# Genetic Consequences of Population Reduction and Geographic Isolation in the Critically Endangered Frog, *Rana sevosa*

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**Anthropogenic habitat fragmentation and reduction are major causes of population declines and extinction. As these processes intensify, our ability to rescue imperiled taxa is critically dependent on an understanding of historical, demographic, and genetic parameters of diminishing populations. We assessed the effects of recent geographic isolation and population reduction on genetic variability for endangered Dusky Gopher Frogs, *Rana sevosa*. Only two populations of *R. sevosa* exist, each is geographically isolated and restricted to a single breeding pond, and one of them may have gone locally extinct. Therefore, we studied the largest and perhaps only population of *R. sevosa*. The only option for comparison to non-isolated populations was of its ecologically similar sister species (other Gopher Frogs, *R. capito*) and of the sister species to *R. sevosa* and *R. capito* (Crawfish Frogs, *R. areolata*). Variation in seven microsatellite DNA loci was assessed for each population to determine the effects of isolation and population bottleneck on *R. sevosa*. In comparison to the average non-isolated population, *R. sevosa* had significantly lower genetic variation and a strong signature of population bottleneck. In fact, *R. sevosa* had $H_O$ that was 72%, $H_E$ that was 81%, and A that was 61% of the average non-isolated population. Results indicated a severe, negative genetic consequence of recent population reduction and geographic isolation via lack of gene flow, enhanced effects of drift, and inbreeding. Extensive demographic data have been collected for *R. sevosa* beginning when the species was rediscovered in 1987 and continuing through our study. These previously collected demographic data aid in interpretation of our genetic data and discussion of implications for conservation and management.**

As human populations continue to expand and encroach on the natural landscape, the severity of habitat fragmentation intensifies and habitat-patch sizes diminish. Consequently, populations of many non-human organisms that were once large and connected by gene flow become subdivided, reduced in size, and typically confined to discrete, small habitat patches (Keller et al., 2004; Honnay et al., 2007; Allentoft et al., 2009). A fundamental issue in conservation biology is to understand how loss of inter-population dynamics affects species persistence. Isolated subpopulations tend to become inbred and genetically differentiated from each other (Van Rossum et al., 2004; Allentoft et al., 2009). Anthropogenic habitat fragmentation and reduction are major causes of population declines and local extinctions. Ameliorating situations in which populations have become completely isolated is necessary for conservation because effective gene flow acts to remedy the negative effects of inbreeding (Spielman and Frankham, 1992; Bouzat et al., 2009). Additionally, regional migration allows recolonization in areas where local extinctions occur (Etienne and Heesterbeek, 2001).

A long-standing debate involves the relative importance of and interaction between genetic and demographic factors for species' survival (Lande, 1988; Frankham, 1995). For example, population bottlenecks severely reduce population sizes and have demographic and genetic consequences. Genetic consequences include loss of variability and increased probability of inbreeding depression. Small size leads to demographic stochasticity and extreme population size fluctuations, which increase the likelihood of local extinction (Alford and Richards, 1999). Genetic data greatly enhance conservation planning, and management decisions can be made that alleviate both demographic and genetic concerns. For example, artificially establishing nearby subpopulations should decrease the likelihood of extinction due to catastrophe, allow for differential juvenile recruitment into the population among ponds, and maintain higher genetic variation over time.

Studies of ecological and genetic dynamics of populations in amphibians are important and play a key role in understanding worldwide amphibian declines (Alford and Richards, 1999; Beebee, 2005). Many amphibians, especially those that breed in ponds, tend to have clumped distributions across the landscape. Maintenance of interconnectedness and population dynamics within this metapopulation setting (in the sense of ''ponds as patches;'' Marsh and Trenham, 2001) is critical if populations are to persist, particularly for rare species (Ficetola and De Bernardi, 2004; Moore et al., 2004; Honnay et al., 2007). Although patch size is positively related to genetic variability and persistence (Knaepkens et al., 2004), the dynamics and connections among patches can have a greater influence on extinction probabilities of populations and species (Drost and Fellers, 1996; Hitchings and Beebee, 1997; Joly et al., 2001; Semlitsch, 2002).

