Journal of Heredity 2009:100(4):424–431
doi:10.1093/jhered/esp033
Advance Access publication June 3, 2009

Population Genetics of *Astragalus bibullatus* (Fabaceae) Using AFLPs

Carol J. Baskauf and John M. Burke

From the Department of Biology, Austin Peay State University, PO Box 4718, Clarksville, TN 37044 (Baskauf); and the Department of Plant Biology, University of Georgia, Athens, GA (Burke).

Address correspondence to Carol J. Baskauf at the address above, or e-mail: baskaufc@apsu.edu.

Abstract

Astragalus bibullatus (Fabaceae) is an endangered plant species endemic to limestone cedar glades in Tennessee. Data from 134 amplified fragment length polymorphism (AFLP) fragments indicate that populations of this species are genetically very similar, with genetic identity values ranging from 0.976 to 0.991. Approximately 10% of the species' total genetic variation is due to differences among populations (F_{ST} = 0.102), and a principle coordinate analysis based on genetic distance among individuals revealed considerable overlap between populations. Averaging across populations, 38.4% of the AFLP markers were polymorphic, and the mean expected heterozygosity was 0.120. These estimates are higher than what has previously been reported for this species based on a survey of allozyme variation. Despite the overall similarity of populations of this species, a model-based clustering approach revealed the presence of 2 (possibly 3) genetically distinct subgroups. The results of this study highlight the utility of DNA-based markers for conservation genetic studies in genetically depauperate species and reveal that detectable levels of genetic substructuring may be present even in relatively undifferentiated species.

Key words: *AFLP, Astragalus bibullatus, cedar glade, endangered species, endemic, population genetics*

Genetic variability is an important resource for species. Populations and/or species with little genetic variability may have reduced fitness in their current environment and may not have the evolutionary potential to adapt to changing environmental conditions (e.g., Frankel 1970; Soulé 1980; Bradshaw 1984; Huenneke 1991; Reed and Frankham 2003). Rare species are of particular concern for conservation biologists, as they often have chronically small populations, or populations that go through periodic "genetic bottlenecks." In either case, affected populations are expected to lose genetic variability through random genetic drift faster than larger, stable populations, or populations that are connected with each other via pollen- or seed-mediated gene flow. Moreover, inbreeding is more likely to occur in small populations, and in such cases, inbreeding depression can reduce survival and/or reproduction, especially in outcrossing species (e.g., Charlesworth and Charlesworth 1987; Barrett and Kohn 1991; Ellstrand and Elam 1993). Although there are exceptions, rare species often do show lower levels of genetic variability as compared with widespread species (e.g., Karron 1987, 1991; Hamrick and Godt 1990; Gitzendanner and Soltis 2000).

Population genetic analyses can provide insight into patterns and levels of genetic diversity within and among populations of a species and can also be used to identify genetically unique subgroups across a species' range. This sort of information can then be used to inform management decisions. For example, knowledge of the distribution of genetic variation can be valuable in determining how many and which populations to protect as well as in guiding policies for seed collection and the establishment of new populations. The research described in this paper examines patterns and levels of population genetic variability in the federally endangered flowering plant *Astragalus bibullatus* Barneby & Bridges (Fabaceae).

Astragalus bibullatus, which was first described in 1987 (Barneby and Bridges 1987), is an herbaceous perennial with a relatively long-lived seed bank (Morris et al. 2002; Baskin and Baskin 2005). Known only from a few sites, *A. bibullatus* is endemic to limestone cedar glades in Tennessee's Central Basin. Population sizes of *A. bibullatus* appear to be quite variable over time and have been extremely small at times (U.S. Fish and Wildlife Service (FWS) 1991; Somers and Gunn 1990).

