ISSN 1479-2621
Plant Genetic Resources: Characterization and Utilization 7(1); 33–41
doi:10.1017/S1479262108014378

Genetic diversity in the USDA *Limnanthes* germplasm collection assessed by simple sequence repeats

Linda M. Donnelly[1,2†], Maria M. Jenderek[1*‡], James P. Prince[2], Patrick A. Reeves[3], Allan Brown[1] and Richard M. Hannan[4]

[1]*National Arid Land Plant Genetic Research Unit, USDA-ARS, Parlier, CA 93648, USA,* [2]*Department of Biology, California State University Fresno, Fresno, CA 93740, USA,* [3]*National Center for Genetic Resources Preservation, USDA-ARS, Fort Collins, CO 80521, USA and* [4]*Western Regional Plant Introduction Station, USDA-ARS, Pullman, WA 99164, USA*

Received 21 February 2008; Accepted 23 May 2008 – First published online 16 June 2008

Abstract

The genus *Limnanthes* (Limnanthaceae), also known as meadowfoam, has attracted attention for industrial use due to the unique characteristics of its seed oil. Samples from wild populations showed variability in agronomically important traits involved in seed oil yield, warranting the establishment and continued development of a germplasm collection. The level of genetic diversity within the United States Department of Agriculture, Agriculture Research Service, National Plant Germplasm System (USDA-ARS-NPGS) *Limnanthes* collection was evaluated using 15 simple sequence repeat (SSR) markers across 62 accessions representing 7 species. Parsimony analysis separated the accessions into two main groups consistent with the traditional taxonomic sections Inflexae and Reflexae, but there was little resolution within groups. These two groups were confirmed using neighbour-joining analysis and principal coordinate analysis. SSR marker variation suggests that the *Limnanthes* germplasm collection is genetically diverse and the accessions within the species likely contain novel alleles, and therefore the collection contributes to the conservation of the wild *Limnanthes* gene pool.

Keywords: conservation genetics; genetic diversity; germplasm resources; meadowfoam; simple sequence repeat

Introduction

The genus *Limnanthes*, first described by Robert Brown in 1833, is native to regions of northern California, southern Oregon, and Vancouver Island, British Columbia (Mason, 1952). Commonly referred to as meadowfoam, these white flowering plants grow in vernal pools and meadows in valley grasslands along the foothills (Jain *et al.*, 1977). *Limnanthes* is a low-growing winter annual that germinates in the early spring in cool, moist environments, maturing rapidly before the climate becomes dry in late spring and early summer (Jolliff *et al.*, 1981). The genus includes nine species separated into two sections based on petal position following fertilization. These are the Inflexae (petals enclose the developing fruit): *Limnanthes alba*, *Limnanthes floccosa*, *Limnanthes gracilis* and *Limnanthes montana*; and the Reflexae (petals reflex as fruit develops): *Limnanthes bakeri*, *Limnanthes douglasii*, *Limnanthes macounii*, *Limnanthes striata* and *Limnanthes vinculans* (Mason, 1952). Research into the genus increased after

* Corresponding author. E-mail: maria.jenderek@ars.usda.gov
† Current address: Crops Pathology and Genetics Research Unit, USDA-ARS, One Shields Avenue, Davis, CA 95616, USA.
‡ Current address: National Center for Genetic Resources Preservation, USDA-ARS, NCGRP, 1111 S. Mason St., Fort Collins, CO 80521, USA.

Limnanthes was identified as a prospective industrial crop due to the presence of long-chain fatty acids in the seed oil which had larger molecular weights than acids found in common domesticated vegetable oil plants (Gentry and Miller, 1965). With the use of sperm whale oil banned in 1972, *Limnanthes* seed oil was proposed as a replacement in lubricants, cosmetics, waxes, detergents and pharmaceuticals (Jain *et al.*, 1977).

Fatty acids comprise 25% of the seed oil (Miwa and Wolff, 1962) with 95% of the oil comprised of four main acids, *cis*-5-eicosenoic (20:1), *cis*-5-docosenoic (22:1), *cis*-13-docosenoic (eruic acid, 22:1) and *cis*-5-*cis*-13-docosadienoic (22:2) acids (Smith *et al.*, 1960; Bagby *et al.*, 1961). The presence of long-chain fatty acids unique to *Limnanthes* (*cis*-5-eicosenoic acid and *cis*-5-docosenoic acid; Smith *et al.*, 1960; Bagby *et al.*, 1961) and the unique position of unsaturation in the *cis*-5-*cis*-13-docosadienoic acid provide stability at high temperatures. Interest in this property fuelled research into the seed oil for use as an industrial lubricant (Jain *et al.*, 1977). Using standard chemical reactions, *Limnanthes* oil can be converted to a compound similar to liquid jojoba wax or a solid wax with a high melting point similar to carnauba and candelilla waxes (Miwa and Wolff, 1962; Gentry and Miller, 1965). *Limnanthes* may have an agricultural advantage over other wax-producing crops as its cultivation is comparatively easy, and it can be double-cropped with rice, an important crop in northern California (Jain *et al.*, 1977), or it can be maintained in surplus fields with winter grains (Gentry and Miller, 1965; Jolliff *et al.*, 1981).

Traits of agronomic importance, including yield (Jain and Abuelgasim, 1981), seed retention (Gentry and Miller, 1965; Higgins *et al.*, 1971), seed oil content (Pierce and Jain, 1977; Jain and Abuelgasim, 1981), uniformity in maturity, growth habit and moisture requirements (Gentry and Miller, 1965), were reported to differ among *Limnanthes* accessions. Breeding for cultivars exhibiting combinations of desirable agronomic qualities was undertaken from various *Limnanthes* species (Gentry and Miller, 1965; Higgins *et al.*, 1971). Research focused on the development of cultivars with high seed oil content that are self-pollinating to avoid the necessity for pollen vector application in production fields (Jolliff *et al.*, 1981).