Amphibians are particularly susceptible to habitat alteration, loss, and fragmentation (Gallant et al., 2007). At least two life history characteristics of amphibians predispose them to failure in fragmented landscapes. First, populations, especially those of pond-breeding amphibians, are characterized by episodic annual reproductive success consisting of fairly infrequent ''boom'' years of high reproductive output interspersed among ''bust'' years of zero or low reproductive output (Semlitsch et al., 1996; Richter et al., 2003; Gibbons et al., 2006). This typically results in multiyear population size declines punctuated by dramatic increases during boom years (Alford and Richards, 1999). Second, relative to other vertebrates, amphibians typically do not move great distances across the landscape (Dodd and Smith, 2003). However, many studies have found that a few individuals move much greater distances than the population mean would suggest (Semlitsch and Bodie, 2003:appendix 1).

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Opportunities to study historical, demographic, and genetic parameters of shrinking populations in natural amphibian populations are infrequent but exist for Dusky Gopher Frogs, *Rana sevosa*. *Rana sevosa* is listed as Endangered under the U.S. Endangered Species Act and Critically Endangered on the Red List of the International Union for Conservation of Nature and Natural Resources (USFWS, 2001; IUCN, 2008). The historical geographic range of these frogs once extended throughout the coastal plain of Louisiana, Mississippi, and western Alabama in upland, Longleaf Pine (*Pinus palustris*) forests (Goin and Netting, 1940). *Rana sevosa* breed in temporary, upland ponds and spend the non-breeding season in adjacent Longleaf Pine forests in small mammal burrows, holes associated with dead trees, and other below-ground refugia (Richter et al., 2001). Annual breeding-migration events between the forest and pond facilitate sampling adults of the population.

Critical habitat has been reduced across the geographic distribution of Dusky Gopher Frogs due to logging and conversion of Longleaf Pine ecosystems to Slash Pine (*P. elliottii*) plantations. In addition to habitat loss, these frogs are threatened by habitat fragmentation, fire suppression, introduction of fish to breeding ponds, and road mortality (Richter and Jensen, 2005). *Rana sevosa* is considered extirpated in Louisiana and Alabama. Although once abundant in coastal Mississippi (Allen, 1932), only two breeding populations are known to exist. Extensive ecological and demographic data exist for one of the populations, which has a population size of 100–200 adults (Glen's Pond; Harrison County, Mississippi; Richter et al., 2001, 2003; Richter and Seigel, 2002). The other population, which is located about 32 km east of Glen's Pond, was recently discovered (March 2004; Mike's Pond; Jackson County, Mississippi), has a small population size (<50 adults) based on egg mass counts, and appears to have recently gone extinct.

The objectives of our study were to assess population genetic variation and consequences of geographic isolation and population reduction in the largest and perhaps only remaining population of *R. sevosa*. To interpret these results, genetic data from non-isolated populations are necessary. The only comparison possible is to populations of closely related frogs with similar life histories (i.e., generation time, ecology, habitat use, etc.). Therefore, we studied non-isolated populations of the sister species of *R. sevosa* (other Gopher Frogs, *R. capito*) and the sister species of *R. capito* and *R. sevosa* (Crawfish Frogs, *R. areolata*). These species are similar morphologically and ecologically and were all considered *Rana areolata* throughout the 1980s until Collins (1990) resurrected *R. capito* for populations throughout the coastal plain. This was later followed by a resurrection of *R. sevosa* by Young and Crother (2001), who found the Glen's Pond population of *R. sevosa* (Mike's Pond was unknown at the time) to be a distinct lineage separate from *R. capito*. Taxonomic, ecological, and demographic affinities among the three support the validity of *R. capito* and *R. areolata* populations to address our objectives. We predict that *R. sevosa* will have lower genetic variability than non-isolated populations of sister species and will have a genetic signature of recent population bottleneck event.

