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Stratified Analysis of the Soil Seed Bank in the Cedar Glade Endemic, *Astragalus bibullatus*: Evidence for Historical Changes in Genetic Structure¹

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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 gene flow. This species has an extremely limited distribution in Central Tennessee where it is found in open areas and along edges of cedar glades. Protein electrophoresis was conducted on seedlings grown from seeds that had been recovered from three 1 cm thick layers of soil sampled from six sites. Analysis of seven polymorphic allozyme loci indicated that there was substantial levels of genetic differentiation both among soil layers and among sites. Seed populations from the uppermost soil layer were more inbred, displayed higher levels of differentiation among sites, and had higher private allele frequencies than seed populations from the lower two soil layers. Higher levels of genetic diversity were found in seed compared to vegetative populations present at the same sites, which had been sampled in a previous study. The patterns of change in levels of inbreeding and gene flow are consistent with 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: Fragmentation, gene flow, inbreeding, population size, private alleles, seed bank, temporal variation.

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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 geographic distributions would disrupt patterns of gene flow among populations, increase the severity of inbreeding within populations, reduce levels of genetic diversity, and ultimately affect the pattern and mode of evolution in these systems (Fahrig and Merriam 1994; Templeton et al. 1990; Young et al. 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 (Hall et al. 1996; Morden and Loeffler 1999; Nason and Hamrick 1997; Prober and Brown 1994; Tumer et al. 2000; reviewed in Young et al. 1996; Young et al. 1993). 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 have not typically been possible. Direct comparisons of temporally separated populations occurring at the same site would provide more accurate assessments of the consequences of landscape processes for the level and distribution of genetic variation and 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 et al. 1982; Cabin 1996; Cabin et al. 1998; McCue and Holtsford 1998; Schneller 1998; Tonsor et al. 1993; Vavrek et al. 1991). 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., McGraw 1993; van der Valk and Davis 1979). 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 (Archbold 1989; Kellman 1970; Leck and Simpson 1987). While it would be feasible to assess historical changes in the level and distribution of genetic variation by sampling seed banks, there have been few attempts to infer temporal variation in population genetic structure from stratified samples of dormant populations (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 an investigation for a temporal analysis of genetic variation because characteristics of its seeds and habitat favor the development of a large, 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 (Baskin and Baskin 1998; Rolston 1978). 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 (Quinlivan 1968; Toole and Brown 1946). Third, soils in the cedar glade habitats consist almost entirely of coarse sand and rocks (Quarterman et al. 1993), which, in combination with repeated frost heaving and sedimentation processes, would 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 would disrupt age stratification of seed banks (Chambers and Macmahon 1994), evidence of soil disturbance by animals is minimal in cedar glade habitats (pers. observ.). Hence, we would 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 assess 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 use estimates of heterozygote deficiency and genetic diversity within populations, and differentiation among populations, to infer historical changes in levels of inbreeding and gene flow. Our temporal analyses of population genetic processes provides 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 Middle Tennessee (Barneby and Bridges 1987). The known distribution of *A. bibullatus* is limited to seven locations within Rutherford County, TN, with two apparently extirpated locations in Rutherford and Davidson counties. 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 (Fig. 1). 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; Fig. 1). At the time that we sampled the Overbridge site it consisted of local 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 Baskauf and Snapp's WO site 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 (Fig. 1). A seventh extant site was not sampled because it was discovered after the completion of our field studies.

The distribution of *A. bibullatus* is limited to cedar glades in Central Tennessee, which are open areas with varying densities of Eastern red cedars (*Juniperus virginiana*) that are dominated by annual or perennial forbs, annual grasses, and cryptogams. These areas are characterized by shallow soils, high levels of irradiance, and temperature extremes (Baskin and Baskin 1999; Quarterman et al. 1993). Within glades *A. bibullatus* is often restricted to transition areas along the edges, or are 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 (Baskin and Baskin 1999; Estill and Cruzan In press; Quarterman et al. 1993).

