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STRATIFIED ANALYSIS OF THE SOIL SEED BANK IN THE CEDAR GLADE ENDEMIC *ASTRAGALUS BIBULLATUS*: EVIDENCE FOR HISTORICAL CHANGES IN GENETIC STRUCTURE[1]

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Persistent seed banks may provide information on historical changes in the genetic composition of populations. We used stratified sampling of the soil seed bank of *Astragalus bibullatus* (Pyne's ground plum) to assess levels of temporal variation in population genetic structure and to infer historical changes in the levels of inbreeding and relative gene flow. This species has an extremely limited distribution in the Central Basin of Tennessee, where it is found in open areas and along the edges of cedar glades. Protein electrophoresis was conducted on seedlings grown from seeds that had been recovered from three successive 1 cm thick layers of soil sampled from six sites. Analyses of seven polymorphic allozyme loci indicated that there were substantial levels of genetic differentiation among soil layers and sites. Higher levels of genetic diversity were found in seed than in vegetative populations that had been sampled in a previous study. Seed populations from the uppermost soil layer had higher heterozygote deficiencies, displayed higher levels of differentiation among sites, and had higher private allele frequencies than seed populations from the lower two layers. The change in heterozygosity and distribution of genetic variation among sites for the youngest soil layer is consistent with a pattern of increased selfing, sib mating, and decreased gene flow among populations. These changes in inbreeding and relative levels of gene flow are corroborated by information on historical land use practices in the region and support the hypothesis that loss of appropriate habitat has led to smaller population sizes and a more fragmented distribution of this cedar glade endemic.

Key words: *Astragalus bibullatus*; fragmentation; inbreeding; landscape genetics; population size; private alleles; relative gene flow; seed bank; temporal variation.

Theoretical analyses indicate that shifts in the geographic distribution of organisms can precipitate changes in the level and distribution of genetic variation. For example, it has been suggested that increased fragmentation of distributions would disrupt patterns of gene flow among populations, increase the severity of inbreeding, reduce levels of local genetic diversity, and ultimately affect the pattern and mode of evolution (Templeton et al., 1990; Fahrig and Merriam, 1994; Young, Boyle, and Brown, 1996). The predicted effects of increased fragmentation have been supported by a number of empirical studies of populations that have recently become isolated from previously contiguous distributions (Young, Merriam, and Warwick, 1993; Prober and Brown, 1994; Hall, Walker, and Bawa, 1996; Young, Boyle, and Brown, 1996; Nason and Hamrick, 1997; Morden and Loeffler, 1999; Turner et al., 2000; Cruzan, in press). While these investigations have made important contributions to our understanding of landscape genetic processes, their conclusions are inferential because comparisons of historical and contemporary distributions of genetic variation for the same populations were not performed. However, direct comparisons of temporally separated populations occurring at the same site could provide more accurate assessments of the consequences of landscape modifications for the level and distribution of genetic variation and could measure the potential for fragmented populations to maintain genetic diversity.

One approach to the examination of historical patterns of population genetic structure would be to analyze the genetic variation present in populations of dormant individuals (e.g., Bosbach, Hurka, and Haase, 1982; Vavrek, McGraw, and Bennington, 1991; Tonsor et al., 1993; Cabin, 1996; Cabin, Mitchell, and Marshall, 1998; McCue and Holtsford, 1998; Schneller, 1998). In particular, temporal analyses may be possible in systems where dormant representatives of past populations are present in vertical strata that allow their relative age to be inferred (e.g., van der Valk and Davis, 1979; McGraw, 1993). Such stratigraphic depositions could provide historical records of changes in populations and communities. For example, stratified sampling of seed banks from different soil depths has been used to infer changes in vegetation composition (Kellman, 1970; Leck and Simpson, 1987; Archbold, 1989). While it would be feasible to use soil seed populations to assess historical changes in the level and distribution of genetic variation, there have been few attempts to infer temporal variation in population genetic structure from stratified samples of dormant individuals (e.g., Schneller, 1998).

Here we use stratified sampling of the soil seed bank to examine historical changes in population genetic structure of the perennial cedar glade endemic, *Astragalus bibullatus* Barneby and E. L. Bridges (Fabaceae). This species is ideal for

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an investigation of a temporal analysis of genetic variation because characteristics of its seeds and habitat favor the development of a persistent seed bank that is stratified by age. First, as with many species in this group, seeds of *A. bibullatus* possess hard, impermeable seed coats that impose a strong physical germination barrier (Rolston, 1978; Baskin and Baskin, 1998). Second, species of legumes that have hard seed coats are relatively long-lived and are known to remain viable in the soil longer than seeds of most other species (Toole and Brown, 1946; Quinlivan, 1968; Baskin and Baskin, 1998). Third, soils in the cedar glade habitats consist almost entirely of coarse sand and rocks (Quarterman, Burbanck, and Shure, 1993), which, in combination with repeated frost heaving and sedimentation processes, may promote the migration of the smooth, hard seeds of *A. bibullatus* down through the soil column. While it has been suggested that the digging activity of rodents and invertebrates can disrupt age stratification of seed banks (Chambers and Macmahon, 1994), evidence of soil disturbance by animals appears to be minimal in cedar glade habitats (M. B. Cruzan, personal observation), probably because the soil is shallow and rocky (Quarterman, Burbanck, and Shure, 1993). Hence, we expect the development of an age-stratified seed bank for populations of *A. bibullatus*, with the most recently produced seeds near the soil surface and average seed age increasing with soil depth.

