GENETIC VARIATION IN THE ILLINOIS-THREATENED HILL PRAIRIE LARKSPUR

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Abstract: *Delphinium carolinianum* Walter is a threatened wildflower in the state of Illinois, where it is limited to isolated hill prairies along the Mississippi River. Isolated populations often experience little to no gene flow with other populations, and this may cause inbreeding depression that ultimately leads to extinction. Through use of amplified fragment length polymorphism (AFLP) molecular markers, we assessed the patterns of genetic variation within and between populations of *D. carolinianum* to better understand the stability of extant populations of this rare wildflower. Our data show that high levels of genetic variation are present within the populations studied, yet it is unclear whether this variation is due to current gene flow or preservation of historic variation.

Key Words / Search Terms: AFLP analyses, *Delphinium carolinianum*, rare plants, hill prairies, fragmented populations, population genetics

INTRODUCTION

Delphinium carolinianum (Ranunculaceae) is commonly called wild blue larkspur and grows in the Midwestern, southeastern, and south central United States (USDA, NRCS 2010). The plant is a perennial, 0.6-0.9 meters tall, which flowers in June, then sets seed and goes dormant for the remainder of the growing season. In Illinois as elsewhere, *D. carolinianum* is commonly called wild blue larkspur (Mohlenbrock 2002). However, in this study, we use hill prairie larkspur as its common name because in Illinois it is found mostly on hill prairies and the flowers are more white than blue. The hill prairie larkspur is currently found in three counties along the Mississippi River but was previously reported to be found in six counties across Illinois (Figure 1). The populations within each county are highly isolated, and many lack long-term protection status because they are located on private property. Our demographic data provided the Illinois Endangered Species Protection Board with the information necessary to place *D. carolinianum* on the state-threatened species list (IESPB 2009). *Delphinium carolinianum* is considered endangered in Florida and threatened in Kentucky (USDA, NRCS 2010).

A hill prairie is a dry grassland patch located on the west or southwest slope of an elevation (Evers 1955). Hill prairies are one of the least disturbed prairies left in the United States because the steep slopes where they are located are not suitable for conversion to farmland. Agriculture and development have replaced the surrounding natural areas, causing the habitat of the hill prairie larkspur to become highly fragmented into small "hill prairie islands." The lack of conservation within and around the hill prairies

Figure 1: Map of Illinois with documented current and historical locations of the hill prairie larkspur. Current locations indicated with circle, historical locations indicated with triangle. Historical location data taken from Mohlenbrock and Ladd (1978).

has allowed invasive species to encroach. Some of these species include dogwood (*Cornus drummondii* C.A. Mey.), bush honeysuckle (*Lonicera maackii* (Rupr.) Maxim.), and red cedar (*Juniperus virginiana* L.). Management and preservation must be done on hill prairies to help prevent *D. carolinianum* from becoming extirpated.

Genetic data provide conservationists and land managers with information to aid in preserving biodiversity within fragmented ecosystems. Fragmentation of populations can result in genetic drift, inbreeding, and reduced gene flow (Young et al. 1996). All of these reduce the amount of genetic variation present in a population, and increase the chances of having deleterious alleles become fixed in the population. The restoration of gene flow between fragmented populations can alleviate these effects by introducing new alleles to those populations lacking genetic diversity. Despite these generally observed trends in population response to fragmentation, fragmentation has sometimes been observed to increase gene flow in plant populations through the exchange of alleles between fragment populations (Hamrick 1994, Young et al. 1993).

We undertook this study to answer three questions: (1) What patterns of variation are present in Illinois populations of *D. carolinianum*? (2) How has habitat fragmentation influenced this variation? and (3) How much gene flow occurs between these populations?

MATERIALS AND METHODS

Fieldwork was conducted during the summers of 2005-2010. When a population of hill prairie larkspur was found, a handheld global positioning system (GPS) was used to mark the location. Throughout the month of June demographic data were collected from known hill prairie sites in Henderson, Pike, and Calhoun counties. We conducted a thorough search of the appropriate habitat (loess hill prairie/limestone glade), including around the edges of these communities. We used two or three people and spread out, conducting informal transect lines while walking across these communities, and taking extra care to look more carefully where plants were found or previously found and in areas with similar conditions and associated plants as those areas where larkspurs were found. Population size, number of flowering individuals, life-cycle stage, seedling recruitments, and species associates were recorded for each population. Leaf material was also haphazardly collected and placed in a paper coin envelope (1 leaf per individual, 30 individuals per population, as allowed by population size). The envelopes were placed in plastic sandwich bags containing Drierite (W. A. Hammond Drierite Co., Xenia, Ohio) to dry leaf material for future DNA analyses.