A previous study of allozyme variability within and among populations of *A. bibullatus* revealed relatively low levels of genetic diversity and minimal differentiation among populations, though this study was limited to 15 allozyme loci, of which only 4 were polymorphic (Baskauf and Snapp 1998). In comparison, the cedar glade "near-endemic" *Astragalus tennesseensis* has relatively high levels of allozyme variability (Edwards et al. 2004). Low levels of allozyme variability have also been reported for some western species

of *Astragalus* with restricted geographic ranges (Karron 1991; Liston 1992; Allphin et al. 2005), although 2 federally endangered western *Astragalus* species have been found to have relatively high levels of genetic variability (Travis et al. 1996; Allphin et al. 2005; Neel 2008).

Here, we use amplified fragment length polymorphism (AFLP) markers to provide a more complete description of the species-wide population genetics of *A. bibullatus* than is currently available. These data not only provide insight into levels of DNA polymorphism but may also provide the ability to identify genetically distinct subgroups across the species' range.

Materials and Methods

Leaves of *A. bibullatus* were collected during the late spring of 2003 from all sites where this species is known to naturally occur, resulting in the sampling of 7 spatially separated clusters of plants referred to here as "populations" (Figure 1). The collection sites included 5 that had previously been studied (WO, WS, AX, D, and V) as well as 2 new study sites (AP and M). Population sizes ranged from extremely small (e.g., V with 21 plants at time of sampling) to hundreds of plants (AP and M being the largest). Population size estimates at the time of sampling were as follows: V (21), D (49), WO (273), AX (198), WS (66), M (424), and AP (≤1000). Sampling of plants was done systematically throughout the population so that sampling intensity reflected plant density. The goal was to sample at least 48 plants per population unless the population was too small, in which case all plants were sampled. Population WS is the sole exception, in that fewer than 48 plants were sampled, even though this population had more than 48 individuals. WS, a small cluster of plants growing in a narrow opening in the woods, is only about 70 m from the relatively large WO population growing in the open. The reduced sample size in this case was due to the spatial proximity of plants within this population and a desire to avoid sampling the same plant twice. Despite their close proximity, WS and WO were shown to differ genetically in an isozyme study (Baskauf and Snapp 1998), so the 2 were treated as separate populations in this study.

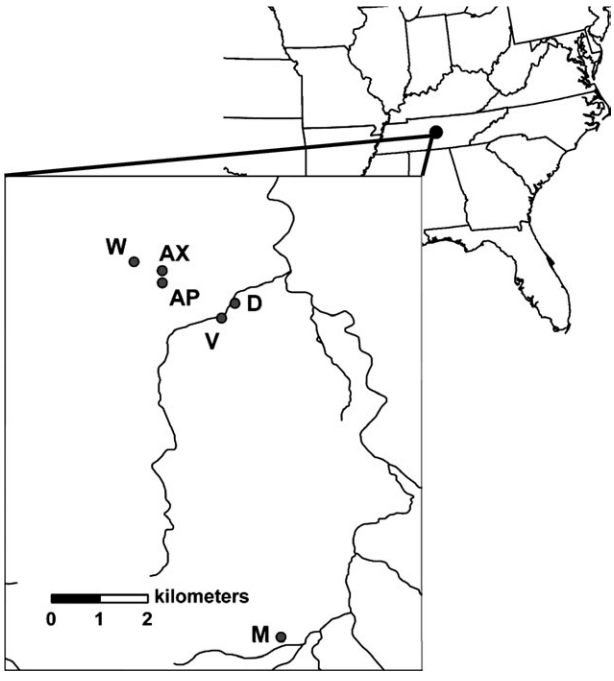

Figure 1. Relative locations of sampled *Astragalus bibullatus* populations. Population names generally follow Baskauf and Snapp (1998), except "AX" corresponds to population "A" in the earlier study. "W" represents both "WO" and "WS," which are within 70 m of each other, and "AP" and "M" are newly discovered populations.

Total genomic DNA was extracted from 100 mg of fresh leaf tissue using the DNeasy plant mini kit (Qiagen, Valencia, CA) after grinding with a Retsch (Newtown, PA) MM301 bead mill. DNA was quantified using a Hoefer DyNA Quant 200 fluorometer (Hoefer Corp., San Francisco, CA), after which samples were stored in an ultracold freezer (−80 °C) until they were used for AFLP analysis. In all, 287 individual plants were assayed for this study.