In this study, we assessed levels of genetic diversity and the genetic structure of past populations of *A. bibullatus* by sampling seed populations at different depths in the soil column. Specifically, we used estimates of heterozygote deficiency and genetic diversity within populations, and differentiation among populations, to infer historical changes in levels of inbreeding and relative gene flow. Our temporal analyses of population genetic processes provide an example of the consequences of the effects of increased fragmentation and habitat loss on the level and distribution of genetic variation.

METHODS

Astragalus bibullatus is an herbaceous perennial endemic to cedar glades of the Central Basin of Tennessee (Barneby and Bridges, 1987), USA. The known distribution of *A. bibullatus* is limited to seven locations within Rutherford County, Tennessee, USA, with two apparently extirpated populations. The sparse distribution of contemporary populations of this species and its limited seed dispersal strongly suggest that it must have been more abundant in the past. The cedar glade habitats of *A. bibullatus* are open areas with varying densities of eastern red cedars (*Juniperus virginiana*) that are dominated by a sparse cover of annual or perennial forbs, annual grasses, and cryptogams. These areas are characterized by shallow soils, high levels of irradiance, and temperature extremes (Quarterman, Burbanck, and Shure, 1993; Baskin and Baskin, 1999). During the winter months soils often remain saturated for long periods and frost heaving is common (A. Shea, Tennessee Department of Environment and Conservation, personal communication). Presumably the expansion that occurs during freezing produces fissures in the soil that promote the burial of seeds. Within glades, *A. bibullatus* is often restricted to transition areas along the edges or associated with scattered trees within glades where they are partially shaded by overstory vegetation. The flora of cedar glades in the Central Basin is relatively rich with a high incidence of endemism and taxa with broader distributions in the grasslands of Midwestern North America (Quarterman, Burbanck, and Shure, 1993; Baskin and Baskin, 1999; Estill and Cruzan, 2001).

Astragalus bibullatus (Pyne's ground plum) is an herbaceous perennial, flowering from late April to early May and fruiting in early June. Plants are acaulescent low-growing rosettes, up to 25 cm in diameter. Their leafy rosettes arise from fleshy roots that may be branched beneath the soil surface, so larger plants may consist of several closely spaced rosettes. Inflorescences remain close to the ground and bear compact racemes of 10–16 pink flowers (~1 cm in length) with darker purple markings. Fruits are inflated pods, 1.5–3 cm in length and 1–1.5 cm in diameter, which acquire a characteristic reddish "plum" color as they mature. Observations indicate that each inflorescence typically produces only one or two fruits. Ovaries have up to 40 ovules, but fruits rarely contain >30 seeds (M. B. Cruzan, unpublished data). The kidney-shaped seeds are 2–4 mm in length and are shiny and black, which facilitate their identification and extraction from soil samples. Primary seed dispersal is by gravity, but secondary dispersal by water is possible during the winter months when surface flow is common in cedar glades. Germination in the field occurs during February and March (M. B. Cruzan, personal observation). Differences in levels of reproduction among sites have been noted and are thought to be a result of shading from the encroachment of woody vegetation (Baskauf and Snapp, 1998). Observations suggest that shaded plants produce greater vegetative growth and fewer fruits, while plants in full sun produce large numbers of fruits and go dormant earlier. The pollinators of *A. bibullatus* have not been identified, but casual observations indicate that small-bodied bees and skippers (Hesperiidae) visit flowers (M. B. Cruzan, personal observation).

Sampling methods—We took stratified samples of the soil seed bank in February 1999. We used six of the known extant sites for this study, all of which were in Rutherford County and within 6 km of each other. Four of these sites are the same as were sampled by Baskauf and Snapp in a study of genetic variation in extant vegetative populations (their WS = Flat Rock B, A = Alexander, D = Davis, and O = Overbridge; Baskauf and Snapp, 1998). At the time that we sampled the Overbridge site, it consisted of native plants plus transplants from Baskauf and Snapp's C site, which has been nearly extirpated by the land owner. Our Flat Rock A site was within 100 m of another one of Baskauf and Snapp's sites (WO) and our Flat Rock B site. The Airfield site was discovered after Baskauf and Snapp's study was completed and is within 100 m of the Alexander site. A seventh extant site was not sampled because it was discovered after the completion of our field studies.

