 374801 (Knapp and Crane, 1997). Fatty acid concentrations (phenotypes) were quantified for 180 progeny, while molecular marker genotypes were measured on and a genetic map was constructed from 100 progeny (Katengam et al., 1999). The significance of mean difference between *E_* and *ee* progeny, and the coefficient of determination ($R^2$) for the effect of *E* locus for 180 $BC_1$ progeny

were determined using one-way ANOVA from SAS PROC GLM (SAS Institute, 1996) (SAS Institute Inc., Cary, NC). Differences were considered significant at $p < 0.05$; actual *p* values are given where appropriate. The original map was comprised of 103 amplified fragment length polymorphism (AFLP) markers and one phenotypic marker (*E*). We selected 66 well-spaced loci from the map for the analyses described in this paper. The map was reconstructed using the methods described by Katengam et al. (1999) (Fig. 4.1)

Backcross seed was germinated at 4°C and transplanted to potting soil (pumice: peat moss: sandy loam) in 7.5 x 7.5 cM plastic pots. The seedlings were grown for 5 weeks in a growth chamber (Model CEL 37-14, Sherer-Gillett Co., Marshall, MI) with daily temperatures at 15°C of 8 h light. Five week-old plants were transferred from the growth chamber to a greenhouse with daily temperatures ranging from 18°C (night) to 25°C (day) and 16 h light. The plants were grown to maturity and harvested at 10 weeks.

Because whole plants were needed to develop the genetic map (Katengam et al., 1999) we performed chemical analyses of the backcross progeny using half- seed samples (Knapp and Crane, 1998). $BC_1$ seeds were sliced into nearly equal apical and basal halves with a scalpel. Fatty acids were extracted from the apical explants as described by Knapp and Crane (1995) and methylated, while the basal explants were germinated and transplanted. Fatty acid concentrations were measured using a Hewlett-Packard gas chromatograph (HP 6890 Series GC system) with a Durabond-23 column (30 meters, 0.25 mm I.D) (J&W Scientific, Folsom, CA). Standards with known 18:1Δ5, 18:1Δ9, 18:3 (linolenic acid), 20:0, 20:1Δ11, 20:1Δ5, 22:1Δ5, erucic acid, and dienoic acid concentrations were used to identify peaks and check measurements. Standards were acquired from Thomas Abbott (USDA-ARS, NCAUR, Peoria, IL).

Histograms (phenotypic distributions) were produced for the four principal fatty acids found in meadowfoam oil (20:1Δ5, 22:1Δ5, erucic acid, and dienoic

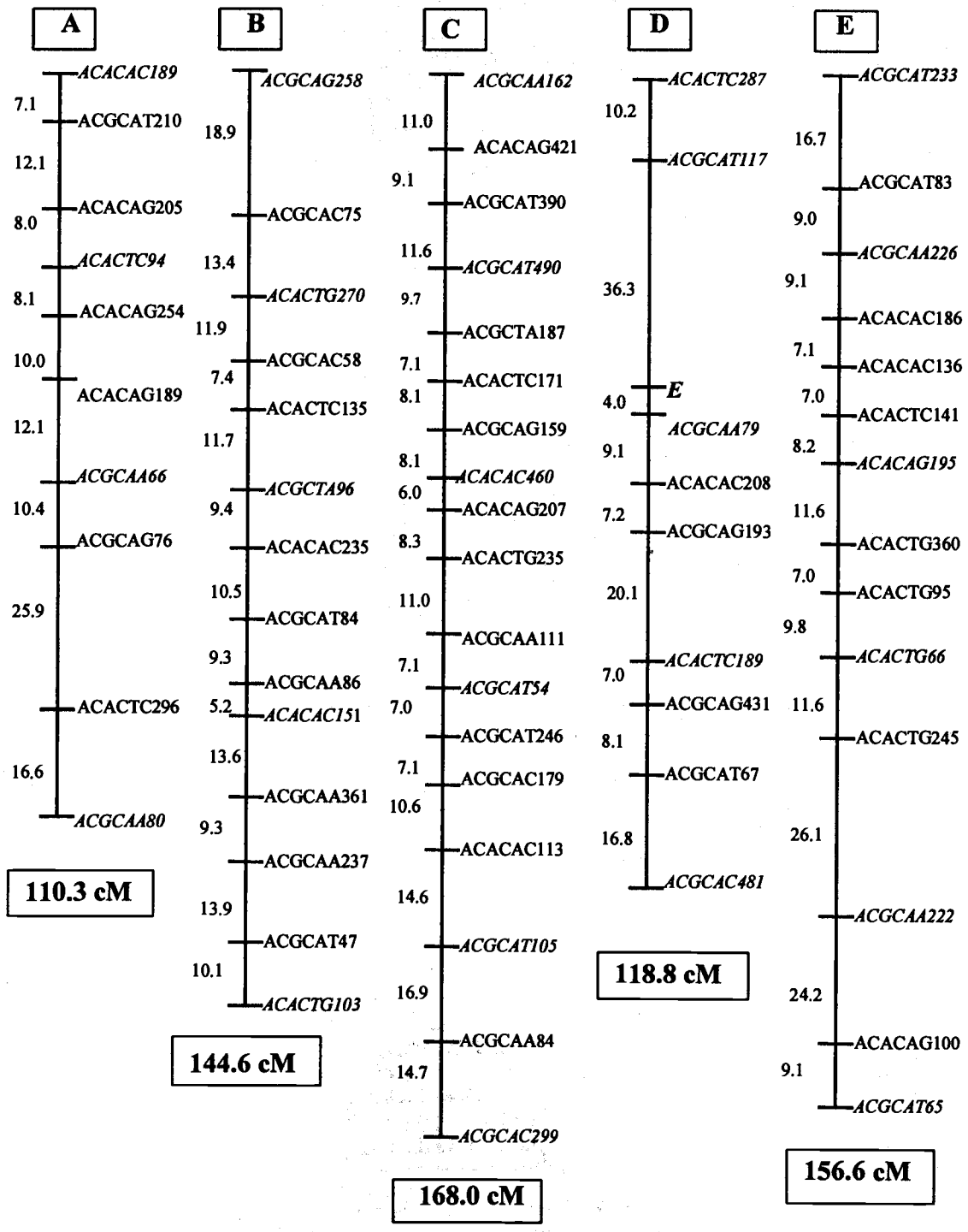


**Figure 4.1** A genetic map of meadowfoam (*Limnanthes alba*) comprised of a subset of 66 AFLP loci and one phenotypic marker (*E*). The map was built using 100 [(OMF40-11 x OMF64) x OMF64] $BC_1$ progeny. Background markers are shown in italic.

acid) and for erucic by dienoic acid concentration. Backcross progeny were assigned to *E_* (low erucic and high dienoic acid concentration) and ee (high erucic and low dienoic acid) classes using the erucic by dienoic acid distribution. The fit of the observed phenotypic distribution to the predicted genotypic distribution for the segregation of a single gene in a backcross population (1:1) was checked using a $\chi 2$ -test with one degree of freedom. The *E* locus was mapped using the methods described by Katengam et al. (1999).