The AFLP genotyping followed the general methods of Vos et al. (1995), and unless otherwise indicated, New England Biolabs (Ipswich, MA) reagents were used. Total genomic DNA (375 ng) was digested with 6.25 units of *Eco*RI and *Mse*I restriction enzymes at 37 °C for 18 h in a 50 µl reaction volume that included 5 µl of 10× NEBuffer 2 and 0.5 µl of 10 mg/ml bovine serum albumin (BSA, 5 µg). After digestion, a 10-µl aliquot of digested DNA from each sample was run on a 2% agarose gel to check for proper digestion.

The restriction digestion was followed by adapter ligation. The ligation reactions were performed in a 50 µl solution comprised of 40 µl of the digested DNA, 15 pmol of the *Eco*RI adapter, 150 pmol of the *Mse*I adapter, 200 units T4 DNA ligase (0.5 µl), 1 µl of 10 mM adenosine triphosphate (Cell Signaling Technologies, Danvers, MA), 0.10 µl of 10 mg/ml BSA (1 µg), and 2 µL 10× NEBuffer 2. These reactions were allowed to run for 3 h at 37 °C.

Preamplification reactions utilized AFLP primers with one selective nucleotide (A) and were performed in a 25-µl volume with 1 µl of the ligation reaction combined with 187.5 ng each of the *Eco*RI+A and *Mse*I+A primers, 2.5 µl tricine buffer (300 mM tricine, 500 mM KCl, 20 mM $MgCl_2$), 0.4 µl dNTPs (each at 25 mM), and 0.5 units of *Taq* DNA polymerase. These reactions were then placed in a thermal cycler and subjected to 20 cycles of 30 s at 94 °C, 30 s at 60 °C, and 60 s at 72 °C.

Selective amplification of the preamplified fragments used primers with 3 selective nucleotides (Table 1). The 5 primer pairs used in this study were chosen based on the number, clarity, and reproducibility of bands produced in a preliminary survey of 48 primer combinations. Reproducibility was tested by repeating the entire protocol 3 times on a common set of 24 individuals. The selective

Table 1. Summary of the number of AFLP fragments (putative loci) scored for different primer pairs used in selective amplifications

*Eco*RI primer	*Mse*I primer	Number of loci	Number of polymorphic loci (%)
E-ACC	M-ACG	17	8 (47.1)
E-ACC	M-AGG	27	12 (44.4)
E-ACC	M-ACA	31	16 (51.6)
E-ACG	M-ACA	30	16 (53.3)
E-AAC	M-ACC	29	11 (37.9)
Total		134	69 (51.5)
Mean		26.8	12.6 (47.0)

amplification reactions were performed in a 10-μl volume containing 2.5 μl of diluted preamplification reaction (diluted 1:20 in 1× Tris–ethylenediaminetetraacetic acid buffer), 5 ng of the *Eco*RI+3 and 15 ng of the *Mse*I+3 selective primers, 1 μl tricine buffer, 0.16 μl dNTPs (each at 25 mM), and 0.5 units of *Taq* DNA polymerase. All but one of the *Eco*RI+3 selective primers were fluorescently labeled with carboxyfluorescein at the 5′ end. The one exception was E-AAC, which was labeled with VIC (Applied Biosystems, Foster City, CA). These reactions were then placed in a thermal cycler and subjected to 10 "touchdown" cycles starting with 30 s at 94 °C, 30 s at 65 °C (reduced by 1 °C per cycle), and 60 s at 72 °C followed by 26 cycles with an annealing temperature of 54 °C.

Shortly before electrophoresis, 1 μl of the selective amplification was combined with 4 μl of formamide mixed with ROX-labeled MapMarker 1000 size standard (BioVentures, Inc, Murfreesboro, TN). The samples were denatured at 95 °C for 5 min and then held at 4 °C until loading. AFLP fragments were separated on polyacrylamide gels (KBB Frag Pack; Cambrex, Rockland, ME) using a BaseStation DNA fragment analyzer (MJ Research, South San Francisco, CA). Gels were visualized using the Cartographer software supplied with the BaseStation, and fragments were scored manually. All unambiguous peaks between 80–350 bp were analyzed, including those that were monomorphic.

