

Notes and Discussion

Conservation Genetic Assessment of the Blue-spotted Salamander in Iowa

ABSTRACT.—Blue-spotted salamanders (*Ambystoma laterale*) are a widespread and relatively common species throughout northeastern North America. The distribution of this species is marked by a pair of peripherally isolated populations at the southwestern boundary of its range in Iowa, a state where these salamanders are endangered. Because small peripatric populations suffer greater risks of extinction, the genetic state of the isolates was compared to that of a reference population that appears to be in geographic contiguity with the primary distribution of the species. Five polymorphic microsatellite loci were used to examine population genetic structure. Whereas allelic richness exhibited by each locus was qualitatively similar across study populations, genetic data indicate that the scaled effective population sizes of the peripheral isolates were demonstrably smaller compared to that of the reference population. One of the Iowa isolates shows evidence of a recent bottleneck and of substantial inbreeding; this population may therefore be subject to a particularly heightened risk of extirpation.

INTRODUCTION

Blue-spotted salamanders (*Ambystoma laterale*, Ambystomatidae) are broadly, but patchily, distributed throughout northeastern North America (Fig. 1), occupying much of the area previously covered by glaciers 18–19 kya at the most recent glacial maximum (Menzel and Goellner, 1976; Pielou, 1991; Yokoyama *et al.*, 2000; Demastes *et al.*, *in press*). Within the last several thousand years, beginning ca. 5.4–6.6 kya (Menzel and Goellner, 1976), the range of blue-spotted salamanders has become increasingly fragmented in the areas that presently correspond to the periphery of this species' distribution (Fig. 1). *Ambystoma laterale* are philopatric and largely dependent on the availability of ephemeral pools associated with woodland or forest habitat (Downs, 1989). Therefore, the advance of prairie, at the cost of lowland coniferous forest, may have contributed to range fragmentation, especially at the periphery of the distribution of *A. laterale* (Menzel and Goellner, 1976).

In 1976 Menzel and Goellner described two isolated populations of *Ambystoma laterale* in Iowa. Despite concerted collecting efforts in more recent years, other populations of *A. laterale* have not been reported and the species is now considered endangered in the state (Iowa Administrative Code, 2002). Today, agricultural land and riparian forest surrounds one of these populations of *A. laterale* (Behrens Ponds and Woodland; BPAW, Fig. 1), whereas a metropolitan area surrounds the other (George Wyth State Park; GWSP, Fig. 1). Moreover, a migrant would need to disperse either ca. 80 km from one of these localities to the other or ca. 150 km from another population (Fig. 1) in order to recolonize GWSP or BPAW. Due to changes in both climate and land use, *A. laterale* at GWSP and BPAW may therefore be increasingly susceptible to extirpation, with little prospect of reversing course via recruitment.

Loss and degradation of habitat are considered foremost causes of global amphibian declines (Collins and Storer, 2003). Whereas blue-spotted salamanders are relatively common in many parts of their geographic range, the acidification or conversion of wetlands has doubtlessly degraded or altogether removed suitable habitat for this and other amphibian species (Storfer, 2003). Therefore, the genetic health of blue-spotted salamander populations, and of many other amphibian populations, is of immediate concern. Peripherally isolated populations are expected to experience even greater demographic and genetic stochasticity than do other populations (Soulé, 1973; Lesica and Allendorf, 1995). Hence, the isolated populations of *Ambystoma laterale* in Iowa are of particular concern. The objective of this study was to compare the genetic status of the two peripheral isolates of *A. laterale* in Iowa with that of a reference population situated well within the main part of the species distribution in Minnesota. For geographically isolated populations, increased risk of genetic bottlenecks, inbreeding and loss of allelic richness may be important genetic concerns, all of which can be assessed using microsatellites.