A. 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. Leaves 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 (ca. 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 color as they mature. Observations indicate that each inflorescence typically produces only up to one or two fruits. Ovaries have up to 40 ovules, but fruits rarely contain more than 30 seeds (unpub. data). The kidney-shaped seeds are 2 - 4 mm in length and shiny black in color, which facilitates 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. 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 amounts 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 (Cruzan, pers. observ.).

Sampling Methods

We took stratified samples of the soil seed bank in February of 1999. At each site, one (five sites) or two (the Airfield site) 5 m transects were placed in areas where plants were known to occur. The seed bank was sampled by collecting three layers of soil from 30 cm square quadrats at one m intervals along the 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), approximately 1 cm in thickness, were carefully removed from each plot using flat masonry trowels. 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 until 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 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 H₂SO₄ for 15 minutes 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 minutes and placed in petri dishes on 1% agar containing Hogland's basal medium (Sigma H-2395). Petri dishes were first stored for one week 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 one week were retreated with sulphuric acid and returned to the growth chamber. Viable seeds generally germinated within two weeks, and any seeds that remained ungerminated after four weeks in the growth chamber were scored as inviable. Viability tests with tetrazolium chloride (Baskin and Baskin 1998) on a subsample of the ungerminated seeds confirmed that they did not contain live embryos (unpub. data). Upon germination, seedlings were transplanted into soil flats and moved to a greenhouse. Leaf material was collected from plants in the greenhouse for allozyme

analysis two to three weeks after transplanting.

Electrophoretic Methods

We used horizontal starch gel electrophoresis to estimate levels and distribution of genetic variation present in different strata of the soil seed bank. Approximately 1 cm² of leaf material was ground in 300 ul of extraction buffer (Cruzan In review) in 1.5 mL microcentrifuge tubes. The extracted materials were stored in centrifuge tubes at -70°C. On each day assays were conducted, frozen samples were thawed and absorbed onto 3 mm by 10 mm wicks cut from Whatman #3 filter paper. We made initial screens of 20 enzymes on 6 gel buffers to identify two buffer systems that clearly and consistently resolved 10 loci: (1) Tris Borate EDTA pH 8.3 for ME (1 locus), LAP (1 locus), PGI (1 locus), and G3PDH (2 loci); (2) L-Histidine pH 5.7 for PGM (2 loci), 6PGD (2 loci), and ADH (1 locus). Tris Borate EDTA gels were run at 55 mA for 5 hours. L-Histidine gels were run at 30 mA for 3.5 hours. 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 was significant levels of genetic differentiation among soil layers and to assess temporal changes in the 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 and B layers only) and stratified (single soil layers compared among sites for all three layers) 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 spatial genetic structure. The 95% confidence intervals for genetic structure parameters were estimated with GDA by bootstrapping across loci. Estimates of gene flow (N_m) among the populations sampled for different seed bank strata were made using both private allele analyses (Slatkin 1985) and F_{ST} methods (Hedrick 1983).

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; $F = 6.01$, $P < 0.0001$, 5/96 df: Table 1). Estimates of the number of seeds per meter square also varied dramatically among sites, from a low of 24 to a high of 753 (Table 1). The viability of soil seeds also differed among sites, ranging from a low of 23%

(Airfield) to a high of 72% (Flat Rock A; $F = 8.25$, $P < 0.0001$, 5/40 df). Differences among layers for the number and viability of seeds were less evident (Fig. 2a). Seed recovery was somewhat greater for the B layer than for the other two soil strata (Fig. 2a). However, this pattern was not significant ($F = 2.85$, $P < 0.630$, 2/96 df) and was primarily due to the large number of seeds collected from the B layer at the Flat Rock B site. Seed viability tended to decline with soil depth (Fig. 2b), but the difference in germination among soil layers was not significant ($F = 0.28$, $P > 0.760$, 2/40 df).