At each site, one (five of the sites) or two (the Airfield site only) 5-m transects were placed in areas where the largest concentrations of plants were known to occur. The seed bank was sampled by collecting three layers of soil from five 30 × 30 cm quadrats at 1-m intervals along each transect. Fruits from the previous season, along with moss, lichens, and debris, were removed from the surface of each plot prior to excavation. Three layers of soil (labeled A, B, and C going from the surface to the deepest layer), ~1 cm in thickness, were carefully removed from each plot using flat masonry trowels. Seeds may have been present in deeper soil strata, but they were not sampled in this study. As lower layers were removed, care was taken to prevent contamination from the upper soil layers. Soil layers were stored in separate resealable plastic bags at 4°C to retard the growth of mold during the 1–3 mo period before they could be processed. Seeds were extracted from soil samples by sifting the soil with a No. 10 soil sieve (2-mm openings). The seeds collected from each layer were stored in separate envelopes at room temperature for several weeks until they were treated for germination trials.

Preliminary tests indicated that seeds of *A. bibullatus* possessed a physical germination barrier (i.e., a hard, impermeable seed coat), but did not require an extended cold treatment. We determined that treatment with concentrated H_2SO_4 for 15 min was the most effective method for rendering seed coats permeable to water after several trials with alternative methods of scarification (Baskin and Baskin, 1998). Treated seeds were rinsed with deionized water for 15 min and placed in petri dishes on 1% agar containing Hogland's basal medium (Sigma H-2395). Petri dishes were first stored for 1 wk at 4°C before transferring them to a growth chamber with a 12/12 h alternating light/dark cycle and a corresponding 20°/10°C alternating temperature. Most seeds quickly imbibed water and nearly doubled their volume within a few days. Any seeds that remained small after 1 wk were retreated with sulphuric acid and returned to the growth chamber. Viable seeds generally germinated within 2 wk, and any seeds that remained ungerminated after 4 wk in the growth chamber were scored as inviable. Viability tests with tetrazolium chloride

TABLE 1. The number of quadrats sampled and seeds collected from each population of *Astragalus bibullatus*. Seeds per square meter were calculated based on the total number of seeds found in all of the 30-cm^2 quadrats sampled at each site. Soil layers A–C (uppermost to the lowest) were pooled to estimate the proportion of polymorphic loci (p), the effective number of alleles per locus (A_e), and the genetic diversity (H_e). No viable seeds were recovered from the missing layers at Davis, Flat Rock A, and Overbridge.

Site	No. quadrats sampled	Layer	No. seeds collected	No. seeds analyzed	No. seeds /m^2	p	A_e	H_e
Airfield	10	A	85	23	170	0.7	2.3	0.12
		B	36	6				
		C	22	4				
Alexander	5	A	12	7	49	0.5	1.6	0.15
		B	7	3				
		C	3	2				
Davis	5	A	5	4	24	0.3	1.5	0.16
		B	4	2				
Flat Rock A	5	A	8	5	40	0.3	1.3	0.15
		B	10	8				
Flat Rock B	5	A	50	28	753	0.6	3.0	0.10
		B	203	144				
		C	86	63				
Overbridge	5	A	28	10	62	0.6	2.1	0.26

(Baskin and Baskin, 1998) on a subsample of the ungerminated seeds confirmed that none of them contained live embryos (unpublished data), so for the purposes of this study we assumed that germination is equivalent to viability. Upon germination, seedlings where transplanted into soil flats and moved to a greenhouse. Leaf material was collected from all seedlings for allozyme analysis 2–3 wk after transplanting.

Electrophoretic methods—We used horizontal starch gel electrophoresis to estimate the levels and distribution of genetic variation present in different strata of the soil seed bank. Approximately 0.5 cm^2 of leaf material was ground in 300 μL of extraction buffer (Cruzan, 2001) in 1.5-mL microcentrifuge tubes with plastic pestles. The extracted materials were stored in microcentrifuge tubes at −70°C. On each day that assays were conducted, frozen samples were thawed and absorbed onto 3 × 10 mm wicks cut from Whatman #3 filter paper. We made initial screens of 20 enzymes on six gel buffers to identify two buffer systems that clearly and consistently resolved ten loci: (1) Tris Borate EDTA pH 8.3 for ME (one locus), LAP (one locus), PGI (one locus), and G3PDH (two loci); (2) L-Histidine pH 5.7 for PGM (two loci), 6PGD (two loci), and ADH (one locus). Tris Borate EDTA gels were run at 55 mA for 5 h. L-Histidine gels were run at 30 mA for 3.5 h. Gels were documented using a video camera fitted with a video copy printer. Genotypes were determined from the video images.

Data analysis—We analyzed the seed sampling data to assess differences in the numbers of seeds recovered and levels of seed viability among sites and soil strata. These data were not normally distributed, so we used Friedman's two-way analysis of ranks blocked by site (SAS, 1989) to test for differences in the number and viability of seeds among soil layers and sites.