## MATERIALS AND METHODS

***Sample collection.***—A population of *R. sevosa*, *R. areolata*, and *R. capito* were sampled as follows (Fig. 1). The *R. sevosa* site is located at the northern edge of United States Forest Service (USFS) property in De Soto National Forest (Harrison County, Mississippi) only 250 m south of the USFS boundary line (Richter et al., 2001). Based on visual and audio surveys performed since the late 1980s (G. N. Johnson and M. A. Sisson, unpubl. data), this population is completely isolated and geographically separated by ca. 32 km from the only other known population. The *R. capito* site was located in contiguous, protected habitat with many breeding ponds at Archbold Biological Station (Highlands County, Florida). The *R. areolata* site was located on private lands within contiguous pastureland with many breeding ponds (Atoka County, Oklahoma).

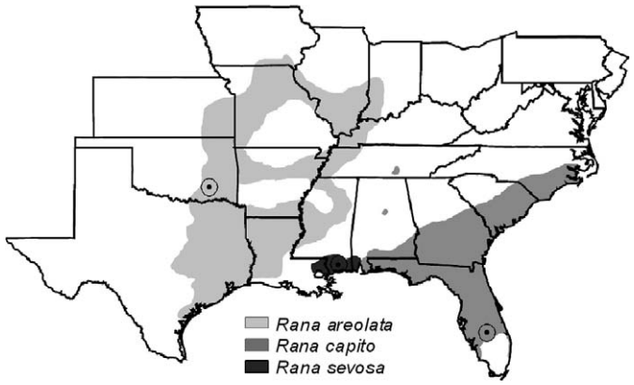


**Fig. 1.** Historical geographic distribution of Gopher Frogs (*Rana capito* and *R. sevosa*) and Crawfish Frogs (*R. areolata*). Study sites are indicated with circles for each: *R. areolata* (Atoka County, OK), *R. capito* (Highlands County, FL), *R. sevosa* (Harrison County, MS).

Individuals were captured by hand (*R. areolata*), via a drift fence with pitfall traps (*R. sevosa*), or by hand and with pitfall-trap arrays (*R. capito*). A single toe was collected from adults of each species (*R. sevosa* $n = 46$; *R. capito* $n = 37$; *R. areolata* $n = 32$). *Rana sevosa* samples were collected prior to the species being listed as federally endangered, so no special permits were required. All toe samples were stored in 95% ethanol until DNA was extracted.

***Genetic data collection.***—DNA was extracted from each individual using Qiagen (Valencia, CA) DNEasy tissue kit and protocol. Loci were selected from a microsatellite library that was developed previously for *R. sevosa* (Richter and Broughton, 2005). For each individual, DNA was PCR amplified for seven microsatellite loci in *R. sevosa* and *R. capito* (*Rs*B12, *Rs*C02, *Rs*Co5, *Rs*Ms3, *Rs*F01, *Rs*E03, *Rs*A05) and for three loci in *R. areolata* (*Rs*F01, *Rs*E03, *Rs*A05). PCR conditions, primer sequences, fragment lengths, and repeat motifs for each locus are described in Richter and Broughton (2005). Genetic data were collected using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA) by pooling samples of PCRs for three loci per individual using different fluorescently labeled primers for each locus. Allele lengths were scored using GeneScan Analysis Software version 3.1.2 (Applied Biosystems, Inc.).

***Genetic analyses.***—The following genetic calculations and analyses were performed using FSTAT Version 2.9.3.2 (Goudet, 1995, 2002). Observed heterozygosity ($H_O$), allelic richness ($A$), expected heterozygosity ($H_E$), and Wright's inbreeding coefficient ($F_{IS}$) were first calculated. Allelic richness was calculated using rarefaction to standardize

**Table 1.** Genetic Variability at Each Microsatellite Locus for *Rana sevosa* at Glen's Pond, Harrison County, Mississippi (*Rs*; $n = 46$), *R. capito* at Archbold Biological Station, Highlands County, Florida (*Rc*; $n = 37$), and *R. areolata* at Atoka County, Oklahoma (*Ra*; $n = 32$). *A* = observed number of alleles per locus; *R* = corrected (via rarefaction) number of alleles per locus; range = size range of alleles; $H_E$ = expected heterozygosity under Hardy Weinberg equilibrium; $H_O$ = observed heterozygosity.