Analysis of allozyme variation indicates 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. Comparison among soil layers indicated that the proportion of polymorphic loci (P), the number of alleles per locus (A_n), the effective number of alleles per locus (A_e), and the level of genetic diversity (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_p) (Weir 1996) was significantly greater than zero for the among soil layers estimate ($\theta_p = 0.148$, 95% CI = 0.226 to 0.055), and nearly significantly greater than zero for the among the sites estimate ($\theta_p = 0.082$, 95% CI = 0.154 to -0.011; based on 1000 bootstraps across loci).

The level of genetic differentiation among sites differed depending on the stratum in the seed bank that was being compared (Fig. 3a). 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 B and C layers (Fig. 3a). The higher level of differentiation for the uppermost soil stratum suggests lower levels of gene flow were prevalent when these seed populations were formed. The same pattern was indicated by private allele estimates of gene flow; where the two 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 (Fig. 3b, Table 2). Both the expected (H_e) and observed (H_o) levels of heterozygosity were higher for the A than for the B and C layers (Fig. 3b). Differences in the level of heterozygote deficiency were also apparent among layers (i.e., $H_e - H_o$; Fig. 3b), resulting in a fixation index (F) that was nearly five times greater for the seed populations in the A layer compared to populations in the lower soil strata (Table 2).

Discussion

Seed populations from the three soil layers of sites occupied by *Astragalus bibullatis* displayed striking differences in their level of interpopulation differentiation and inbreeding. 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 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 conditions of higher rates of gene flow. A reduction

in the level of gene flow among seed populations in the youngest soil layer appears to have been coincident with an increase in the level of local inbreeding. These most recent seed populations displayed relatively high levels of heterozygote deficiency compared to the seed populations from the older soil strata, indicating that higher frequencies of selfing and local inbreeding were common at the time 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 have been subjected to changes in aspects of the physical and biological environments that have affected basic population genetic processes.

As the conclusions indicated by this study may have broad implications for our understanding of population genetic processes and for the management of this endemic taxon, 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 has not been significant amounts of soil disturbance, which would homogenize the seed bank. While it is possible that there has been some mixing, the high level of genetic differentiation among soil layers indicates that these seed populations in different layers have remained largely distinct. Second, because of the high variance often apparent in soil seed densities (Baskin and Baskin 1998; Leck et al. 1989), the sample sizes for some of the populations studied were minimal. However, reanalysis of the data using only the three populations with the largest sample size yields results that are qualitatively the same as those presented (i.e., gene flow was substantially lower and inbreeding higher for the youngest seed populations: unpub. data). 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 in the rates of seed migration through the soil column among sites. Assuming temporal variation in allele frequencies in the vegetative population (e.g., Cabin et al. 1998), different rates of seed migration 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 highest differences in allele frequencies found among populations in the uppermost soil layer, so it is unlikely that variation in the migration rates of seeds through the soil had a substantial impact on the temporal differences in population genetic structure detected.

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). Moreover, this difference was maintained even when the seed population data were restricted to a 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 $A_n = 3.9$ for the restricted sample of soil seed populations). However, it is not clear that this pattern is general since some studies have reported equal levels of genetic variation in seed bank compared to vegetative populations (e.g., Mahy et al. 1999; Tonsor et al. 1993).

The capacity of seed banks to retain higher levels of genetic diversity may be dependent on seed dormancy characteristics. Species with strong germination barriers will tend to have seed populations that contain a wider range of seed age classes and will contain genetic variants from a larger number of vegetative generations than species with seeds that germinate within a few years (Templeton and Levin 1979). Since 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 large 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 (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., Mahy et al. 1999; Tonsor et al. 1993). It is notable in this context that both of the studies that did find seed banks that were genetically diverse compared to the extant vegetative populations were on endemic species with relatively small population sizes (i.e., the present study and McCue and Holtsford 1998). In both of these cases it is possible that higher seed bank genetic diversities reflect historically greater abundances and broader distributions than are evident from contemporary populations.

Variation among soil strata. The *A. bibullatus* seed populations from different soil strata differed for 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 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 et al. 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 reduced compared to historical levels. Alternatively, frost heaving may lead to more rapid migration rates of seeds through the upper soil and accumulation in lower layers. However, this is unlikely since it would lead to homogenization of the soil seed bank, which is inconsistent with the observed high levels of genetic differentiation among soil layers.

