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Temporal Comparisons on the Genetic Variation of the Dusky Gopher Frog (Lithobates sevosus)

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TEMPORAL COMPARISONS ON THE GENETIC VARIATION OF THE DUSKY GOPHER FROG (*LITHOBATES SEVOSUS*)

By

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ABSTRACT

Monitoring temporal changes in genetic diversity within populations can provide vital information on future viability. The dusky gopher frog, *Lithobates sevosus,* exists in isolation with an estimated population size of 100–200 individuals, and previous research has shown that low genetic variability exists as a consequence of isolation and population size reduction. However, the temporal changes in genetic variation are not known. Therefore, my objectives were to (1) determine temporal trends in population genetic variation and implications for long-term viability of *L. sevosus* and (2) estimate effective population size. To accomplish these objectives, egg samples collected from 1997 to 2014 were genotyped for nine microsatellite loci. Observed and expected heterozygosity, allelic richness, and Wright's inbreeding coefficient were calculated for each year and differences between sample years were assessed. Additionally, overall and pair-wise F_{ST} values were calculated to test for temporal genetic structuring. To estimate effective population size, two single-sample estimators (Tallmon et al. 2008; Waples and Do 2008) and two temporal estimators (Waples 1989; Wang 2001) were used. The results show a stable, but low, level of genetic variation. Weak genetic structure (F_{ST} = 0.023 (95% CI 0.006–0.043)) was found among years, which can be attributed to the increased effects of genetic drift in small populations. *L. sevosus* currently has an estimated effective population size between 32.99–58.6 individuals. The ratios of effective size to census size per year are fairly large (~0.5) and exhibit an increasing trend over time, which could possibly be explained by genetic compensation—or the lessening of genetic variation reduction in times with low population numbers. This research indicates the current management programs in place for *L. sevosus* have been effective at maintaining the genetic diversity present in the population; however, additional strategies need to be implemented to increase genetic diversity.

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INTRODUCTION

The maintenance of genetic variation within populations and species is a primary concern in conservation biology because this variation allows species and populations to adapt to their changing environments (Frankham et al. 2004). Without this adaptive potential, populations are at an increased risk of endangerment and extinction. Habitat loss and fragmentation are major threats to genetic variation because they result in small, isolated populations (Keller et al. 2004; Smith et al. 2009; Richter et al. 2009). Ultimately, the persistence of these populations depends on the retention of genetic diversity. However, small, isolated populations have lower amounts of genetic variation and are more prone to inbreeding depression (Frankham 1996; Richter and Nunziata, 2014). Species residing in wetland habitats, especially amphibians, are particularly vulnerable because most natural wetlands have been destroyed or degraded, causing a reduction in habitat and wetland species' population sizes (Dahl 2000; Beebee 2005; Curado et al. 2011).

Assessing temporal change in genetic variation of populations provides both historic and contemporary information and can reveal previous population bottlenecks, effects of genetic drift, and whether genetic variation is increasing or decreasing within a population (Heath et al. 2002; Bouzat 2010; Cuveliers et al. 2011). Many populations have decreased in genetic diversity over time due to anthropogenic and ecological factors (Arnaud and Laval 2004; Gomaa et al. 2011). Therefore, monitoring a species' genetic variation over time can serve as a necessary and valuable indicator of extinction risk and efficacy of current conservation management programs.

An informative parameter in evaluating maintenance of genetic variation both temporally or at a single point in time is effective population size (N_e), which can be estimated through the use of microsatellite markers. Effective population size is defined as the size of the ideal population that experiences genetic drift at the same rate as the population of interest (Wright 1931). More generally, effective population size is the number of reproductively successful and genetically contributing individuals in a population. Effective population size provides insight to the amount of genetic variation being retained within a population, as N_e is inversely related to amount of inbreeding and loss of genetic diversity (Beebee 2009; Hoehn et al. 2012).

There are two types of effective population size estimates—single sample and temporal. Single-sample estimators require one generation of microsatellite genotypes in order to calculate an effective population size estimate (Tallmon et al. 2008). They are advantageous for long-lived species and for short-term projects. Previously, single-sample estimates were rarely used because they were found to be imprecise and biased (England et al. 2006). However, recent improvements in computer algorithms coupled with widespread availability of microsatellite DNA databases have made single-sample estimators more useful and accurate (Waples 2006; Tallmon et al. 2008; Luikart et al. 2010).

Temporal estimators require two temporally separated samples of allele frequency data (Waples 1989). These samples are used to detect changes in microsatellite allele frequency over a known set of generations. As allele frequency changes between generations increase, N_e is expected to decrease (Beebee 2009). Temporal samples can be either moment-based or likelihood-based, with likelihood-based models using more information from the dataset than moment-based. Because of this difference, likelihood-based models are presumed to be more precise and accurate (Berthier et al. 2002). No matter the estimator used, effective population size estimates offer information on the genetic viability of a population, and they can serve as the basis for predictions on future viability (Hare et al. 2010).