QTL analyses were performed using genotypes (AFLP marker scores) and phenotypes (fatty acid concentrations) recorded on 100 backcross progeny. Simple interval mapping (SIM) and simplified composite interval mapping (sCIM) analyses were performed with MQTL (Tinker and Mather, 1995). sCIM analyses were performed using 27 'background' markers, which are used to account for possible variation in regions of the genome other than that under test which can refine the location of QTLs reveals by SIM (Tinker and Mather, 1995). The *E* locus was one of the background markers used in this analysis. The background markers were spaced every ~30 cM, start from both ends of each linkage group. SIM and sCIM test statistics and tests of the null hypothesis (no QTL) were performed using permutation tests (Churchill and Doerge, 1994) with 1,000 permutations per test. Tests were performed for every 5 cM interval in the genome. Thresholds for hypothesis tests were estimated from the empirical distribution by setting a genome-wise Type I error threshold of 0.05. Primary QTL locations were established where peaks for SIM and sCIM coincided (Tinker and Mather, 1995). In additional, 'secondary' QTL locations were declared where either SIM or sCIM, but not both gave evidence for QTLs (Tinker et al., 1996). The phenotypic variance explained ($R^2$) of QTLs was estimated by MQTL (Tinker and Mather, 1995). The QTL effect was estimated by regression analysis.

## RESULTS AND DISCUSSION

Two continuous, non-overlapping erucic acid distributions were observed (Fig. 4.2). The dienoic acid distribution was continuous with perhaps two modes, but with no discontinuities. The 20:1Δ5 and 22:1Δ5 distributions were continuous (Fig. 4.3). The erucic by dienoic acid distribution showed that the backcross progeny segregated into two distinct phenotypic classes (Fig. 4.4), one with low erucic and high dienoic acid and the other with high erucic and low dienoic acid, as previously reported for this inter-subspecific cross (Knapp and Crane, 1998). There were 96 progeny in the low erucic, high dienoic (dominant or *E_*) class and 84 progeny in high erucic, low dienoic (recessive or *ee*) class (Fig. 4.4). The fit of the observed phenotypic distribution was not significantly different from 1:1 ($p = 0.55$), the expected distribution for a single gene segregating in a backcross population. Tests for independent assortment were performed between the *E* locus and 103 AFLP loci on the genetic map (Katengam et al. 1999). The *E* locus mapped to linkage group four near the AFLP locus ACG_CAA_79 (Katengam et al. 1999) (Fig. 4.4).

OMF40-11, the *E_* inbred line produced 95 g $kg^{-1}$ erucic and 204 g $kg^{-1}$ dienoic acid, whereas OMF64, the *ee* inbred line 203 g $kg^{-1}$ erucic and 92 g $kg^{-1}$ dienoic acid (Table 4.1). *E_* progeny produced 90 g $kg^{-1}$ erucic and 227 g dienoic acid $kg^{-1}$, whereas *ee* progeny produced 227 g $kg^{-1}$ erucic and 106 g $kg^{-1}$ dienoic acid (Table 4.2). The fatty acid concentration means for *E_* and *ee* progeny were significantly different for 18:1Δ5 and all of the VLCFs (Table 4.2); thus the *E* locus pleiotropically affected the concentrations of most the fatty acids produced in meadowfoam seed oil.

Our hypothesis is that the *E* locus encodes a Δ5 desaturase. The concentrations of 22:1Δ13 (erucic acid) and 22:1Δ5Δ13 (dienoic acid) were nearly perfectly negatively correlated ($p = -0.90$) among the backcross

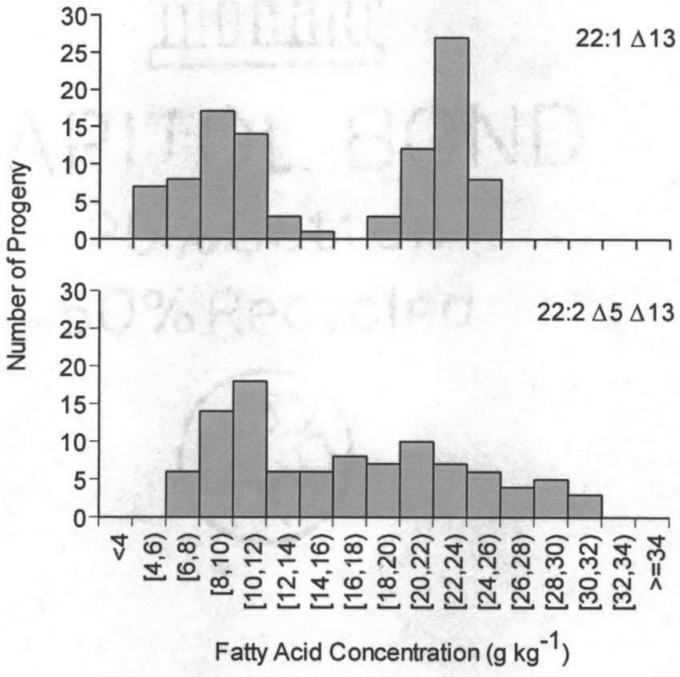


**Figure 4.2** Phenotypic distribution for fatty acid content in erucic acid (22:1Δ13) and dienoic acid (22:1Δ5, Δ13) for 180 $BC_1$ progeny derived from the cross between [(OMF40-11x OMF64) x OMF64]