DNA was extracted from dried leaf tissue using a Wizard Kit (Promega, Madison, WI). Voucher specimens for each population will be deposited in the Field Museum of Natural History, Chicago. AFLP molecular markers were used to assess patterns of variation within and between fragmented larkspur populations. Methods for AFLP analysis generally followed Vos et al. (1995) and a modified protocol of the Applied Biosystems Plant Genome kit developed by M. Gitzendanner (U of FL; personal communication), with the following modifications: Genomic DNA, 2 µl of a 1:20 dilution with water, was digested for 3 hrs at 37˚C with 0.25 µl of *EcoRI* (12U/µl; Fisher Scientific, Pittsburgh, PA ,or New England Biolabs, Beverly, MA), 0.25 µl *MseI* (10U/µl; New England Biolabs), 0.5 µl each of the supplied enzyme buffers, and 1 µl BSA (1 mg/ml) brought to a final reaction volume of 10 µl. The digest reaction was stopped with a 65˚C soak for 20 min. A 10 µl ligation reaction containing 0.5 µl of T4 DNA ligase (3U/µl; Fisher Scientific or New England Biolabs), 2 µl 10X T4 Ligase buffer, 1.8 µl *MseI* adapter (50 µM; 5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3'), and 1.8 µl *EcoRI* adapter (5 µM; 5'-CTCGTAGACTGCGTACC-3' and 5'-AATTGGTACGCAGTCTAC-3') was then added to the completed restriction digest and run at 25˚C for 3 hrs followed by 10 min at 70˚C. These reactions were then diluted with 120 µl of sterile water.

The first selective amplification was conducted in 20 µl reaction volumes containing 4 µl of the diluted restriction-ligation reaction, 0.2 µl Taq DNA polymerase (5U/µl; Sigma Chemical Co., St. Louis, MO), 2 µl 10X PCR buffer (Sigma), 2.8 µl 25mM MgCl2, 1.6 µl 10mM dNTPs, 1.2 µl *EcoRI*+1A primer (5µM; 5'-GACTGCGTACCAATTCA-3'), and 1.2 µl *MseI* +1C primer (5µM; 5'-GACGATGAGTCCTGAGTAAC-3'). Reactions were heated to 72˚C for 2 min, then cycled 20 times at 94˚C for 30 s, 56˚C for 30 s, 72˚C for 120 s, and then held at 60˚C for 30 min. These reactions were diluted in 250 µl of sterile water and used in the second selective amplification step.

The second amplification was performed in 10 µl reactions containing 2.5 µl diluted +1 PCR product, 0.1 µl Amplitaq Gold DNA polymerase (5U/µl Applied Biosystems, Foster City, CA), 1 µl 10X Amplitaq PCR Buffer (Applied Biosystems), 1.2 µl 25mM MgCl2, 0.8 µl 10mM dNTPs, 0.3 µl of each *EcoRI*+4 primer (1.65µM each; 5'-[6-FAM] GACTGCGTACCAATTCACAT-3'; 5'-[NED] GACTGCGTACCAATTCAAGT-3'; 5'-[VIC] GACTGCGTACCAATTCAACT-3'; 5'-[PET] GACTGCGTACCAATTCACCT-3'), and 0.25 µl *MseI*+4 primer (5µM; 5'-GACGATGAGTCCTGAGTAACTTA-3').

We chose +4 primers because *Delphinium* has a large genome, making a more selective primer necessary to avoid homoplasy (Althoff et al. 2007). The *EcoRI* +4 primers were fluorescently labeled for visualization on an automated DNA analyzer. Single reactions contained four primer combinations because each primer was labeled with a different wavelength of dye (i.e., each *EcoRI* +4 primer with the *MseI* +4 primer). The reactions were held at 94˚C for 2 min, then cycled 10 times starting at 94˚C for 30 s, 65˚C for 30 s, 72˚C for 2 min, with a reduction in the annealing temperature by 1˚C per cycle. Reactions were then cycled 36 times at 94˚C for 30 s, 56˚C for 30 s, 72˚C for 2 min, followed by a 30-min 60˚C hold.

