

ISSR Marker Diversity of the Threatened *Astragalus crassicaarpus* var. *trichocalyx* in Illinois Populations

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ABSTRACT

Astragalus crassicaarpus Nutt. var. *trichocalyx* (Nutt.) Barneby is a perennial plant that occurs in the southeastern part of the Great Plains on prairies, open oak woodlands, limestone hills and riverbanks. The species was considered extirpated from Illinois until it was rediscovered in 1987 on a limestone bluff in Beaver Dam State Park in Jersey County. It is now on the state list of endangered plant species. Presently three populations exist in Macoupin County, Illinois within isolated glacial till prairie sites. In Illinois *A. crassicaarpus* populations are small and restricted to areas within the prairie sites. The three existing populations are separated by geographical barriers, which severely restricts gene flow or seed dispersal between populations. The sites themselves are fragile due to the encroachment of woody species. All of these factors increase the threat of extinction for the species in Illinois. Conversely, *A. crassicaarpus* exists in several large populations in Missouri such as Painted Rock Conservation Area in Osage County. If conservation strategies are to be developed, an understanding of the genetic variation of the species in large and endangered populations is essential. One molecular marker that has proven useful in genetic diversity studies are ISSR markers. We compared the large population in Missouri to the small endangered populations in Illinois using ISSR markers. Painted Rock Conservation Area had the highest within population diversity value (0.574) of all sampled populations. On average, the Illinois populations had a lower diversity value than the Missouri population (0.482). Interestingly, Roderick Prairie, the smallest population in Illinois, had a diversity value of 0.525. Every Illinois population sampled contained unique genetic variation not found in the Missouri population. Because of this diversity and how it is partitioned, it is important to preserve as many Illinois populations as possible, large or small.

INTRODUCTION

Astragalus crassicaarpus Nutt. var. *trichocalyx* (Nutt.) Barneby is a perennial plant that occurs in the southeastern part of the Great Plains on prairies, open oak woodlands, limestone hills and riverbanks (McGregor 1986). It is a member of the Fabaceae family and is commonly known as prairie groundplum or milkvetch (USDA 2007). It is native to North America and ranges from Wisconsin to Texas (USDA 2007). *Astragalus crassicaarpus* var. *trichocalyx* is endangered in Illinois and Wisconsin (USDA 2007).

The species was considered extirpated from Illinois until it was rediscovered in 1987 on a limestone bluff in Beaver Dam State Park in Jersey County, and it is now on the state list of endangered plant species (Herkert and Ebinger 2002). *Astragalus crassicaarpus* var. *trichocalyx* occurs in three localities in the state, all of them on glacial drift prairies in Macoupin County (McClain et al. 2002). *Astragalus crassicaarpus* exists in several large populations in Missouri such as Painted Rock Conservation Area in Osage County (USDA 2007).

There are five varieties of *A. crassicaarpus* (USDA 2007). Variety *berlandieri* occurs in Texas, and variety *cavus* occurs in New Mexico and Arizona (USDA 2007). Varieties *crassicaarpus* and *paysonii* are widespread from Montana to Texas (USDA 2007). The variety examined in this study is *trichocalyx*, which occurs in seven states from Illinois south and west to Texas (USDA 2007).

The genus *Astragalus* contains two other species which are listed as endangered in Illinois (INHD 2007). *Astragalus distortus*, also known as Ozark milkvetch, occurs in four counties (INHD 2007). *Astragalus tennesseensis*, or Tennessee milkvetch, is currently found in two counties (INHD 2007).

Illinois contains many prairie inclusions, but glacial drift prairies are not common throughout the state (McClain et al. 2003). They are sometimes called gravel, dolomite, or glacial till prairies (IDNR 2007). Glacial drift prairies contain sediment deposited from the last ice age (McClain et al. 2003). In Illinois at the end of the Pleistocene, 126,000 years ago to 5,000 years ago, the glacier masses retreated to leave what we now know as glacial drift prairies (IDNR 2007). Glacial drift prairies are dry, well drained, and slightly alkaline (McClain et al. 2003). Many in Illinois have been destroyed by modern development and agriculture (IDNR 2007).

Glacial drift prairies tend to occur on slightly rolling, rocky terrain (McClain et al. 2003). Fires and nutrient-poor soil helped to keep these prairie areas free from invasion by woody species in pre-settlement times (Ebinger and McClain 1991). Their range in Illinois, however, has decreased because of fire suppression by European settlers (McClain et al. 2002). Glacial drift prairie remnants are rare and small (less than one hectare in size) and, therefore, have not received as much attention as other communities in the state (McClain et al. 2002). There is no known reason for the restriction of *A. crassicaarpus* to glacial till prairies in Illinois.