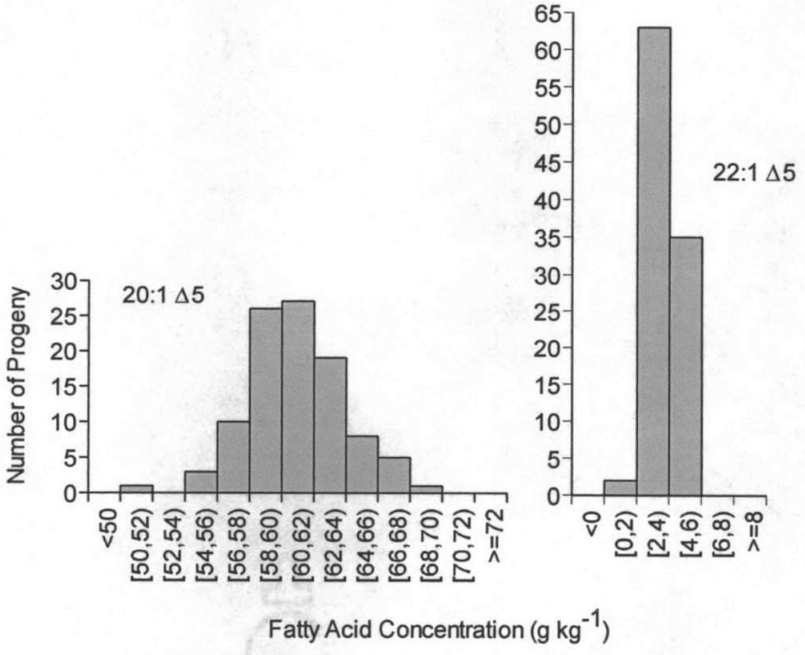


**Figure 4.3** Phenotypic distribution for fatty acid content in 20:1Δ5 and 22:1Δ5 for 180 $BC_1$ progeny derived from the cross between [(OMF40-11x OMF64)x OMF64]

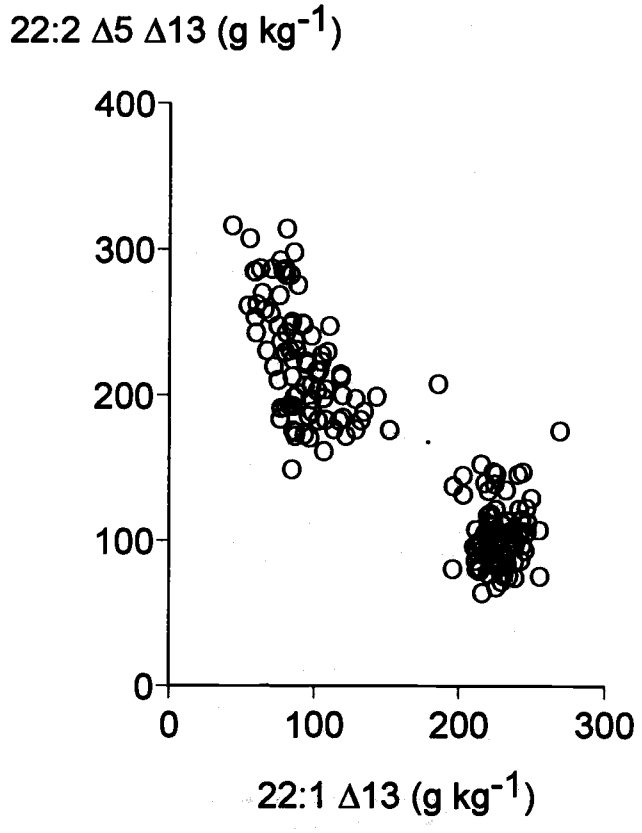


**Figure 4.4** Joint phenotypic distribution of erucic acid (22:1Δ13) by dienoic acid (22:2Δ5, Δ13) concentration (g $kg^{-1}$) for 180 [(OMF40-11 x OMF64) x OMF64] $BC_1$ progeny.

progeny (Table 4.3). The Pollard and Stumpf (1980b) pathway for VLCFs biosynthesis in meadowfoam predicts that 22:1Δ13 and 22:2Δ5, Δ13 are sequentially produced in one branch of the pathway from elongated 18:1 substrate, while 20:1Δ5 and 22:1Δ5 are sequentially produced in another branch of the pathway from elongated 20:0 substrate. As predicted by this model, reduced activity of the Δ5 desaturase on 22:1Δ13 (erucic acid) substrate should increase 22:1Δ5, Δ13 (dienoic acid) concentration (the phenotypic change produced by the *E* locus). Whether or not the same desaturase has activity on 20:0 and 22:0 substrates is uncertain. Although

**Table 4.1** Mean seed oil fatty acid concentrations for OMF40-11 and OMF64, minimum and maximum seed oil fatty acid concentrations for 180 [(OMF40-11 x OMF64) x OMF64] $BC_1$ progeny of meadowfoam

| | | | $BC_1$ | |
|---|---|---|---|---|
| Fatty Acid | OMF40-11 | OMF64 | Minimum | Maximum |
| | | g $kg^{-1}$ | | |
| 18:1Δ5 | 5.1 | 3.4 | 0 | 8.9 |
| 18:1Δ9 | 10.3 | 14.0 | 7.1 | 41.8 |
| 18:3 | 4.7 | 11.3 | 0 | 33.8 |
| 20:0 | 7.0 | 12.6 | 0 | 16.5 |
| 20:1Δ5 | 628.8 | 610.5 | 466.2 | 696.1 |
| 20:1Δ11 | 6.5 | 8.6 | 0 | 28.5 |
| 22:1Δ5 | 38.2 | 44.0 | 13.6 | 55.2 |
| 22:1Δ13 | 95.2 | 203.5 | 42.3 | 268.7 |
| 22:2Δ5Δ13 | 204.2 | 92.1 | 66.0 | 317.8 |

**Table 4.2** Seed oil fatty acid concentrations, probability (*p*) of tests of significance of mean differences between *E_* and *ee* progeny, and coefficient of determination ($R^2$) for the effect of the *E* locus for 180 [(OMF40-11 x OMF64) x OMF64] $BC_1$ progeny of meadowfoam

| | Mean | | | |
|---|---|---|---|---|
| Fatty Acid | *E_* | *ee* | *p* | $R^2$ |
| | g $kg^{-1}$ | | | |
| 18:1Δ5 | 3.9 | 2.4 | 0.0001 | 0.27 |
| 18:1Δ9 | 13.5 | 11.9 | 0.0217 | 0.03 |
| 18:3 | 7.2 | 6.4 | 0.165 | 0.01 |
| 20:0 | 1.6 | 3.4 | 0.0048 | 0.04 |
| 20:1Δ5 | 621.5 | 596.7 | 0.0001 | 0.14 |
| 20:1Δ11 | 2.8 | 6.1 | 0.0001 | 0.12 |
| 22:1Δ5 | 32.3 | 39.6 | 0.0001 | 0.20 |
| 22:1Δ13 | 90.0 | 227.3 | 0.0001 | 0.94 |
| 22:2Δ5Δ13 | 227.2 | 106.2 | 0.0001 | 0.77 |

numerous desaturases have been cloned from plants, cDNA or genomic sequences for Δ5 desaturases from meadowfoam have not yet been published, so the number, specific activities, and expression patterns of the novel meadowfoam desaturases are not known. The concentrations of 20:1Δ5 and 22:1Δ5 were significantly negatively and positively correlated with 22:1Δ13 concentration among the backcross progeny, respectively (Table 4.3). Although the *E* locus significantly affected 20:1Δ5 and 22:1Δ5 concentration, this locus was only associated with 14 and 20% of the phenotypic variance for 20:1Δ5 and 22:1Δ5 concentration, respectively (Table 4.2)