MATERIALS AND METHODS

For the present study, 100 individuals of *Ambystoma laterale* were sampled from three localities (Fig. 1), which are all state-protected areas: George Wyth State Park (GWSP, 186 ha.), Carlos Avery Wildlife

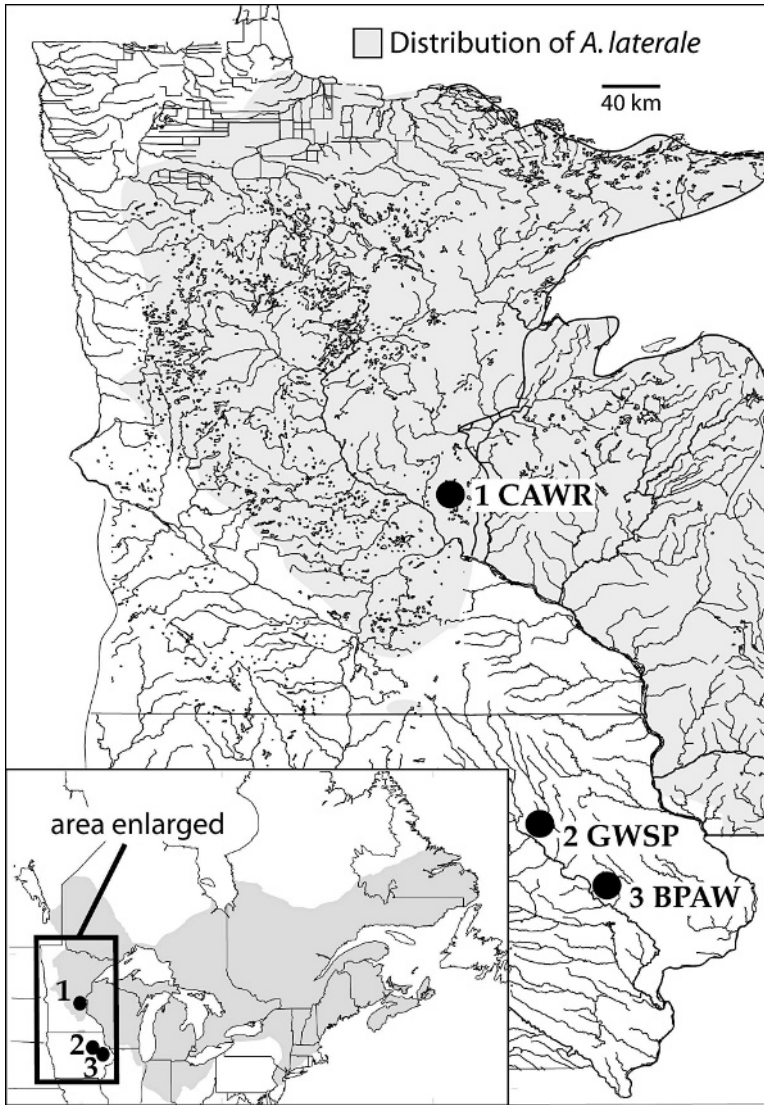


FIG. 1.—Sampling localities and the geographical range of *Ambystoma laterale*. Sampling sites include GWSP (George Wyth State Park, Black Hawk county, Iowa; $n = 29$), BPAW (Behrens Ponds and Woodland, Linn county, Iowa; $n = 45$) and CAWR (Carlos Avery Wildlife Refuge Area, Anoka county, Minnesota; $n = 26$)

Refuge (CAWR, 9300 ha.) and Behrens Ponds and Woodland (BPAW, 13 ha.). All samples were collected either by hand or by pit-fall traps. Nearly all tissue acquisitions were toe clips, though eight voucher specimens were collected at CAWR where these salamanders are not considered endangered.

Whole-genomic DNA was extracted and purified using the Qiagen DNeasy™ (Qiagen, Valencia, California) protocol. Five polymorphic microsatellite loci were used: AjeD75, AjeD283, AjeD346,

AjeD422 (Julian *et al.*, 2003b); and AmaD367 (Julian *et al.*, 2003a). These loci were amplified separately by polymerase chain reaction (PCR) in 20 μL reactions. JumpStart RED *Taq* DNA PolymeraseTM (Sigma, St. Louis, Missouri) was used in concentrations of 0.05 units/ μL . Final concentrations of PCR reagents were as follows: 1.0 μM each primer, 1X PCR buffer (included with DNA polymerase), 1.25 μM each dNTP and an estimated 1–100 ng/ μL template DNA. Thermal cycles were performed on a T-gradient machine (Biometra, Göttingen, Germany) and were as follows: initial denaturation at 95 C for 2 min; 36 cycles consisting of denaturation at 95 C for 1 min, primer annealing at either 55 C (AjeD283, AjeD346 and AmaD367) or 60 C (AjeD75 and AjeD422) for 1 min and extension at 72 C for 1 min; a final extension period of 30 min at 72 C was conducted to promote the completion of PCR products. PCR products were size-fractionated using an ABI Prism 377 Genetic Analyzer (Applied Biosystems, Foster City, California) at the Iowa State University Sequencing and Synthesis Facility. GENOGRAPHER 1.6 (Benham, 2001) was used to score genotypes.