Conservation efforts for the populations of *Limnanthes* were undertaken due to the infringement of urban development and livestock grazing (Dole and Sun, 1992; California Native Plant Society (CNPS), 2005). Four *Limnanthes* species are listed as endangered: *L. douglasii* ssp. *sulphurea* and *L. gracilis* ssp. *parishii* by the state of California; and *L. floccosa* ssp. *californica* and *L. vinculans* by both state and the federal governments (CNPS, 2005). Some species of *Limnanthes* have limited ranges, e.g. *L. macounii*, which was once believed to be extinct but has since been found in Victoria, British Columbia (Higgins *et al.*, 1971). To preserve wild populations, a germplasm collection of the genus was established in 1972 at the University of California, Davis, California, to maintain genetically diverse resources for breeding (Jain *et al.*, 1977). However, the seed in that collection is no longer viable.

The utility of a germplasm collection relies on its ability to represent the genetic diversity present in the corresponding natural populations. Until now, the diversity of the USDA *Limnanthes* collection has only been assessed using morphological traits. For confirmation of morphological diversity data as well as for taking preliminary steps in marker-assisted breeding programs for crop improvement, it is also necessary to document genetic diversity at the molecular level. Many types of molecular markers are currently available. Simple sequence repeat (SSR) markers were chosen for this work. Due to the wide distribution in the genome and high levels of polymorphism exhibited by SSR markers, they have been successfully employed to determine genetic diversity between accessions in other germplasm collections (Godwin *et al.*, 2001; McLauchlan *et al.*, 2001; Baranger *et al.*, 2004; Dillon *et al.*, 2005). In 2004, Kishore *et al.* identified SSR markers in *Limnanthes* and used them to screen intra- and interspecific variation in 14 accessions, including 4 inbred lines, 2 open pollinated breeding selections and 8 open pollinated wild species. The objective of the current study was to describe genetic diversity at the molecular level in the *Limnanthes* germplasm collection preserved by the United States Department of Agriculture, Agriculture Research Service, National Plant Germplasm System (USDA-ARS-NPGS).

Materials and methods

Plant material and DNA extraction

Genomic DNA was extracted from leaf tissue of 62 *Limnanthes* germplasm accessions maintained as open-pollinated populations isolated by caging at the National Arid Land Plants Genetic Research Unit (NALPGRU), Parlier, California. The 62 accessions belonged to 7 species: *L. alba*, *L. douglasii*, *L. bakeri*, *L. floccosa*, *L. gracilis*, *L. montana* and *L. striata*. Accessions designated 'a' or 'b' of the same identification number were collected from the same population but exhibited morphological differences in leaf and stem colour ('b' – smaller leaves and purple stems; Table 1). Genomic DNA extractions were performed on a composite sample of 10 plants of each accession using the cetyltrimethylammonium bromide protocol from Lodhi *et al.* (1994). A 24:1 solution

https://doi.org/10.1017/S1479262108014378 Published online by Cambridge University Press

Table 1. *Limnanthes* germplasm accessions evaluated for simple sequence repeat marker diversity