**Table 4.3** Simple phenotypic correlations between major fatty acid concentrations in a meadowfoam $BC_1$ mapping population

| | Fatty acid | | |
|---|---|---|---|
| Fatty Acid | 22:1Δ5 | Erucic acid | Dienoic acid |
| 20:1Δ5 | 0.33** | -0.41** | 0.02 |
| 22:1Δ5 | | 0.43** | -0.67** |
| Erucic acid | | | -0.90** |

**Correlations marked with double asterisks were highly significantly different from 0.00 ($p$ = 0.01).

By contrast, the *E* locus was associated with 94% of the phenotypic variance for erucic and 77% of the phenotypic variance for dienoic acid concentration; thus, for erucic acid concentration, most of the phenotypic variance was genetic (the heritability for this trait is greater than or equal to 0.94) (Table 4.2). There was greater dispersion in the dienoic than the erucic acid distribution (Fig. 4.3). Dienoic acid concentrations ranged from 151 to 318 g $kg^{-1}$ among *E_* and 66 to 209 g $kg^{-1}$ among *ee* progeny (the dienoic acid

distributions of the two classes overlapped) (Fig. 4.3). This variation might have arisen from the cumulative effects of numerous genes, each having a small effects (QTL), or by non-genetic factors, or by both.

QTL affecting 20:1Δ5, 22:1Δ5, 22:1Δ13 (erucic acid), and 22:2Δ5, Δ13 (dienoic acid) concentrations mapped to linkage group four and were centered or near the *E* locus accounted for 19%, 16%, 88% and 74% of total phenotypic variation, respectively (Table 4.4, Fig. 4.5- 4.7). Our hypothesis was that QTL were associated with some fraction of the phenotypic differences within *E* locus classes; however, simplified composite interval mapping searches of the genome did not uncover any QTL other than the QTL peak for 22:1Δ13 (erucic acid), and 22:2Δ5, Δ13 (dienoic acid) concentrations centered on or nearly on the *E* locus on linkage group four (Fig. 4.5 -4.6). QTL effects on linkage group four for the different fatty acids were presumably caused by the effect of the *E* locus alone. The coincident of QTL localization at the *E* locus affecting these fatty acids (multiple traits) suggests pleiotropic effects (Falconer, 1989; Paterson et al., 1991; Xiao et al., 1996).

QTL affecting 20:1Δ5, 22:1Δ5 mapped near the *E* locus accounted for small proportion of total phenotypic variation (19% and 16%, respectively). The unexplained remainder of phenotypic variation might be due to undetected QTLs or environmental effect or both. Moreau et al. (1981) characterized Δ5 fatty acid desaturase activity in extracts of developing meadowfoam seed and reported that the highest activity was obtained from eicosanoyl-CoA (20:0) which elongated from palmitate but the activity of Δ5 desaturase was not detected for erucic acid. Erucic acid is one of the major VLCFs which accounts for ~15% of total seed oil. The detected Δ5 desaturation of erucic acid *in vivo* have been demonstrated by Pollard and Stumpf (1980b). Since the *E* locus had major effect for 22:1Δ13, and 22:2Δ5, Δ13 whereas less effect for 20:1Δ5 and 22:1Δ5, we speculated that there

**Table 4.4** Genomic locations, allele phase, phenotypic effect and phenotypic variation explained ($R^2$) of putative QTLs for fatty acid composition of meadowfoam (*Limnanthes alba*) seed oils in 100 [(OMF40-11 x OMF64) x OMF64] $BC_1$ progeny.

| Fatty acids | LG† | NML‡ | Distance of NML (cM) | DPE§ (%) | Phenotypic Effect¶ (%) | $R^{2\#}$ (%) | Total $R^{2\dagger\dagger}$ (%) |
|---|---|---|---|---|---|---|---|
| 22:2Δ5Δ13 | 4 | E‡‡ | 0 | $P_1$ | 10.99 | 74 | 74 |
| | 4 | ACACTC189‡‡ | 10 | $P_1$ | 1.36 | 36 | |
| 22:1Δ13 | 4 | E‡‡ | 0 | $P_2$ | 13.04 | 88 | 90 |
| | 4 | ACACTC189‡‡ | 10 | $P_2$ | 1.23 | 45 | |
| 22:1Δ5 | 4 | E§§ | 0 | $P_2$ | 0.4 | 19 | 27 |
| | 3 | ACGCAC179§§ | 0 | $P_2$ | 0.6 | 11 | |
| 20:1Δ5 | 4 | E§§ | 10 | $P_2$ | 2.9 | 16 | 24 |
| | 4 | ACGCAA79§§ | 0 | $P_1$ | 5.5 | 23 | |

† = Putative genetic linkage group in meadowfoam map
‡ = Nearest marker locus of putative QTL
§ = Direction of phenotypic effects ($P_1$= OMF40-11, $P_2$ = OMF64 $S_5$), indicating allele increase that fatty acid content
¶ = Phenotypic effect accounted for the QTL
# = Phenotypic variation accounted for the QTL
†† = Total phenotypic variation accounted for the QTLs affecting that fatty acid (Multi-locus $R^2$)
‡‡ = Primary QTL
§§ = Secondary QTL