Effective population size estimates and census sizes (N_c) can be used together to create a N_e/N_c ratio. This ratio is useful because census size is easier to measure than effective population size (Ardren and Kapuscinski 2003). Therefore, further understanding of this ratio could help predict N_e by estimating N_c, thus predicting rate of genetic loss (Ardren and Kapuscinski 2003). This ratio is actively pursued in research; however, no consensus has been reached on what constitutes a typical range of N_e/N_c for natural populations (Nunney 1993; Scribner et al. 1997; Hoffman et al. 2004). Further, few studies have examined the temporal stability of N_e/N_c estimates (Arden and Kapuscinski 2003). Therefore, there is a need for improved understanding of this ratio (Luikart et al. 2010).

Many effective population size estimators share common assumptions (i.e. the population is sampled at random, there is no immigration, there is no selection, and there are no overlapping generations) (Waples 1989; Berthier et al. 2002; Tallmon et al. 2008; Waples and Do 2008). Of these assumptions, the need for discrete generations is often violated in natural populations, specifically in anurans (Beebee 2009). Research has shown that ignoring this assumption and proceeding with N_e estimates can lead to biased results because changes in allelic frequencies are not solely due to genetic drift but also to age-specific birth and survival rates (Waples and Yokota 2007; Robinson and Moyer

2012; Jorde and Ryman 1995). An estimator created by Jorde and Ryman (1995) does not require discrete generations and instead necessitates detailed inputs such as age-specific birth and survival rates and the grouping of samples into cohorts. Because much of this information is not known/readily available for natural populations, this estimator is rarely utilized. Instead, researchers have found procedures that minimize biases associated with overlapping generations. Waples and Yokota (2007) found that in temporal estimates, the bias largely disappears when sample points are 5–10 generations apart and suggest future studies can eliminate bias by sampling at least 3–5 generations apart. Further, Scribner et al. (1997) suggested that a population with high adult mortality is equivalent to discrete generations. And finally, Rowe and Beebee (2004) used larvae in each temporal sample and claimed that this method circumvented the need for discrete generations. However, this method has been contested and shown to cause a downward biased N_e estimate (Waples and Yokota 2007). With all things considered, there is no perfect N_e estimator for natural populations—all results should be interpreted with caution. Additionally, it is important to note that for species with overlapping generations, such as frogs, single-sample effective population size estimates most directly reflects the effective number of breeders for one reproductive season (N_b), as opposed to N_e (Waples 2005). The relationship between N_b and N_e can be complex, but in many cases N_b serve as comparable measure to N_e (Waples 2005).

Dusky gopher frogs (*Lithobates sevosus*), which are listed as Endangered by the United States Fish and Wildlife Service (2001) and Critically Endangered by the International Union for Conservation of Nature and Natural Resources (2008), function as a prime study species to determine the effects of population isolation on genetic diversity and population structure. First described by Goin and Netting (1940), the historic geographic range of *L. sevosus* occurred from the coastal plains of Louisiana and Mississippi to the western corner of Alabama, preferring longleaf pine (*Pinus palustris*) forests (Richter and Jensen 2005).

Now extirpated from the majority of its historical range, *L. sevosus* is only known to exist in two populations in coastal Mississippi. The population at Glen's Pond in Harrison County has a census population size estimate of 100–200 individuals, while the population in Mike's Pond in Jackson County appears to have fewer than 20 adults and could possibly be extinct based on egg counts (Richter et al. 2001; Richter et al. 2009). Additionally, in 2013 some individuals dispersed to Pony Ranch—a restored pond 1.3km from Glen's Pond—and natural breeding occurred in both 2013 and 2014 (Pechmann and Tupy 2013; Stephen Richter, personal communication) (Figure 1). Previous research documented a farthest distance travelled of 299 meters; however, the movement to Pony Ranch indicates a greater dispersal capability (Richter et al. 2001).

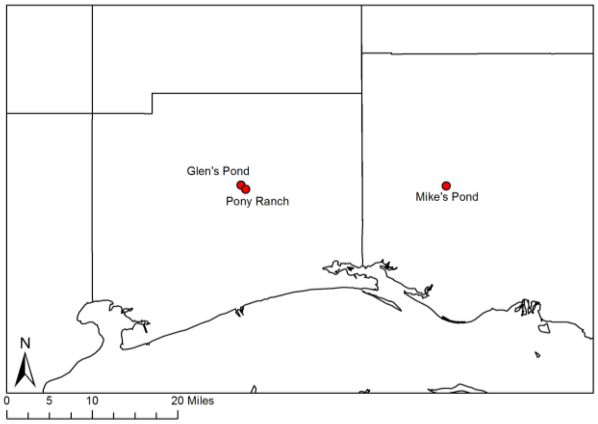

Figure 1. Map of approximate locations of Glen's Pond, Pony Ranch, and Mike's Pond in Harrison and Jackson County, Mississippi.

Extensive genetic and demographic research has been conducted with *L. sevosus*, and many conservation management strategies (i.e. headstarting which involves farm-rearing tadpoles to a certain age for release back into the wild) have been suggested and implemented (Richter et al. 2001; Richter and Seigel 2002; Richter et al. 2003; Richter et al. 2009). However, studies have yet to address how implemented conservation management activities, inbreeding, genetic drift, and lack of gene flow caused by population isolation have affected changes in genetic variability of *L. sevosus* over the past 18 years. Therefore my objectives were to (1) determine temporal trends in population genetic variation and implications for long-term viability of *L. sevosus* and (2) estimate effective population size (or effective number of breeders). This information will allow an evaluation of the effectiveness of current management strategies in terms of preserving and enhancing genetic variability. Furthermore, this study hopes to inform broadly applicable questions in the field of conservation biology, including 1) Is there temporal genetic structuring in isolated populations? 2) How do conservation management programs, like headstarting, impact genetic diversity over time? and 3) Are N_e/N ratios stable overtime in small, isolated populations?