Population genetic statistics were primarily calculated using Tools for Population Genetic Analyses version 1.3 (Miller 1997). Estimates of genetic similarity were based on Nei's (1978) unbiased identity and minimum distance, and a neighbor joining tree (Saitou and Nei 1987) was constructed from the distance values using PAUP* ver. 4.0b (Swofford 2003). Expected heterozygosity was likewise estimated following Nei (1978). F_{ST} was estimated by the method of Weir and Cockerham (1984), and a 95% confidence interval for the mean was generated by 1000 iterations of bootstrapping over polymorphic loci. Expected heterozygosity and F_{ST} calculations assumed Hardy–Weinberg equilibrium conditions for each of the populations. This appears to be a reasonable assumption, considering that Baskauf and Snapp (1998) found allozyme data for the 4 variable loci conformed to Hardy–Weinberg expectations for this species.

Two additional analyses were carried out to further investigate the genetic structuring of *A. bibullatus*. First, a principal coordinate (PCO) analysis was performed using GENALEX ver. 6.2 (Peakall and Smouse 2006) to transform the multidimensional genetic distances between all *A. bibullatus* individuals into a 2-dimensional representation that explains as much of the observed variance as possible. Second, the Bayesian clustering program STRUCTURE ver. 2.2 (Pritchard et al. 2000; Falush et al. 2003) was used to further investigate the genetic structuring of *A. bibullatus*. This analysis consists of 2 main phases. First, the most likely number of unique population genetic clusters (K) within the data set is estimated without prior information on collection locations. This is done by estimating $P(X|K)$, the posterior probability; the data fit the hypothesis of K clusters. Second, the software estimates fractional membership of each individual in each cluster (Q). We performed these analyses using the default settings over a range of $K = 2$–7 with a burn-in period of 20 000 replicates and 50 000 Markov chain Monte Carlo iterations.

Results

The 5 AFLP primer pairs chosen for use in this study resulted in data for 134 fragments (Table 1). Estimates of genetic variability are listed in Table 2. Of the 134 fragments

Table 2. Genetic diversity estimates for 7 *Astragalus bibullatus* populations, based on 134 AFLP fragments ("loci")

Population	Average sample size per locus	Expected heterozygosity[a]	% Polymorphic loci (no minimum criterion)	% Polymorphic loci (95% criterion)
WO	46.3	0.128	41.8	38.1
WS	22.6	0.102	32.8	30.6
AX	45.1	0.131	41.8	39.6
AP	47.3	0.118	38.8	34.3
D	42.7	0.131	41.0	35.1
V	18.6	0.107	30.6	28.4
M	45.7	0.125	41.8	36.6
Mean	38.3	0.120	38.4	34.7
Standard error	4.6	0.004	1.8	1.5

[a] Nei's (1978) unbiased averages, assuming Hardy–Weinberg equilibrium conditions.

Table 3. Pairwise genetic distances (above the diagonal) and identities (below the diagonal) for the 7 *Astragalus bibullatus* populations, based on 134 AFLP markers (following Nei 1978)

Population	WO	WS	AX	AP	D	V	M
WO	—	0.014	0.017	0.017	0.018	0.020	0.020
WS	0.987	—	0.018	0.016	0.017	0.024	0.024
AX	0.984	0.982	—	0.019	0.013	0.024	0.020
AP	0.984	0.984	0.981	—	0.009	0.015	0.011
D	0.982	0.984	0.987	0.981	—	0.013	0.009
V	0.980	0.976	0.977	0.986	0.987	—	0.014
M	0.981	0.977	0.980	0.989	0.991	0.986	—

