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V. K. Kishore · P. Velasco · D. K. Shintani · J. Rowe · C. Rosato · N. Adair · M. B. Slabaugh · S. J. Knapp

Conserved simple sequence repeats for the Limnanthaceae (Brassicales)

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Abstract The Limnanthaceae (Order Brassicales) is a family of 18 taxa of *Limnanthes* (meadowfoam) native to California, Oregon, and British Columbia. Cultivated meadowfoam (*L. alba* Benth.), a recently domesticated plant, has been the focus of research and development as an industrial oilseed for three decades. The goal of the present research was to develop several hundred simple sequence repeat (SSR) markers for genetic mapping, molecular breeding, and genomics research in wild and cultivated meadowfoam taxa. We developed 389 SSR markers for cultivated meadowfoam by isolating and sequencing 1,596 clones from *L. alba* genomic DNA libraries enriched for AG_n or AC_n repeats, identifying one or more unique SSRs in 696 clone sequences, and designing and testing primers for 624 unique SSRs. The SSR markers were screened for cross- taxa utility and polymorphisms among ten of 17 taxa in the Limnanthaceae; 373 of these markers were polymorphic and 106 amplified loci from every taxon. Cross-taxa amplification percentages ranged from 37.3% in *L. douglasii* ssp. *rosea* (145/389) to 85.6% in *L. montana* (333/389). The SSR markers amplified 4,160 unique bands from 14 genotypes sampled from ten taxa (10.7 unique bands per SSR marker), of which 972 were genotype-specific. Mean and maximum haplotype heterozygosities were 0.71 and 0.90, respectively, among six *L. alba* genotypes and 0.63 and 0.93, respectively, among 14 genotypes (ten taxa). The SSR markers supply a critical mass of high-throughput DNA markers for biological and agricultural research across the Limnanthaceae and open the way to the development of a genetic linkage map for meadowfoam ($x = 5$).

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V. K. Kishore · P. Velasco · M. B. Slabaugh · S. J. Knapp (✉)
Department of Crop and Soil Science,
Oregon State University,
Corvallis, OR 97331, USA
e-mail: steven.j.knapp@orst.edu
Tel.: +1-541-7375842
Fax: +1-541-7371334

D. K. Shintani
Department of Biochemistry,
University of Nevada,
Reno, NV 89557, USA

J. Rowe
Nevada Genomics Center,
University of Nevada,
Reno, NV 89557, USA

C. Rosato · N. Adair
Center for Gene Research and Biotechnology,
Oregon State University,
Corvallis, OR 97331, USA

Present address:
V. K. Kishore, Sunseeds,
8850 59th Avenue NE, Brooks, OR 97305, USA

Present address:
P. Velasco, Misión Biológica de Galicia (CSIC),
Aptdo 28, 36080 Pontevedra, Spain

Introduction

The Limanthaceae (Order Brassicales) is a family of 18 species and subspecies of *Limnanthes* native to Pacific Western North America (Mason 1952; Ornduff and Carvello 1968; Ornduff 1971) and one species, *Floerkea proserpinacoides* (false mermaid), native to the north-eastern United States and Canada (Houle et al. 2000). Wild *Limnanthes* (meadowfoam) populations fluorish in temporally wet habitats (vernal pools) west of the Sierra Nevada mountain range in California and the Cascade mountain range in Oregon and British Columbia (Mason 1952). Several taxa in the family are threatened or endangered and native habitats are rapidly disappearing, particularly in urban growth boundaries. Twelve taxa in

the family (*L. bakeri*, *L. douglasii* ssp. *sulphurea*, *L. floccosa* ssp. *bellingeriana*, *L. floccosa* ssp. *californica*, *L. floccosa* ssp. *grandiflora*, *L. floccosa* ssp. *pumila, L. gracilis* ssp. *gracilis*, *L. gracilis* ssp. *parishii*, *L. macounii*, *L. montana*, *L. striata*, and *L. vinculans*) are listed as rare, vulnerable, or endangered (Eastman 1990; Dole and Sun 1992; Walter and Gillett 1998). The greatest threat to the family is human activity, particularly agricultural and urban development. Cultivated meadowfoam (*L. alba* ssp. *alba* and *L. alba* ssp. *versicolor*) and three subspecies of *L. douglasii* do not seem to be seriously threatened or endangered (Walter and Gillett 1998). Nevertheless, several wild populations of *L. alba*, a native of California (Mason 1952), are known to be threatened (Knapp and Crane 1998).