MATERIALS AND METHODS

Sample Collection

Lithobates sevosus egg samples were previously collected from individuals at Glen's Pond in De Soto National Forest in Harrison County, Mississippi. The collection period spanned from 1997 to 2014 (Appendix A). A subset of years during the collection period was selected for analysis: 1997, 2005, 2008, 2013, and 2014. These years were selected to address the efficacy of current conservation management programs, with 1997 and 2005 largely representing pre-conservation management activities, and 2008, 2013, and 2014 representing post-conservation management activities. Individuals were genotyped for nine previously developed microsatellite loci, four loci from Richter and Broughton (2005) and five loci from Nunziata et al. (2012). Samples collected in 1997 and 2005 were previously genotyped for four microsatellite loci from Richter and Broughton (2005). These same samples were then genotyped for the additional five loci from Nunziata et al. (2012). For 2008, 2013, and 2014 samples, DNA was extracted using the Qiagen DNeasy Blood and Tissue protocol (Qiagen, Inc., Valencia, CA). All individuals were genotyped for nine microsatellite loci via PCR. The resulting PCR products were genotyped using an ABI 3100 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA). Allele lengths were scored using Gene Mapper version 4.0 (Applied Biosystems, Inc).

Statistical Analyses

Tests for linkage disequilibrium and Hardy-Weinberg equilibrium were conducted using FSTAT Version 2.9.3.2 (Goudet 1995). Tests between all pairs of loci were assessed for linkage disequilibrium, and deviations from Hardy-Weinberg equilibrium were assessed per locus per sample at the 5% nominal level with Bonferroni corrections.

The computer program MICROCHECKER was used to test for null alleles (Van Oosterhout et al. 2004). To determine the amount of genetic variation within *L. sevosus* for each year, observed (H_o) and expected heterozygosity (H_e) were calculated using GenAlex Version 6.501, and allelic richness using rarefaction and Wright's inbreeding coefficient (F_{IS}) were calculated using FSTAT Version 2.9.3.2 (Goudet 1995; Peakall and Smouse 2006, 2012). Friedman's tests were run to determine if significant differences in H_o, H_e, allelic richness, and F_{IS} existed between the sample years. Wilcoxon signed-rank tests for related samples were used to determine what specific pairs of years significantly differed. Simple linear regression was performed to determine if a correlation existed between the number of clutches deposited each year and the allelic richness and observed and expected heterozygosities. Appropriate data transformations were made to meet the assumptions of normality of residuals (square root for allelic richness and arcsine for H_o and H_e). Overall and pair-wise F_{ST} comparisons were calculated to determine if significant genetic differences existed temporally using FSTAT Version 2.9.3.2, with 95% confidence intervals calculated for the overall value via bootstrapping over loci (Weir and Cockerham 1984; Goudet 1995). I computed the regression of $F_{ST}/(1-F_{ST})$ on temporal distance, and a Mantel test was performed using FSTAT Version 2.9.3.2 to assess significance levels (Goudet 1995).

***N_b* and *N_e* Estimates**

Four methods to estimate effective size were used—two single-sample estimators and two temporal estimators. The single-sample estimates were calculated with Bayesian partial likelihood analysis using ONeSAMP 1.2 (Tallmon et al. 2008) and through a bias corrected linkage disequilibrium method (Waples and Do 2008; Peel et al. 2013), implemented using NeEstimator V2.01 (Do et al. 2014). The computer program

ONeSAMP 1.2 generated 50,000 simulated populations with effective population sizes drawn at random from previously specified upper and lower N_b limits ($4>N_b>500$). The N_b of simulated populations with summary statistics resembling that of the test population were used to infer N_b of the target population (Tallmon et al. 2008). The linkage disequilibrium method operates under the assumption that linkage disequilibrium (LD) of alleles will increase due to genetic drift. This increase is expected to create a greater amount of nonrandom associations between unlinked loci in small populations than in large populations (Hill 1981). Low frequency microsatellite alleles are shown to bias N_b estimates, so allele frequencies of 0.02 or less were removed prior to computing $N_{b,}$ as recommended by Waples and Do (2010). Effective number of breeders for both estimators were calculated for years 1997, 2005, and 2014 to represent effective population sizes pre and post farm-reared tadpole supplementation.

The temporal estimates of N_e were found using a pseudolikelihood method (Wang 2001) and a moment-based estimator (Waples 1989). Calculations for the pseudolikelihood method were computed using the computer program MNE 1.0 (Wang 2001). This model assumes that populations are closed, and that allele frequency changes are only caused by drift. Calculations for the moment-based estimator were computed using NeESTIMATOR V2.01 (Do et al. 2014). This program uses Waples' (1989) moment-based estimator, while providing three separate options for computing the standard variance in temporal changes in allele frequency (Do et al. 2014). Standard variance was computed using Nei and Tajima's (1981) method. This model has two sampling plans: in Plan I individuals are taken after a reproductive event; in Plan II individuals are taken before reproduction (Waples 1989). Plan II was implemented, as all of the samples are eggs. Effective population sizes for both estimators were determined for the pre-farm-reared tadpole supplementation phase (1997–2005), the post-farm-reared tadpole supplementation phase (2005–2014), and the entire sample period (1997–2014).