that were scored, 69 (51.5%) were polymorphic across the species. Within populations, an average of 38.4 ± 1.8% of the fragments were polymorphic, though this value dropped to 37.4 ± 1.5% under a strict 95% criterion (i.e., when considering only those loci with a major allele frequency of ≤0.95 to be polymorphic). Expected levels of heterozygosity within populations ranged from 0.102 to 0.131 with a mean of 0.120 (±0.004). No private (unique) alleles were identified for any of the populations. Genetic identity values range from 0.976 to 0.991 with the corresponding distance values ranging from 0.009 to 0.024 (Table 3). The neighbor joining tree based on the genetic distance among populations is presented in Figure 2. The mean F_{ST} was 0.102 (95% confidence interval: 0.080–0.124), indicating that about 10% of the total genetic variability of the species is due to differences among populations (data not shown).

The results of the PCO are presented in Figure 3. The first coordinate (x axis) accounted for 38.4% of the variance in genetic distance among individuals, whereas the second coordinate (y axis) accounted for 14.8% of the variance. Consistent with the relatively low estimate of F_{ST}, there was considerable overlap among individuals from different populations. However, differentiation along the y axis (positive vs. negative values) is roughly concordant with the 2 genetic subgroups identified by STRUCTURE (WO/WS/AX vs. AP/D/V/M; see below).

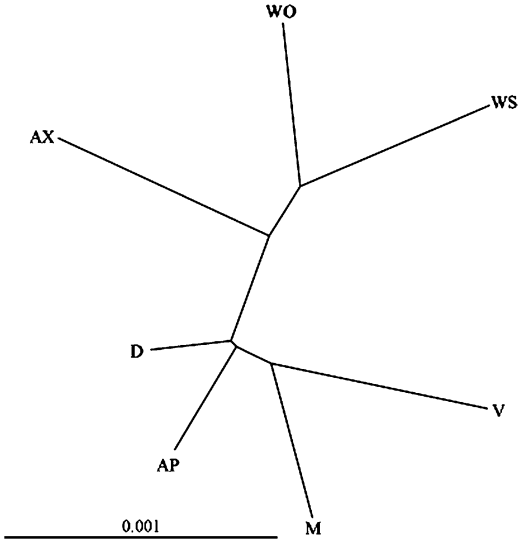

Figure 2. Unrooted neighbor joining tree of the 7 *Astragalus bibullatus* populations based on Nei's (1978) genetic distance. Population abbreviations are as in Figure 1.

The STRUCTURE analysis revealed that $P(X|K)$ is maximized at $K = 3$, suggesting the presence of 3 genetically distinct subgroups. However, it has been argued that this approach to estimating the most likely number of subgroups is prone to overestimation (Evanno et al. 2005). Rather, Evanno et al. (2005) have demonstrated that the rate of change in the log probability of the data between successive K values is a much more reliable indicator of the true number of genetic subgroups (Evanno et al. 2005). As such, we used ΔK, a measure of the second-order rate of change in the likelihood of K, to identify the most likely number of subgroups. This calculation resulted in the identification of $K = 2$ genetically distinct subgroups (Figure 4). In general terms, one subgroup was largely comprised of individuals from populations WO, WS, and AX, whereas the other subgroup was largely comprised of individuals from populations AP, D, V, and M.

Discussion

The AFLP-based diversity estimates reported herein are substantially higher than previous allozyme-based estimates for *A. bibullatus* (Baskauf and Snapp 1998). Whereas 51.5% of the AFLP markers were polymorphic at the species level, 27% of allozymes were previously found to be polymorphic. Likewise, at the population level, 38.4% of the AFLP fragments were polymorphic as compared with 25.6% for allozyme loci. Expected heterozygosity estimates were nearly twice as high for the AFLP data as compared with the allozyme data (0.120 vs. 0.063). Given the dominant nature of AFLPs, it is impossible to directly count heterozygotes as can be done with the codominant allozyme data. Rather, heterozygote frequencies must be estimated by assuming that the populations under consideration are at Hardy–Weinberg equilibrium. Given that the allozyme data conform to Hardy–Weinberg expectations (Baskauf and Snapp 1998), this assumption is likely met in these populations, such that valid heterozygosity estimates can be derived from the AFLP data.