| | | Locus | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Populations | | *Rs*B12 | *Rs*C02 | *Rs*C05 | *Rs*Ms3 | *Rs*F01 | *Rs*E03 | *Rs*A05 |
| *Rs* | *A (R)* | 7 (6.8) | 2 (2.0) | 2 (2.0) | 5 (4.8) | 4 (3.6) | 5 (4.8) | 3 (3.0) |
| | range | 8 | 3 | 3 | 17 | 4 | 5 | 3 |
| | $H_E$ | 0.779 | 0.502 | 0.196 | 0.783 | 0.715 | 0.678 | 0.423 |
| | $H_O$ | 0.826 | 0.435 | 0.174 | 0.761 | 0.609 | 0.674 | 0.435 |
| *Rc* | *A (R)* | 7 | 5 | 4 | 9 | 9 (8.8) | 10 (9.7) | 9 (8.8) |
| | range | 8 | 5 | 5 | 14 | 9 | 16 | 15 |
| | $H_E$ | 0.791 | 0.777 | 0.531 | 0.801 | 0.847 | 0.622 | 0.681 |
| | $H_O$ | 0.838 | 0.703 | 0.649 | 0.703 | 0.946 | 0.595 | 0.487 |
| *Ra* | *A* | — | — | — | — | 9 | 7 | 6 |
| | range | — | — | — | — | 9 | 12 | 6 |
| | $H_E$ | — | — | — | — | 0.841 | 0.732 | 0.650 |
| | $H_O$ | — | — | — | — | 0.875 | 0.625 | 0.688 |

based on the size of the smallest sample (El Mousadik and Petit, 1996). For loci that amplified only in *R. sevosa* and *R. capito*, the smallest sample size was for *R. capito* ($n = 37$). For the loci that amplified in all three species, *R. areolata* had the smallest sample size ($n = 32$). To evaluate deviations from Hardy Weinberg Equilibrium (HWE) for each locus and population, global tests (1000 permutations) with Bonferroni corrections were used (Weir, 1996).

The following measures of genetic variability were compared among populations as follows. For each measure, differences were statistically evaluated between *R. sevosa* and *R. capito* and qualitatively compared among all three species due to low sample size (3 loci) in *R. areolata*. Differences in unbiased $H_E$ were evaluated using a paired *t*-test on arcsine-transformed data (Nei, 1987). Differences in $F_{IS}$ over all loci were compared based on 28,000 randomizations and Bonferroni corrections. Differences in *A* were evaluated using a Wilcoxon's signed-rank test.

Loss of alleles in populations was quantified using Garza and Williamson's (2001) *M* statistic, which is calculated by dividing the number of alleles by the range in size of alleles for each locus averaged over all loci (i.e., *M* takes advantage of the number of alleles being reduced at a higher rate than the size range). *M* values range from a maximum of one (no alleles missing) and approach zero as alleles are lost. It has been shown to be an accurate estimator of population history and is smaller in recently reduced populations than in populations at equilibrium (Garza and Williamson, 2001).

Two analyses implemented in BOTTLENECK Version 1.2.02 (Cornuet and Luikart, 1996; Piry et al., 1999) were used to test for recent population bottlenecks. The first test was designed to take advantage of allelic diversity being reduced at a faster rate than heterozygosity shortly after severe reductions in population size. For each locus, heterozygosity excess was evaluated by calculating $H_E$ and then estimating heterozygosity expected at mutation-drift equilibrium ($H_{EQ}$), which is based on observed number of alleles and sample size. Populations that have not undergone a recent change in population size should have $H_E = H_{EQ}$ (Piry et al., 1999).

$H_{EQ}$ was estimated under three models of mutation: infinite alleles model (IAM), stepwise mutation model (SMM), and two-phase mutation model (TPM) based on 5000 iterations. The TPM was used with 95% single-step mutations (5% multi-step) and a variance among multiple steps of 12% as recommended by Piry et al. (1999). Following estimation of $H_{EQ}$, a one-tailed Wilcoxon's signed-ranks test was used to test the prediction that $H_E > H_{EQ}$ in *R. sevosa* and that $H_E = H_{EQ}$ in the non-isolated populations of sister species. This test appears to be robust for tests using <20 polymorphic loci (Piry et al., 1999).