N_b/N_c estimates

Census size (N_c) values were estimated from demographic data based on the number of adults captured during each breeding season (Richter and Seigel 1996, 1997,1998; Seigel and Kennedy 2000; Seigel et al. 2001; Thurgate et al. 2002; Sisson 2003, 2004, 2005; Baxley and Qualls 2006, 2007; Sisson et al. 2008; Lee 2011; John Tupy, personal communication). Effective size to census size ratios were calculated for estimates derived from both the single-sample and temporal estimators. Ratios were calculated via the discrete generation method suggested by Waples (2005). However, amphibians have overlapping generations, and recognized bias and inaccuracies could be introduced into these estimates.

RESULTS

Genetic variation and structure

Null alleles were present in three years of the sampling period. Data were reanalyzed upon removal of loci with null alleles, and the results were similar. Additionally, there was no consistent pattern across years or loci, so all loci were included in final analyses. Deviations from Hardy-Weinberg Equilibrium occurred at locus Lica 41 in the year 2013. No pairs of loci exhibited linkage disequilibrium. The amount of genetic variation within *Lithobates sevosus* remained relatively stable throughout the sample period, with the exception of year 2013. Observed and expected heterozygosity ranged from 0.591 to 0.385 and 0.589 to 0.511, respectively, with significant differences occurring in observed heterozygosity (Figure 2A; Table 1) (observed heterozygosity: X^2= 13.422, d.f.= 4, P=0.009; expected heterozygosity: X^2= 4.899, d.f.= 4, P=0.298). After correcting alpha-values with Bonferroni corrections, the Wilcoxon signed-rank test did not indicate any significant differences between the pairs of years. However, without these conservative corrections, observed heterozygosity values in 2013 were different from all years with $p < 0.05$. Allelic richness ranged from 3.95 to 4.726, with a non-significant increase found over the sample period (Figure 2B; Table 1) (X^2= 2.416, d.f.= 4, P=0.660). Wright's inbreeding coefficient values ranged from 0.007 to 0.278, with an increase found over the sample period (Figure 2C; Table 1) (X^2= 10.044, d.f.=4, P=0.040). As with observed heterozygosity, year 2013 was different from other years. All populations exhibit a heterozygote deficiency, with F_{IS} values greater than 0. Simple linear regression showed no significant relationship between number of clutches deposited and allelic richness using rarefaction or number of clutches deposited and observed heterozygosity (Figure 3; F_{stat}=1.158, P=0.361, R^2=0.278;

F_{stat}=2.482, P=0.213, R^2= 0.453). A weak relationship was found between number of clutches deposited and expected heterozygosity (Figure 3; $F_{stat=}$ 8.884, P=0.059, R^2=0.748).

The overall F_{ST} value was 0.023 (95% CI 0.006–0.043), indicating weak genetic differentiation among years. Significant differentiation ($F_{ST} > 0$) existed between all pairs of sample years, with the exception of years 2008 vs. 2014 and 2005 vs. 2008 (Table 2). Pairwise F_{ST} comparisons did not show a significant trend of increasing genetic differentiation with time (Mantel test with 10000 permutations, R^2= 0.2598, P= 0.1297). However, a positive correlation was present—indicating that approximately one-fourth of the variation in genetic distance can be attributed to temporal distance (Figure 4).