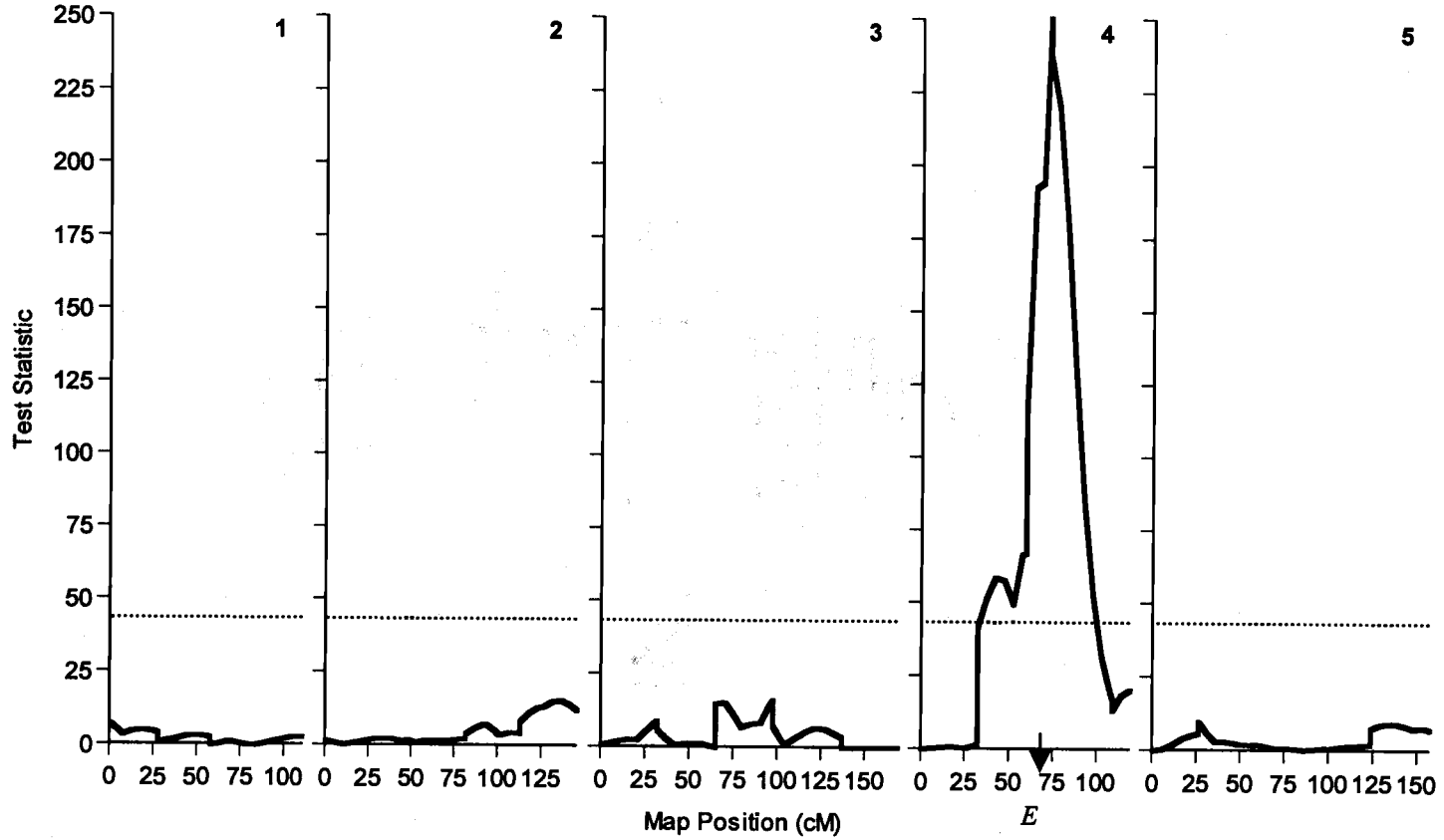


**Figure 4.5** sCIM test statistics from the QTL analysis for erucic acid (22:1 Δ13) concentration for 5 cM intervals for each linkage group (x = 5) in the meadowfoam genome. The significance threshold for composite interval mapping is shown as a dotted horizontal line for an empirical genome-wise Type I error probability threshold of 0.05. The position of the *E* locus in linkage group four is shown as a vertical arrow.