Genetic data were analyzed to determine whether there were significant levels of genetic differentiation among soil layers and sites to assess temporal changes in population genetic parameters. The distribution of genetic variation within and among sites was analyzed using both hierarchical (soil layers nested within each site for the A layer and the B and C layers combined) and stratified (the A layer and the combined B and C layers compared among sites) designs with the Genetic Data Analysis (GDA) and PopGene (Yeh and Boyle, 1997) software packages. These programs use Weir and Cockerham's (1984) and Nei's (1973) methods, respectively, to examine genetic structure (see Weir, 1996 for a comparison of these methods). The 95% confidence intervals for genetic structure parameters were estimated with GDA by bootstrapping (1000 replications) across loci. Estimates of gene flow (Nm) among the sites sampled for different seed bank strata were made using both private allele analyses (Slatkin, 1985) and F_{ST} methods (Hedrick, 1983). Note that gene flow estimates are generally not accurate (Whitlock and McCauley, 1999) and are used here strictly for comparative purposes. Genetic diversity and differentiation parameters were compared among layers using Friedman's two-way analysis of ranks blocked by locus with ranks weighted by each sample size (SAS, 1989).

RESULTS

The number of seeds recovered from soil samples and the average viability of seeds varied among sites and soil layers. A total of 561 seeds were extracted from the 35 quadrats sampled across six sites. Of these, 311 (55%) germinated and were used in allozyme assays. The number of *A. bibullatus* seeds found in soil samples varied substantially among sites, ranging from a low of 11 (Davis) to a high of 339 (Flat Rock B; $F = 6.01$, df = 5,96, $P < 0.0001$; Table 1). Estimates of the total number of seeds per square meter also varied dramatically among sites, from a low of 24 to a high of 753 (Table 1). The viability of soil seeds differed among sites, ranging from a low of 23% (Airfield) to a high of 72% (Flat Rock A; $F = 8.25$, df = 5,40, $P < 0.0001$). Differences among layers for the number and viability of seeds were less evident (Fig. 1a). Seed recovery was somewhat greater for the B layer than for the other two soil strata (Fig. 1a). However, this pattern was not significant ($F = 2.85$, df = 2,96, $P < 0.630$) and was primarily due to the relatively large number of seeds collected from the B layer at the Flat Rock B site. Seed viability tended to decline with soil depth (Fig. 1b), but the difference in germination among soil layers was not significant ($F = 0.28$, df = 2,40, $P > 0.760$).

Analysis of allozyme variation indicated that relatively high levels of genetic diversity were present in the seed bank populations sampled. Out of the ten allozyme loci assayed, only the two G3PDH loci and the ME locus were monomorphic for all of the populations sampled, leaving a total of seven polymorphic loci. Levels of genetic diversity as measured by the proportion of polymorphic loci (p), the effective allele number (A_e), and the expected heterozygosity (H_e) were relatively high and varied to some degree among sites (Table 1). Since sample sizes for the lower soil layers were relatively small (Table 1), and because allele frequency differentiation between them was not significantly different from zero ($\theta_p = 0.018$, and the 95% confidence interval [CI] of 0.045 to −0.002 overlaps zero; Fig. 2a), we pooled the B and C soil layers for genetic analyses.

Comparison among soil layers indicated that the effective number of alleles per locus (A_e) and the level of genetic di-

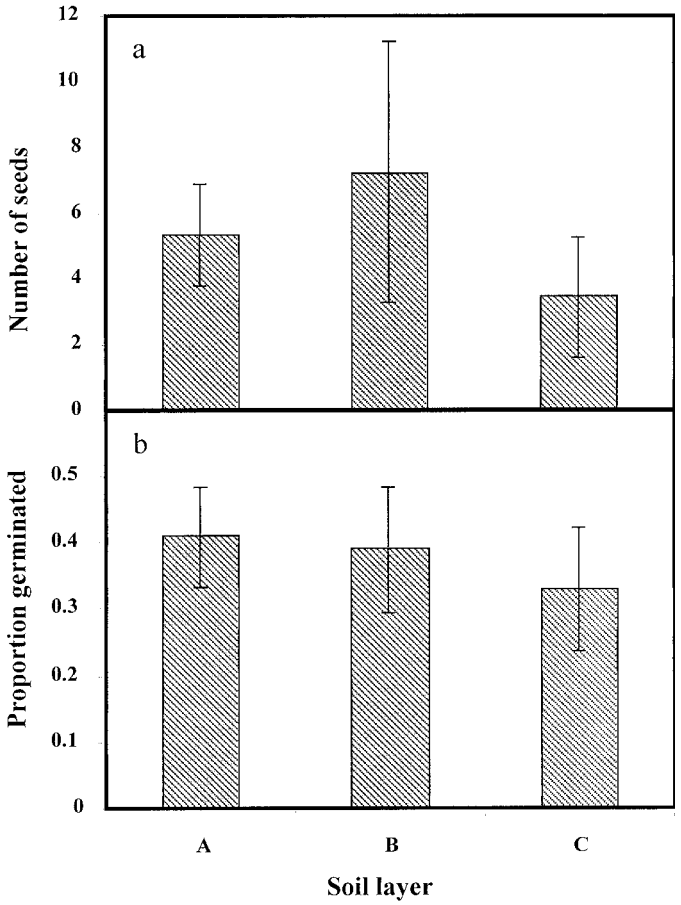

Fig. 1. (a)Average number of seeds recovered from each 1 cm thick soil layer of individual 30 × 30 cm quadrats and (b) the proportion of seeds that germinated across sites of *Astragalus bibullatus*. Vertical lines represent the standard error of each mean.