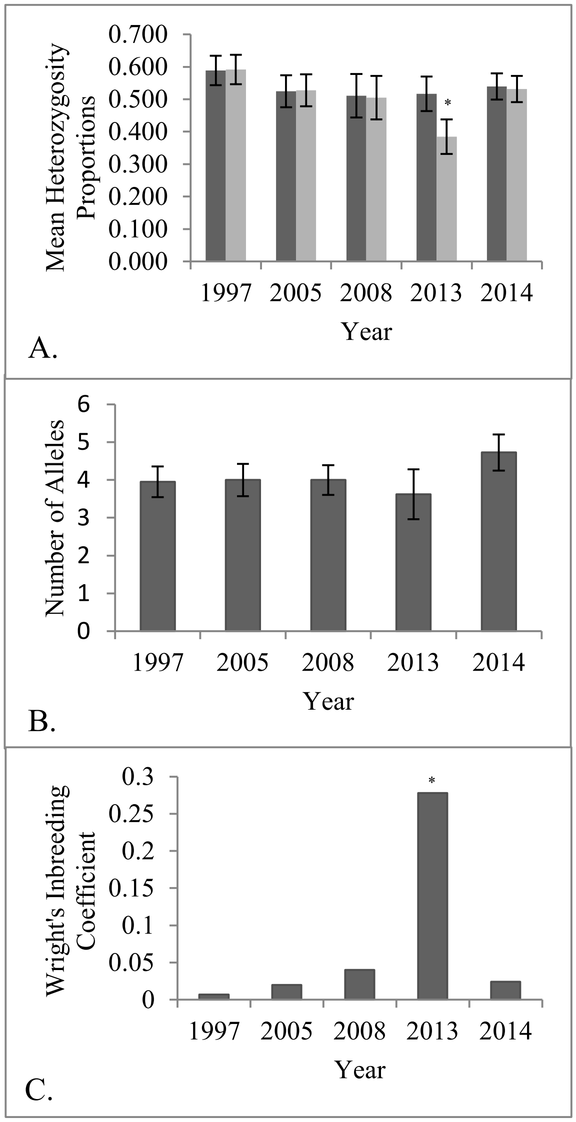

Figure 2. Average observed and expected heteroyzgosities (A), allelic richness using rarefaction (B), Wright's inbreeding coefficient, F_{IS} (C) values across nine microsatellite loci for *Lithobates sevosus* from 1997–2014. Error bars denote + 1 standard error. Asterisks (*) indicate significance between pairwise comparisons.

Table 1. Summary descriptive population genetic statistics for *Lithobates sevosus* from 1997–2014 based on nine microsatellite loci. Statistics include number genotyped (N), observed and expected heterozygosity (H_o and H_e), allelic richness corrected using rarefaction (AR), and Wright's inbreeding coefficient (F_{IS}). Values are mean (one standard error). Asterisks (*) indicate significance between pairwise comparisons.

Year	N	H_o	H_e	AR	F_{IS}
1997	49	0.591 (0.045)	0.589 (0.045)	3.950 (0.405)	0.007
2005	20	0.528 (0.049)	0.525 (0.049)	4.000 (0.427)	0.02
2008	19	0.505 (0.067)	0.511 (0.053)	4.000 (0.395)	0.04
2013	21	0.385 (0.053)*	0.516 (0.053)	3.619 (0.658)	0.278*
2014	51	0.532 (0.040)	0.539 (0.040)	4.726 (0.479)	0.024

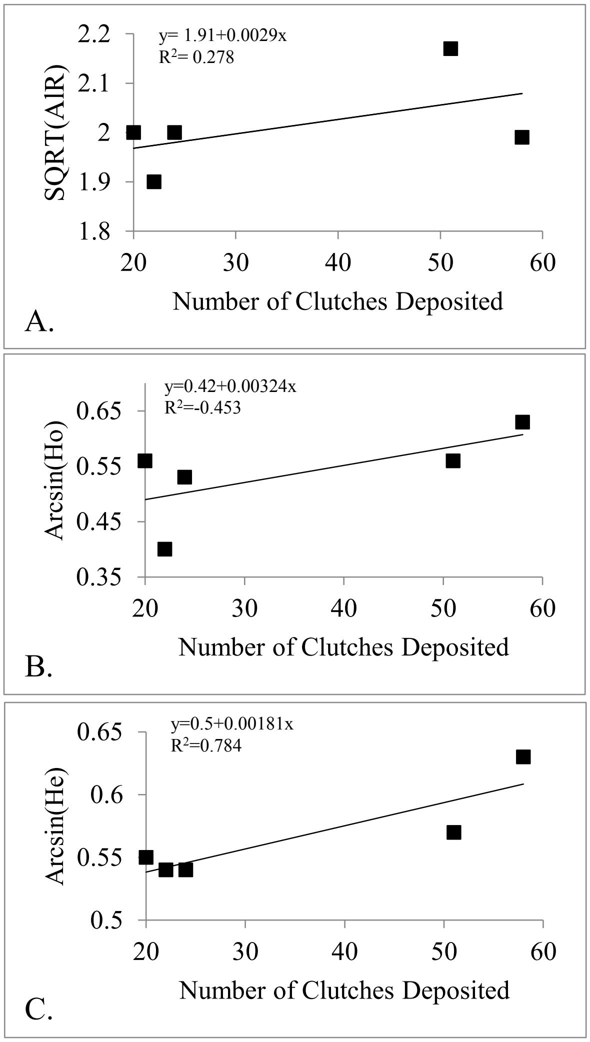

Figure 3. Simple linear regression of number of clutches deposited and square root transformed allelic richness (A), number of clutches deposited and arcsine transformed observed heterozygosity (B), and number of clutches deposited and arcsine transformed expected heterozygosity (C) for *Lithobates sevosus* from 1997–2014 based on nine microsatellite loci.

Table 2. Pairwise F_{ST} values for *Lithobates sevosus* from 1997–2014 based on nine microsatellite loci. Pairwise F_{ST} values calculated using FSTAT Version 2.9.3.2 (Goudet 1995).