Cultivated meadowfoam has been the focus of research and development as an industrial oilseed for three decades, particularly the last (Knapp and Crane 1998). The seed oil of meadowfoam is a rich source of novel very long-chain fatty acids (C_{20} and C_{22}) (Smith et al. 1960; Bagby et al. 1961). The oil has unusually high concentrations of Δ5 unsaturated (typically 86%) and C_{20} and C_{22} (typically 96%) fatty acids and extraordinarily low concentrations of saturated fatty acids (typically less than 2%) (Knapp and Crane 1995). The novelty of the oil has been the primary factor behind the development of the meadowfoam oil industry and, more recently, the development of novel chemical derivatives for industrial markets (Isbell 1997).

The prospect for developing meadowfoam into an economically significant oilseed crop hinges on the development of genetically superior cultivars, and on conserving genetic diversity in the Limnanthaceae. Meadowfoam germplasm has not been collected from the wild on a significant scale for more than 20 years, and a recent census of wild populations has not been performed (Knapp and Crane 1998). The early wild collections and a few domesticated germplasm accessions are maintained by the United States Department of Agriculture National Plant Germplasm System (http://www.ars-grin.gov/npgs), and have supplied genetic diversity crucial for meadowfoam cultivar development and, consequently, for the development of the meadowfoam oil industry. Genetic diversity for several biologically and agriculturally important traits has been surveyed and cataloged in cultivated meadowfoam (Knapp and Crane 1995, 1997, 1998); however, molecular genetic diversity has not been surveyed, essentially because high-throughput, meadowfoam-specific DNA markers have not been developed.

Simple sequence repeats (SSRs) are logical targets for DNA marker development in species lacking high-throughput, sequence-tagged-site (STS) DNA markers (Weber and May 1989; Morgante and Olivieri 1993; Rafalski and Tingey 1993; Powell et al. 1996). SSRs can be rapidly isolated in significant numbers from SSR-enriched genomic DNA libraries (Rae et al. 2000; Ramsay et al. 2000; Tang et al. 2002), are often highly polymorphic, and can be rapidly and efficiently assayed. While our primary goal was to develop SSR markers for cultivated meadowfoam (*L. alba* ssp. *alba* and *L. alba* ssp. *versicolor*), the utility and polymorphisms of cultivated meadowfoam SSR markers across wild meadowfoam taxa was assessed. We developed 389 SSR markers for cultivated meadowfoam and identified 106 conserved SSR markers for the Limnanthaceae. The cross-taxa utility and polymorphisms of the SSR markers are described herein.

Materials and methods

Genomic DNA library development and screening

Genomic DNA was isolated from OMF40–11 (*L. alba* ssp. *alba*) and OMF64 (*L. alba* ssp. *versicolor*) (Crane and Knapp 2000) leaf tissue as described by Lodhi et al. (1994). The two DNA samples were pooled for library construction. Genomic DNA libraries enriched for AG_n, AC_n, AAT_n, or ATC_n repeats, where n is the number of repeats, were constructed by Genetic Identification Services (Chatsworth, Calif., USA), essentially as described by Karagyozov et al. (1993) and Edwards et al. (1996). DNA fragments in the 350 –800 bp range were cloned into pUC19 plasmids. The latter were transformed into the *Escherichia. coli* strain DH5α (yields ranged from 5,000 to 10,000 recombinant cells/1.4 ml).

Fifty to 100 μl of the original stock solutions of each of the four genomic DNA libraries were spread on LB-agar media with 75 μg/ml of ampicillin. Recombinant clones were isolated using the IPTG and X-Gal procedures. White colonies were transferred to ampicillin-LB agar medium in 96-well format plates. The plates were incubated at 37°C overnight. Clone scrapes were used as templates for colony PCRs. Meadowfoam genomic DNA inserts were amplified using universal M13 forward (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and reverse (5′- TCACACAGGAAACAGCTATGAC-3′) primers. PCR reactions were performed with Perkin Elmer 9600 or MJ PTC 200 thermocyclers using reaction mixtures in a total volume of 35 μl containing 1x PCR buffer, 2 mM Mg^{2+}, 0.2 μM each of dNTPs, 1% Tween 20, 1.5 mM Cresol Red, 3% sucrose, 0.5 μM per primer, and 0.75 U *Taq* polymerase (Qiagen, Valencia, California, USA). PCRs were performed by denaturing at 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 1 min, and a final 10 min. extension. We estimated insert lengths on 1.5% agarose gels and selected inserts in the 350 – 800 bp range for DNA sequencing and SSR marker development. The selected PCR products were purified using QIAquick PCR purification kits. Half of the purifications were processed on a Qiagen BioRobot 3000. DNA sequencing was performed using 1.5–3.0 pmol of the M13 forward primer and 25 – 50 ng of purified PCR products on ABI 377 or 3700 automated sequencers (Perkin Elmer, Foster City, Calif., USA). Dimethyl sulfoxide (5%) was added as denaturant to increase DNA sequence quality, especially for clones harboring long repeats.