Other species are restricted to glacial drift prairies in Illinois as well. *Lespedeza leptostachya* or prairies bush clover, *Dalea foliosa* or leafy prairie clover, and *Hymenoxys acaulis* var. *glabra* or lakeside daisy are all endangered in Illinois (USFW 2007). The natural populations of lakeside daisy were extirpated when a mining company destroyed its site (USFW 2007). Preserving the glacial till prairie habitats of Illinois will help conserve not only *A. crassicaarpus* var. *trichocalyx*, but all of the species mentioned above.

In a 2006 thesis by Wells, some *A. crassicaarpus* var. *trichocalyx* life history characteristics are presented. All of these following factors may contribute to its

endangerment in Illinois. Soil cores were taken in all three Illinois prairies to assess the seed bank (Rogenski et al. 2004). The soil seed bank is small to nonexistent (Rogenski et al. 2004). Seed scarification experiments were performed on *A. crassicaarpus* seeds (Wells 2006). Scarification is rupturing of the seed coat by scratching or softening to encourage germination and is required for germination of *A. crassicaarpus* seeds (Baskin & Quaterman, 1969). Disturbance, such as burnings, may aid in scarification and thus germination. If burnings are not done annually, other species or invasive species may out compete *A. crassicaarpus*. Other members of the genus are buzz pollinated (Kearns et al. 1998). *Astragalus crassicaarpus* var. *trichocalyx* is most likely buzz pollinated by *Bombus perplexus*, which requires the insect to grab onto the flower and move their flight muscles which causes the flower and anthers to vibrate, releasing the pollen. This vibration is called buzz pollination. It is not known if it is self incompatible, because self incompatibility varies in the genus. *Astragalus crassicaarpus* does not asexually reproduce (Reveal 1978). If there are not enough compatible mating types available there may be reduced seed production, and population size could even further decrease.

Understanding genetic variation is essential to the population structure and rarity of *A. crassicaarpus* var. *trichocalyx*. The more diversity within a taxon, the better it can deal with evolutionary and environmental changes (Godt et al. 1996). In order to safeguard the Illinois sites, genetic understanding of the species in both large and endangered populations is key. Response of a species to environmental changes, disease resistance, and climatic conditions depends on the amount of genetic variation within a population (Godt et al. 1996).

One way to estimate the amount of genetic diversity in populations is through the use of molecular markers. Molecular markers are DNA markers that assess variation based on differences in the DNA sequence. One molecular marker that has proven useful in genetic diversity studies is ISSR (inter-simple sequence repeats) markers (Esselman et al. 1999; Alexander et al. 2004). Unlike other molecular markers, ISSRs require no prior knowledge of the exact DNA sequence (Godwin et al. 1997). Also, ISSRs show more genetic diversity than other molecular markers and have been used to examine genetic diversity of imperiled species (Nagaoka and Ogihara 1997; Esselman et al. 1999; McGlaughlin 2002; Alexander et al. 2004). They require less time, money, and labor than other methods and exhibit Mendelian inheritance (Harris 1999; Alexander et al. 2004). The technique behind ISSR markers makes use of PCR (polymerase chain reaction) and ISSR primers. The primers bind to sequence repeats in the genome, these regions get amplified via the PCR reaction and create DNA fragments of various sizes. The fragments produced are separated by size on an agarose gel by passing a current through the gel, a process known as gel electrophoresis. The Missouri population was chosen because of the large number of individuals and relatively close proximity to Edwardsville, Illinois. Permission was given by Tim Smith of the Missouri Department of Conservation to collect individuals from this conservation area.

We hypothesized that the isolated populations of *Astragalus crassicaarpus* var. *trichocalyx* in Illinois will have lower levels of ISSR marker diversity than a larger population in Missouri. Loss of habitat, smaller populations, and restriction of Illinois populations to glacial drift prairies all predict lower diversity (Godt et al. 1996). Because of geographical barriers between populations, human interference, such as cattle grazing and

restriction to glacial drift prairies, the Illinois populations should contain lower levels of diversity. This lack of diversity will affect the way *A. crassicaarpus* responds to environmental and evolutionary change (Godt et al. 1996). *Astragalus crassicaarpus* exists in 54 counties throughout the state of Missouri (USDA 2007). The population at Painted Rock Conservation Area is large and should contain more ISSR diversity because it is less restricted by glacial till prairies, endemic habitats, and small population size.