Accession ID	Species	Subspecies	Section	Origin
PARL 429a	*L. alba*	*alba*	Inflexae	Lincoln, CA
PARL 429b	*L. alba*	*alba*	Inflexae	Lincoln, CA
PARL 430a	*L. alba*	*alba*	Inflexae	Lincoln, CA
PARL 430b	*L. alba*	*alba*	Inflexae	Lincoln, CA
PARL 431a	*L. alba*	*alba*	Inflexae	Lincoln, CA
PARL 431b	*L. alba*	*alba*	Inflexae	Lincoln, CA
PARL 432a	*L. alba*	*alba*	Inflexae	Sheridan, CA
PARL 432b	*L. alba*	*alba*	Inflexae	Sheridan, CA
PARL 433	*L. alba*	*alba*	Inflexae	Sheridan, CA
PARL 434a	*L. alba*	*alba*	Inflexae	Wheatland, CA
PARL 434b	*L. alba*	*alba*	Inflexae	Wheatland, CA
PI 283701	*L. alba*		Inflexae	Wheatland, Yuba Co., CA
PI 283704	*L. alba*	*alba*	Inflexae	Perkins, Sacramento Co., CA
PI 283705	*L. alba*	*versicolor*	Inflexae	Redding, CA
PI 367900	*L. alba*		Inflexae	Perkins, Sacramento Co., CA
PI 374790	*L. alba*		Inflexae	Redding, Shasta Co., CA
PI 374791	*L. alba*		Inflexae	Palo Cedro, Shasta Co., CA
PI 374792	*L. alba*		Inflexae	Millville, Shasta Co., CA
PI 374793	*L. alba*		Inflexae	Lincoln, Placer Co., CA
PI 374794	*L. alba*		Inflexae	Sheridan, Placer Co., CA
PI 374795	*L. alba*		Inflexae	Sheridan, Placer Co., CA
PI 374796	*L. alba*		Inflexae	Oroville, Butte Co., CA
PI 374798	*L. alba*		Inflexae	Palermo, Butte Co., CA
PI 374799	*L. alba*		Inflexae	Redding, Shasta Co., CA
PI 374800	*L. alba*		Inflexae	Round Mt., Shasta Co., CA
PI 374801	*L. alba*		Inflexae	Palo Cedro, Shasta Co., CA
PI 374802	*L. alba*		Inflexae	Bella Vista, Shasta Co., CA
PI 374803	*L. alba*		Inflexae	California
PI 374804	*L. alba*		Inflexae	California
PI 374805	*L. alba*		Inflexae	California
PI 543894	*L. alba*	*alba*	Inflexae	Oregon
PI 604600	*L. alba*		Inflexae	Oregon
PI 608039	*L. alba*	*versicolor*	Inflexae	Oregon
PI 283706	*L. bakeri*		Reflexae	Willits, Mendocino Co., CA
PI 283707	*L. bakeri*		Reflexae	Willits, Mendocino Co., CA
PARL 423	*L. douglasii*	*nivea*	Reflexae	Chico, Butte Co., CA
PARL 424	*L. douglasii*	*nivea*	Reflexae	Chico, Butte Co., CA
PARL 426	L. douglasii	*rosea*	Reflexae	Chico, Butte Co., CA
PARL 427	*L. douglasii*	*nivea*	Reflexae	Chico, Butte Co., CA
PARL 428	*L. douglasii*	*rosea*	Reflexae	Chico, Butte Co., CA
PI 283709	*L. douglasii*	*douglasii*	Reflexae	Nicasio, Marin Co., CA
PI 283710	*L. douglasii*	*nivea*	Reflexae	Oakville, Sonoma Co., CA
PI 283711	*L. douglasii*	*nivea*	Reflexae	Calistoga, Napa Co., CA
PI 283712	*L. douglasii*	*nivea*	Reflexae	Nice, Lake Co., CA
PI 283713	*L. douglasii*	*nivea*	Reflexae	Willits, Mendocino Co., CA
PI 283714	*L. douglasii*	*nivea*	Reflexae	Calistoga, Napa Co., CA
PI 283715	*L. douglasii*	*rosea*	Reflexae	Snelling, Merced Co., CA
PI 283716	*L. douglasii*	*rosea*	Reflexae	Chico, Butte Co., CA
PI 283718	*L. douglasii*	*douglasii*	Reflexae	Pt. Reyes Peninsula, Marin Co., CA
PI 349685	*L. douglasii*		Reflexae	France
PI 374806	*L. douglasii*		Reflexae	Lakeport, Lake Co., CA
PI 283719	*L. floccosa*		Inflexae	Bella Vista, Shasta Co., CA
PI 283720	*L. floccosa*	*bellingerina*	Inflexae	Pinehurst, Jackson Co., OR
PI 283721	*L. floccosa*	*pumila*	Inflexae	Jackson Co., OR
PI 420129	*L. floccosa*		Inflexae	Jackson Co., OR
PI 420132	*L. floccosa*	*bellingerina*	Inflexae	Butte Falls, Jackson Co., OR
PI 420134	*L. floccosa*		Inflexae	Jackson Co., OR
PI 420135	*L. floccosa*	*pumila*	Inflexae	Jackson Co., OR
PI 283723	*L. gracilis*	*gracilis*	Inflexae	Kirby, Josephine Co., OR
PI 420137	*L. gracilis*		Inflexae	Josephine Co., OR

https://doi.org/10.1017/S1479262108014378 Published online by Cambridge University Press

Table 1. *Continued*

Accession ID	Species	Subspecies	Section	Origin
PI 283725	*L. montana*		Inflexae	Mariposa, Mariposa Co., CA
PI 283727	*L. striata*		Reflexae	La Grange, Tuolumne Co., CA
PI 283728	*L. striata*		Reflexae	Sonora, Tuolumne Co., CA

Inventory of the USDA-ARS-NALPGRU-Parlier *Limnanthes* collection can be accessed at http://www.ars-grin.gov

of chloroform to isoamyl alcohol was used instead of 24:1 chloroform to octanol solution in the organic extraction step. DNA concentrations were determined using a Shimadzu UV-1601 spectrophotometer (Kyoto, Japan).

PCR amplification and PCR product visualization

Fifteen primer pairs developed by Kishore *et al.* from SSRs of the species *L. alba* (2004) were used to screen the *Limnanthes* DNA pools for polymorphism (Table 2). These primers were sub-selected based on high levels of polymorphism observed across 17 taxa in the Limnanthaceae (Kishore *et al.*, 2004). Touchdown PCR was performed according to Kishore *et al.* (2004) in 20 μL reactions containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.03% BSA (bovine serum albumin), 15 pmol of forward and reverse primers (Operon Technologies, Alameda, CA), 0.75 U of Taq polymerase (Invitrogen, Carlsbad, CA) and 10 ng of genomic DNA. Reactions without DNA were run as negative controls. PCRs were carried out in an Eppendorf Mastercycler Gradient thermocycler using an initial denaturation step of 94°C for 3 min, followed by 1 cycle of 94°C for 30 s,

Table 2. Primer, annealing temperature, G/C content, expected and observed size ranges and the number of amplicons across all *Limnanthes* accessions analyzed