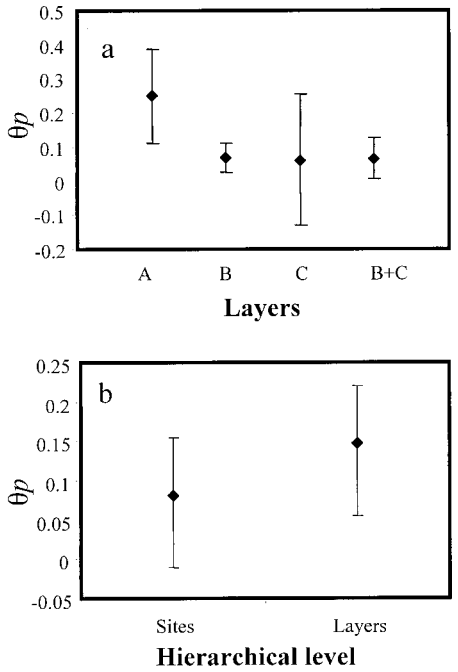

Fig. 2. (a)Levels of differentiation among sites stratified by soil layer and (b) hierarchical analysis of genetic differentiation among sites and soil layers (b) in *Astragalus bibullatus*. The letters A–B represent seed populations recovered from successively deeper 1 cm thick soil layers collected at six different sites. Values of θ_p are based on allozyme data and were calculated with the GDA software package (Genetic Data Analysis; Lewis and Zaykin, 2001) using methods described by Weir (1996). Vertical bars represent 95% confidence intervals that are from 1000 bootstraps of the data across loci. Values of θ_p for which the confidence intervals do not overlap zero are considered significantly different from zero.

versity (H_e) tended to be highest in the A layer and declined with increasing soil depth (Table 2). Hierarchical analysis of genetic variation with the GDA program indicated that genetic differentiation (θ; Weir, 1996) was significantly greater than zero for the among-soil-layers estimate ($\theta_S = 0.148$, 95% CI = 0.221–0.049 for differentiation among subpopulations), and nearly significantly greater than zero for the among-sites estimate ($\theta_p = 0.082$, 95% CI = 0.159 to −0.015 for differentiation among populations; Fig. 2b). Confidence intervals are based on 1000 bootstraps across loci.

The level of genetic differentiation among sites differed depending on the stratum in the seed bank being examined (Table 2). Both the F_{ST} and θ_p estimates of population differentiation from stratified analyses indicated that differences in allele frequencies among populations were much higher for the A than for the combined B and C layers (Table 2; Fig. 2a). The higher level of differentiation for the uppermost soil stratum suggests lower levels of gene flow among sites were prevalent when these seed populations were formed. The same pattern was indicated by private allele estimates of gene flow; the two

TABLE 2. Levels of genetic diversity, inbreeding, gene flow, and differentiation among sites for soil seed bank layers collected from populations of *Astragalus bibullatus*. Soil layers B and C were combined for analyses. Measures of genetic variation include the proportion of polymorphic loci across all sites (*p*), effective allele number (A_e), and expected heterozygosity (H_e). Inbreeding is indicated by the degree of heterozygote deficiency, i.e., $(H_e - H_o)/H_e$, or the fixation index, *F*.

Layer	*p*	A_e	H_e	*F*	N_{samp}	N_p	P_I	Nm_{priv}	Nm_{adj}	Nm_{FST}	F_{ST}	θ_p
A	0.70	1.29	0.190	0.100	12.83	8	0.134	0.43	0.83	0.16	0.605	0.172*
B + C	0.70	1.11	0.093	0.007	19.50	13	0.046	3.61	4.62	1.49	0.144	0.032*
F	—	7.68*	∞***	0.76	—	—	—	—	—	—	∞***	0.89

Note: Gene flow (Nm) was estimated using the level of genetic differentiation (Nm_{FST}) and by private allele analysis (Nm_{priv}). The latter is calculated from the average frequency (P_I) of alleles found for each soil layer that were unique to one site (N_P = the total number of private alleles; Slatkin 1985). These gene flow estimates were adjusted (Nm_{adj}) to account for differences in the average sample size per population (N_{samp}). Levels of genetic differentiation were estimated using both Weir and Cockerham's (θ_p:1984) and Nei's (F_{ST}:1973) methods. Tests for differences between layers (*F* values) were made with Friedman's two-way analysis of ranks (∞ = *F* value too large to be defined, — = single values per layer so no test was conducted). Asterisks associated with θ_p values indicate 95% confidence intervals based on 1000 bootstraps across loci that did not overlap zero (* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

lower soil strata displayed much lower private allele frequencies, and hence higher levels of gene flow, than seed populations in the A layer (Table 2).

There were differences in the overall level of heterozygosity and the apparent level of inbreeding among the three soil strata (Table 2). The expected (H_e) was higher for the A than for the combined B and C layers (Table 2). Differences in the level of heterozygote deficiency were also apparent among layers, resulting in a fixation index (i.e., the inbreeding coefficient: $F = (H_e - H_o)/H_e$; Hedrick, 1983) that was more than ten times greater for the seed populations in the A layer, but the high variance among loci rendered this difference insignificant (Table 2).