Allelic richness and the probabilities of genotypic disequilibria among pairs of microsatellite loci were calculated using FSTAT v.2.9.3.2 (Goudet, 2002). Measures of expected (H_e) and observed heterozygosity (H_o) were determined for each population at each locus. These measures, and conformity of each locus and population to Hardy-Weinberg equilibrium (HWE) expectations, were assessed using ARLEQUIN v. 2.000 (Schneider *et al.*, 2000). For HWE expectation tests, 10^5 steps were run through a Markov Chain Monte Carlo simulation, which involved 10^3 dememorization steps.

Genetic partitioning within and among populations was determined by the parameter estimators \hat{f} (for F_{IS}) and $\hat{\theta}$ (for F_{ST}), using FSTAT. Permutation tests, using 300 and 1023 randomizations, respectively, were employed using ARLEQUIN to determine if the estimated \hat{f} and $\hat{\theta}$ values differed significantly from zero. Bootstrapping was implemented in order to determine the 95% confidence interval containing the global $\hat{\theta}$ estimate. For populations within which at least two sublocalities were sampled (GWSP and CAWR), population substructuring was examined by ad hoc assignment of individuals to pseudo-populations, which were consistent with the sampled sublocalities. Calculations of $\hat{\theta}$ were then repeated to assess the possibility of the Wahlund effect in each population. Pairwise F_{ST} for all possible population pairs was calculated using ARLEQUIN, with \hat{D} as the parameter estimator (Reynolds *et al.*, 1983). Genetic differentiation calculations and pairwise F_{ST} estimates were permuted with 10^3 steps to test for values that significantly differed from zero. Simultaneous tests for significance were corrected using the sequential Bonferroni method where applicable (Rice, 1989).

Probability of a recent population bottleneck as measured by heterozygosity excess was determined using BOTTLENECK v.1.2.02 (Cornuet and Luikart, 1997). Estimations were performed with the assumption of a two-phase model (TPM, with default parameters), which may most accurately model mutation in most populations (Di Rienzo *et al.*, 1994; Beck *et al.*, 2003). To determine if results were model dependent, the stepwise mutation model (SMM) was also used, this being the most statistically conservative model in BOTTLENECK (Cornuet and Luikart, 1997). A one-tailed Wilcoxon sign-rank test was employed in BOTTLENECK to determine the probability of a bottleneck in each study population. The mode-shift descriptor in BOTTLENECK also was utilized as a measure of population history.

A coalescent-based maximum likelihood estimation of the scaled effective size of each study population was determined using the composite parameter estimator θ (equivalent to $4N_e\mu$ and not to be confused with $\hat{\theta}$) in MIGRATE 2.0.3, which uses an approximation of the SMM (Beerli and Felsenstein, 1999, 2001). The search conditions were as follows: three short chains sampled 10,000 genealogies each, ten long chains sampled 100,000 genealogies each and 100 genealogies per chain was used as the burn-in value. A total of 20,000 genealogies were recorded, and the last 5000 trees of the final long chain were used for parameter estimation. The magnitude and directionality of recent gene flow between pairs of populations also was determined with MIGRATE by the migration quantity M , which is the migration rate scaled by the multiplicative inverse of the mutation rate. The quantity M was used to estimate the number of migrants for each possible direction of migration, by the following relationship: $(M\theta)/4 = N_m$, where θ is the scaled effective population size of the receiving population and N_m is the effective number of migrants from the source population (Beerli and Felsenstein, 1999, 2001).

TABLE 1.—Number of individuals sampled from each population (n), scaled effective population size (θ) with 95% confidence interval, inbreeding coefficient (\hat{f} ; * indicates value significantly different from zero), average observed heterozygosity among loci (H_o) for each study population and estimated number of migrants per generation from source population into another population (N_m)

Source	n	θ	\hat{f}	H_o	N_m		
					BPAW	GWSP	CAWR
BPAW	45	0.40 (0.37, 0.44)	0.09	0.48	—	0.72	0.06
GWSP	29	0.37 (0.33, 0.41)	0.20*	0.60	1.27	—	0.24
CAWR	26	0.58 (0.51, 0.66)	0.16	0.61	0.05	0.17	—

RESULTS

Private alleles accounted for greater than half of all detected alleles (28 of 48): 6 were observed in GWSP, 8 in BPAW and 14 in the population at CAWR. The number of alleles per locus per population ranged from 1 to 8. All loci examined were polymorphic in BPAW. At GWSP and CAWR, all loci were polymorphic except for Amd367. No evidence of genotypic disequilibrium was detected for any loci within any population ($0.05 < P < 1.00$).