MATERIALS AND METHODS

Study Sites

Plants were collected in 2005 from Beaver Dam State Park, Humphries Prairie, and Roderick Prairie Nature Preserve, all of which occur within 16 km of each other on glacial till prairies in Macoupin County Illinois (Figure 1). Individuals were also collected in 2005 from Painted Rock Conservation Area located in Osage County Missouri, approximately 225 km from Edwardsville, Illinois. With thousands of individuals, it is a very large population, and will provide a genetic comparison to the much smaller endemic populations of Illinois.



Figure 1. Location of sites: Macoupin County, Illinois and Osage County, Missouri.

Beaver Dam State Park is the largest of the Illinois populations with 278 individuals in 2004 (Wells 2006). The site is divided into two sections by a four-wheel track. It has been subjected to burns approximately every two to three years in spring (Mark Phipps, Illinois Department of Natural Resources, personal communication with Wells 2006).

Humphries Prairie is located on private land and from 1999-2000 was grazed by cattle (Wells 2006). Cattle grazing may continue, but it is not documented. In 2004, Humphries Prairie had a total of 167 individuals (Wells 2006).

Roderick Prairie Nature Preserve is the smallest of the Illinois populations. This site is also located on private land and is partially bordered by agricultural land. In 2004, the population consisted of 67 individuals (Wells 2006).

Method of Collection

Samples were collected in mid to late summer of 2005 along transects at each site. Samples from Beaver Dam State Park, Humphries Prairie, and Roderick Nature Preserve were collected on the same day. Collection from Painted Rock Conservation Area occurred at a later date in summer of 2005. During collection the leaf tissue was placed in a small bag with silica gel and numbered. Samples consisted of leaf tissue, where younger tissue was preferred to older tissue.

DNA Extraction and Isolation

A DNeasy Qiagen kit and protocol was used in 2007 to isolate the DNA from dried material. Approximately 0.04 mg of leaf tissue was used in each extraction. Because of potential DNA degradation, the gels were run within one week of isolation.

PCR Procedures

Three primers were utilized in this study: 17898, 17899, and 17901 (Esselman et al. 1999). All were obtained from GenoSys. Reactions were carried out in a volume of 23 μ L consisting of 3 μ L $MgCl_2$, 2 mM dNTPs, 1 μ L primer, a volume of DNA, 0.7 μ L Taq DNA polymerase and 2.5 μ L x Taq DNA polymerase buffer. A Strategene Robocycler was used with the thermocycle program set at 1.5 min at 94°C, 35 x 40 s at 94°C, 45 s at the annealing temperature, 1.5 min at 72°C; 45 s at 94°C, 5 min at 72°C, 6°C soak. The primer designations and compositions were 17898 (CA)₆RY, 17899(CA)₆RG, and 17901(GT)₆YR.

ISSR Analysis

The gels were run at 100 V (approximately 65 mA) until the marker dye moved a total of 7 cm. The PCR generated ISSR markers were separated out on 1.4% agarose gel in 1X TBE Buffer. The gels were stained with ethidium bromide. A 100 bp ladder (Gibco/BRL) was run with the PCR generated fragments to determine sizes of the fragments. The gels were read and analyzed using the Bio-Rad Gel Doc XR Molecular Imager. The banding patterns for each gel were read as present (1) or absent (0). Only band presence values were entered into a program written by Vera Ford, UC-Davis, revised by Timothy York, to generate average pairwise similarity values (Esselman et al. 1999).

The similarity values were recorded and then entered into NTSYSpc Version 2.02 (Rohlf 1998) for UPGMA (unweighted pair group using arithmetic average) cluster analysis and to generate the phenogram. The similarity and clustering methods of analysis were done to determine the amount of variation within and among populations. The number of unique bands and the percent of variable bands were tabulated for each population.

RESULTS

A total of 50 bands were scored. Primer 17898 had a total of 22 bands. Primer 17899 had a total of 17 bands. Eleven bands were scored for primer 17901.

Table 1. Distance values for Missouri and Illinois populations. Within population distances values are italicized.

	Missouri	Beaver Dam	Humphries	Roderick
Missouri	<i>0.574</i>			
Beaver Dam	0.715	<i>0.491</i>		
Humphries	0.712	0.659	<i>0.429</i>	
Roderick	0.692	0.627	0.647	<i>0.525</i>

Table 1 indicates the distance values for all populations analyzed using ISSR markers. A distance value of one indicates complete ISSR marker similarity among compared individuals. Between the Illinois populations, Humphries has the lowest within population diversity (0.429). Beaver Dam Prairie has a distance value of 0.491, slightly higher than Humphries Prairie. Roderick has a distance value of 0.525. These values indicate slightly lower levels of diversity than the largest population, Painted Rock Conservation Area in Missouri. The Missouri population has the highest within population distance value (0.574). In comparisons between populations (Table 1, Figure 2), the three Illinois populations are more similar to one another than either is to the Missouri population.