DNA sequence analyses and SSR marker development

DNA sequences were searched for the presence of dinucleotide, trinucleotide, and tetranucleotide repeat motifs. SSRs with five or more repeat units were selected for further analysis and SSR marker development. Sequence analyses were performed using the SEQLAB module of the Genetics Computing Group (GCG) Software (Madison, Wis., USA). Sequences harboring common repeat motifs were trimmed to remove the plasmid sequence and checked for redundancy using the GCG PILEUP function. Unique sequences were divided into perfect, imperfect, and compound repeat classes according to Weber (1990).

PRIMER 3.0 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) was used to design primers complementary to

Table 1 Taxonomic and historical background for 14 *Limnanthes* germplasm accessions

Accession name	Section	Scientific name	Plant introduction number	Origin
OMF40–11	Inflexae	*L. alba* spp. *alba*	–	F_6 line originating from Mermaid (PI 601232)
OMF64	Inflexae	*L. alba* spp. *versicolor*	PI 608039	F_6 line originating from PI 374801
Wheeler	Inflexae	*L. alba* spp. *alba*	PI 620761	Open-pollinated cultivar
OMF156	Inflexae	*L. alba*	–	Open-pollinated population
LE76	Inflexae	*L. alba* spp. *alba*	–	F_6 line originating from Mermaid (PI 601232)
OMF109–2	Inflexae	*L. alba* spp. *versicolor*	–	F_6 line originating from (Mermaid/OMF62//OMF64)
LFF	Inflexae	*L. floccosa* spp. *floccosa*	PI 283719	Wild species
LFG	Inflexae	*L. floccosa* spp. *grandiflora*	PI 420133	Wild species
LGG	Inflexae	*L. gracilis* spp. *gracilis*	PI 420137	Wild species
LGP	Inflexae	*L. gracilis* spp. *parishii*	PI 283724	Wild species
LM	Inflexae	*L. montana*	PI 283725	Wild species
LDD	Reflexae	*L. douglasii* spp. *douglasii*	PI 278170	Wild species
LDN	Reflexae	*L. douglasii* spp. *nivea*	PI 283713	Wild species
LDR	Reflexae	*L. douglasii* spp. *rosea*	PI 283715	Wild species

DNA sequences flanking the SSRs. DNA sequences for reference alleles and primers for the SSR markers have been deposited in GenBank (http://www.ncbi.nlm.gov); BV007038-BV007426. We designed primers in the 20 – 27 bp range with G-C contents ranging from 35 to 60% and annealing temperatures (T_m) ranging from 59 to 63°C. The other parameter settings for primer design were a T_m variance of 2.0°C, maximum 3′ stability of 9.0, maximum self-complementarity of 6.0, maximum 3′ self-complementarity of 2.0, and max ploy-X of 4.0. To facilitate multiplexing, predicted PCR product lengths were systematically varied from 108 to 554 bp and the 5′ ends of forward primers were labeled with one of three fluorophores (6-FAM, HEX, or TET) in combinations to minimize overlap in multiplexed genotyping assays. Primers were produced and supplied by MWG Biotech (High Point, N.C., USA).