In comparing the results of the current AFLP study of *A. bibullatus* with those of the previous allozyme study, it should be noted that the populations sampled for the allozyme work overlapped with, but were not identical to, the set of populations studied herein. The AP and the M sites were not known at the time of the allozyme study, and one population included in that earlier study ("C") has been destroyed since that time. Also, a small number of individuals from the now extirpated C population were transplanted into population V following the allozyme analysis. Nonetheless, the populations included in these 2 studies are similar enough that differences in the results are likely primarily due to the type of markers used rather than the specific populations included in each study.

Although relatively few plant species have been subjected to population genetic analyses using both AFLPs

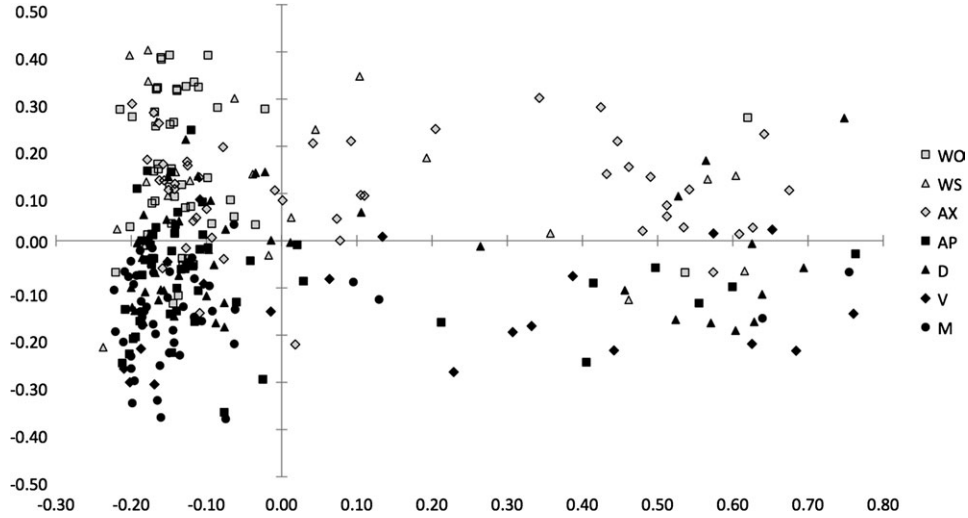

Figure 3. Genetic structure across 287 *Astragalus bibullatus* individuals based on a PCO. The first coordinate (*x* axis) accounts for 38.4% of the variance in genetic distance among individuals, whereas the second coordinate (*y* axis) accounts for 14.8% of the variance. Population abbreviations are as in Figure 1.

and allozymes, a few points for comparison are available. As was the case for *A. bibullatus*, AFLP diversity estimates are higher than allozyme estimates for some species (e.g., Waycott and Barnes 2001; Huh and Ohnishi 2002; Pérez-Collazos and Catalán 2006). However, allozyme variability has been found to be higher than AFLP variability in other cases (Chung et al. 2004; see also Travis et al. 1996 vs. Allphin et al. 2005 below).