Table 2. Number of individuals sampled, unique bands, and percent unique bands.

Population	Number of Individuals	Number of Unique Bands	% Unique Bands
Missouri	16	7	14
Beaver Dam	22	2	4
Humphries	13	1	2
Roderick	18	3	6

There were unique ISSR marker bands (Table 2) in every population. Missouri, with seven, had the most unique bands (14%). Roderick, the smallest Illinois population, had a total of three unique bands (6%). Beaver Dam, the largest Illinois population, had a total of two unique bands (4%). Humphries Prairie contained only one unique band (2%). All unique band percentages of the Illinois populations were lower than the Missouri population.

DISCUSSIONS, CONCLUSIONS, RECOMMENDATIONS

Astragalus crassicaarpus populations in Illinois are small, with fewer than 400 plants (Wells 2006). Obstacles such as roads, fields, farms, and bodies of water severely restrict gene flow or seed dispersal between populations. Dispersal by larger animals from one prairie to another may not be realistic because of these geographical barriers. The sites themselves are fragile due to the encroachment of woody species (Wells 2006), and all sites occur within a small geographic range, referred to as endemism by conservation biologists (Anderson 1994). All of these factors increase the threat of extirpation for the species in Illinois (Anderson 1994).

Based on the results of this ISSR study, every measure should be taken to protect *A. crassicaarpus* populations in Illinois both large and small. Painted Rock Conservation Area had a distance value of 0.574 (Table 1). The average distance value of the Illinois populations was 0.482. This supports our hypothesis that there is more ISSR marker diversity in the larger population of Missouri as compared to the endangered, endemic populations of Illinois.

In Illinois we found that population size did not correlate with high levels of diversity. The largest population in Illinois, Beaver Dam State Park, had an intermediate distance value of 0.491. Although this site contains more individuals, it is in close contact to human traffic including four wheelers and grazing cattle. This disturbance may affect flowering, seedling survival, or seed dispersal and results in lower diversity values. Roderick, the smallest population, had the highest diversity value (0.525). Because the smaller Illinois populations have high levels of diversity, every existing population needs to be preserved.

In comparisons between populations, the three Illinois sites are more similar to each other than any of them are to the Missouri population (Table 1, Figure 2). All populations sampled contained unique bands (Table 2). Unique bands are those that only exist in one population and are a measure of unique genetic information. As we expected, the largest population sampled, Missouri, had the most unique markers (7). Illinois had an average of two unique bands per population. Roderick, the smallest Illinois population, had a total of three unique bands (6%).

The percent unique bands would be expected to decrease as population size decreases. This was not supported as Roderick, the smallest population, contained the most unique bands of all Illinois populations. This is important because even the smallest Illinois population has the most unique genetic information which could be important to the survival of the species in the state.

Similar to *A. crassicaarpus* var. *trichocalyx*, *Trifolium stoloniferum* or running buffalo clover is a member of the Fabaceae family (Crawford et al. 1998). It is restricted to five states and is currently federally endangered (Crawford et al. 1998). Crawford et al. (1998) sampled 390 individuals from 21 populations. The average distance value of *T. stoloniferum* is 0.048 while the average distance value of *A. crassicaarpus* in Illinois populations is 0.482 (Crawford et al. 1998). This suggests that *A. crassicaarpus* has

maintained a high level of diversity in its small Illinois populations compared to another member of the legume family.

Karron et al. (1988) investigated the population genetic structure of two locally endemic *Astragalus* species with enzyme markers. He found that these restricted populations were not genetically depauperate. Karron et al. (1988) suggests that diversity in these endemic populations is the result of changes in distribution ranges. *Astragalus crassicaarpus* is widespread from Wisconsin to Texas, but the species is endemic in Illinois. This suggests that the remaining Illinois populations and the diversity they contain are remnants of a once larger distribution range. At this time, there are no historical records of the species distribution in Illinois.

Future research on *A. crassicaarpus* var. *trichocalyx* should involve genetic comparisons of endangered Illinois populations with successful populations in more states, including other habitats similar to glacial drift prairies. Another study that should be undertaken is to determine if *A. crassicaarpus* is self-incompatible. Understanding the reproductive biology of this species is crucial to developing management plans.