The SSR markers were screened for polymorphisms among 14 *L. alba*, *L. floccosa*, *L. gracilis*, *L. montana*, and *L. douglasii* germplasm accessions (Table 1). We screened four inbred lines of *L. alba* (OMF64, OMF40–11, LE76, and OMF109–2) and ten randomly selected individuals from open-pollinated populations of *L. alba* (Wheeler and OMF156), *L. flocossa* ssp. *flocossa* (LFF), *L. flocossa* ssp. *grandiflora* (LFG), *L. gracilis* ssp. *gracilis* (LGG), *L. gracilis* ssp. *parishii* (LGP), *L. montana* (LM), *L. douglasii* ssp. *douglasii* (LDD), *L. douglasii* ssp. *nivea* (LDN), and *L. douglasii* ssp. *rosea* (LDR) (Table 1) (http://www.ars-grin.gov/npgs; Crane and Knapp 2000, 2002). DNA samples for the inbred lines were produced from leaf samples collected from ten greenhouse grown plants per inbred, whereas DNA samples for open-pollinated populations were produced from leaf samples collected from a single greenhouse grown plant per population. Genomic DNA was isolated from the leaf tissue as described by Lodhi et al. (1994). PCRs were performed in a 20 μl volume containing 1x PCR buffer, 2.5 mM Mg^{2+}, 0.2 μM each of dNTPs, 0.1% Tween 20, 5 – 7.5 pmol of each primer, 0.75 U *Taq* polymerase, and 15 – 20 ng of meadowfoam genomic DNA. We used a 'touchdown' PCR protocol (Don et al. 1991) to minimize non-specific PCR amplification. PCRs were performed using an initial denaturation step at 95°C for 3 min, followed by one cycle of 94°C for 30 s, 68°C for 30 s, and 72°C for 1 min. Subsequent cycles were performed by decreasing the annealing temperature by 1°C to a base temperature of 53 – 58°C (annealing temperatures varied from 53° to 58°C as a function of the T_m of each primer pair). The annealing temperature for each SSR primer pair is listed in the SSR primer database supplied as supplemental data. Finally, PCRs were continued for 30 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 1 min, and a final 20 min extension.

SSR genotyping assays were performed on sequencing gels run on an ABI 377 equipped with the GeneScan and Genotyper software and filter set C (Applied Biosystems Incorporated, Foster City, Calif.) by post-PCR multiplexing three SSR markers per lane labeled with different fluorophores. The pooled amplicons were diluted 20-fold. Samples containing 0.5 μl of the diluted PCR products, 0.2 μl GeneScan 500 internal lane standard labeled with TAMRA, and 50% formamide were heated to 92°C for 5 min and chilled on ice for 5 min before gel loading. SSR allele calling was performed using GeneScan and allele lengths were estimated using Genotyper. We manually checked the output and corrected allele lengths as necessary.

Statistical analyses

Because more than half of the SSR markers amplified multiple loci and the allelism of bands amplified by multilocus SSR markers could not be ascertained, we calculated the number of alleles per locus, heterozygosities (H), and other statistics from a subset of 90 single-locus SSR markers, in addition to calculating the number of bands per marker and haplotype heterozygosities (H_h) from the genotypes of the complete set of 389 single-locus and multilocus SSR markers. H was estimated from SSR allele frequencies as described by Ott (1991), whereas H_h was estimated by substituting haplotype frequencies for allele frequencies in H, where each haplotype is a unique SSR banding pattern. Hence, if each of 14 genotypes have unique haplotypes, then $H_h = 1 - [14 \times (1/14)^2] = 0.929$. H estimates the probability of observing an allelic polymorphism between two individuals (genotypes) drawn at random, whereas H_h estimates the probability of observing a minimum of one allelic polymorphism between two genotypes drawn at random. We estimated the number of loci amplified by each SSR marker from the genotypes of two F_6 lines developed by single-seed descent (OMF40–11 and OMF64). Statistics were estimated using PROC FREQ, GLM, and CORR of the Statistical Analysis System (http://www.sas.com).

Results

SSR marker development

Thirty-six clones from the AG_n, AC_n, AAT_n, and ATC_n-enriched genomic DNA libraries, where n is the number of repeat units, were sequenced to check for SSR enrichment. Nine clones from each library were analyzed. SSRs were not found in DNA sequences from the AAT_n and ATC_n libraries, whereas SSRs were found in ten of the 18 DNA sequences from the AG_n and AC_n libraries, equally split between the two. We performed colony PCR on 3,264 recombinant clones from the AG_n and AC_n-enriched genomic DNA libraries and selected 1,596

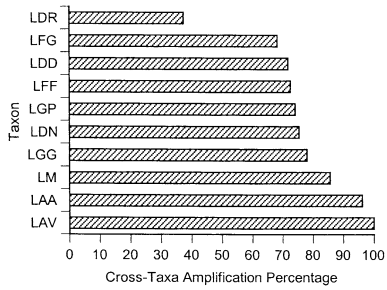

Fig. 1 Cross-taxa amplification percentages for 389 SSR markers genotyped in *Limnanthes alba* ssp. *alba* (*LAA*), *L. alba* ssp. *versicolor* (*LAV*), *L. floccosa* ssp. *floccosa* (*LFF*), *L. floccosa* ssp. *grandiflora* (*LFG*), *L. gracilis* ssp. *gracilis* (*LGG*), *L. gracilis* ssp. *parishii* (*LGP*), *L. montana* (*LM*), *L. douglasii* ssp. *douglasii* (*LDD*), *L. douglasii* ssp. *nivea* (*LDN*), and *L. douglasii* ssp. *rosea* (*LDR*)