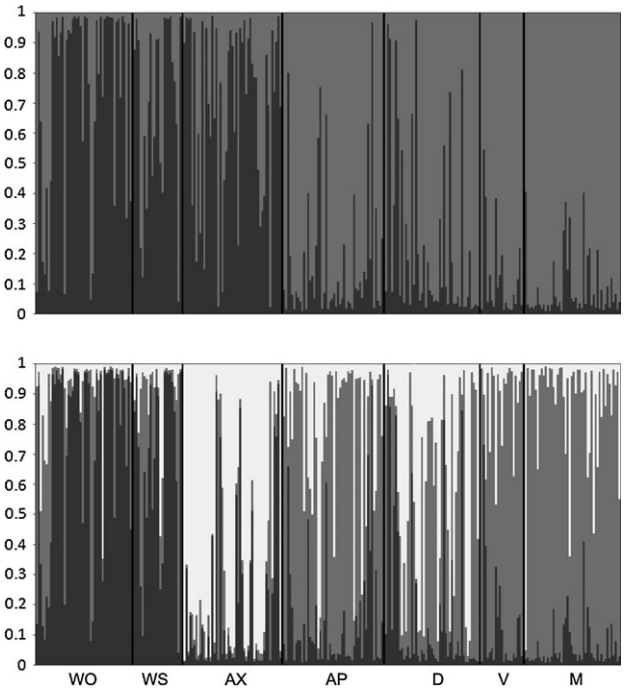

Figure 4. Genetic structure across 287 *Astragalus bibullatus* individuals using the model-based Bayesian algorithm implemented in the program STRUCTURE. The upper panel corresponds to $K = 2$ subgroups, whereas the lower panel corresponds to $K = 3$ subgroups. Each individual is represented by a vertical bar, and fractional membership in each of the subgroups is indicated by color/shade. The 7 collection locales are separated by vertical black bars. Population abbreviations are as in Figure 1.

Despite its rarity, it seems clear that *A. bibullatus* is not completely bereft of genetic variability. Nonetheless, AFLP variability estimates for *A. bibullatus* are somewhat low compared with AFLP studies of many other rare plant species, both in terms of the percentage of polymorphic loci and expected heterozygosity (e.g., Drummond et al. 2000; Gaudeul et al. 2000; Evans et al. 2001; Ronikier 2002; Rottenberg and Parker 2003; Tero et al. 2003; Kreivi et al. 2005; Tang et al. 2006; Vilatersana et al. 2007; Prentis and Mather 2008), although some other species have lower diversity estimates than *A. bibullatus* (e.g., Peakall et al. 2003; Sgorbati et al. 2004; Armstrong and De Lange 2005 for heterozygosity). The relatively low overall levels of diversity found in *A. bibullatus* are likely due, at least in part, to the small and fluctuating population sizes that have been reported for this species (U.S. Fish and Wildlife Service 1991; Morris et al. 2002; Somers and Gunn 1990). However, the fact that *A. bibullatus* is a perennial and likely has a long-lived seed bank (Morris et al. 2002; Baskin and Baskin 2005) may have helped to prevent an even greater loss of genetic variability within populations of this species. Indeed, an earlier allozyme-based study revealed that the seed bank of *A. bibullatus* is considerably more variable than the vegetative population (Morris et al. 2002).

Both the AFLP and allozyme data agree that populations of *A. bibullatus* are genetically very similar to each other. Genetic identity values for AFLP data range from 0.976 to 0.991 (Table 3), as compared with 0.981–1.000 for the allozyme data (Baskauf and Snapp 1998). Estimates of population genetic differentiation (i.e., F_{ST}) tell a similar

story with about 10% of AFLP diversity residing among populations as compared with about 9% for allozymes. This low level of differentiation is somewhat surprising given that cedar glade species such as *A. bibullatus* typically reside in island-like forest openings, creating the potential for greater population differentiation due to habitat isolation. Nonetheless, most *A. bibullatus* populations are geographically quite close to each other (all but M are less than 2.5 km apart, and most are much closer). Based on our data, it appears that the distances between the glades do not prohibit pollen and/or seed dispersal, such that the different populations have remained in reproductive contact. Although specific pollinators have not been identified for *A. bibullatus*, insects have been observed to visit flowers of this species (Morris et al. 2002). An allozyme-based study of the endangered *Echinacea tennesseensis*, an insect-pollinated cedar glade endemic from the same region, found similarly low levels of population differentiation (F_{ST} = 0.092; Baskauf et al. 1994). Likewise, the nearly endemic (Baskin and Baskin 1989) *A. tennesseensis*, which not only co-occurs with *A. bibullatus* but is also found on most other limestone glades in Tennessee, shows low levels of allozyme differentiation in Tennessee (G_{ST} = 0.059), though population differentiation across the range of the species was higher (Tennessee, Alabama, and Illinois, G_{ST} = 0.217; Edwards et al. 2004).