	2014	2013	2008	2005	1997
2014	-	0.0308	0	0.0009	0.03
2013		-	0.0196	0.0241	0.0488
2008			-	0	0.022
2005				-	0.0302
1997					-

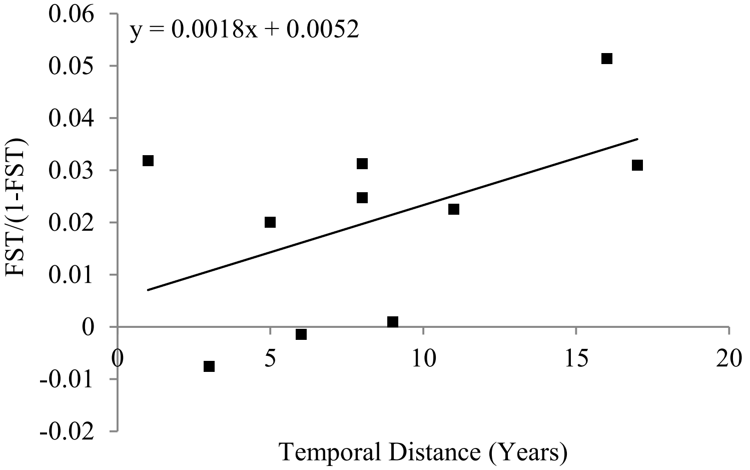

Figure 4. Isolation by temporal distance for pairwise F_{ST} comparisons for *Lithobates sevosus* from 1997-2014 based on nine microsatellite loci.

N_b and N_e Estimates

The two single sample estimators generated values with different trends over time (Table 3). The Bayesian partial likelihood method of Tallmon et al. (2008) produced estimates ranging from 30.5–58.6 over the sample period, with 2005 containing the smallest value and 2014 containing the largest. In contrast, the linkage disequilibrium method of Waples and Do (2008) produced estimates ranging from 56.4–∞, with 2014 containing the smallest value and 1997 containing the largest. An estimate of infinity reflects a lack of statistical power in estimating N_b, and is therefore omitted from interpretation (Waples 1989). The analyses for the Bayesian partial likelihood method omitted loci RsevC02 and RsevF01 due to missing data. To test for the effects of excluding these loci on the single-sample N_b estimates, the linkage disequilibrium method was rerun also excluding RsevC02 and RsevF01. The results produced N_b estimates around 1.2–1.7 times larger than those with nine loci.

The two temporal estimators produced somewhat similar results in the 1997–2005 and 1997–2014 sample periods (Table 4). However, estimates in the 2005–2014 sample period produced largely different results. In all instances, the pseudolikelihood method produced smaller estimates than the moment-based. Despite differences, both estimators exhibited a trend in which N_e increased over the study period.

Table 3. Effective number of breeders estimates with 95% confidence intervals via single-sample methods (Bayesian partial likelihood analysis (BPLA) of Tallmon et al. (2008) and linkage disequilibrium (LD) method of Waples and Do (2008)) for *Lithobates sevosus* based on nine microsatellite loci. N_b/N_c ratios estimated using the Bayesian partial likelihood analysis of Tallmon et al. (2008).

Year	BPLA	LD	N_b/N_c
1997	45.5 (31.4–54.9)	67.7 (32.3–381.4)	0.484
2005	30.5 (23.7–66.0)	∞ (31.4–∞)	0.526
2014	58.6 (43.0–144.0)	56.4 (29.9–165.3)	0.771

Table 4. Effective population size estimates with 95% confidence intervals via temporal methods (moment-based method of Waples (1989) and pseudolikelihood method of Wang (2001)) for *Lithobates sevosus* based on nine microsatellite loci. N_e/N_c ratios estimated using the pseudolikelihood method of Wang (2001).

Year	Moment-based	Pseudolikelihood	N_e/N_c
1997-2005	30.5 (12.3–91.4)	25.54 (13.87–62.64)	0.369
2005-2014	112.7 (29.3–∞)	46.63 (22.27–166.19)	0.707
1997-2014	51.6 (25.4–100.8)	32.99 (21.06–53.98)	0.484

N_b/N_c and N_e/N_c estimates

The estimates from the Bayesian partial likelihood analysis and the pseudolikelihood method were used to calculate the N_b/N_c and N_e/N_c ratios, respectively, because of the presence of finite estimates and finite confidence intervals for each sample year. N_b/N_c estimates ranged from 0.484–0.771, with estimates increasing with time. N_e/N_c estimates ranged from 0.369–0.707 and exhibit the same trend of increasing with time.

DISCUSSION

Genetic variation and structure

Population isolation and small population size are known drivers for reduction in genetic variation (Frankham 1996). Previous research found critically reduced levels of genetic variation in *L. sevosus* when compared to sister species *L. capito* and *L. areolatus* (Richter et al. 2009). The findings of this study are consistent with Richter et al. (2009) and suggest that these reduced levels have remained generally stable over the sample period 1997–2014.

In terms of trends in genetic variation, both levels of observed and expected heterozygosities decreased and then increased during the sample period. This trend could be attributed to the implementation of various management programs aimed at rescuing *L. sevosus* from extinction. Since 2002, a subset of eggs laid in Glen's Pond have been transferred to cattle tanks and reared until metamorphosis for release into the population (J. H. Tupy, unpublished data). Further, in years with short hydroperiods, water from an onsite well was used to prevent complete drying (Seigel et al. 2006; J. H. K Pechmann, unpublished data). These efforts have likely assisted in maintaining the levels of genetic variation and preventing further genetic erosion. Additionally, allelic richness remained relatively stable throughout the sample period, which provides further indication of the success of management programs.