Primer[a]	Primer sequence 5′–3′	Annealing temp. (°C)	Primer G/C content (%)	Expected size[a] (bp)	Observed size (bp)	No. of amplicons
Ls-90	F: GGAGGACGTATTGGCAGGTG	57	60.0	293–316	294–306	3
	R: TGAGACGTGAGCTGTGTTTCC		52.4			
Ls-107	F: CAGTGGGGTATCAGGGGATAG	53	57.1	202–218	195–213	6
	R: GTTACAGCCCCCGTTCAATTT		47.6			
Ls-266	F: GCAACGCTAAAATCAGAGGTAAA	63	39.1	226–238	218–237	9
	R: CTCATCACTCAAAGCACCAAGAT		43.5			
Ls-273	F: AAGCTCTGATCCGCATAGCATT	61	45.5	388–427	378–437	9
	R: TTCCAAAATTGCCCCTTTAAGTT		34.8			
Ls-276	F: CTCAAAAACTCGAAGAACCATCG	61	43.5	175–436	144–199	24
	R: TTCCAAAGATACCCCCGATATAAA		37.5			
Ls-278	F: ATTCTGAGTTTGTCTCTTCTGCTTT	59	36.0	162–178	157–178	14
	R: ATCATACCACCAATAGCCTTCAA		39.1			
Ls-315	F: ATATGATGTGTGCAACCCAATCC	61	43.5	270–279	227–284	6
	R: TTGAAACAGGACCAGGAGTAGTTC		45.8			
Ls-318	F: AAAGGGTCAAACACAAACTCTCTACA	59	38.5	361–406	360–380	2
	R: GAATATGGTTCAAATGCAGGGTA		39.1			
Ls-339	F: ACGAATCAGTGGAGAACGAAGAG	59	52.2	313–335	312–336	8
	R: CGGTTCATATACTGCCTTGGTTG		47.8			
Ls-371	F: TCAGTGACTACTGGTGATCTGACAA	63	44.0	364–392	377–408	6
	R: GAACCAACATAGGGAGTTTGTGG		47.8			
Ls-402	F: CCCCTCTCATCTTCACATTTTCA	63	43.5	283–298	287–308	6
	R: CGGCGATTGTAGTCATCTTTACTT		41.6			
Ls-466	F: GTAGCCCTAAAATGCAGCAATCC	59	47.8	169–379	174–294	8
	R: TTTGGGTGAGTTGTTCTCCCTTT		43.5			
Ls-483	F: GTCGATGGAGTGCTGACTGATTT	57	47.8	187–324	205–241	10
	R: CCCAATTCGTTTCTTACCTTGTTC		41.7			
Ls-484	F: CAGAGGTTGAAAGAGTAGCATCC	53	47.8	235–316	191–313	14
	R: ATCATCTTGTACGTCCTCGGTCT		47.8			
Ls-566	F: GTGTTTCCAATCTGTGTTGATGC	57	43.5	194–453	185–322	18
	R: CACACCTGCAAATATCACAAACC		43.5			

[a] Primers used to screen *Limnanthes* cultivars by Kishore *et al.* (2004).

https://doi.org/10.1017/S1479262108014378 Published online by Cambridge University Press

68°C for 30 s and 72°C for one min. Subsequent cycles were performed with the annealing temperature decreasing by 1°C to a base temperature between 53 and 63°C depending on the optimum annealing temperature for the primer pair used. Reactions were continued for 30 cycles: (94°C for 30 s, the optimum annealing temperature for the respective primer pair for 30 s, and 72°C for 1 min), followed by a final elongation step of 72°C for 20 min.

PCR products were diluted 1:2 with 2× Novex, TBE-Urea Sample Buffer (Invitrogen). The samples and 40 ng/μL solutions of 10 and 50 bp standards (Invitrogen) were denatured at 95°C for 5 min. The samples and standards were immediately placed on ice to prevent strand re-annealing for a minimum of 5 min prior to gel loading. The gel was loaded with 4 μL of each standard and 5.5 μL of each sample. Samples were then run on a 6% (w/v) polyacrylamide sequencing gel to determine amplicon sizes. Electrophoresis was performed at 1800 V, 90 W and 200 mA, between 2.5 and 3.5 h depending on expected amplicon sizes. The gels were stained using 0.2% silver nitrate solution following the Promega Silver Sequence DNA Sequencing System protocol (Promega, Madison, WI).

The gels were visually scored based on the presence or absence of the same size amplicons among the *Limnanthes* samples for each primer set, regardless of the band intensity.

Cluster analysis

A binary data matrix representing presence and absence of bands in each pooled DNA sample was assembled. The dataset contained 62 accessions and 140 simple-sequence length polymorphisms (SSLPs), each of them treated as a separate character in the analysis. Approximately, 23% of the matrix contained missing data. A series of detailed simulation studies has shown that phylogenetic analysis methods are robust to missing data and, in most cases, can yield accurate results with much higher levels of missing data than that seen here, although resolution may suffer (Wiens, 2006). Treebased analyses of genetic diversity among samples were performed using PAUP*4.0 (Swofford, 2002). A parsimony bootstrap tree was generated using 10,000 replicates and the TBR-M search strategy of Debry and Olmstead (2000). A neighbour-joining bootstrap tree was also generated in order to confirm the parsimony results.

Principal coordinate analysis of the binary matrix was performed with NTSYS (Exeter Software) using Jaccard distances. In order to determine whether any statistically significant clusters of principal coordinates existed, we used PCOMC (Reeves and Richards, 2007; http://lamar.colostate.edu/~reevesp/PCOMC/PCOMC.html). PCOMC uses a nonparametric density clustering procedure as an objective means to identify natural discontinuities in multidimensional principal coordinate data. All principal coordinate axes were considered simultaneously.

Results

Amplification and polymorphism

SSR-based PCR was used to identify SSLPs between the 62 accessions of the NPGS *Limnanthes* germplasm collection. Statistics were calculated for SSLPs and haplotypes instead of alleles and genotypes due to the amplification of multiple loci with the SSR markers used in this study. A total of 140 SSLPs were identified using 15 SSR markers, ranging from 144 to 437 bp in size. Eleven of the fifteen SSR primer pairs amplified products within 10 bp of the expected size ranges reported by Kishore *et al*. (2004). The remaining four primers amplified products within 50 bp of the expected ranges (Table 2).

The number of scorable SSLPs observed with the primers ranged from 2 to 24 per marker with an average of 9.5 SSLPs per marker. The total number of bands amplified for the different species was 126 for *L. alba*, 54 for *L. bakeri*, 105 for *L. douglasii*, 89 for *L. floccosa*, 60 for *L. gracilis*, 40 for *L. montana* and 44 for *L. striata*. The mean number of bands for accessions in the Section Inflexae (*L. alba*, *L. floccosa*, *L. gracilis* and *L. montana*) was 79, and a mean of 68 bands amplified among the accessions in the Section Reflexae (*L. bakeri*, *L. douglasii* and *L. striata*) (Table 3). The percentage of the total bands amplified that were polymorphic within each species was 92% in *L. alba*, 52% between the two *L. bakeri* accessions, 90% in *L. douglasii*, 89% in *L. floccosa*, 18% in *L. gracilis*, and 23% between the two *L. striata* accessions. Only one accession from *L. montana* was available and therefore no statistics were calculated. The mean polymorphism observed in the Inflexae accessions was 66%, greater than the mean observed in the Reflexae at 55% (Table 3).