DISCUSSION

Seed populations of *Astragalus bibullatus* from the three soil layers displayed differences in their levels of among-population differentiation. Stratified sampling of the soil seed bank has allowed us to analyze both spatial and temporal variation in population genetic structure and has provided an historical perspective on the ecological factors that may have led to the restricted distribution and reduced abundance of this cedar glade endemic. The observation of more similar allele frequencies among populations from the older (lower) soil strata indicates that those seeds were formed under the conditions a of higher rate of gene flow. A reduction in the level of gene flow among seed populations in the youngest soil layer may have been coincident with an increase in the level of local inbreeding. These most recent seed populations tended to have higher levels of heterozygote deficiency, indicating there may have been higher frequencies of selfing and sib mating at the time that they were produced. These data provide evidence of historical changes in the level and distribution of genetic variation in *A. bibullatus* and indicate that these populations may have been subjected to changes in aspects of their physical and biological environments that have affected basic population genetic processes.

The results of this study have potentially important implications for our understanding of population genetic processes and for the management of this endemic taxon. However, it is important to recognize the assumptions and limitations of the data presented. First, we are assuming that each soil layer contains seeds of different age and that there have not been significant amounts of soil disturbance, which would have homogenized the seed bank. While it is possible that there has been some mixing, the high level of genetic differentiation among soil layers indicates that seed populations in different layers have remained largely distinct. Ideally, we would like to obtain the actual ages of seeds from different soil depths. The methods to conduct such assays are available (Moriuchi et al., 2000), but it would be prohibitively expensive to obtain estimates for reasonable samples of seeds from different layers.

Second, because of the high variance often apparent in soil seed densities (Leck, Parker, and Simpson, 1989; Baskin and Baskin, 1998), the sample sizes for some of the populations studied were minimal. Reanalysis of the data using only the two populations with the largest sample size yields results that are qualitatively the same as those presented; there was strong differentiation among layers ($\theta_p = 0.103$, 95% CI = 0.199–0.007) and gene flow was substantially lower among the youngest (Nm = 1.08) than among the older (Nm = 2.60) seed populations.

Third, we are assuming that soil strata at different sites represent equivalent seed age ranges. For example, it is possible that differences in soil structure could have resulted in variation among sites in the rates of seed migration down through the soil column. Different rates of vertical seed migration among sites would be expected to produce the largest errors in the relative age, and hence the highest level of genetic differentiation, for the deepest seed populations. However, the pattern found in the present study was just the opposite, with the strongest differences in allele frequencies among populations found in the uppermost soil layer, so it is unlikely that variation in the vertical migration rates of seeds through the soil had a substantial impact on the stratigraphic differences in population genetic structure detected.

While the pattern of genetic differentiation among soil layers is striking, it is possible that these differences represent the effects of differential selection rather than a temporal record of differences in relative levels of gene flow among populations. For example, seed germination has been reported to be nonrandom with respect to genotype (Cabin, Mitchell, and Marshall, 1998), which could affect allele frequencies in older seed populations. Hence, the older (deeper) seeds could become genetically differentiated from seeds in the upper layer because of differential germination. However, note that lower layers were also more homogeneous across sites. While genotype-dependent germination may be expected to be neutral or to increase the level of differentiation, it is extremely unlikely that it would result in a conversion of allele frequencies among sites. Since the primary homogenizing force that is generally recognized in population genetics is gene flow, it is much more reasonable to assume that similarity in allele frequencies among sites in the deeper soil layers is the result of historically higher rates of dispersal.

Seed bank genetic diversity—It has been suggested that seed banks may act as reservoirs of genetic variation that would buffer populations from the loss of genetic diversity during bottlenecks (Templeton and Levin, 1979). Seed bank populations of *A. bibullatus* are apparently consistent with this expectation and contain higher levels of genetic variation than vegetative populations. Comparison of seed populations of *A. bibullatus* to adult individuals sampled in the same region (Baskauf and Snapp, 1998) indicates that seed banks contained higher levels of genetic diversity ($H_e = 0.063$ for vegetative vs. 0.156 for seed populations), a larger proportion of polymorphic loci ($p = 25.6\%$ for vegetative vs. 50.4% for seed populations), and averaged more alleles per locus ($A_n = 1.4$ for vegetative vs. 1.97 for seed populations). Note that individual plants of *A. bibullatus* can live for several years (A. Shea, Tennessee Department of Environment and Conservation, personal communication), so these studies should be comparable because sampling for both was done within a single generation. Moreover, the difference in genetic diversity was maintained even when the seed population data were restricted to the same subset of allozyme loci and sites used in the Baskauf and Snapp study of the vegetative populations (i.e., $H_e = 0.147$, $p = 75.0\%$, and $A_e = 3.9$ for the restricted sample of soil seed populations). However, it is not clear that this pattern is general because some studies have reported equal levels of genetic variation in seed bank and vegetative populations (e.g., Tonsor et al., 1993; Mahy, Vekemans, and

Jacquemart, 1999). In *A. bibullatus*, the seed bank appears to represent a significant genetic reservoir that may help preserve genetic diversity when vegetative populations are small or absent.