Wright's inbreeding coefficient (F_{IS}) within *L. sevosus* increased over the sample period, with the largest amount of inbreeding occurring in 2013. These values represent the probability that two alleles are identical by descent—with a value of 0.278 in 2013 at the level expected of full sib (brother x sister) mating. This increase is due to the ongoing lack of gene flow, small population size, and life history (Richter et al. 2009; Frankham et al. 2004). The cause for this large amount of inbreeding in 2013 followed by an immediate reduction in 2014 is unknown, but likely due to the stochastic nature of *L. sevosus*' highly variable reproductive success and output (Richter et al. 2003). Few adults breed more than once, with an average rate of return between 16–22% (Richter and Seigel 2002). Therefore, each breeding season is largely dependent on new breeders. Sample year 2013 represents a subset of reproductively mature individuals with a reduced amount of genetic variation. Additionally, Richter and Nunziata (2014) found that inbreeding depression may be higher in some years and not others. Estimates of inbreeding in *L. sevosus* are in line with these findings.

As mentioned above, sample year 2013 serves as somewhat of an outlier to the other sample years, and it illustrates how populations with low amounts of genetic diversity also have high amounts of inbreeding. It is difficult to pinpoint a specific cause for this reduction in genetic diversity; however it is likely due to a suite of factors. First, a low reproductive effort (31 egg masses) could cause a drop in genetic diversity by having only a subset of the population represented. Also, reductions in genetic diversity may also experience time lags. This is important to note because only a small breeding event occurred in 2011 due to drought, and no tadpoles were available for headstarting or translocation (Lee 2011). The substantial drop in genetic diversity and increase inbreeding in year 2013 could be a delayed effect of the environmental stress in year 2011. Year 2013 serves as a reminder of the effects of reproductive and environmental stochasticity on genetic variation.

Throughout the sampling period, a variable number of clutches were deposited in each breeding season. In some cases, number of clutches deposited can affect genetic variation estimates. However, no significant correlation was found between allelic richness and number of clutches deposited or observed heterozygosity and number of clutches deposited. Alternatively, a positive relationship was found between expected heterozygosity and the number of clutches deposited—accounting for 74.8% of the variation within the expected heterozygosity estimates.

Richter et al. (2009) found evidence of population bottlenecks in *L. sevosus* due to significant heterozygote excess and a mode-shift in allele frequencies. Although bottlenecks can severely reduce the population size and amount of genetic variation within a population, the data from this study suggest extensive monitoring and management programs for *L. sevosus* have succeeded in generally maintaining or even increasing genetic variation over time.

The temporal stability in population structure is not often measured due to the large amount of data required; however, there is an acknowledged need for these estimates (Hoffman et al. 2004). Previous research found temporally stable genetic structure within other ranids, specifically in *Rana pipiens.* However, our findings suggest a weak genetic structuring over time in the dusky gopher frog. This weak temporal structuring is likely the result of random genetic drift and inbreeding caused by nonrandom mating due to a small population size (Frankham et al. 2004).

N_b and N_e Estimates

Effective population size is a significant parameter that influences population viability and conservation management decisions. This estimate can be interpreted both at the global and the local scale, depending on the scope of the project. At the global scale, information such as long-term viability, adaptive potential, and maintenance of genetic variation can be gained. At the local scale, immediate threats to population persistence can be revealed (Luikart et al. 2010). Because *L. sevosus* exists as one population, N_e (or N_b) estimates can be interpreted at both scales. Additionally, it is important to note that the N_e and N_b estimates violate the assumption of discrete generations, which could result in biased or inaccurate results. However, a combination of low adult survivorship and low rate of return after the first breeding event help to eliminate the biases associated with this assumption violation (Scribner et al. 1997; Richter and Seigel 2002). Further, while cognizant of possible limitations, few species have discrete generations, and these models have been widely applied systems with overlapping generations (Scribner et al. 1997; Robinson and Moyer 2002; Hoffman et al. 2004; Waples 2005).

In general, the two temporal methods produced comparable results, with a notable exception occurring in the 2005–2014 time period. During this period, estimates for the moment-based method produced results more than twofold higher than the psuedolikelihood method. This discrepancy could be caused by an upward bias in moment-based estimates in the presence of many rare alleles, while likelihood-based methods have been shown to be less biased and more precise in such situations (Wang 2001). Furthermore, sample sizes less than 25 individuals are more likely to produce estimates with large biases (Waples and Yokota 2007). The 2005 reproductive event consisted of only 20 egg masses, which is likely the cause of wide confidence intervals reaching infinity in Waples (1989) moment-based estimator and estimates of infinity in Waples and Do (2008) LD method.