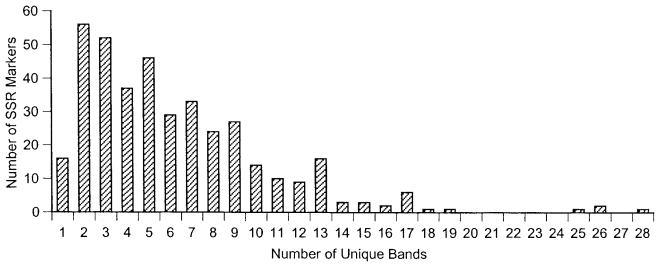

Fig. 2 The number of unique bands per SSR marker for 389 SSR markers among 14 meadowfoam germplasm accessions

clones for sequencing, 817 from the AC_n and 779 from the AG_n library. The selected clones had inserts ranging in length from 350 to 800 bp. Colony PCR products from the selected clones were purified, sequenced, and screened for all possible dinucleotide, trinucleotide, and tetranucleotide repeats. SSRs with a minimum of five repeat units (n>5) were identified in 1,237 clone sequences, 620 from the AC_n and 617 from the AG_n library. DNA sequences harboring common motifs were aligned and screened for redundancy. We identified 541 redundant and 696 unique SSR sequences, 281 from the AC_n and 415 from the AG_n library. The total and unique SSR enrichment percentages were 77.5% and 43.6%, respectively. The most common repeat motifs found in the selected sequences were AG_n (328) and AC_n (136), the repeats targeted for enrichment; however, 59 different perfect, imperfect, and compound repeat motifs were isolated and are listed in the SSR primer database (supplied as supplemental data).

We designed and tested primers for 624 unique SSRs (544 dinucleotide, 75 trinucleotide, and five tetranucleotide repeats), but could not design primers for the other 72 unique SSRs because of short flanking sequences. Of the 624 SSR primers, 389 amplified bands of the lengths predicted from OMF40–11 or OMF64 reference allele sequences (110 from the AC_n and 279 from the AG_n library), 24 produced multiple bands, and 211 failed to amplify bands from any of the 14 genotypes. Hence, 389 SSR markers were developed for cultivated meadowfoam from a starting point of 1,596 genomic DNA clone sequences and 696 unique SSR sequences. Fifteen of the SSR markers failed to amplify bands from OMF40–11 (*L. alba* ssp. *alba*) (Fig. 1).

Cross-taxa utility and polymorphisms

The SSR markers were screened for cross-taxa utility and intra- and interspecific polymorphisms among 14 genotypes from ten taxa (Table 1). Of 389 SSR markers, 106 amplified bands from every taxon, whereas 283 failed to amplify bands from one or more taxa (Fig. 1). Cross-taxa amplification percentages were greater for section Inflexae (82.0%) than section Reflexae (61.4%) taxa; however, bands were amplified from two out of three section Reflexae taxa (*L. douglasii* ssp. *douglasii* and *L. douglasii ssp. nivea*) as often as from wild section Inflexae taxa. The lowest cross-taxa amplification percentage was observed for *L. douglasii* ssp. *rosea* (37.3%). The cross-taxa amplification percentages for taxa other than *L. douglasii* ssp. *rosea* ranged from 68.1% for *L. floccosa* ssp. *grandiflora* to 85.6% for *L. montana* (Fig. 1). The utility of individual SSR markers within and between taxa can be ascertained from the SSR primer and polymorphism databases supplied as supplemental data.

One to 28 unique bands were amplified by each SSR marker among the 14 genotypes (Fig. 2). Sixteen of the SSR markers (4.1%) were monomorphic. One SSR marker produced two unique bands per genotype (14 unique haplotypes). The number of loci amplified from *L. alba* was estimated from the number of bands amplified from two inbred lines (OMF40–11 and OMF64); 171 SSR markers amplified a single locus (single band) and 218 SSR markers amplified two or more loci (two or more bands). Of the former, 90 amplified one or two bands from non-inbred, non-reference *L. alba* genotypes and were selected for calculating allele numbers, heterozygosities, and other single-locus SSR marker statistics (Table 2).