The capacity of seed banks to retain higher levels of genetic diversity may be dependent on seed dormancy characteristics. Species with strong germination barriers would be expected to have seed populations that contain a wider range of seed age classes and to contain genetic variants from a larger number of vegetative generations than species with seeds that germinate within a few years (Templeton and Levin, 1979). As the seeds of *A. bibullatus* have thick, impermeable seed coats, they are likely to persist in the soil for a long period of time, producing seed banks that contain a wide range of seed ages. Unfortunately, the dormancy characteristics of the other species studied are not readily available, so it is difficult to determine whether the lower levels of seed bank diversity reported in some studies are due to differences in the ages of seeds assayed.

The relative size of the seed bank population compared to the vegetative population may also determine its capacity to act as a genetic reservoir. Depending on dormancy characteristics, large seed bank populations may be more likely to sequester rare genetic variants that are not present in the respective vegetative population. However, as pointed out by Cabin et al. (1998), the large size and aggregated spatial distribution of seed bank populations render them inherently difficult to sample. Hence, it is likely that even relatively large sample sizes will miss much of the variation present, and this may explain why some studies have not detected higher levels of seed bank genetic diversity (e.g., Tonsor et al., 1993; Mahy, Vekemans, and Jacquemart, 1999). It is notable that the studies that did find seed banks that were genetically diverse compared to the extant vegetative populations (i.e., the present study and McCue and Holtsford, 1998) were on endemic species with relatively small population sizes. In both cases it is possible that higher seed bank genetic diversities reflect historically greater abundances than are evident from the distributions of contemporary populations.

Variation among soil strata—The *A. bibullatus* seed populations from different soil strata differed with respect to their seed densities, levels of among-site genetic differentiation, expected heterozygosity, and heterozygote deficiency. In particular, the uppermost soil layer contained lower densities of seeds than expected (see below) and seed populations from this stratum had the highest levels of among-site differentiation and the highest heterozygote deficiencies. As seed populations age, their numbers would be expected to decline as individuals are lost through germination and mortality (Leck, Parker, and Simpson, 1989). Hence, with a constant rate of input, we would expect that the youngest seed banks would be the largest and that seed numbers would decrease with soil depth. In *A. bibullatus*, the observation that the youngest seed populations were smaller than populations from the second layer suggests that contemporary rates of seed input have been lower than earlier seed input rates.

An alternative explanation for the higher number of seeds in the lower soil layers is variation in vertical migration rates among soil strata. For example, frost heaving may lead to more rapid migration rates of seeds through the upper soil and accumulation in lower layers. However, this is unlikely as it would lead to homogenization of the soil seed bank, which is inconsistent with the observed high levels of genetic differentiation among layers. Furthermore, if seeds were accumulating in lower soil layers, then it would be difficult to explain the observation of high frequencies of unique alleles in the upper soil layer that were not present in lower layers at the same sites. While it is not possible to entirely exclude the possibility that the patterns of seed abundance and genetic variation are due to the accumulation of seeds in deeper soil, the level of genetic variation and distribution of unique alleles among layers and sites is more consistent with the hypothesis that these patterns are due to historical changes in mating patterns and gene flow among populations.

Hierarchical analysis of genetic variation among sites and soil strata indicated that the level of differentiation among soil layers was as great or greater than the level of differentiation among sites sampled. Similar patterns of genetic differentiation between seed bank and seedling populations (Cabin, 1996), seed bank and vegetative populations (McGraw, 1993), and among seed bank populations of different age (Bennington, McGraw, and Vavrek, 1991) have been observed in other species. Such variation in the genetic composition of soil seed banks may be due to fluctuations in allele frequencies in vegetative populations (Templeton and Levin, 1979) and nonrandom patterns of germination with respect to seed genotype (Cabin, Mitchell, and Marshall, 1998). In the case of *A. bibullatus*, the genetic differentiation among seed bank populations may also have been influenced by historical changes in mating patterns. The apparent increase in inbreeding in the uppermost soil seed populations would be expected to decrease effective population sizes and increase the probability of local fixation of alleles. Furthermore, our sampling design may have been particularly sensitive to the effects of increased levels of selfing and biparental inbreeding. With very restricted seed dispersal, our relatively small quadrats (30×30 cm) would have included seeds from only a few individual plants. Hence, reduced outcrossing would be expected to lead to increased levels of differentiation among quadrats at a site (i.e., a Walhund effect; Hedrick, 1983). Such small-scale differentiation due to rates of inbreeding would also help explain the higher level of expected heterozygosity (i.e., because of higher variation in the frequencies of alleles among quadrats within sites) and the greater heterozygote deficiency observed in the youngest seed populations. However, note that even if a Walhund effect were responsible for a portion of the observed heterozygote deficiency, this pattern is still indicative of increased levels of inbreeding for the youngest seed populations.