Dendrograms and principal coordinate analysis

A maximum parsimony tree based on the SSLP data is shown in Fig. 1. The accessions split into two principal groups with little resolution within groups. The first group (Group I) included all of the accessions of *L. alba*, *L. floccosa*, *L. gracilis* and *L. montana*. This group corresponds to Section Inflexae. The second group (Group II) included all of the accessions of *L. douglasii*, *L. bakeri* and *L. striata*, equivalent to Section

https://doi.org/10.1017/S1479262108014378 Published online by Cambridge University Press

Table 3. Simple sequence repeat amplification in the analyzed *Limnanthes* germplasm collection. Number of accessions, number of amplified bands, percentage of total bands, and the percentage of bands polymorphic within each species

Taxa	No. of accessions within group	No. of bands amplified	Percentage of total bands amplified	No. of SSLPs amplified in the species	Percentage of SSLP bands observed in the species
Section Inflexae					
L. alba	33	126	92	116	92
L. floccose	7	89	64	79	89
L. gracilis	2	60	43	11	18
L. montana	1	40	29	N/A	N/A
Mean		79	57	69	66
Section Reflexae					
L. bakeri	2	54	39	28	52
L. douglasii	16	105	75	94	90
L. striata	2	44	31	10	23
Mean		68	48	44	55

SSLP, simple-sequence length polymorphism.

Reflexae given current sampling. The split between Group I and Group II was confirmed by neighbour-joining bootstrap analysis (results not shown). The separation of the 62 accessions into the two groups was also supported by PCO-MC analysis. Two statistically significant clusters ($P < 0.05$) found using PCO-MC (Fig. 2) matched exactly with Groups I and II from the dendrogram analyses.

Fingerprinting accessions

Five of the SSR markers (Ls-402, Ls-466, Ls-483, Ls-484 and Ls-278) were able to distinguish 59 (95.2%) of the 62 bulked accession fingerprints from each other. Two additional SSR markers were needed to fully distinguish all accessions. Ls-107 was needed to distinguish PARL 428 *L. douglasii rosea* from PARL 423 *L. douglasii nivea* as well as PARL 430b *L. alba alba* from PARL 431b *L. alba alba*. Ls-273 was needed to distinguish PI 374802 *L. alba* from PI 608039 *L. alba versicolor*.

Discussion

In this study, all of the SSR primer pairs amplified multiple products in the *Limnanthes* accessions. The amplification of multiple loci in *Limnanthes* using SSR markers was also observed in a study by Kishore *et al.* (2004), in which 1–28 unique bands were amplified for each SSR marker, with 56% of the primers amplifying multiple loci. Here, where samples were bulked, the level of allelic richness of 9.5 alleles per SSR primer set is comparable to the average number of alleles found in the survey of individual genotypes (10.7 per marker) in Kishore *et al.* (2004). In other studies of SSRs in higher plants, where specific allelic status was unknown because of polyploidy, bulked samples found comparable levels of allelic richness. For example, mean allelic richness was 8 per marker in cultivated sugarcane cultivars (Cordeiro *et al.*, 2000), 12.6 per marker in open-pollinated *Zea* sp., 6.9 per marker in inbreds in *Zea* sp. (Matsouka *et al.*, 2002) and 11.8 per marker in *Sorghum* sp. (Dillon *et al.*, 2005).

Pooled DNA samples have been used successfully in prior studies of genetic variability in plant collections (Havey, 1995; Charters *et al.*, 1996; Gilbert *et al.*, 1999; Sham *et al.*, 2002). When accessions are represented using SSR fingerprints from bulked samples, special care is necessary during analysis. SSR fingerprints produced from bulked samples comprise a composite phenotype of the alleles sampled within an accession. Because of this, commonly used genetic distances based on allele frequencies cannot be used. Here, we have chosen to use parsimony as a conservative criterion to classify accession fingerprint data. In addition, we used a non-hierarchical ordination method to cluster fingerprints and employed a quantitative metric to identify significant clusters.

The SSR fingerprint data were sufficient to distinguish all samples from one another. This suggests that all of the *Limnanthes* accessions in the NPGS collection contain unique alleles and are thus distinct. However, the possibility that this finding is a sampling artefact due to the sample pooling strategy cannot be categorically eliminated. While the maximum parsimony tree showed limited substructure among SSR fingerprint phenotypes, the division of accessions into Group I and Group II is clear and is supported by neighbour-joining as well as by principal coordinate analysis (Fig. 2). The lack of substructure within Group I and II observed in the dendrogram may be due to a variety of factors, including

https://doi.org/10.1017/S1479262108014378 Published online by Cambridge University Press

