GENETIC VARIATION IN RARE AND WIDESPREAD *LOMATIUM* SPECIES (*APIACEAE*): A COMPARISON OF AFLP AND SSCP DATA

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Plant conservation genetics has been hampered by a lack of markers for studies of levels and patterns of variation in rare species. We investigated the levels of variation in several rare and widespread species of the western North American genus *Lomatium* Raf. (*Apiaceae*) using two relatively new molecular markers: AFLPs and single-strand conformation polymorphisms (SSCPs). For each species, approximately 150 AFLP loci have been scored, yielding estimates of species-level percent polymorphic loci in rare species ranging from near zero to over 80%. Levels of AFLP diversity were similar in two of the rare species, *L. bradshawii* (Rose ex Mathias) Mathas & Constance and *L. ochocense* Helliwell & Constance, and the widespread species. The third rare species, *L. cookii* Kagan, which has small populations, has low levels of diversity based on AFLPs. We also examined nucleotide diversity at the single-copy nuclear-DNA locus glyceraldehyde 3-phosphate dehydrogenase (Gap-C). PCR-amplified segments were analysed for allelic variation using SSCPs, and intrapopulational nucleotide polymorphisms were identified in both *L. bradshawii* and *L. cookii*. In the 211bp segment of Gap-C analysed, five nucleotide sites were segregating within populations of *L. bradshawii* and two in *L. cookii*.

Keywords. *Apiaceae*, coalescence, conservation genetics, *Lomatium*, molecular markers, nuclear-DNA sequencing, *Umbelliferae*.

Introduction

Molecular data frequently contribute to conservation planning for rare and endangered species, and the types of information that can be gleaned from these data are increasingly detailed and informative about processes that cannot otherwise be observed. Along with the theoretical and analytical tools for analysing genetic data has come the desire for additional molecular markers that can reveal more details about the variation present within and among populations of a species. We present data from two promising molecular markers for population and conservation genetics: AFLPs[1] (Vos *et al.*, 1995) and single-strand conformation polymorphism (SSCP, Orita *et al.*, 1989), a rapid method of screening for DNA sequence variation, which we have used to examine nucleotide diversity in single-copy nuclear-DNA (nDNA) genes and their introns.

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[1] Despite the common use of the term AFLP as an acronym for 'Amplified Fragment Length Polymorphism', the initial description of the technique (Vos *et al.*, 1995: 4413) stated that 'The name AFLP, however, should not be used as an acronym, because the technique will display presence or absence of restriction fragments rather than length differences'.

We emphasize that (i) molecular data from a rare species cannot, in the absence of other forms of data, form the basis of a conservation program, and (ii) molecular data from a rare species, in the absence of data from a close relative, offer only limited information. By studying a widespread congener, in addition to a rare species, researchers have a point of reference with which to compare their data on rare species (Gitzendanner & Soltis, 2000).

Plant population biologists generally cannot use two of the most informative molecular markers in animals, mitochondrial DNA (mtDNA) sequences and microsatellites (Avise, 1994). The plant mitochondrial genome has low rates of nucleotide substitution, is subject to frequent genome rearrangements, and frequently integrates portions of other genomes (Palmer, 1992) and is therefore of little use in population genetics. Additionally, even in animals, where some sequences are informative at the individual level, the mitochondrial genome, which does not generally recombine (but see Awadalla *et al.*, 1999), must be viewed as a single locus, and any analyses based on mtDNA data will be influenced by the particular evolutionary distortions at that one locus. The chloroplast genome, while valuable for higher-level phylogenetics and some interpopulational studies, generally has too little variation to be useful for analyses of within-population variation (Soltis & Soltis, 1998). The development of microsatellite markers in plants has been much slower than in animals, and there are few loci that can be readily amplified across a diverse array of taxa. Additionally, recent work has demonstrated several problems in the interpretation of homology of microsatellite loci (Ortí *et al.*, 1997; Viard *et al.*, 1998). However, there have been several recent studies using microsatellites in plants (Naito *et al.*, 1998; Vendramin *et al.*, 1998; Burke & Arnold, 1999), including the successful demonstration of interspecific and even intergeneric amplification with *Glycine max* microsatellite primers (Peakall *et al.*, 1998).