The second analysis was the qualitative evaluation of allele frequency distributions, which was originally described by Luikart et al. (1998). This mode-shift indicator test was used to determine the shape of the frequency distribution of alleles and inspect it for a signature distortion. Allelic frequency distributions in healthy populations should be L-shaped because of the presence of many low-frequency alleles. Flattening of this L-shape in populations that have undergone a bottleneck results from a greater probability of loss for low-frequency alleles (Luikart et al., 1998).

## RESULTS

Genetic variation was lower in the geographically isolated population of *R. sevosa* than the non-isolated populations of sister species. Severe reductions in polymorphism were found at three loci for *R. sevosa* (*Rs*C02 and *Rs*C05 had only two alleles and *Rs*A05 had three) compared to much higher levels in the non-isolated population of *R. capito* (five, four, and nine alleles, respectively; Table 1). For individual loci, genetic variation was generally less in the isolated population of *R. sevosa*. Observed heterozygosity ($H_O$) and expected heterozygosity ($H_E$) were lower for six of seven loci in *R. sevosa*. $H_O$ ranged from 0.174 to 0.826 for *R. sevosa*, 0.595 to 0.946 for *R. capito*, and 0.625 to 0.875 for *R. areolata* (Table 1). $H_E$ ranged from 0.196 to 0.783 for *R. sevosa*, 0.531 to 0.847 for *R. capito*, and 0.650 to 0.841 for *R. areolata* (Table 1). No loci deviated significantly from HWE at the 5% significance level after Bonferroni correction. Mean $H_E$ and $H_O$ were much lower in *R. sevosa* (Table 2). Differences were found between *R. sevosa* and *R. capito* for $H_O$ (paired *t*-statistic 2.22, df = 6, $P = 0.068$) and $H_E$ (paired *t*-statistic

**Table 2.** Mean Data and Inbreeding Coefficient ($F_{IS}$) for Seven Microsatellite Loci for *Rana sevosa* at Glen's Pond, Harrison County, Mississippi (*Rs*), *R. capito* at Archbold Biological Station, Highlands County, Florida (*Rc*), and *R. areolata* at Atoka County, Oklahoma Site (*Ra*). $n$ = # of individuals sampled; $A_{TOT}$ = total # alleles observed for all loci; $A$ = observed mean # of alleles per locus; $R$ = corrected (via rarefaction) mean # of alleles per locus; $PA$ = mean # of private alleles per locus; $S$ = mean size range of alleles; $M$ = ratio of corrected mean # alleles to size range of alleles; $H_O$ = observed heterozygosity; $H_E$ = expected heterozygosity under Hardy-Weinberg equilibrium. Standard errors are indicated in parentheses. * Because only three loci amplified for *R. areolata*, $A_{TOT} = A \times 7$ for this population.

| Population | $n$ | $A_{TOT}$ | $A$ | $R$ | $PA$ | $S$ | $M$ | $H_O$ | $H_E$ | $F_{IS}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| *Rs* | 46 | 28 | 4.00 (0.69) | 3.85 (0.66) | 1.3 (0.7) | 8.3 (2.2) | 0.661 (0.088) | 0.518 (0.086) | 0.598 (0.084) | 0.122 |
| *Rc* | 37 | 53 | 7.57 (0.87) | 7.48 (0.84) | 3.7 (1.0) | 10.3 (1.8) | 0.784 (0.066) | 0.703 (0.040) | 0.729 (0.057) | 0.026 |
| *Ra* | 32 | 51* | 7.33 (0.88) | 7.33 (0.88) | 1.7 (0.9) | 9.0 (1.7) | 0.861 (0.138) | 0.729 (0.055) | 0.746 (0.075) | 0.016 |

2.46, df = 6, $P = 0.049$). $F_{IS}$ was significantly higher in *R. sevosa* ($P < 0.05$; Table 2).