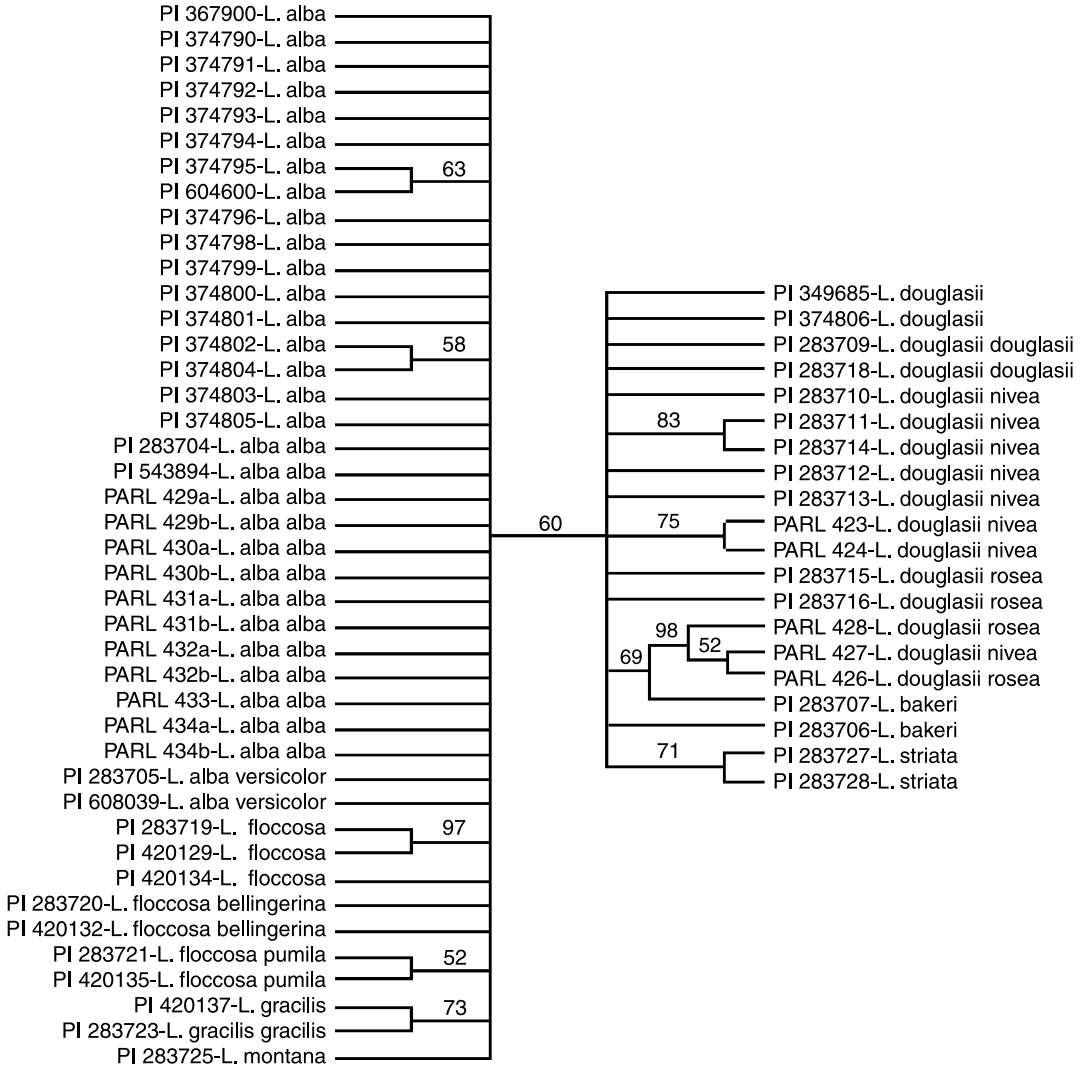

Fig. 1. Maximum parsimony tree based on simple-sequence length polymorphisms identified for accessions in the USDA-NPGS *Limnanthes* germplasm collection. Numbers at internodes are bootstrap support values. Branches with <50% bootstrap support have been collapsed. Tree is displayed as unrooted to emphasize the central branch, which splits the accessions into two groups also found to be significantly distinct using principal coordinate analysis. Group I (Inflexae) is on the left; Group II (Reflexae) is on the right.

bulking, missing SSLP data, lack of sufficient DNA marker resolution, scoring of non-allelic fragments of the same size as allelic, and gene flow in the wild. The first three issues may be resolved by other approaches that could improve genetic resolution in the data including addition of loci or marker density and the collection of individual genotypes. Avoiding scoring of non-allelic fragments of the same size as allelic could be dealt with by sequencing of *Limnanthes* amplicons of interest to ensure that the SSLPs amplified with each primer are truly allelic, as seen in rice by Panaud *et al.* (1996). The fifth issue, natural gene flow between taxa, would probably require extensive DNA sequencing to detect.

Within the USDA collection, the *L. alba*, *L. floccosa* and *L. douglasii* accessions showed the highest level of genetic diversity (92, 89 and 90% of all alleles found experiment-wide, respectively) based on the SSR analysis (Table 3). These taxa have shown the greatest promise for cultivar development (Jolliff *et al.*, 1981) and, therefore, have been sampled extensively and are represented by a large number of accessions in the NPGS collection (33, 7 and 16, respectively). The number of accessions for *L. bakeri*, *L. gracilis* and *L. striata* (2 for each species) was low as was the percentage of total allelic diversity observed (52, 18 and 23%, respectively). These findings support the need for increased sampling of wild *L. bakeri*, *L. gracilis*, *L. striata* and *L. montana* populations (which is represented by only one accession in the NPGS collection). Further study may find that the genetic diversity truly is lower in the *L. bakeri*, *L. gracilis*,

https://doi.org/10.1017/S1479262108014378 Published online by Cambridge University Press