An increase in the apparent frequency of heterozygous genotypes in older seed populations could also be due to higher mortality rates for more homozygous seeds (e.g., Del Castillo, 1994), possibly because they were produced through selfing rather than by outcrossing. However, the alternative hypothesis that differences in the level of heterozygote deficiency is due to changes in historical levels of inbreeding is also supported by the distribution of private alleles among the soil seed populations. Both of the older soil layers contained larger numbers of private alleles that were present at lower frequencies than in the youngest seed populations. This pattern is consistent with a recent increase in the level inbreeding since higher frequencies of selfing and sib mating would lead to the random loss of some rare alleles and the development of local patches with higher frequencies of other rare alleles. While it is possible that some of the change in the relative number of heterozygous genotypes among soil strata is due to different rates

of mortality of inbred and outbred seeds, the observed differences in unique allele frequencies suggests that at least a portion of the increase in heterozygote deficiency in younger seed populations is due to an increase in the level of inbreeding within populations.

It is notable that some unique alleles in the oldest seed populations could be the result of novel somatic mutations, which are known to occur at relatively high frequencies in aged seeds (Levin, 1990). For example, 8 out of 24 private alleles in the two older seed populations were only found once and could be due to mutations that arose after the seeds were produced. However, removing these alleles does not produce substantial changes in our gene flow estimates for the two older soil layers (Nm_{adj} becomes 5.14 and 6.02 for the B and C layers, respectively). Hence, the relatively high frequency of rare alleles in the youngest seed populations is most likely due to a recent history of increased levels of inbreeding and restricted gene flow among populations.

Temporal variation—The stratified analysis of seed bank genetic diversity in *A. bibullatus* has provided insights into possible historical changes in processes affecting population genetic structure. The lack of genetic differentiation among sites for the oldest soil seed layers indicate that levels of gene flow were probably higher in the past and that populations have recently become isolated. Decreased levels of gene flow among cedar glade populations could be the result of several factors. For example, it is likely that cedar glades were historically more widespread and had lower densities of trees (DeSelm, 1994; Heikens and Robertson, 1994). Several lines of evidence suggest that aboriginal inhabitants of this region may have used fire to clear these areas of woody vegetation (Delcourt, 1987; Delcourt et al., 1998). Fire suppression policies in the last century have apparently led to higher densities of cedar trees (DeSelm, 1994), and may have increased the levels of fragmentation of *A. bibullatus* populations as the habitat quality eroded due to increased shading.

The apparent effects of woody vegetation encroachment on the viability of *A. bibullatus* populations can be seen in some of the extant populations. For example, the Flatrock B site has one of the lowest census population sizes (A. Shea, Tennessee Department of Environment and Conservation, personal communication) and the highest soil seed density, suggesting that plants in this area were much more abundant in the past. The plants at this site are located along an abandoned road bed that is surrounded by dense stands of cedars. Numbers of plants at this site have decreased in recent years, and flowering rates of these plants are generally very low compared to populations at more open locations (A. Shea, Tennessee Department of Environment and Conservation, personal communication). Such extensive overgrowth by woody species may eventually lead to extinction of vegetative populations of *A. bibullatus*, suggesting that residual seed populations may exist at many sites in this region where habitat conditions are currently inhospitable to their growth and survival.

A possible example of the recovery of such a cryptic population is evident at the Airfield site, where a large population of *A. bibullatus* was only recently discovered. Sites in the local area (e.g., Alexander and the Flat Rock sites) were regularly counted and surveys made for additional populations in this area since 1979. However, the large abundance of *A. bibullatus* plants at the Airfield site only became apparent in 1996 after the land owner commenced regular mechanical removal of the woody vegetation in the area. Whether or not a few vegetative individuals had persisted at this site and were simply overlooked, the high density of seeds throughout the soil strata indicates that a large population of *A. bibullatus* was present at this site at some point in the past and that the majority of the extant population was probably derived from the soil seed bank within the last few years.

The temporal changes in the population genetic structure of *A. bibullatus* observed in this study are consistent with patterns expected under increased fragmentation (Templeton et al., 1990; Fahrig and Merriam, 1994; Young, Boyle, and Brown, 1996). While the absolute timescale of these changes is unknown, based on the studies of seed longevity in the soil for other species (reviewed in Baskin and Baskin, 1998) and the thick seed coat of this species, we can surmise that the oldest seeds in this study could have been produced as much as a 100 yr ago. In any case, it is probable that intrusion by woody vegetation and increased urbanization of cedar glades have contributed to the decreased rates of gene flow and reduced population sizes inferred for the uppermost seed layer. However, the relatively low seed densities and increased inbreeding apparent in the youngest seed populations suggest that pollinator availability may also have changed in recent years. Lack of adequate pollinator service would be expected to result in lower levels of seed production, higher frequencies of selfed seeds, and lower rates of gene flow among populations (Kearns, Inouye, and Waser, 1998), all of which are consistent with the changes observed in the uppermost seed soil layer compared to the older layers. Additional studies on the reproductive biology of *A. bibullatus* may help elucidate the possible contribution of pollination conditions to the historical changes in the mating patterns and the prospects for continued maintenance of genetic diversity in this endemic species.

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