Hierarchical analysis of genetic variation among sites and soil strata indicated that the level of differentiation among soil populations was as great or greater than the level of differentiation among sites sampled. Similar patterns of genetic differentiation between seed bank and seedling (Cabin 1996), seed bank and vegetative (McGraw 1993), or among seed bank populations of different age (Bennington et al. 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 non-random patterns of germination with respect to seed genotype (Cabin et al. 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 cm²) 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: \]. 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) and the greater heterozygote deficiency observed in the youngest seed populations. Note however 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.

The effects of differences in levels of inbreeding among soil seed populations is also apparent in the change in the number and frequency of private alleles among the soil seed populations. Both of the older layers contained larger numbers of private alleles that were present at lower frequencies than in the youngest seed populations. This pattern is consistent with increased levels of local inbreeding; higher frequencies of selfing and sib mating would lead to the loss of some rare alleles and the development of local patches with higher frequencies of other rare alleles. Some of the unique alleles in the oldest seed populations could also be due to 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 are 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 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 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, pers. comm.), 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, Pers. comm.). 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 censused 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 in 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 (Fahrig and Merriam 1994; Templeton et al. 1990; Young et al. 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 a century or more in the past. In any case, it is probable that intrusion by woody vegetation and increased urbanization of cedar glades have contributed to 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 has also 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, 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 prospects for the continued maintenance of genetic diversity in this species.

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Table 1. The number of quadrats sampled and seeds collected from each population of *Astragalus bibullatus*. Seeds per meter square was calculated based on the number of seeds found in the total number of 30 cm² quadrats. Soil layers A - C (uppermost to the lowest) were pooled to estimate the proportion of polymorphic loci (P), the average number of alleles per locus (A_n), and the genetic diversity (H_e).

Site	Quadrats Sampled	Seeds Collected	Seeds /m ²	Layers Analyzed	P	A_n	H_e
Airfield	10	143	170	A/B/C	0.7	2.3	0.12
Alexander	5	22	49	A/B/C	0.5	1.6	0.15
Davis	5	11	24	A/B	0.3	1.5	0.16
Flat Rock A	5	18	40	A/B	0.3	1.3	0.15
Flat Rock B	5	339	753	A/B/C	0.6	3.0	0.10
Overbridge	5	28	62	A	0.6	2.1	0.26

Table 2. Levels of genetic diversity, inbreeding, and gene flow among sites for each soil seed bank layer collected from populations of *Astragalus bibullatus*. Measures of genetic variation include the proportion of polymorphic loci (P), average number of alleles per locus (A_n), and expected heterozygosity (H_e). Inbreeding is indicated by the degree of heterozygote deficiency (i.e., expected - observed heterozygosity, or the fixation index: F). 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 ($P_{(l)}$) 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 the average sample size (N_{sam}).

Layer	P	A_n	H_e	F	N_{sam}	N_p	$P_{(l)}$	$Nm_{(priv)}$	$Nm_{(adj)}$	$Nm_{(FST)}$
A	0.35	1.51	0.124	0.057	12.83	8	0.134	0.43	0.83	0.16
B	0.38	1.76	0.134	0.012	27.33	14	0.026	11.28	10.31	2.46
C	0.33	1.67	0.084	0.013	11.67	10	0.041	4.41	9.45	1.81

Fig. 1. The distribution of the six *Astragalus bibullatus* sites (populations) sampled in Rutherford County, Tennessee. The scale indicates distance in kilometers.

Fig. 2. Number of seeds recovered from soil samples (A) and the proportion of seeds that germinated (B) for each soil layer across sites of *Astragalus bibullatus*. Vertical lines represent the standard error of each mean.

Fig. 3. Levels of genetic differentiation among sites (A) and levels of heterozygosity as observed (H_o) and as expected under conditions of random mating (H_e) for each soil layer across sites of *Astragalus bibullatus*. Levels of genetic differentiation was estimated using both Weir and Cockerham's (θ_p :1984) and Nei's (F_{ST} :1973) methods.