The +4 PCR reactions were sent to the Biotechnology Resource Center, Cornell University, Ithaca, NY, to be run on an Applied BioSystems 3730xl DNA Analyzer. Samples were prepared by mixing 1 µl of the +4 reaction with 0.2 µl LIZ 500 size standard (Applied Biosystems) and 9.8 µl HiDi formamide (Applied Biosystems).

Results were analyzed with GeneMapper (v4.0; Applied Biosystems). The program's default settings were used, except only fragments from 100-500 base pairs (bp) were analyzed, the allele calling threshold was set to 50, common alleles were not deleted, and advanced peak detected was selected.

DATA ANALYSIS

Fragments smaller than 100 bp were removed from the study to avoid fragment-size homology (Althoff et al. 2007). In the entire data set fragments present in more than 95% of individuals were removed from the analysis as well. We used these strict culling measures to assure we removed fixed fragments from the analysis. Other AFLP studies involving plants followed similar culling measures to assure meaningful polymorphism data were studied (Zhan et al. 2009). After culling, the remaining fragments were analyzed with AFLP-SURV (Vekemans 2002) and Structure v.2.2.3 (Pritchard et al. 2000, following analysis design of Hipp and Weber 2008). AFLP-SURV was used to calculate F-statistics between and within counties. Structure was used to look at how the variation of AFLP markers cluster individuals into populations.

RESULTS AND DISCUSSION

Our fieldwork resulted in our finding two additional populations (North New Canton and Kopp's Glade; Table 1). Kopp's Glade in Calhoun County is unique because it is a limestone glade and not a hill prairie. The total number of Illinois populations currently known is nine.

Table 1. Sites sampled and *D. carolinianum* population counts of number of individual plants by year

COUNTY	POPULATION	OWNERSHIP	2005	2006	2007	2008	2009	2010
Henderson	Bald Bluff	Private			136	60	165	12
Henderson	Tartan Bluff	Private	2	30	128	295	286	29
Henderson	Rt 34	Private	60			53		10
Henderson	Dallas City	Public	30			18		0
Pike	North New Canton	Private					2	0
Pike	Grubb Hollow	INPC/DNR	22	2	38	85		83
Pike	Houson	Private	0	0	50		107	
Pike	Walnut Grove	Private	0	4				
Calhoun	Kopp's Glade	Private				250		27

Population size fluctuated dramatically from year to year (Table 1). This could be due to individuals staying dormant for a season, as has been demonstrated in California (Lewis and Epling 1959) After the culling measures 337 AFLP markers remained from the 1,023 generated. The percentage of polymorphic makers in the hill prairie larkspur in Illinois ranged from 58.2% to 77.2%. Pike County had the lowest percentage of polymorphic markers (58.2%), followed by Calhoun (71.8%), and then Henderson (77.2%). While these levels were higher than those of other *Delphinium* species studied (Koontz et al. 2001), AFLPs generate higher levels of polymorphism than the allozymes used in previous studies on *Delphinium* (e.g., Koontz et al. 2001, Richter et al. 1994). The levels of polymorphism provide baseline data on the genetic diversity of the Illinois populations and can be used in future studies of this species.

The average Fst value across all populations of hill prairie larkspur studied was 0.0606 (range: 0.0285-0.1046). This indicates that genetic variation is partitioned among populations and not within an individual population. Comparisons within counties show higher levels of gene flow (Table 2) than populations in different counties. This signifies that there is or has been gene flow occurring between populations.

Table 2. The average Fst values between counties

POPULATION	TARTAN BLUFF	DALLAS CITY	GRUBB HOLLOW	HOUSON	KOPP'S GLADE
Bald Bluff	.0480	.0285	.1046	.0718	.0518
Tartan Bluff		.0533	.1027	.0685	.0688
Dallas City			.1023	.0734	.0611
Grubb Hollow				.0354	.0880
Houson					.0493

The Structure results show variable patterns between populations (Figure 2). The clustering of individuals from different counties supports gene flow between counties, but some of the clustering does not follow what would be expected by isolation-by-distance models. For example, clustering of individuals from Calhoun County with individuals from Henderson County and vice versa is nonintuitive, because the counties are separated by a large geographic distance. This brings up the question of how far apart *Delphinium* populations can be and still experience gene flow.