Allelic richness per locus was less in *R. sevosa* for all loci, both for observed numbers ($A$) and estimates corrected via rarefaction ($R$; Table 1). $R$ was significantly lower in *R. sevosa* than in *R. capito* (Wilcoxon's signed-rank test; Z = 2.37; $P = 0.018$). Allelic size ranges varied widely among loci and populations, and no clear pattern was detected among populations (Table 1). A total of 67 distinct alleles were revealed for the seven loci. Of the 67 total alleles, 38 (57%) were unique to individual populations: 7 (10%) to *R. sevosa*, 26 (39%) to *R. capito*, and 5 (7%) to *R. areolata*.

Although populations differed considerably in allelic composition, only one locus (*Rs*Ms3) had private alleles that were outside the allelic range of other species by more than one repeat motif. Private alleles were represented at fairly low frequencies within each population (mean ± SE = 0.111 ± 0.03 for *R. sevosa*, 0.114 ± 0.03 for *R. capito*, and 0.110 ± 0.03 for *R. areolata*). The high number of private alleles can be explained in part by the apparent high frequency of allelic loss in *R. sevosa*, which was more severe than for other species in terms of allelic richness and $M$ (Table 2).

Analyses of heterozygote excess indicated that a recent population bottleneck event occurred in *R. sevosa*. Under the IAM and TPM, *R. sevosa* showed a significant heterozygote excess ($P < 0.05$; i.e., significant deviation from mutation-drift equilibrium). Neither of the non-isolated populations of sister species exhibited significant heterozygote excess under the IAM, SMM, or TPM mutation models ($P > 0.05$). Additionally, *R. sevosa* (but not *R. areolata* or *R. capito*) exhibited an allele frequency shift as a result of loss of low-frequency alleles, which also indicated that a recent bottleneck occurred in *R. sevosa*. This is evidenced by a comparison of frequency distribution of allelic proportions in the populations (Fig. 2).

## DISCUSSION

We found that population genetic diversity has been severely reduced in critically endangered Dusky Gopher Frogs, *Rana sevosa*. The apparent cause for this is a recent reduction in population size and geographic isolation from other sources of alleles, which results in inbreeding and loss of alleles. All genetic analyses that were performed support our predictions, including observed and expected heterozygosity, inbreeding coefficient ($F_{IS}$), allelic richness, allelic loss, test of heterozygote excess, and mode-shift of allele frequency distribution. We attribute the rapid rate of genetic erosion primarily to the naturally small size of local populations and life history of Gopher Frogs. Studies of non-isolated populations of *R. capito* suggest that Gopher Frog populations were historically located within metapopulations (in the sense of ''ponds as patches;'' Marsh and Trenham, 2001), where individual breeding populations had small sizes (<200–300 adults) but the overall population size across the landscape was large (Semlitsch et al., 1995; Palis, 1998; Greenberg, 2001). As subpopulations of *R. sevosa* became fragmented and isolated, overall population sizes rapidly diminished. What remains is a small population (<200 adults) with a single breeding pond that was once a subpopulation within a larger complex of subpopulations and breeding ponds. These findings may be general for amphibians with similar life histories and for many other organisms that use the same breeding sites.

Species may respond differently to various levels of habitat fragmentation (DiBattista, 2008). Habitat specialists, which are typically rare, will be impacted more severely than habitat generalists, which are typically common. Whereas generalists may lose connections among a few populations as the habitat is subdivided, specialists have a much greater probability of having populations that are completely isolated. All three species in this study represent habitat specialists and so should be impacted by habitat fragmentation similarly. This study addressed a worst-case extreme in range of habitat fragmentation—complete isolation from other *R. sevosa* populations. Populations that become completely isolated have high probabilities of extinction (Westemeier et al., 1998; Richter et al., 2003).

Although habitat specialists are predisposed to extinction by human encroachment, non-isolated populations are buffered more against demographic, genetic, or cataclysmic extinctions than isolated populations of species with similar life histories. In a multi-year, multi-pond study of *R. capito* in the Ocala National Forest (Ocala, Florida), Greenberg (2001) found that all ponds had some level of recruitment across the five years of the study. Annual variation in recruitment was high among the eight ponds, as a few ponds had reproductive success in all five years, while others had success in only two of five years. Many unmarked, recently metamorphosed frogs entered most ponds in years with high landscape-level reproductive success. Even though many ponds had no reproductive success, the presence of multiple ponds allowed reproductive recruitment into the population for all five years. A complex of *Rana capito* on the Savannah River Site (Aiken, South Carolina) appears subject to rapid local extinction due to small population sizes and infrequent recruitment of juveniles (Semlitsch et al., 1995). Nevertheless, these populations appear to be composed of multiple (sub)populations at multiple ponds and continue to persist (Semlitsch et al., 1995; J. W. Gibbons, unpubl. data).