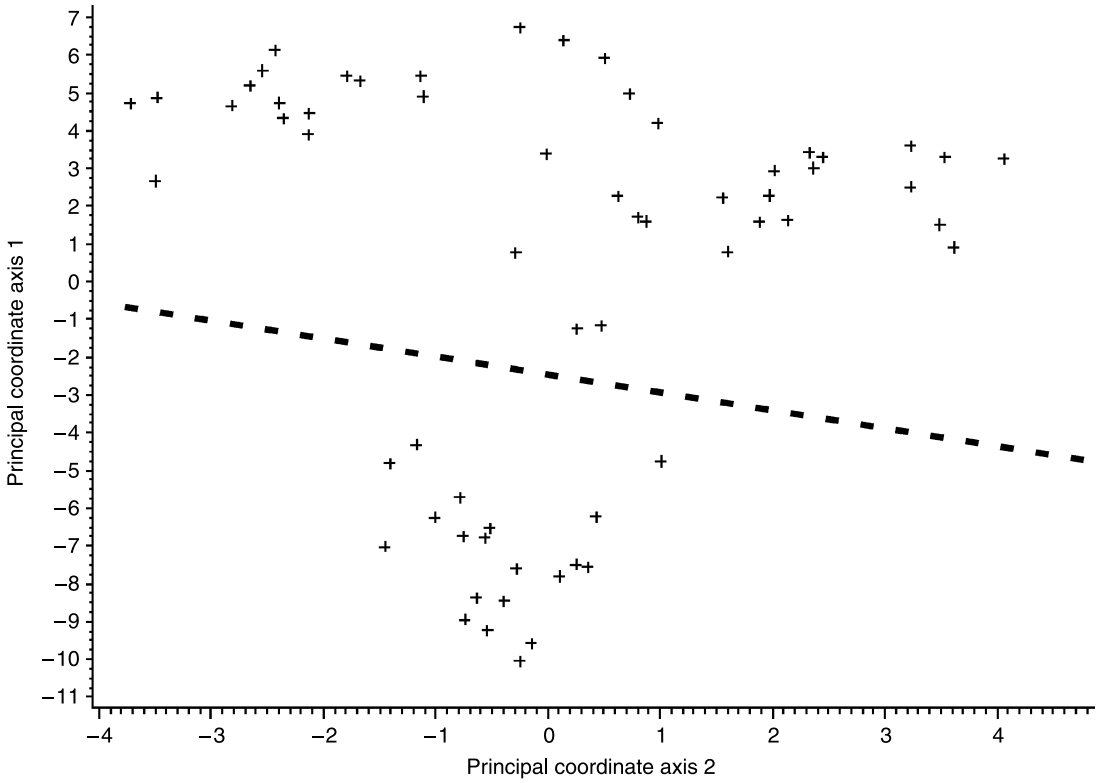

Fig. 2. Plot of sampled accessions along the first two principal coordinate axes. Using PCO-MC and data from all principal coordinate axes, two statistically significant clusters ($P < 0.05$) that were consistent with parsimony analysis were identified. A dashed line is used to indicate the discontinuity between significant clusters. Group I (Inflexae) is above the line; Group II (Reflexae) is below the line.

L. striata and *L. montana* taxa compared with other *Limnanthes* species, but at present, assumptions cannot be made due to small sample size. Representatives of *L. macounii* and *L. vinculans* should also be included in the collection to cover all taxa of the genus.

In this study, taxa of the Inflexae exhibited more diversity than taxa of the Reflexae, with 66% of the amplified products observed to be polymorphic in the Inflexae compared with 55% in Reflexae (Table 3). This may prove to be important in cultivar development due to the taxa in the Inflexae having superior seed retention qualities than the Reflexae taxa (Gentry and Miller, 1965; Higgins *et al.*, 1971). For breeding purposes, having a large repertoire of accessions with traits of agronomic value is of importance. The data suggest that accessions of the NPGS germplasm collection within the Inflexae are of diverse genetic backgrounds and therefore may provide a suitable platform for cultivar development.

Conservation of wild relatives of cultivated crops is necessary to prevent the loss of genetic diversity with repeated breeding. In domesticated crops, genetic variation is often subjected to a 'domestication bottleneck' when selection for desirable characteristics results in the loss of diversity. Such loss of variation occurred in maize (Matsuoka *et al.*, 2002) and wheat (McLauchlan *et al.*, 2001), where wild relatives showed greater diversity among SSR markers than did cultivated varieties. Breeding efforts aimed at the development of new cultivars may lead to losses in *Limnanthes* genetic diversity. Hence, the development of a germplasm collection with high genetic diversity is important. As this study showed, the *Limnanthes* species most used in cultivar development, *L. alba*, *L. douglasii* and *L. floccosa*, are the species most highly represented in the NPGS germplasm collection and have the greatest amount of intraspecific diversity. In comparison, species *L. bakeri*, *L. gracilis*, *L. striata* and *L. montana* show little intraspecific variation and should be collected more extensively to ensure the genetic diversity of the genus and the respective species in the collection.

Diverse germplasm collections provide a range of traits for use in breeding programs from increased yield to traits that may be required in the future due to unforeseen abiotic and biotic challenges. The data from the current study showed that the NPGS *Limnanthes* germplasm collection is genetically diverse and that the accessions within the species likely contain novel alleles. The collection therefore forms an important contribution to the conservation of the *Limnanthes* gene pool.

https://doi.org/10.1017/S1479262108014378 Published online by Cambridge University Press

Acknowledgements

This work was supported in part by USDA CRIS # 5302-21000-008-00D. We would like to thank Dr Christopher Richards (USDA-ARS-NCGRP, Fort Collins, CO), Dr Jefferey Dole (The University and Jepson Herbaria, UC, Berkeley, CA) and Jeremy Ross (CSU Fresno, CA) for their technical assistance, and the CSU Fresno, College of Science and Mathematics for supporting J. Prince during the preparation of this manuscript.

References

Bagby MO, Smith CR, Miwa TK, Lohmar RL and Wolff IA (1961) A unique fatty acid from *Limnanthes douglasii* seed oil: the C_{22} diene. *Journal of Organic Chemistry* 26: 1261–1265.

Baranger A, Aubert G, Arnau G, Laine AL, Denoit G, Potier J, Weinachter C, Lejeune-Henaut I, Lallemand J and Burstin J (2004) Genetic diversity within *Pisum sativum* using protein- and PCR-based markers. *Theoretical and Applied Genetics* 108: 1309–1321.