AFLPs are dominant molecular markers (Vos *et al.*, 1995) that can be analysed with standard frequency-based methods developed for allozymes, with some adjustments for their dominant nature. AFLPs are generally analysed using the assumption that the populations sampled are in Hardy–Weinberg equilibrium, and violations of this assumption may bias interpretations. AFLPs have been successfully used in several conservation studies (e.g. Travis *et al.*, 1996; Palacios *et al.*, 1999) and are becoming more common in analyses of plant population genetics. We chose AFLPs because the technique combines many of the benefits of RAPDs (random amplified polymorphic DNA) and RFLPs (restriction fragment length polymorphisms), and because a large number of loci can be assayed. AFLP analysis involves the digestion of genomic DNA with two restriction endonucleases, ligation of adapters of known sequence to the sticky ends left by the enzymes, and amplification using the adapter sequence and up to three additional random nucleotides to select for subsets of fragments.

It has been over 15 years since the first population-level studies using DNA sequencing (e.g. Kreitman, 1983), and technological advances have made DNA sequencing considerably easier. Additionally, with the rapid development of coalescent theory

(started by Kingman, 1982a,b), many authors have pointed out the benefits of DNA sequencing and the potential utility of the data in population genetics in general and conservation biology in particular (Milligan *et al.*, 1994; Moritz, 1995; Karp *et al.*, 1996). To facilitate gathering sequence data, several methods have been developed that allow rapid identification of sequence variation in PCR-amplified segments of DNA without actually sequencing each sample (reviewed by Lessa & Applebaum, 1993). One such method is SSCP (Orita *et al.*, 1989), which separates PCR fragments in a non-denaturing polyacrylamide gel based on the sequence-dependent secondary structure of single-stranded DNA (Fig. 1). With this method, individuals can be screened for allelic variation, and sequence can be inferred for all individuals by sequencing only a few representatives of each allele identified on the basis of mobility.

Lomatium Raf. (*Apiaceae*) is endemic to western North America and contains over 90 species, many of which are rare (Constance, 1993). Most *Lomatium* species are andromonoecious, perennial herbs, are pollinated by generalists (Diptera, Hymenoptera and Coleoptera), and have high outcrossing rates (Hardin, 1929; Schlessman, 1982). This study, which is part of a larger project to study genetic diversity in over 10 species of *Lomatium*, is focused on three rare and three widespread species.

Lomatium bradshawii (Rose ex Mathias) Mathias & Constance is a Federally listed

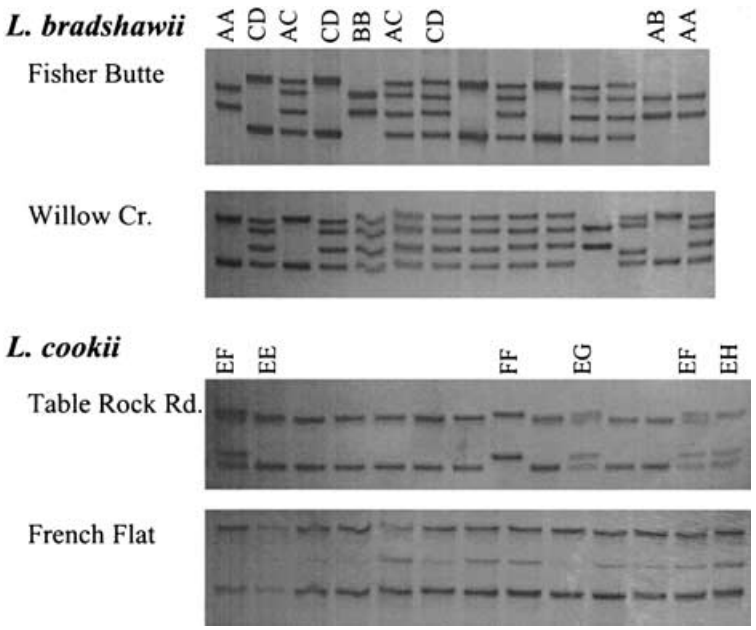

FIG. 1. SSCP profiles of *Gap-C* alleles amplified from individuals in two *Lomatium bradshawii* and two *L. cookii* populations. Letters indicate genotypes of each individual for which sequence data is available. Alleles A and B in *L. bradshawii* differ at nucleotide position 87, yet appear indistinguishable on the gels. Similarly, alleles F and G in *L. cookii* differ by one nucleotide, and are indistinguishable.