Evidence of genetic substructure was not apparent within any study population. This is not surprising given that collecting localities at CAWR were situated ca. 900 m or less from each other, and collecting localities at GWSP were 686 m or less from each other. Therefore, it is not likely our analyses of population structure were confounded by the Wahlund effect.

The estimate of diversity partitioning among populations $\hat{\theta}$ was 0.262 (95% confidence interval = 0.201–0.333). This value is significantly different from zero ($P < 0.001$) and suggests a substantial degree of genetic differentiation among populations. Pairwise \hat{D} comparisons were consistent with this result; each value was significantly different from zero ($P < 0.001$). Estimates of gene flow also indicated a degree of genetic exchange between these populations, albeit at generally low rates. The greatest amount of gene flow appeared to be from the population at GWSP into BPAW, at roughly one migrant per generation (Table 1).

Allelic richness, averaged over all loci, was slightly lower in the Iowa populations than in the reference population: 4.15 (BPAW), 4.74 (GWSP) and 5.20 (CAWR). Average observed heterozygosity was lowest in the BPAW population of *Ambystoma laterale* (Table 1), which may be due in part to the lower allelic richness of this population.

Estimates of inbreeding, from the estimator \hat{f} (for F_{IS}), were positive for each population, but only for GWSP was this value significantly greater than zero (Table 1; $P = 0.0033$, where the adjusted significance level was 0.0033). Inbreeding measures and estimates of observed heterozygosity both are vulnerable to the influence of undetected null alleles; however, we have no evidence suggesting the presence of null alleles for any of the loci examined. DNA from all study individuals amplified equally well for all microsatellite loci.

Genetic data from GWSP indicated significant deviation from HWE expectations in two of the four loci that were polymorphic in this population, in a manner consistent with an inbred population. All deviations from expectation were unidirectional for GWSP: for all polymorphic loci, H_e exceeded H_o . The population at CAWR exhibited a similar trend, with H_e exceeding H_o for all polymorphic loci. However, only one locus differed significantly from HWE expectations, and the \hat{f} statistic for this population was not significantly greater than zero ($P = 0.0067$, where 0.0033 was the adjusted significance level). Four of the five loci in BPAW were consistent with HWE expectations and nonsignificant deviations from expectation in this population were bi-directional.

Scaled effective population sizes of both peripheral isolates (BPAW and GWSP) were demonstrably smaller than that for the reference population (θ ; Table 1). One population, GWSP, appears to have experienced a recent genetic bottleneck based on measures of heterozygosity excess ($P = 0.03$ for both TPM and SMM). Corroborating evidence for a genetic bottleneck in the GWSP population was supplied by the mode-shift descriptor, where a shifted mode in allele frequency distributions is indicative of a very recent bottleneck (Luikart *et al.*, 1998). Probabilities of a recent bottleneck for the other populations

under both models were as follows: for CAWR, $P_{TPM} = 0.84$ and $P_{SMM} = 0.90$; for BPAW, $P_{TPM} = 0.84$ and $P_{SMM} = 0.84$.

DISCUSSION

The significant degree of genetic subdivision observed for the study populations and the low rate of gene flow between these populations suggests that these populations are and have been genetically independent. Given that these populations are genetically differentiated, they could be considered distinct management units (*sensu* Moritz, 1994). Putatively, one migrant per generation (*i.e.*, $N_m = 1$) is required to attenuate any adverse effects of genetic drift, while still allowing genetic divergence between populations (Wright, 1951; Mills and Allendorf, 1996). Estimates of gene flow were well below this threshold for all population pairs (Table 1), with a single exception, which may be explained by historical gene flow between the GWSP population and the BPAW population via extirpated stepping-stone populations.