The number of loci amplified from OMF40–11 and OMF64 was estimated to be 421–431, respectively; 374 SSR markers amplified loci from OMF40–11, whereas 389 SSR markers amplified loci from OMF64. The number of bands amplified from non-reference *L. alba* genotypes ranged from 276 for LE76 to 347 for OMF156. SSR amplification rates dropped off for wild taxa in both sections (Fig. 1). The mean number of bands amplified from different germplasm or taxonomic groups were 353.3 for the six *L. alba* genotypes, 317.8 for the four non- reference *L. alba* genotypes, 267.8 for wild section Inflexae taxa (LFF, LFG, LGP, LGG, and LM), and 232.7 for wild section Reflexae taxa (LDD, LDN, and LDR). SSR markers failed to amplify bands from 0.74 out of six *L. alba* genotypes and 1.37 out of 14 genotypes across the ten taxa.

Of the 4,160 unique bands amplifed by the SSR markers from the 14 genotypes (10.7 unique bands per

Table 2 Mean heterozygosities (H), mean number of alleles per SSR marker locus (n_A), and maximum number of alleles per SSR marker locus (max[n_A]) for 90 single-locus SSR markers and mean haplotype heterozygosities (H_h), mean number of bands per SSR marker (n_B), and maximum number of bands per SSR marker (max[n_B]) for 389 single- or multilocus SSR markers genotyped on 14 meadowfoam germplasm accessions

Group	n	H	n_A	max [n_A]	H_h	n_B	max[n_B]
L. alba	6	0.45	2.53	6	0.71	4.56	14
Section Inflexae	5	0.42	2.35	5	0.73	6.54	28
L. douglasii	3	0.40	1.94	3	0.35	3.09	11
Total	14	0.49	3.26	7	0.63	10.69	28

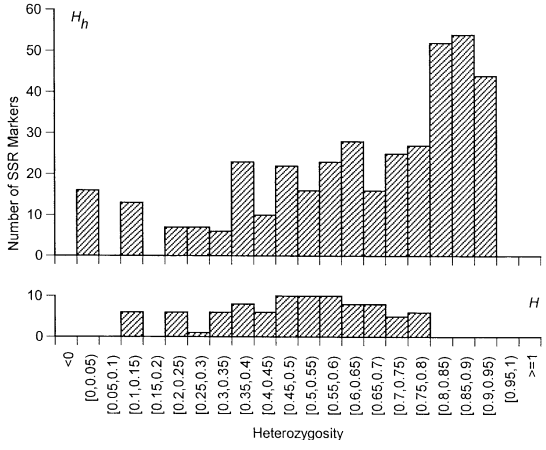

Fig. 3 Heterozygosity (H) distribution for 90 single-locus SSR markers and haplotype heterozygosity (H_h) distribution for 389 SSR markers genotyped on 14 meadowfoam germplasm accessions

SSR marker), 972 were genotype-specific. We identified 410 genotype-specific bands among the six *L. alba* genotypes, 52 from LE76 to 130 from OMF40–11. The number of genotype-specific SSR bands amplified from wild meadowfoam taxa ranged from 37 for *L. douglasii* ssp. *rosea* to 89 for *L. gracilis* ssp. *parishii*. The number of genotype-specific bands was positively correlated (0.71) with the number of bands amplified from each genotype (the sum of shared and genotype-specific bands).

Ninety-six percent of the SSR markers (373 out of 389) were polymorphic, whereas 100% of the SSR markers in the selected single-locus subset (90 out of 389) were polymorphic (Fig. 3). Mean and maximum heterozygosities for the single-locus subset were 0.45 and 0.80, respectively, among the six *L. alba* germplasm accessions and 0.49 and 0.80, respectively, among the 14 genotypes (Fig. 3, Table 2). Similarly, mean and maximum H_h for the complete set of SSR markers were 0.71 and 0.90, respectively, among the six *L. alba* germplasm accessions and 0.63 and 0.93, respectively, among the 14 genotypes. The H_h mean was lower across taxa than within *L. alba* because null allele frequencies were greater for the former than the latter (thereby reducing allelic diversity) (Table 2). By contrast, the H mean was slightly greater across taxa than within *L. alba* because null allele frequencies were greatly reduced in the single-locus SSR marker subset.