Amphibian breeding populations that are part of a larger metapopulation complex should have higher genetic vari-

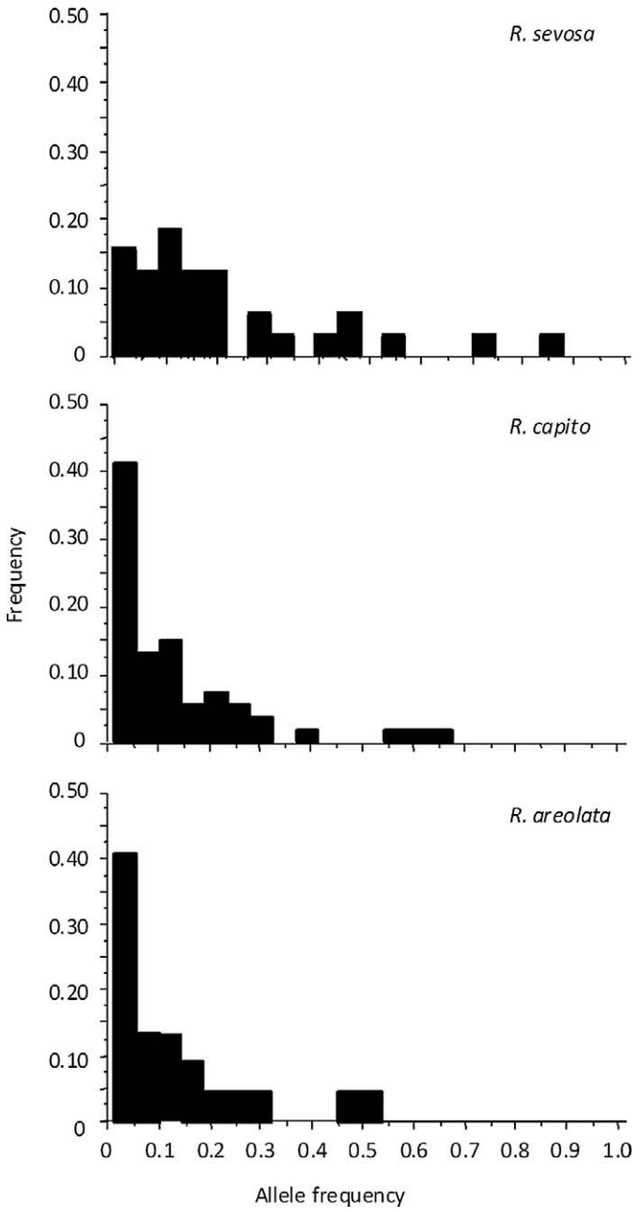


**Fig. 2.** Histogram of population allele frequencies for all genotyped loci in *Rana sevosa*, *R. areolata*, and *R. capito*. The x-axis depicts allele frequencies grouped into 0.05 class intervals (e.g., 0–0.5, 0.5–1.0, etc.). Note the increased frequency of rare alleles in *R. capito* and *R. areolata* as opposed to *R. sevosa*.

ation and would be less likely to go extinct than isolated populations where no recruitment from outside sources is possible (Skelly et al., 1999; Marsh and Trenham, 2001; Brede and Beebee, 2004). Causes of extinction could be genetic (e.g., mutational meltdown or reduction of genetic variation and consequently fitness), demographic (e.g., severe reduction in reproductive recruitment and population size), or catastrophic (e.g., a single event that extirpates an entire population). In a metapopulation, demes typically have reduced inbreeding, increased recruitment from nearby demes, and can be recolonized from a nearby source following a local extinction (Skelly et al., 1999; Marsh and Trenham, 2001). This contrasts strongly to the population of *R. sevosa* at Glen's Pond because the geographic isolation of this population eliminates the possibility of a rescue effect occurring naturally and enhances inbreeding and demographic difficulties (Wilson et al., 2005).