California Native Plant Society (CNPS) (2005) Inventory of rare and endangered plants [online edition v6-05c]. Sacramento, CA: California Native Plant Society, http://www.cnps.org/inventory

Charters YM, Robertson A and Wilkinson MJ (1996) PCR analysis of oilseed rape cultivars (*Brassica napus* L. ssp. *oleifera*) using 5′-anchored simple sequence repeat (SSR) primers. *Theoretical and Applied Genetics* 92: 442–447.

Cordeiro GM, Taylor GO and Henry RJ (2000) Characterization of microsatellite markers from sugarcane (*Saccharum* sp.), a highly polyploidy species. *Plant Science* 155: 161–168.

Debry RW and Olmstead RG (2000) A simulation study of reduced tree-search effort in bootstrap resampling analysis. *Systematic Biology* 49: 171–179.

Dillon SL, Lawrence PK and Henry RJ (2005) The new use of *Sorghum bicolor*-derived SSR markers to evaluate genetic diversity in 17 Australian *Sorghum* species. *Plant Genetic Resources* 3(1): 19–28.

Dole JA and Sun M (1992) Field and genetic survey of the endangered Butte County meadowfoam – *Limnanthes floccosa* ssp. *californica* (Limnanthaceae). *Conservation Biology* 6(4): 549–558.

Gentry HS and Miller RW (1965) The search for new industrial crops IV. Prospectus of *Limnanthes*. *Economic Botany* 19(1): 25–32.

Gilbert JE, Lewis RV, Wilkinson MJ and Caligari PDS (1999) Developing an appropriate strategy to assess genetic variability in plant germplasm collections. *Theoretical and Applied Genetics* 98: 1125–1131.

Godwin ID, Mace E and Nurzuhairawaty (2001) Genotyping Pacific Island taro (*Colocasia esculenta* (L.) Schott) germplasm. In: Henry RJ (ed.) *Plant Genotyping: The DNA Fingerprinting of Plants*. New York: NY: CAB International, pp. 109–128.

Havey MJ (1995) Identification of cytoplasms using the polymerase chain reaction to aid in the extraction of maintainer lines from open-pollinated populations of onion. *Theoretical and Applied Genetics* 90: 263–268.

Higgins JJ, Calhoun W, Willingham BC, Dinkel DH, Raisler WL and White GA (1971) Agronomic evaluation of prospective new crop species II. The American *Limnanthes*. *Economic Botany* 25: 44–54.

Jain SK and Abuelgasim EH (1981) Some yield components and ideotype traits in meadowfoam, a new industrial crop. *Euphytica* 30: 437–443.

Jain SK, Pierce RO and Hauptli H (1977) Meadowfoam: potential new oil crop. *California Agriculture* 31: 18–20.

Jolliff GD, Tinsley IJ, Calhoun W and Crane JM (1981) Meadowfoam (*Limnanthes alba*) Its research and development as a potential new oilseed crop for the Willamette Valley of Oregon. *Corvallis, Agriculture Experiment Station, Oregon State University, Corvallis Station Bulletin* 648: November 1981: 17

Kishore VK, Velasco P, Shintani DK, Rowe J, Rosato C, Adair N, Slabaugh MB and Knapp SJ (2004) Conserved simple sequence repeats in the Limnanthaceae (Brassicales). *Theoretical and Applied Genetics* 108(3): 450–457.

Lodhi MA, Ye GN, Weeden NF and Reisch BI (1994) A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and *Amplelopsis*. *Plant Molecular Biology Reporter* 12(1): 6–13.

Mason CT Jr (1952) A systematic study of the genus *Limnanthes*. *University of California Publication in Botany* 25: 455–512.

Matsuoka Y, Mitchell SE, Kresovich S, Goodman M and Doebley J (2002) Microsatellites in *Zea* – variability, patterns of mutations, and use for evolutionary studies. *Theoretical and Applied Genetics* 104: 436–450.

McLauchlan A, Henry RJ, Isaac PG and Edwards KJ (2001) Microsatellite analysis in cultivated hexaploid wheat and wild wheat relatives. *Plant Genotyping: The DNA Fingerprinting of Plants*. New York: NY: CAB International, pp. 147–159.

Miwa TK and Wolff IA (1962) Fatty acids, fatty alcohols, and wax esters from *Limnanthes douglasii* (Meadowfoam) seed oil. *Journal of the American Oil Chemists' Society* 39: 320–322.

Panaud O, Chen X and McCouch SR (1996) Development of microsatellite markers and characterization of simple sequence length polymorphism (SSLP) in rice (*Oryza sativa* L.). *Molecular Genetics and Geneticist* 252: 597–607.

Pierce RO and Jain SK (1977) Variation in some plant and seed oil characteristics of meadowfoam. *Crop Science* 17: 521–526.

Reeves PA and Richards CM (2007) Distinguishing terminal monophyletic groups from reticulate taxa: performance of phenetic, tree-based, and network procedures. *Systematic Biology* 56(2): 302–320.

Sham P, Bader JS, Craig I, O'Donovan M and Owen M (2002) DNA pooling: a tool for large-scale association studies. *Nature Reviews Genetics* 3: 862–871.

Smith CR, Bagby MO, Miwa TK, Lohmar RL and Wolff IA (1960) Unique fatty acids from *Limnanthes douglasii* seed oil: the C_{20}- and C_{22}-monoenes. *Journal of Organic Chemistry* 25: 1770–1774.

Swofford DL (2002) *PAUP*: Phylogenetic Analysis using Parsimony and Other Methods (software)*. Sunderland, MA: Sinauer Associates.

USDA, ARS National Plant Germplasm System. Germplasm Resources Information Network (GRIN) [online database]. http://www.ars-grin.gov/cgi-bin/npgs/html

Wiens JJ (2006) Missing data and the design of phylogenetic analyses. *Journal of Biomedical Informatics* 39: 34–42.