Fig. 1

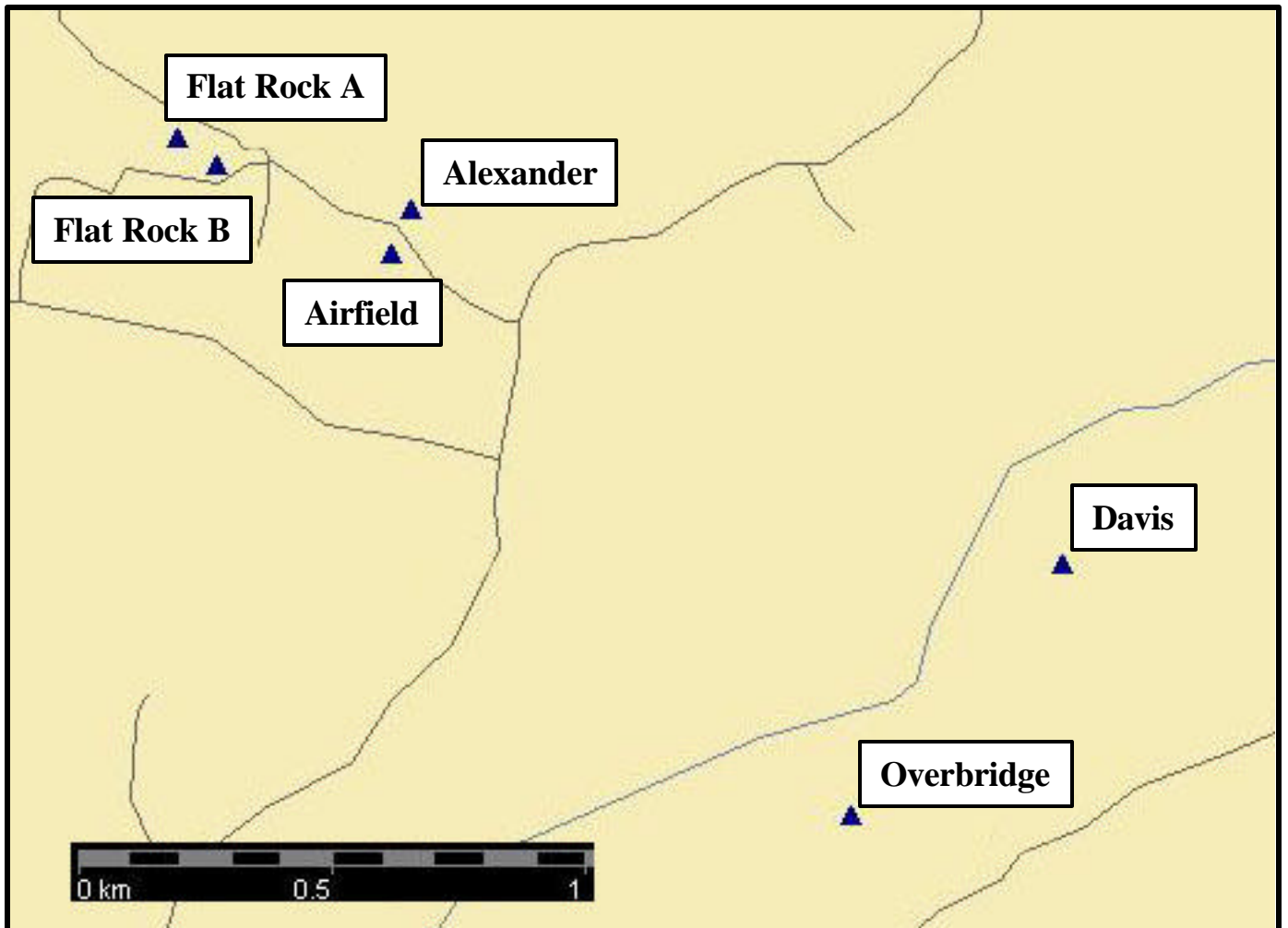