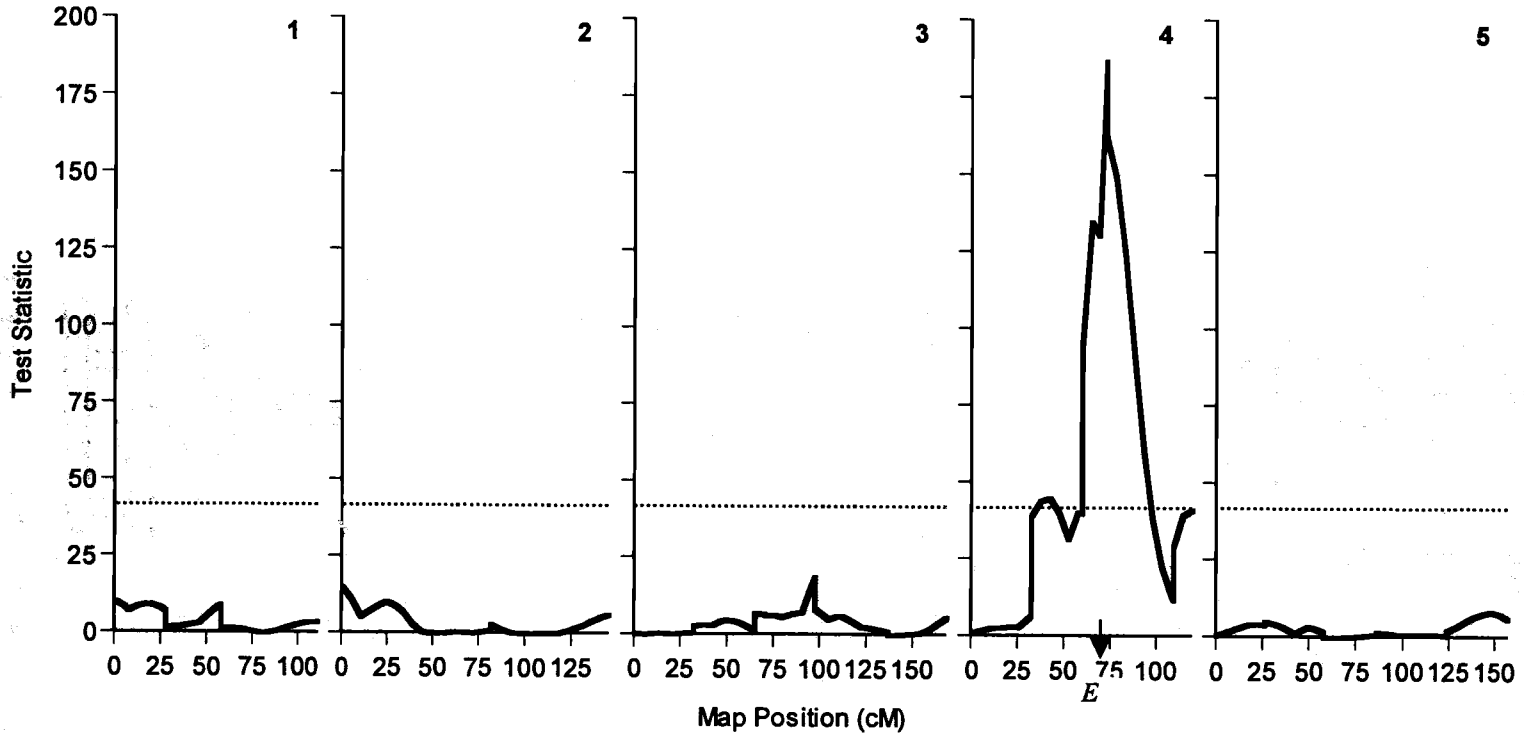


**Figure 4.6** sCIM test statistics from the QTL analysis for dienoic acid (22:2 Δ5 Δ13) concentration for 5 cM intervals for each linkage group (x = 5) in the meadowfoam genome. The significance threshold for composite interval mapping is shown as a dotted horizontal line for an empirical genome-wise Type I error probability threshold of 0.05. The position of the *E* locus in linkage group four is shown as a vertical arrow.

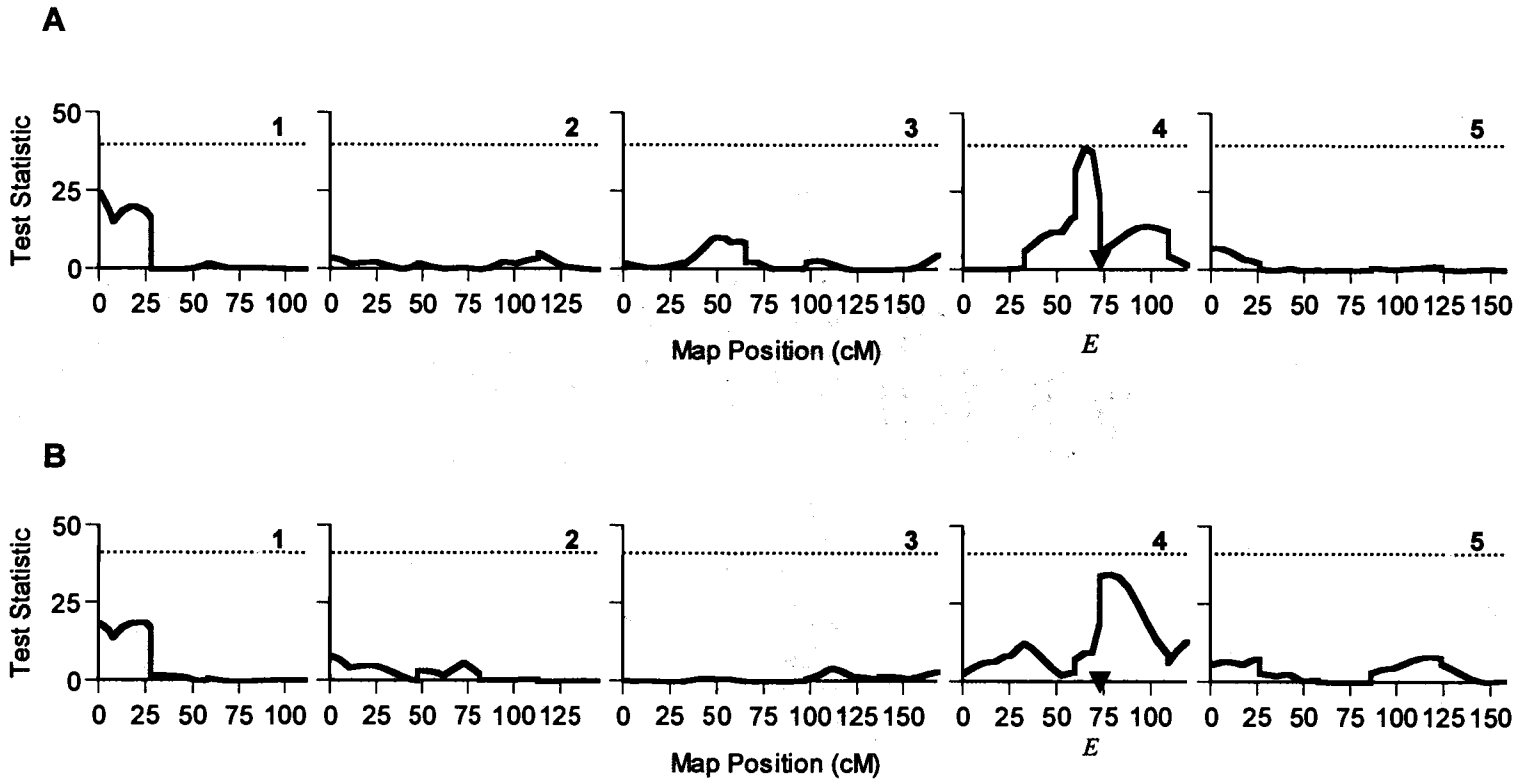


**Figure 4.7** sCIM test statistics from the QTL analysis for (A) 20:1 Δ5, (B) 22:1 Δ5 concentration for 5 cM intervals for each linkage group (x = 5) in the meadowfoam genome. The significance threshold for composite interval mapping is shown as a dotted horizontal line for an empirical genome-wise Type I error probability threshold of 0.05. The position of the *E* locus in linkage group four is shown as a vertical arrow.

might be more than one Δ5 desaturase genes underlying Δ5 desaturation for 20:1 and 22:1.

We showed that QTL underlying erucic and dienoic acid was mapped at the *E* locus. The identified QTL may be allelic to a major gene (*E*) affecting the phenotypic difference between these two subspecies. Several studies have been reported on the coincidence of QTLs mapped to the same location of major genes affecting plant height in maize (Beavis et al., 1991; Edwards et al., 1992; Veldboom et al., 1994). These studies supported the hypothesis proposed by Robertson (1985) that QTLs are allelic with the major genes affecting the same trait.