Despite the mentioned differences, the estimates of Waples (1989), Wang (2001), and Tallmon et al. (2008) all followed similar, positive trends. Effective size estimates increased with the implementation of conservation efforts, like supplementation of farm-reared tadpoles. This trend suggests that the conservation management programs in place for *L. sevosus* are effective at both conserving and increasing effective population size. The Waples and Do (2008) LD method, however, did not exhibit increasing estimates following tadpole supplementation. To elucidate this difference, it is important to distinguish between two effective size concepts. Both models of Tallmon et al. (2008) and Waples and Do (2008) are considered inbreeding effective population size estimates (N_{eI}), or in our case N_{bI}. These estimates are concerned with the loss of heterozygosity, while variance effective population sizes (N_{eV}) (i.e. Waples 1989; Wang 2001) are concerned with changes in allele frequency over time (Crow and Denniston 1988). N_{eV} tends to be more sensitive to early population size changes, while N_{eI} will not change until inbreeding accumulates (Luikart et al. 2010). Thus, the N_{eV} models are more likely to recognize the recent population changes due to the new conservation management practices. Additionally, due to the difference in concept, our temporal and single-sample estimates could be based off of different time periods, making comparisons difficult and impractical. However, despite these caveats, the estimates produced by the single-sample Bayesian partial likelihood method are similar to demographic data collected over the sample period. During the middle of the sample period, there were successive years with little to no reproductive output because of drought and an emergent disease—*Dermomycoides* sp., a pathogen which caused major mortalities in 2003. Following conservation management programs, reproductive output increased. (Richter et al. 2003; Thurgate et al. 2002; Sisson 2003, 2004, 2005; Baxley and Qualls 2006, 2007; Sisson et al. 2008; Lee 2011; John Tupy, personal communication; J. H. K. Pechmann, unpublished data). The single-sample Bayesian partial likelihood method estimates follow the same decreasing and then increasing trend, bolstering our confidence in the accuracy of this estimate. Additionally, this increase in N_b and N_e estimates with time further demonstrates that the reduction in genetic diversity in year 2013 does not appear to be affecting the number of genetically contributing individuals each breeding season.

N_b/N_c and N_e/N_c estimates

Frankham (1995) cites three main factors influencing effective size to census size ratio estimates: fluctuations in population size, variance in individual reproductive success, and unequal sex ratios. There is no consensus on what is "normal" for natural populations, with Frankham (1995) suggesting ratios of around 0.11, while Nunney (1993) proposes that theoretically values should be around 0.5. Specific to anurans, Hoffman et al. (2004) estimated N_e/N ratios of at least 0.10 and indicated that this ratio is probably higher. However, few additional studies have examined what is a typical ratio for anurans, if these ratios vary for different groups of anurans, and if ratios are temporally stable. Our estimates (0.369–0.771) are in line with the theoretical values of Nunney (1993). Jehle et al. (2005) found similar, large N_e/N_c ratios in amphibians (specifically newts) with small population sizes. Similarly, species of conservation concern are seen to have larger N_e/N_c (or N_b/N_c) ratios than stable populations (Palstra and Ruzzante 2008). This phenomenon was first described by Ardren and Kapuscinski (2003) as genetic compensation—an increase in N_e/N_c (or N_b/N_c) ratios at low population sizes. Genetic compensation suggests that when population sizes are low, most individuals participate in breeding activities. In an evolutionary context, this compensation would lessen genetic variation reduction in times with low population numbers (i.e. bottlenecks) by reducing inbreeding. These results suggest that *L. sevosus* might be experiencing genetic compensation in concert with effective conservation management programs that have stabilized N_e/N_c (and N_b/N_c) ratios and genetic variation over the sample period.

CONCLUSIONS

Overall, this study illustrates three major findings. First, the current management programs in place for *L. sevosus* appear to be successful at preventing further genetic erosion. The addition of headstarting tadpoles appears to be an effective way of maintaining genetic variation in study systems subject to variable reproductive output due to environmental stochasticity or disease. However, further efforts are still needed to continue restore genetic variation to higher levels. Supplementing the natural population with the captive zoo populations could aid in this effort, although careful consideration must be taken to eliminate the risk of transferring pathogens from captive to natural populations. And, more research is needed to determine the best life stage for release to ensure site fidelity. Second, *L. sevosus* exhibits weak temporal genetic structuring. This differentiation is likely due to increased effects of genetic drift and inbreeding in small, isolated populations. Future studies should continue to focus on temporal genetic structuring, as it provides genetic information on microevolutionary changes. Third, the effective population size and effective number of breeders estimates can be variable, but overall, effective population size of *L. sevosus* is around half that of the estimated census size. These N_e/N_c and N_b/N_c estimates bolster the literature on anuran effective population size, and suggest that genetic compensation might be acting within this study system, aiding in the maintenance of genetic diversity. Temporal estimates demonstrated that N_e increased, potentially because of headstarting efforts, indicating further success of the conservation plans. Despite the success of multiple management programs in place, *L. sevosus* still has a reduced amount of genetic variation in comparison to its sister species, and would benefit from the establishment of nearby populations in order to improve the future viability of this species. The recent breeding events at Pony Ranch provide encouraging possibilities for the establishment of a functioning metapopulation.

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APPENDIX

APPENDIX A

Tissue samples of *Lithobates sevosus* collected at Glen's Pond in Harrison County, Mississippi, from 1997 to 2014. Egg samples were collected from each clutch deposited. NB = no breeding. Adult samples are toe clips collected from each adult entering the pond to breed.

Year	Tissue Type	#Collected	Year	Tissue Type	#Collected
1997	Eggs	58	**2006**	NB	0
1998	Eggs	37	**2007**	Eggs	6
1999	NB	0	**2008**	Eggs	50
2000	NB	0	**2009**	Eggs	30
2001	Adults	46	**2010**	Eggs	39
2002	NB	0	**2011**	NB	0
2003	Adults	85	**2012**	Eggs	54
2004	Adults	48	**2013**	Eggs	31
2005	Eggs	20	**2014**	Eggs	51