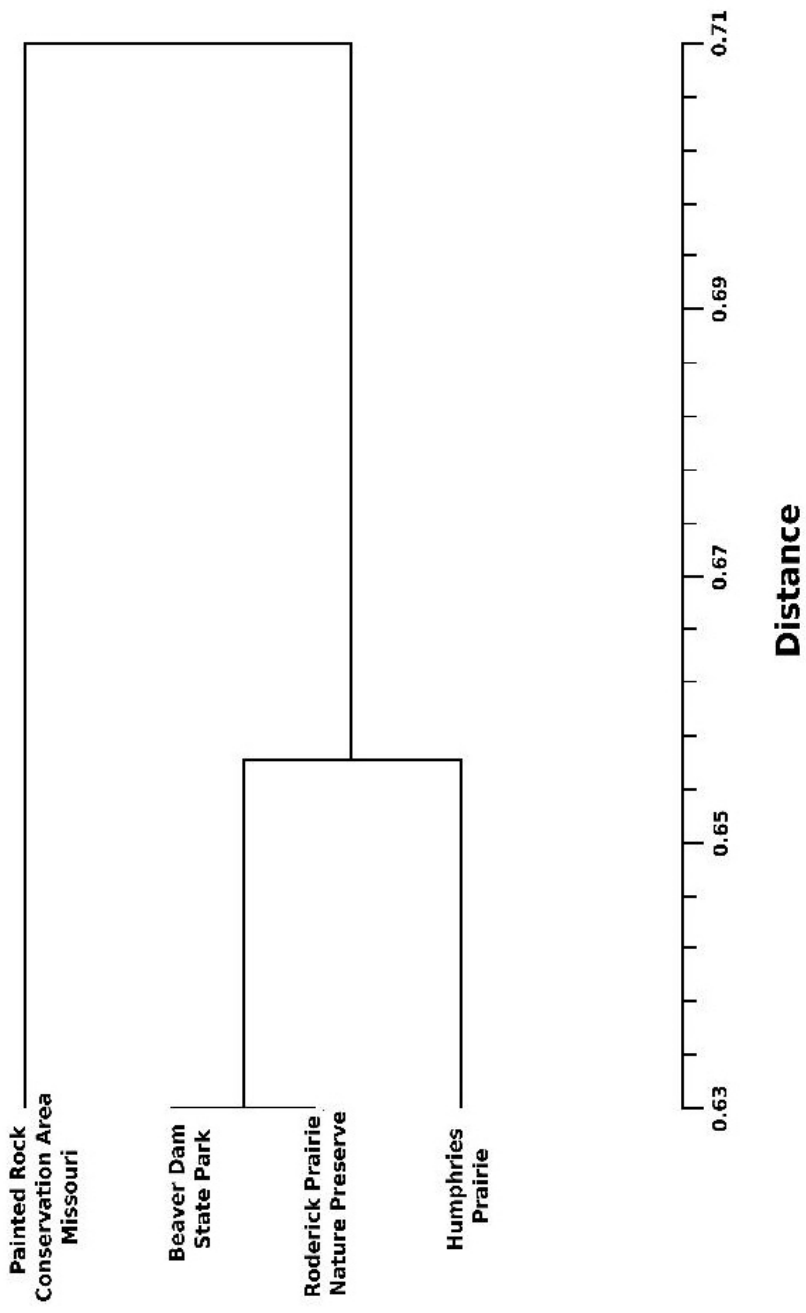
With over 99.9% prairie habitat loss in Illinois, *Astragalus crassicaarpus* var. *trichocalyx* is a remaining representative of this once abundant ecosystem (IDNR 2007). *Astragalus crassicaarpus* has maintained ISSR diversity in the endangered Illinois populations even with limited habitat and individuals. This diversity gives hope to reviving the populations.

Future conservation efforts may include establishing more plants in the Illinois populations and maintaining the habitats with consistent burnings. Other members of the genus *Astragalus*, *A. purshii* and *A. oniciformis*, have been successfully reintroduced into areas by seeding or by transplanting (Wirth and Pyke 2003; Popovich and Pyke 1997). Seedling survival and reduction in the age to first flowering are enhanced by burning in *A. purshii* (Wirth and Pyke 2003). These factors may promote population fitness and increase population sizes over time (Wirth and Pyke 2003). Experimentation with prescribed burns and re-establishing plants in the Illinois sites could successfully increase plant population numbers and survival over time.

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Figure 2. Phenogram of *Astragalus crassicaarpus* var. *trichocalyx* based on ISSR distance values.



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