The H distribution was platykurtic and approximately normal, whereas the H_h distribution was right-skewed (Fig. 3). The skewness was produced by hypervariable, multilocus SSR markers. The heterozygosities for single-locus and haplotype heterozygosities for multilocus SSR markers are listed in the SSR primer database (supplied as supplemental data). Haplotype heterozygosities were calculated to supply a complete summary of the data and show that a many of the more complex, multilocus SSR markers were hypervariable. The probability of observing a *minimum* of one allelic difference between two individuals (H_h) was greater than 0.80 for 150 multilocus SSR markers (Fig. 3). Slightly more than half of the SSR markers (202/389) had haplotype heterozygosities ranging from 0.70 to 0.93 and 60% of the SSR markers uniquely identified a minimum of one out of 14 genotypes. Moreover, individual SSR markers uniquely identified up to 11 genotypes, e.g., LS196 distinguished 11 out of 14 and LS356 distinguished 10 out of 14 genotypes. When combined, LS196 and LS356 distinguished 14 out of 14 genotypes.

Discussion

The SSR markers described herein supply a critical mass of hypervariable, high-throughput DNA markers for biological and agricultural research in the Limnanthaceae, and are the first DNA markers developed specifically for meadowfoam. Heretofore, 18 allozyme markers had been described for meadowfoam (Brown and Jain 1979; Kesseli and Jain 1985; McNeill and Jain 1985; Ritland and Jain 1984). The meadowfoam SSR markers were designed for multiplex genotyping by systematically varying SSR allele lengths. Typically, 6–14 meadowfoam SSR markers can be multiplexed when the allele lengths of the genotypes are known a priori. The multiplexing density depends on the allelic diversity of the genotypes sampled, the genotyping or assay method, and number of loci amplified by the individual SSR markers. Because the allele lengths were not known in the initial screening, we multiplexed three SSR markers, each labelled with a different fluorophore, so that the bands amplified by each SSR marker could be unequivocally identified. The SSR allele length database has been supplied as supplemental data for planning new experiments and selecting SSR markers for multiplex genotyping.

The meadowfoam SSR markers were found to be extraordinarily polymorphic (Fig. 3, Table 2). Most importantly, 373 (96.1%) were polymorphic in *L. alba*, the domesticated species targeted for commercial development and genetic mapping (Knapp and Crane 1998; Katengam et al. 2002). The range of heterozygosities we observed were similar to the ranges reported for SSR markers isolated from unenriched and SSR-enriched genomic DNA libraries in other plant taxa (Akkaya et al. 1992; Morgante and Olivieri 1993; Wu and Tanksley 1993; Saghai Maroof et al. 1994; Kresovich et al. 1995; Plaschke et al. 1995; Röder et al. 1995; Rongwen et al. 1995; Powell et al. 1996; Taramino and Tingey 1996; Innan et al. 1997; Olufowote et al. 1997; Smulders et al. 1997; Loridon et al. 1998; Cho et al. 2000; Smith et al. 1997, 2000; Temnykh et al. 2000; Yu et al. 2002; Tang and Knapp 2003). Because wild and domesticated genotypes were sampled (Table 1), the haplotype heterozygosity distribution was right-skewed: nearly two-thirds of the SSR markers had haplotype heterozygosities greater than 0.6 (Fig. 3). By contrast, slightly less than one-third of the single-locus SSR markers (29/90) had heterozygosities greater than 0.6. H and H_h was greater than the probability of observing a polymorphism between any of the loci amplified by a multilocus SSR marker, which was greater than the probability of observing a polymorphism between the locus amplified by a single-locus SSR marker. Because cultivated meadowfoam has not yet passed through severe domestication or breeding bottlenecks (Knapp and Crane 1998), SSRs seem to be as polymorphic among cultivated meadowfoam germplasm accessions as among wild taxa (Table 2).

Despite a propensity for redundantly amplifying DNA sequences, SSR-enrichment procedures yield genomic DNA libraries greatly enriched for SSR sequences and facilitate the development of SSR markers on a large scale (Ostrander et al. 1992; Karagyozov et al. 1993; Edwards et al. 1996; Rae et al. 2000; Ramsay et al. 2000; Tang et al. 2002). The meadowfoam genomic DNA libraries were greatly enriched for SSR sequences (SSRs were found in 77.5% of the genomic DNA clones); however, 43.7% of the SSR sequences were redundant (541/1,237), primers could not be designed for 10.3% of the unique SSR sequences (72/696), and 37.6% of the primers failed to amplify SSR alleles from any of the genotypes (235/624), primarily because of DNA sequence quality. Of the original 1,596 meadowfoam DNA sequences we produced, 6.3% yielded SSR markers that amplified loci from every taxon, whereas 25.3% yielded SSR markers that amplified loci from *L. alba*. Similarly, of 696 unique meadowfoam SSR sequences, 15.2% yielded SSR markers that amplified loci from all of the taxa, 20.8 to 47.8% yielded SSR markers that amplified loci from taxa other than *L. alba*, and 55.9% yielded SSR markers that amplified loci from *L. alba*. Hence, aside from DNA sequence redundancy, the attrition we observed was primarily caused by SSR primer failures, a common problem in SSR marker development (Kresovich et al. 1995; Liu et al. 1995; Plaschke et al. 1995; Röder et al. 1995; Rongwen et al. 1995; Szewc-Mcfadden et al. 1996; Olufowote et al. 1997; Kubik et al. 1999, Ramsay et al. 1999; Saal and Wricke et al. 1999; Cho et al. 2000; Temnykh et al. 2000; Tang et al. 2002; Yu et al. 2002).