Allelic richness and loss are more sensitive indicators of population bottlenecks and genetic erosion in small populations than is heterozygosity (i.e., small population size reduces heterozygosity less than it does allelic richness; Allendorf, 1986; Cornuet and Luikart, 1996; Garner et al., 2003; Kang et al., 2008). That is, levels of heterozygosity are relatively insensitive to the actual number of different genotypes at a locus (Allendorf, 1986). In fact, populations could have similar levels of heterozygosity and greatly different allelic diversity and patterns of allelic loss (Barker, 2001). While sufficient heterozygosity is necessary for short-term population survival, long-term persistence requires allelic diversity (in terms of both frequencies and numbers) because this is the variation upon which selection can act in the future (Kimura and Crow, 1964; Allendorf, 1986; Petit et al., 1998). In *R. sevosa*, heterozygosity and allelic richness are low. Many studies have found significant, negative relationships between low genetic diversity of neutral loci and fitness-related traits (Coltman et al., 1998; Hannson et al., 2004; Mainguy et al., 2009). Therefore, likelihood of long-term persistence for *R. sevosa* is low without human intervention.

*Rana sevosa* exhibited high allelic loss and consequently a significant heterozygote excess (Cornuet and Luikart, 1996), mode-shift in allele frequencies (Luikart et al., 1998), and low *M* (mean ratio of number of alleles to range in size of alleles; Garza and Williamson, 2001). Garza and Williamson (2001) found that *M* for stable populations ranged from 0.823–0.926 (mean ± SE = 0.873 ± 0.011), whereas for reduced populations it ranged from 0.599–0.693 (mean ± SE = 0.641 ± 0.010). In this study, *M* for *R. sevosa* (0.645) was just above the average for reduced populations and well within the range. Conversely, *M* for *R. capito* (0.784) and *R. areolata* (0.861) were much higher and above the range for reduced populations.

These three genetic analyses corroborate support for population bottleneck. This genetic signature could be due to historic bottlenecks associated with the loss of nearby breeding ponds and subpopulations, to historic bottlenecks within the remaining population, and to a recent bottleneck associated with a seven-year drought (Richter et al., 2003). Following population bottlenecks, populations should return to a balanced state in which allelic diversity and frequency distributions are in mutation-drift equilibrium. Rate and possibility of recovery is highly dependent on population size.

***Implications for conservation and management.***—Current conservation efforts for *Rana sevosa* include continued monitoring of the demographic status of populations, continuing surveys for unknown populations, restoring historic sites with potential for translocations, implementing periodic controlled burning of ponds and upland habitats, maintaining buffer zones around current and potential breeding ponds, and ''farming'' tadpoles in mesocosms to be released at breeding sites to supplement natural reproductive recruitment. A potential management strategy is to use stock from the one known extant population to reintroduce the species to historic localities in which it has gone locally extinct.

Based on the genetic variation of the Glen's Pond population, the following management strategies should be incorporated into the long-term recovery plan for *R.*

*sevosa*. Eggs selected for tadpole farming should be chosen to maximize genetic diversity by sampling each egg mass deposited in Glen's Pond. Genetic diversity of the eggs should be determined by genotyping a few eggs of each egg mass. The other population of *R. sevosa* (Mike's Pond), which was discovered recently (May 2004), is located in an upland that is bisected by a road and other human development and also has a single breeding pond. Based on recent field data, the population is at high risk of extinction and may have gone extinct (M. Sisson, pers. comm.). If the population persists, genetic comparisons between the populations need to be performed, followed by careful consideration of the potential to transplant eggs from Mike's Pond to Glen's Pond to enhance genetic variability in the primary breeding population (Tallmon et al., 2004; Johnson and Dunn, 2006). It may be useful to use captive populations (currently maintained in zoos) to supplement natural populations, and individuals should be genotyped as they are brought into captivity in the event that reintroduction or supplementation is necessary. If reintroductions occur, genetic monitoring of populations should be used to determine the effectiveness of this conservation strategy (Latch and Rhodes, 2005).

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