The genetic boundary subsuming the *Ambystoma laterale* population at CAWR likely extends far beyond the limited area sampled, which may explain the larger effective size of this population relative to the other study populations (0; Table 1). Although the estimated effective population size of the CAWR population is near the middle of the range of values reported in a similar study of recently and historically bottlenecked populations of the congeneric tiger salamander (*A. tigrinum melanostictum*, Spear *et al.*, 2006), there is no evidence of a bottleneck or of inbreeding at CAWR based on our microsatellite data. As long as the integrity of the connection between this population and the remainder of the primary distribution of *A. laterale* remains sound, it is unlikely that the CAWR population requires management.

The smaller effective population size seen in each of the Iowa isolates relative to the reference population (0; Table 1) is of concern. Scaled effective population size for these populations also are on the lower end of a range of values reported for recently bottlenecked populations of tiger salamanders (*Ambystoma tigrinum melanostictum*, Spear *et al.*, 2006). Several factors suggest that the apparent bottleneck of the GWSP population, evidenced by both heterozygosity excess and shifted allele frequency distributions, was recent. These factors include the relatively high level of H_o , the relatively small effective size, and the significantly positive inbreeding coefficient detected for the GWSP population (Table 1). Furthermore, Spear *et al.* (2006) found that heterozygosity excess and shifted allele frequency distributions do not remain detectable in tiger salamander populations that are thought to have been bottlenecked more than 50 y ago. Luikart *et al.* (1998: p. 238) also showed that shifts in allele frequency distributions reveal bottlenecks that have occurred "within the past several dozen generations." Therefore, all evidence points to a relatively recent bottleneck in the GWSP population.

If the GWSP population of *Ambystoma laterale* experienced a bottleneck recently, it is possible that it was induced by habitat fragmentation from human activity. Road development in and near the park prior to 1990 transected the woodlands used by *A. laterale* (Wilson, 1990). In 1992 further highway construction occurred near the park and a road was constructed within the park leading to a borrow-pit (now Alice Wyth Lake; Lori Eberhard, GWSP Manager, pers. comm.). The road within the park transects the area from which our current samples of *A. laterale* were drawn. Whether or not this activity is responsible for the apparent genetic bottleneck in this population, it is likely that road construction has impacted the GWSP population of *A. laterale* in a number of ways (reviewed by Marsh *et al.*, 2004).

It also is quite likely that the GWSP population has experienced one or more bottlenecks in the past that are unrelated to human activities. Most of the available breeding ponds at GWSP are shallow and we have observed them to remain dry for periods of two to five years. Although one breeding pond at GWSP is less shallow and has not been observed to go completely dry, this site is not a center of salamander density in the park. Furthermore, this deeper pond is frequently connected to a larger body of water during periodic spring floods, exposing salamanders to predation by fish.

Given the unreliable nature of the breeding pools at GWSP, the future of *Ambystoma laterale* at this site seems tenuous. A few years of drought followed by a spring flood, for example, could be all that is required for extirpation of the population. It also is possible that the isolated population at GWSP may suffer further inbreeding and, perhaps, mutational meltdown (Kimura *et al.*, 1963). If this is to happen, the effective population size will become further reduced, resulting in intensified genetic drift and an

increased risk of extinction due to demographic or environmental stochasticity (Cassel and Tamaru, 2003). Therefore, most, if not all, novel alleles (whether selectively neutral or not) might be eliminated from this population and consequently would be lost from the gene pool of this species.

Justification for management.—Whereas GWSP and BPAW might seem to be a negligible subset of *Ambystoma laterale* given the seeming success of the species as a whole, this judgment, it is argued, would be shortsighted. Populations at the margins of a species range may be evolutionarily valuable as unique reservoirs of genetic diversity, because isolates often possess novel alleles that either originated exclusively in the isolate or that have been lost by the more central populations (Lesica and Allendorf, 1995; Mayr and Diamond, 2001). Given the general acceptance of the maxim regarding the conservation value of preserving the greatest possible genetic diversity of a species, peripherally isolated populations are often, and ought to be, of highest management concern (Lesica and Allendorf, 1995). In fact, Ehrlich (1988: p. 22) suggested that, “The loss of genetically distinct populations within species is...at least as important a problem as the loss of entire species.”

It is therefore recommended that a concerted monitoring and management effort commence for *Ambystoma laterale* at GWSP and BPAW to preserve populations of blue-spotted salamanders at these localities, thereby preserving the greatest longterm potential for genetic diversity in *A. laterale* as a whole and maintaining the evolutionary potential of each distinct lineage. Data from this study could serve as a genetic baseline to measure potential shifts in genetic diversity in the future.

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