endangered species (US Fish and Wildlife Service, 1993) endemic to the Willamette Valley region of Oregon and southern Washington, USA. Much of its habitat has been converted to agricultural uses, and fire suppression in the remaining habitat has often led to its exclusion by woody species (Pendergrass *et al.*, 1999). *Lomatium ochocense* Helliwell & Constance was discovered in 1994 and is endemic to five populations in the Ochoco Mts of central Oregon. *Lomatium cookii* Kagan is an Oregon State listed species, which is being considered for Federal listing as endangered. This species is found in two geographically separate localities with distinct habitats, and Kagan (1986) considered naming two separate species. *Lomatium cous* (S. Watson) J. M. Coult. & Rose, *L. grayi* J. M. Coult. & Rose, and *L. triternatum* (Pursh) J. M. Coult. & Rose are common and widely distributed throughout the Pacific Northwest of the USA.

In this paper we present the AFLP data for six *Lomatium* species (data from additional species are presented in Gitzendanner, 2000) and SSCP data from two rare species.

Materials and Methods

Leaf tissue was collected from up to 32 individuals from each sampled population (Table 1; for additional collection information see Gitzendanner, 2000). After removal from the plant, leaves were immediately dried using Drierite (W.A. Hammond Drierite Co., Xenia, OH; 8 mesh). DNA extractions were performed using the standard plant tissue protocol of the Wizard DNA extraction kit (Promega Corporation, Madison, WI). Approximately 20mg of dried leaf tissue were used for the extractions. Precipitated DNA was resuspended in 100μl of TE buffer. No attempt was made to standardize DNA concentrations.

AFLP analysis generally followed standard protocols (Vos *et al.*, 1995, PE Applied Biosystems, Foster City, CA). Our complete protocol can be downloaded from http://www.wsu.edu/~soltilab/aflp.html (see also Gitzendanner, 2000). Adapter and primer sequences were identical to those of Vos *et al.* (1995). The main modifications were the use of fluorescently labelled *Eco*RI+3 primers, and electrophoresis of the products on an ABI 377 DNA 'Sequencer' (PE Applied Biosystems, Foster City, CA). The +3 PCR reactions were performed with Ampli*Taq* Gold™ (PE Applied Biosystems, Foster City, CA) and were multiplexed (i.e. three different *Eco*RI +3 selective primers were used, each with a different fluorescent label). Products were run on 5% Long Ranger gels (FMC, Rockland, ME). All data were analysed with GeneScan and Genotyper software (PE Applied Biosystems, Foster City, CA).

We present SSCP results from the cytosolic glyceraldehyde 3-phosphate dehydrogenase gene (*Gap-C*). Using primers from Strand *et al.* (1997, GPDX7F) and a primer that we designed (GapCx8R: 5′-AGTTTTTCATTCTTTTTATACCTT), we amplified a 211bp region of *Gap-C* spanning the intron between exons 7 and 8. PCR products were mixed 1:1 with 80% formamide loading dye and denatured by heating to 95°C for 5min. After snap cooling on ice, the products were loaded on a 7.5% 49:1 (acrylamide:bis-acrylamide) gel containing 10% glycerol, and run for 16h at 35mAmps at 4°C. The gels were silver stained using the method of Sanguinetti *et al.* (1994). Both strands of the samples were sequenced using the same primers as above and standard automated sequencing protocols on the ABI 377 (PE Applied Biosystems, Foster City, CA), using the Big Dye chemistry.