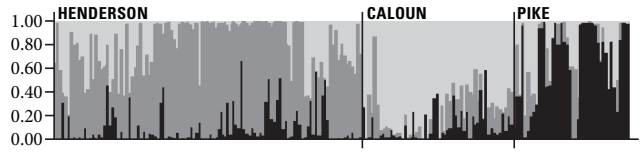

Figure 2: STRUCTURE Clustering with K=3. Each bar represents an individual sampled and shading shows portion of sampled AFLP markers that correspond to a given cluster. Samples generally clustered by their county of origin as shown by the predominance of a particular shade for each county; however some individuals within a county cluster with individuals from other counties.

Hill prairie larkspur can experience gene flow via pollination or seed dispersal. Little is known about seed dispersal in hill prairie larkspur regarding vectors or ranges

for these species. However, pollination biology is known. The larkspur is primarily pollinated by bumblebees (*Bombus* spp.). A survey by Greenleaf et al. (2007) of foraging distance of five different species of bumblebees showed foraging distances ranging from 0.1-10 km. The studied bumblebees were of similar size to the native species from Illinois hill prairies. Some populations within counties fall into the 10 km range but most do not. This makes current pollen-mediated gene flow seem to be a highly unlikely source of gene flow. Furthermore, a negative correlation with distance would discern pollen-mediated gene flow but there seems to be no trend between similar genetic variation and distance between sites.

The results of this study suggest two possibilities: (1) Shared variation is the result of current gene flow by an unknown agent, or (2) shared variation is the result of preserved historical variation prior to population fragmentation. In order to conserve this threatened species, we must find out which possibility is the reality.

If current gene flow is the source of shared variation, we would expect these populations to remain genetically stable in the future. Previous studies have shown fragmentation to result in increased variation between populations. Small amounts of gene flow between populations can greatly reduce the loss of alleles due to genetic drift (Young et al. 1996). Bee foraging-distance data indicate that many of the distances are too great for bumblebees to be the source of pollen-mediated gene flow. More data are required to determine if gene flow is current and by which vector this flow is occurring.

If the gene flow is not current and is a result of preserved historic gene flow, we would expect the fragmented populations to succumb eventually to loss of diversity due to allele fixation and a loss of heterozygosity as a result of genetic drift. This could ultimately have a fatal effect on the fragmented populations from an accumulation of deleterious alleles. If this is the case, management must to be applied to the fragmented populations to restore gene flow or maintain the levels of genetic variation.

Previous studies have shown that *Delphinium* may have the ability to maintain sufficient genetic diversity under fragmented populations (Koontz et al. 2001, Richter et al. 1994). It appears that some individuals use different methods of coping with the effects of fragmentation. Lewis and Epling (1959) observed that several Californian *Delphinium* species remain dormant as seeds or rootstocks. This could serve as a means of maintaining genetic diversity by allowing different plants to breed between generations (Lewis and Epling 1959).

Delphinium carolinianum populations in Illinois appear to have high levels of genetic variation. The source of diversity does not seem to be current, at least not via pollen-mediated gene flow. Application of more-sensitive tests and codominant markers to these populations will allow us to determine heterozygosity levels. We may also be able to determine whether this species has mechanisms for preservation of genetic diversity. This information will be valuable for the conservation of native biodiversity for this species as well as for other plant species.

FUTURE STUDIES

While AFLP markers provide excellent data on distribution of variation between populations, their dominant nature (inability to detect heterozygous individuals) makes them problematic for determining gene flow. In populations at equilibrium between mutation and drift, AFLP data can be used to calculate gene flow via the indirect method. The fragmented sites investigated are almost certainly not at equilibrium between drift and gene flow, so conventional population genetic formulae cannot be used to approximate current rate of gene flow (Young et al. 1996, Ouborg et al. 1999). Thus, estimations of gene flow require direct measurements of heterozygosity with codominant (heterozygotes can be distinguished from dominant homozygotes) markers such as allozymes or microsatellites. For example, microsatellites detect more variation and can provide estimates of gene flow over the past 10-100 generations (Selkoe and Toonen 2006).

Microsatellites are currently being developed. We hope these data will allow us to determine heterozygosity and ultimately whether or not gene flow is current or historical. Field observations will also be continued to document new populations and to monitor the highly fluctuating numbers from year to year. Ecological studies are needed to determine what is triggering the variation in population size. Given the range of *Delphinium carolinianum* we would like to expand the sampling outside of Illinois to see whether our Illinois data are normal or the exception for the species as a whole.

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