Fig. 2

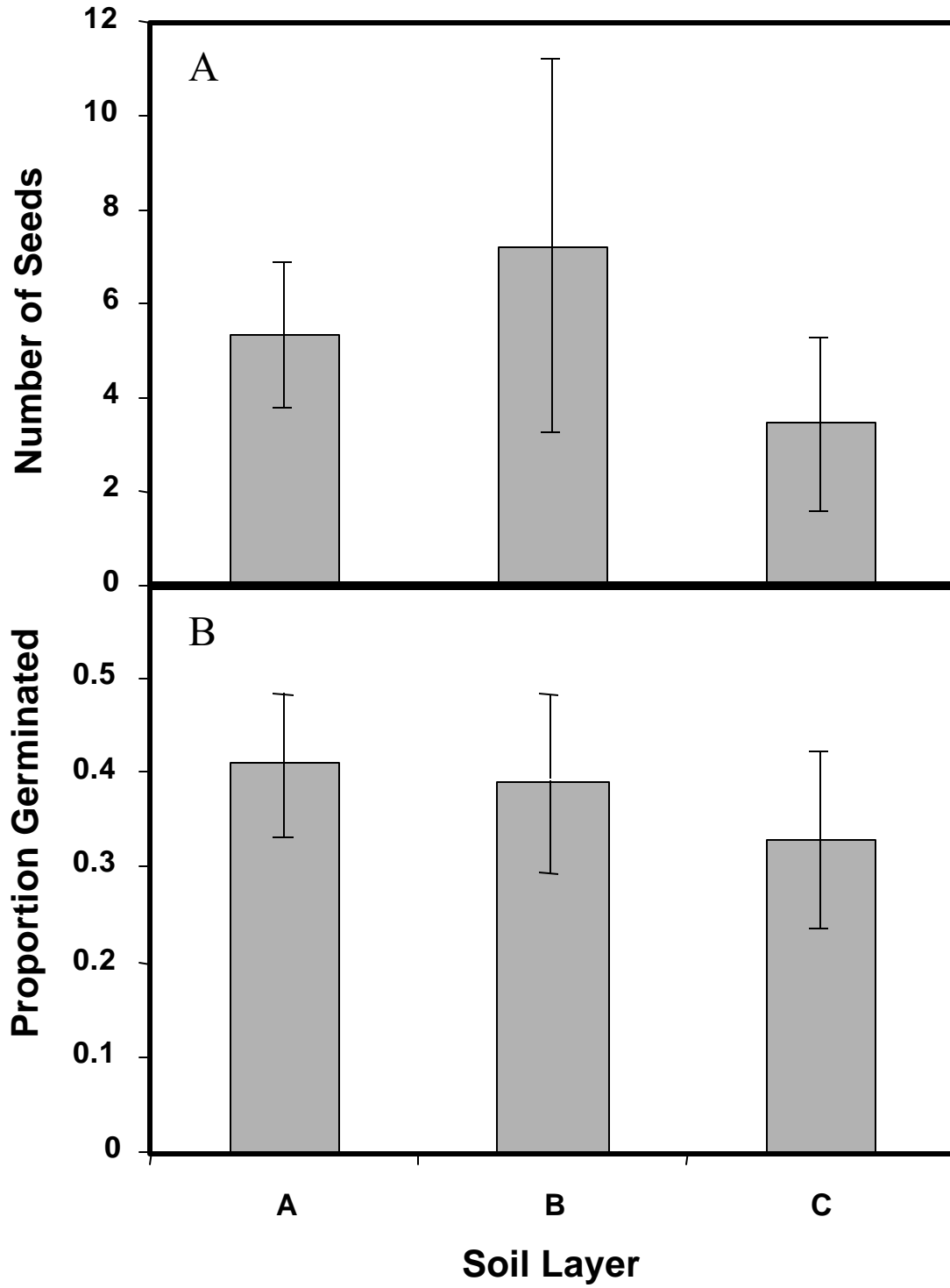


Fig. 3