Data were analysed using POPGENE 1.21 (Yeh *et al.*, 1997) and Tools for Population

TABLE 1. Percentage polymorphic loci (*%P*) and expected heterozygosity (H_e) found in *Lomatium bradshawii*, *L. cookii*, *L. ochocense*, *L. cous*, *L. grayi* and *L. triternatum* based on AFLP data. Number of loci examined from each species listed in parentheses. Population-level means and species-level values are in bold

Species/population	Sample size	*%P*	H_e
L. bradshawii (149 loci)			
West Green Hill Prairie	15	49.7	0.195
Kingston Prairie	30	30.9	0.074
Willow Creek	30	58.4	0.180
Long Tom ACEC	29	55.7	0.199
Green Mountain	26	28.2	0.095
Population-level means		**44.6**	**0.149**
Species-level values		**67.8**	**0.236**
L. cookii (112 loci)			
Table Rock Rd	28	6.3	0.015
Medford-Jackson Co. Airport	31	1.8	0.010
French Flat	30	6.3	0.017
Population-level means		**4.8**	**0.014**
Species-level values		**7.1**	**0.021**
L. ochocense (147 loci)			
Long Ridge 1	29	37.4	0.131
Long Ridge 2	30	45.6	0.104
Cabin Butte	19	59.9	0.241
Battle Point	20	62.6	0.255
Oscar Canyon	29	57.6	0.196
Indian Trail Butte	5	36.7	0.155
Population-level means		**48.4**	**0.177**
Species-level values		**83.7**	**0.280**
L. cous (154 loci)			
Long Ridge	22	46.8	0.173
Cabin Butte	23	61.7	0.220
Population-level means		**54.2**	**0.197**
Species-level values		**57.8**	**0.215**
L. grayi (133 loci)			
Grande Ronde River	32	47.4	0.181
Lower Granite Dam	28	38.3	0.138
S of John Day	30	71.4	0.261
Population-level means		**52.4**	**0.194**
Species-level values		**80.5**	**0.296**
L. triternatum (90 loci)			
Storm Gulch	25	77.8	0.274
Turnbull National Wildlife Refuge	27	53.3	0.222
W of Cedar Ridge	26	66.7	0.263
Population-level means		**65.9**	**0.253**
Species-level values		**84.4**	**0.335**

Genetic Analysis (TFPGA) (Miller, 1998) with the assumption that the populations were in Hardy–Weinberg equilibrium. There are no other data available from these populations with which to test this assumption; however, only the results for expected heterozygosity will be affected by deviation from Hardy–Weinberg equilibrium. Data were analysed with both the square-root-q^2 and Taylor expansion (Lynch & Milligan, 1994) options of TFPGA. As results were similar, and the Taylor expansion is more accurate (Lynch & Milligan, 1994), the latter values are reported. Although we cannot, at this time, test the validity of the Hardy–Weinberg assumption, data from our SSCP analyses will be able to address the issue in the future. For each population, the percentage of polymorphic loci (using a 95% criterion for polymorphism) and expected heterozygosity were calculated. For each species, population-level means were calculated as the mean value of each of the individual population estimates. Species-level means were calculated from all samples, regardless of their population of origin.

Results

We scored 90 to 154 AFLP loci in each of six *Lomatium* species – three rare and three widespread (Table 1). Levels of genetic diversity were similar in *L. ochocense* ($\%P_{spp}$, 84%) and two of the widespread species, *L. grayi* and *L. triternatum* ($\%P_{spp}$, 81% and 84%, respectively). *Lomatium bradshawii* and *L. cous* had somewhat lower levels of diversity ($\%P_{spp}$, 68% and 58%, respectively). *Lomatium cookii* had very low levels of variation detectable with AFLPs ($\%P_{spp}$, 7%). Data from the widespread species are based on two or three populations; they are thus likely underestimates of species-level diversity and should be considered minimum estimates. This is especially true for *L. cous*, for which we have data from two adjacent populations.

Population-level measures of diversity were slightly lower in *L. bradshawii* and *L. ochocense* ($\%P_{pop}$, 45% and 48%, respectively) than in the widespread species (*L. cous* $\%P_{pop}$, 54%; *L. grayi* $\%P_{pop}$, 52%; and *L. triternatum* $\%P_{pop}$, 66%). *Lomatium cookii* had very low levels of population-level diversity ($\%P_{pop}$, 5%).