We empirically tested for the presence of genetic effects other than the *E* locus by selecting and crossing *E_* individuals from the tails of the 22:2Δ5, Δ13 (dienoic acid) distribution from the backcross population. The erucic and dienoic acid concentrations $F_2$ progeny produced from this cross are shown in Fig. 4.8. One of the selected individuals (the $P_1$ parent) produced 93.6 g $kg^{-1}$ erucic and 176.8 g $kg^{-1}$ dienoic acid, while the other selected individual (the $P_2$ parent) produced 62.1 g $kg^{-1}$ erucic and 284.3 g $kg^{-1}$ dienoic acid. $F_2$ progeny replicated the original phenotypic distribution for the *E_* class (Fig. 4.3 and 4.8) and spanned the phenotypic range between the parents ($P_1$ and $P_2$)—erucic acid concentrations ranged from 31 to 135 g $kg^{-1}$ and dienoic acid concentrations ranged from 151 to 347 g $kg^{-1}$ (Fig. 4.8). While not definitive, these data suggest that genetic factors (QTL in this cross) were associated with erucic and dienoic acid concentration differences, especially the latter, among *E_* progeny. Our QTL mapping study was not powerful enough to uncover the putative QTL.

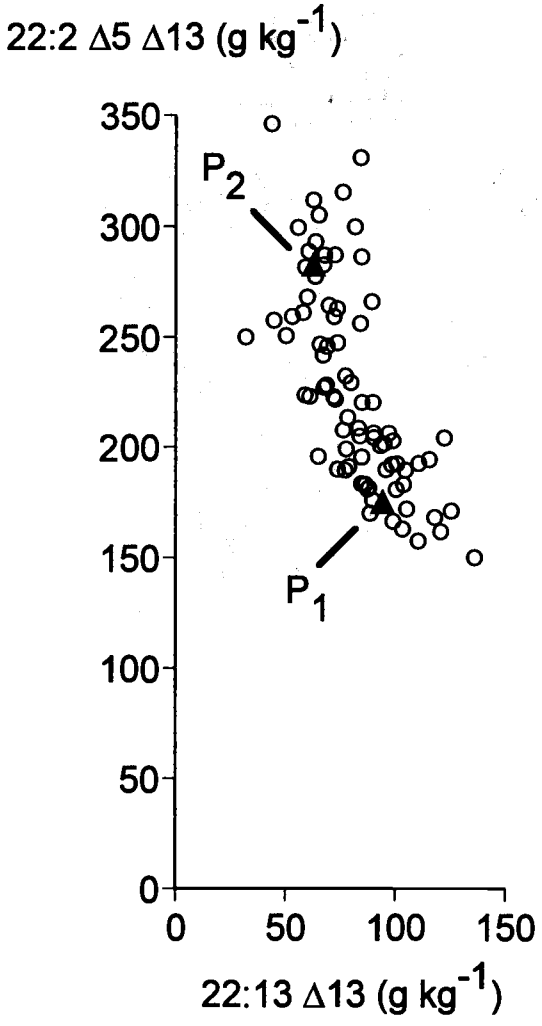


**Figure 4.8** Joint phenotypic distribution for erucic acid (22:1Δ13) concentration by dienoic acid (22:2Δ5Δ13) concentration (g $kg^{-1}$) for 100 $F_2$ progeny from a cross between $P_1$ and $P_2$ (shown as filled triangle).

## ACKNOWLEDGMENTS

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# CHAPTER 5

## CONCLUSION

Molecular marker information is rapidly becoming an essential tool to expedite the process of crop improvement and cultivar development. Although enormous molecular marker and genetic mapping information has been generated for most food and industrial crops, such information is lacking in meadowfoam, a recently domesticated source of novel very long-chain seed oils ($C_{20}$ and $C_{22}$) with low concentrations of saturated fatty acids (typically less than 2%). We employed molecular markers to expedite meadowfoam breeding and cultivar improvement. The objectives of this study were to assess the genetic diversity and relationships patterns of elite and exotic meadowfoam germplasm in the Inflexae section, to construct a genetic linkage map, and to map genes and quantitative trait loci (QTL) underlying fatty acid profile differences in the very long-chain oilseed meadowfoam. AFLP, a relatively high throughput marker, was chosen to facilitate these molecular breeding projects owing to its outstanding features such as being highly abundant, reproducible,with relatively high multiplex ratio, no prior sequence information required, and a large number of polymorphic markers can be produced in a short period of time.

A study of genetic diversity was undertaken to gain an understanding of the pattern of genetic relationships among elite and exotic germplasm accessions of Inflexae section. We fingerprinted 41 accessions including nine inbred lines, eight open-pollinated cultivars, and 24 wild populations and species using AFLP markers. A total of 176 polymorphic markers was generated from six primer combinations. Even though a large number of polymorphic markers were produced, only unambiguous markers across all genotypes were included in our genetic analysis. Out of 176 polymorphic markers tested on all genotypes, 175 were polymorphic among wild populations and species

whereas 142 were polymorphic among inbred lines and 138 for open-pollinated cultivars. The polymorphic information content (PIC) varied from 0.0 to 0.5 with an average of 0.39. Forty-two percent of the markers showed high PIC scores in a range between 0.45 to 0.5, indicating that meadowfoam germplasm in Inflexae section gene pool was diverse. The genetic distance estimated by Roger-W ranged from 0.14 to 0.55 with an average of 0.44. The UPGMA cluster analysis showed concordance with species, subspecies, geographic, and breeding origin. The first principal component analyses accounted for 37% of the total variation of genetic distance estimated. There were three diverse clusters. Cluster and principal component analysis clearly separated *L. floccosa* from *L. alba*. Within *L. alba*, subspecies *alba* and *versicolor* were distinctly separated into two groups. Inbred lines derived from *L. alba* spp. *alba* were clustered with *L. alba* spp. *alba*. Similarly, inbred lines derived from *L. alba* spp. *versicolor* were clustered with *L. alba* spp. *versicolor*. Open-pollinated cultivars were clustered to *L. alba* spp. *alba*. Our results suggested that the *L. alba* gene pool was found to be genetically diverse and most accessions were separated by great genetic distances.