Slightly more than one-fourth of the *Limnanthes* SSR markers (106/389) were conserved across the ten taxa (Figs. 1, 3). Predictably, fewer amplified alleles (bands) from every taxon (106) than from each of the wild taxa (145 to 333). While cross-taxa amplification rates generally decreased as taxonomic differences increased, null allele frequencies were greater for two non-reference *L. alba* genotypes (Wheeler and LE76) than for two wild species from section Inflexae (*L. gracilis* ssp. *gracilis* and *L. montana*) and one wild species from section Reflexae (*L. douglasii* ssp. *douglasii*) (Fig. 1). The failure of SSR markers to amplify loci, both within *L. alba* and across wild meadowfoam taxa, was undoubtedly caused by polymorphisms in the DNA sequences targeted by the SSR primers (Mogg et al. 2002). BLAST analyses of the *Limnanthes* DNA sequences identified no known gene sequences; hence, the SSRs undoubtedly reside in mutation-rich retrotransposon and dispersed repetitive element DNA (Ramsay et al. 1999, 2000).

SSRs generally seem to be less conserved among plants than animals (Schlötterer et al. 1991; Fitzsimmons et al. 1995; Garza et al. 1995; Brown et al. 1996; Garza and Freimer 1996; Primmer et al. 1996; Rico et al. 1996; Whitton et al. 1997; Peakall et al. 1998; Reed et al. 2000; Smulders et al. 2000; Cordiero et al. 2001; Kutil and Williams 2001). Schlötterer et al. (1991), Fitzsimmons et al. (1995), and Rico et al. (1996) identified SSRs that amplified loci across fish, turtle, or whale taxa sharing common ancestors 450, 300, or 40 million years before the present, respectively. Similarly, half of the SSRs tested by Primmer et al. (1996) amplified loci from birds sharing common ancestors ten million years before the present. SSRs frequently amplify loci from closely related plant taxa, but seldom from diverse plant taxa (Röder et al. 1995; Brown et al. 1996; Provan 1996; Whitton et al. 1997; Peakall et al. 1998; Smulders 2000; Kutil and Williams 2001). The interspecific SSR amplification rates we observed in *Limnanthes* (37.3–85.6%) (Fig. 1) were similar to interspecific SSR amplification rates in *Glycine* (48.4–61.3%) (Peakall et al. 1998) and intergeneric SSR amplification rates between tomato (*Lycopersicon esculentum* L.) and potato (*Solanum tuberosum* L.) (Provan et al. 1996; Smulders et al. 1997), among wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and rye (*Secale cereale* L.) (Röder et al. 1995), and between sorghum [*Sorghum bicolor* (L.) Moench.] and maize (*Zea mays* L.) (Brown et al. 1996).

We could not locate seed of and hence could not screen 9 of 19 taxa in the Limnanthaceae, specifically *F. proserpinacoides* and eight meadowfoam taxa, four from section Inflexae (*L. bakeri*, *L. floccosa* ssp. *bellingeriana*, *L. flocossa* ssp. *californica*, and *L. flocossa* ssp. *pumila*) and four from section Reflexae (*L. douglasii* ssp. *sulphurea*, *L. macounii*, *L. striata*, and *L. vinculans*). Because the tested and untested meadowfoam taxa are

closely related (Mason 1952; Ornduff and Crovello 1968; Ornduff 1971) and 145 to 279 of the cultivated meadowfoam SSR markers amplified loci from *L. douglasii* (Fig. 1), the species most distantly related to *L. alba*, 100 or more should amplify loci from each of the untested meadowfoam taxa and supply a significant number of conserved SSR markers for genetic conservation, phylogenetic, phylogeographic, ecological, and evolutionary genetics research in the Limnanthaceae.

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