Sequence data from *L. bradshawii* and *L. cookii* indicate intrapopulational nucleotide variation (Fig. 1), with five and two segregating sites in each species, respectively. Sequencing of individuals confirmed that the differences shown on the SSCP gel are due to differences in the DNA sequences among individuals. *Lomatium bradshawii* showed polymorphism at five sites, four of which are detected by SSCPs. We are currently investigating using restriction enzymes to detect variation at the fifth site, and have identified an enzyme (*Pst*I) with a recognition sequence matching the region of the polymorphism. *Lomatium cookii* has only two polymorphic sites, both in the Table Rock Rd population; the French Flat population is monomorphic for the common allele in the Table Rock Rd population. Variation at one of these sites was not detected with SSCPs; again, restriction site analysis may be useful in detecting this variation.

Discussion

Levels of AFLP diversity in two of the rare species, *L. bradshawii* and *L. ochocense*, are nearly as high as those found in the widespread species examined. Noteworthy

is the fact that these two rare species, despite being restricted to 17 and five populations, respectively, have several large populations (up to 25,000 individuals in *L. bradshawii* – US Fish and Wildlife Service, 1993). The third rare species, *L. cookii*, which generally has much smaller populations, has low variation with AFLPs (Table 1). This result, that population size has a major impact on levels of genetic diversity, is consistent with previous results (e.g. Barrett & Kohn, 1991; Ellstrand & Elam, 1993), and with our own simulation studies designed specifically to address the relative effects of geographic range, habitat specificity, and population size on genetic diversity (Gitzendanner, 2000). Additional data from more *Lomatium* species indicate that geographic range also affects levels of genetic diversity (Gitzendanner, 2000).

The northernmost populations of *L. bradshawii* (Green Mt and an adjacent population not sampled) are approximately 100km away from the next nearest population of the species. Levels of genetic diversity in the Green Mt population were the lowest of all populations in the species ($\%P = 28\%$, $H_e = 0.095$), perhaps indicating a loss of diversity in these isolated northern populations.

Our AFLP data do not offer support for dividing *L. cookii* into two species or subspecies. Levels of AFLP diversity are extremely low in the species ($\%P_{spp} = 7\%$), and at the few variable loci, genetic distances based on allele frequencies indicate a closer relationship between populations in different regions than populations within a region.

The *Gap-C* data show significant levels of nucleotide diversity within populations of *Lomatium* even in the small (211bp) fragment that we examined. SSCPs were able to detect all but one of the differences identified via sequencing among the individuals in the test populations of *L. bradshawii* and *L. cookii*. While it is difficult to make comparisons between the AFLP data set and the limited SSCP data, relative levels of genetic diversity are consistent between AFLPs and SSCPs in *L. bradshawii* and *L. cookii*. As more sequence data are gathered for *L. cookii*, the power of the coalescent approach will be especially clear, where it will be possible to look at the relationship of alleles in each region where *L. cookii*, rather than just the AFLP allele frequencies, providing a more powerful interpretation of the genetic relationships within and between the regions where this plant grows.

Further data from this and other loci are clearly necessary, but we are hopeful that SSCPs will prove very powerful in population genetics. The costs associated with sequencing in most cases prohibit large-scale population-level analyses of sequence variation; the SSCP approach allows analysis of sequence variation among large numbers of individuals at a fraction of the cost. Based on our preliminary *Gap-C* data, we feel that the chances of missing alleles with SSCP analysis are lower than the chances of missing alleles due to undersampling the population.

Our studies indicate that both AFLPs and SSCPs are potentially useful markers for population and conservation genetics. The large numbers of loci that can be examined with AFLPs provide robust estimates of population genetic parameters. However, the dominant nature of the markers is problematic as Hardy–Weinberg

equilibrium must be assumed unless F_{IS} values can be obtained from other data. With the rise of comparative genomics and the availability of large sequence databases with information from many organisms, it is now easier to identify candidate genes for sequencing at the population level and to use the databases to construct primers for amplification (Strand *et al.*, 1997). Additionally, coalescent-based tools, which incorporate the genealogical histories of alleles (see Hudson, 1990), are being refined and are more readily applicable to non-model systems (e.g. Schaal and Olsen, 2000). SSCPs are one of several methods that will allow efficient collection of large amounts of sequence data. Our preliminary results indicate that the technique is capable of resolving very small differences in sequence, allowing for rapid identification of nucleotide variation. Combined with demographic and other data, genetic data, such as those presented here, can provide valuable information for land managers for the conservation of rare species.

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