DNA marker resources and molecular breeding tools are limited in meadowfoam. The aim of the second study (chapter 3) was, therefore, to develop a genetic linkage map for meadowfoam to facilitate molecular breeding and genome mapping. The initial linkage map was built using intersub-specific backcross progeny derived from a cross between OMF40-11 and OMF64 with AFLP markers. The inbred parent lines, OMF40-11 (*L. alba* ssp. *alba*) and OMF64 (*L. alba* ssp. *versicolor*) segregate for self-pollination, fatty acid content, seed oil content, growth habit, seed yield, and variety of morphological traits. Sixteen primer combinations were used to screen these two parents. These primers produced 425 polymorphic and 1376 monomorphic bands. These two parents are highly polymorphic and have greatly facilitated the development of the map and should be useful for marker-assisted selection in inter-subspecies crosses. Nine primer

combinations were chosen based on polymorphic rate to generate AFLP markers for constructing genetic map. One hundred and seven segregating AFLP loci were scored and mapped. The initial AFLP genetic map comprised of 104 markers, assigned to 5 linkage groups which correspond to five haploid chromosomes (x = 5) of *L. alba*. The framework map covers 698.3 cM with a mean density of 6.7 cM. Our results show that this map had excellent genome coverage. The lack of information on the position of telomeres, however prevents us from considering this map complete. Mapping telomeres would help us to define the boundaries of this genetic map. With this information the total genetic distance and potential genetic expansion for each chromosome can be accurately estimated.

The AFLP markers were evenly distributed among the five linkage groups. This finding is contrary to that in several other plant species in which AFLP markers tended to be clustered in some regions particularly around centromeric and telomeric areas (Tanksley et al. 1992; Becker et al. 1995; Qi et al. 1998; Jan et al. 1998). The suppression of recombination in centromeric areas may result from both a centromere effect and (or) a lower level of recombination in heterochromatin around the centromeres (Tanksley et al. 1992). Heterochromatin reduces levels of recombination presumably due to its more condensed during meiosis at the time of crossing over (Roberts 1965).

Although most of the loci were evenly distributed, there were some regions where the distance between pairs of markers exceeded 20 cM. These gaps were probably caused by a lack of polymorphic markers in the genome, representing areas of high recombination (Becker et al. 1995; Berry et al. 1995; Keim et al. 1997). These gaps could be filled as more polymorphic markers are identified and added to the map. Saturating this existing meadowfoam map with a variety of codominant markers, for example, simple sequence repeat (SSR) and intron fragment length polymorphism (IFLP) markers, provides the flexibility for utilization of this molecular genetic map for

different purposes such as for marker-assisted selection (MAS) and map-based cloning. The development of SSR and IFLP markers for meadowfoam is underway in our laboratory. This will provide opportunities not only to anchor the locus specific markers to each linkage group but also to saturate the existing meadowfoam map with multi-allelic, highly polymorphic SSR and gene specific IFLP markers. SSR markers are highly polymorphic and can be multiplexed and automated for high throughput genotyping, and convenient to exchange between laboratories (Powell et al. 1996a, 1996b; Senior et al. 1996). IFLPs are gene specific markers which are developed from unique sequences flanking introns of specific genes (Slabaugh et al. 1997; Hongtrakul et al. 1998). Polymorphisms are caused by intron fragment length differences. IFLP markers for meadowfoam have been developed in our laboratory for fatty acid gene families, for example FatB (thioesterases) and SAD (Δ9-stearoyl ACP desaturase) genes (unpublished data). These PCR-based markers would be useful in synteny mapping and candidate gene analysis as well.

AFLPs have several outstanding features and provide efficient high throughput markers for meadowfoam genome mapping and molecular breeding. The utility of AFLP markers is greatest where scoring as dominant markers is sufficient, for instance mapping in backcross (BC), double haploid (DH), or recombinant inbred lines (RIL), and marker-assisted backcross breeding (Knapp et al. 1999). AFLPs can be scored as co-dominant markers by estimating allele dose using densitometry and the difference of doses is great enough to distinguish between homozygotes and heterozygotes (Hongtrahul 1997, Knapp et al. 1999). The utility of AFLPs as co-dominant markers would be recognized as the hardware and software for co-dominant scoring AFLPs become more available. AFLPs are visually scored as dominant markers. An automated gel scoring system was successfully used to analyze the complex AFLP patterns obtained from sugarcane (Besse et al. 1998). The autoradiographs were scanned using a large BioRad scanner with the GelCompare software (Molecular Analyst Fingerprinting Software, BioRad)

and the gel images can be analyzed and scored using this software. Perkin Elmer has developed an automated AFLP analysis system that requires the use of Applied Biosystem Inc (ABI) sequencer and fluorochomes. The release of software for automated AFLP acquisition and analysis including co-dominant scoring would enhance the utility of AFLP markers for molecular breeding and genome mapping. We showed here that the genetic linkage maps for meadowfoam can be rapidly constructed using small set of AFLP primer combinations. AFLPs should have tremendous utility for molecular breeding, especially marker-assisted backcross breeding, in meadowfoam.

Genetic linkage maps are powerful tool for localizing genes underlying both simple and complex traits. We utilized the AFLP genetic linkage map to locate genes and quantitative trait loci (QTLs) affecting fatty acid profile differences in oilseed meadowfoam (chapter 4). The QTL analyses were performed using both interval mapping and simple regression procedures. Both simple interval mapping (SIM) and simplified composite interval mapping (sCIM) procedures in MQTL were performed for QTLs searching. In the latter, 27 background markers were used to account for possible variation in the regions of the genome other than those under test which can refine the location of QTLs revealed by SIM or reveal additional QTL (Tinker and Mather 1995). We showed here that QTL underlying erucic and dienoic acids were mapped to linkage group four at the *E* locus. The identified QTLs may be allelic to a major gene (*E*) affecting the phenotypic difference between these two subspecies. Our results suggested that the effect of the *E* locus was pleiotropic since the QTLs at this locus was associated with multiple traits, for instance erucic and dienoic acid levels. This study did not uncover QTL underlying erucic and dienoic acid concentration differences within the *E_* and *ee* classes. The result of this study confirmed that the *E* locus has a direct effect on the erucic and dienoic acid content in meadowfoam and we speculated that the *E* locus encoded a $\Delta$ 5 desaturase.

Molecular genetic linkage maps appear to be the greatest tool available to elucidate the Mendelian genetic underlying quantitative traits. In this study we simultaneously determined the number, genome location, and effect of loci underlying fatty acid profile differences in oilseed using interval mapping. However, we could not find other QTLs, which have significant effect on erucic and dienoic acids other than the *E* locus as we expected.

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