In a similar study, Travis et al. (1996) assayed AFLP diversity in a different federally endangered *Astragalus* taxon, *Astragalus cremnophylax* var. *cremnophylax*, which is endemic to the Grand Canyon in Arizona (2 populations on the south rim and 1 population on the north rim of the canyon). Due to the fact that the 2 south rim populations had extremely low variability levels, expected heterozygosity (0.018–0.134) and the percentage of polymorphic loci per population (4.3–38.4%; 95% criterion) ranged much more widely than for the Tennessee cedar glade endemic studied herein. When averaging across the 3 populations, expected heterozygosity was lower for populations of *A. cremnophylax* var. *cremnophylax* (0.063) than for *A. bibullatus* (0.120). Similarly, the mean percentage of polymorphic loci per population (95% criterion) was lower for the Grand Canyon endemic (17.6%) than for *A. bibullatus* (34.7%). At the species level, however, 62.5% of the AFLP markers were polymorphic for the Grand Canyon endemic, as compared with 47% for *A. bibullatus*. This difference results from a much greater level of genetic structuring in the Grand Canyon species as compared with *A. bibullatus*—in fact, Allphin et al. (2005) argued that the genetic distinctiveness of the north rim population may merit separate species status. In contrast to the results for *A. bibullatus*, estimates of allozyme variability for *A. cremnophylax* var. *cremnophylax* (Allphin et al. 2005) were higher than those based on AFLP markers (Travis et al. 1996).

Despite the relatively low F_{ST} value and high genetic identities among populations, as well as the observed overlap among individuals from the various populations (Figure 3), our STRUCTURE analysis resulted in the identification of 2 (possibly 3) genetically distinct subgroups (Figure 4). Although there is some admixture apparent within each of the populations, the 2 genetic subgroups are largely comprised of individuals from populations WO/WS/AX and AP/D/V/M, respectively. This result is in close agreement with the overall topology of the neighbor joining tree (Figure 2), and the split roughly corresponds to individuals with positive versus negative values along the y axis of the PCO plot (Figure 3). From a geographic perspective, this clustering reveals a north/south split (Figure 1). The close genetic association between WO, WS, and AX is not surprising given their close proximity to one another. However, AX and AP are likewise very close to one another, separated only by a country road, and yet our analysis shows these 2 populations to be genetically distinctive, with AP showing a stronger affinity for the more distant populations D, V, and M. Increasing the number of subgroups from $K = 2$ to $K = 3$, as might be suggested by the fact that $P(X|K)$ is maximized at $K = 3$, results in the identification of population AX as a relatively distinct subgroup, though a number of individuals from population D also show an affinity for this third subgroup.

Increasingly, cedar glade habitat is disappearing because of rapid development in the Central Basin of Tennessee. Although the underlying cause of the observed north/south differentiation in this species remains unclear, this sort of substructuring should be kept in mind when making management decisions, notwithstanding the relatively high genetic identity values among populations. The most diverse populations within the northern subgroup are WO and AX, whereas the most diverse populations in the southern subgroup are D and M. M is also one of the largest populations. Thus, these populations should perhaps be targeted as seed sources for future efforts aimed at population reestablishment. Moreover, because WO is still located on private property, and because multiple lines of evidence indicate that this is one of the most diverse *A. bibullatus* populations available, securing some sort of protection for this site seems warranted.

Funding

Tennessee Department of Environment and Conservation contracts ED-03-01164-00 and ED-05-01734-00, which were funded using a portion of US Fish and Wildlife Service Traditional Section 6 grants (TN-E-4-17 and TN-E-4-19); Center of Excellence for Field Biology at Austin Peay State University.

Acknowledgments

The authors thank Tennessee Department of Environment and Conservation staff who helped C.J.B. to locate *A. bibullatus* sites. Steve Baskauf created the map figure. David Baron helped C.J.B. to collect leaves. Mark Chapman assisted with the data analyses. Vanderbilt University provided access to the BaseStation DNA sequencer and laboratory space for this project prior to J.M.B.'s departure.

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Received January 31, 2009; Revised April 17, 2009; Accepted April 23, 2009

Corresponding Editor: Brian Murray