Conservation Genetics of Louisiana Pine Snakes, *Pituophis ruthveni*

Final Report to the Louisiana Department of Wildlife and Fisheries

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INTRODUCTION

The Louisiana Pine Snake (*Pituophis ruthveni*) persists as small, fragmented populations scattered across its' historic range (Rudolph et al. 2006). Most, if not all, of these populations are vulnerable to habitat degradation and the impacts of fragmentation (Rudolph and Burgdorf 1997; Himes et al. 2006; Rudolph et al. 2006). Substantial data now exists to suggest that habitat conversion, vehicle related mortality, and especially, alteration of the fire regime, are responsible for population decline and local extirpation of Louisiana Pine Snakes (Rudolph and Burgdorf 1997; Himes et al. 2002; Ealy et al., 2004; Rudolph et al. 2006).

There is growing concern that inbreeding and loss of genetic diversity may be one of the problems faced by Louisiana Pine Snakes. Wild populations are experiencing increasing fragmentation and the 4-7 existing populations are widely separated geographically with no possibility of demographic or genetic exchange. Trapping surveys and historical data suggest that population numbers are declining in all areas of the species' distribution (LPS meetings 2014, Rudolph et al. In Prep). Modeling based on these data indicates that all populations are in severe decline and strongly suggests extirpation in the range of 2000-2020. The models suggest that the Texas populations will be the earliest to reach extirpation, and in fact, no new animals have been detected in Texas since 2008. The authors conclude that there is a high probability that all wild populations will be extirpated within the decade. In addition, due to the intractable nature of several of the major threats, there is a very low probability of management actions that will be sufficient to prevent extirpation. Consequently, the future survival of the Louisiana Pine Snake will likely be dependent on captive breeding and reintroduction to the small fragments of available habitat that can best be managed to mitigate for the known threats.

As populations fragment, movement and migration is reduced, effectively decreasing gene flow (Keller and Largiadèr 2003; Wendeler et al. 2003; Andersen et al. 2004; Clark et al. 2010). Habitat fragmentation also decreases effective population size (N_e) that, when coupled with reduced gene flow, results in smaller populations that are more susceptible to genetic drift, inbreeding, and loss of heterozygosity (Lacy 1987; Lande, 1995). For example, timber rattlesnake populations isolated and fragmented by roads exhibit lower genetic diversity than contiguous populations (Clark et al. 2010). In the common European adder, *Vipera berus*, isolated populations had higher levels of inbreeding and lower

genetic diversity than non-isolated populations (Madsen et al. 1996; Ursenbacher et al. 2009). The isolated populations also exhibited smaller litter sizes and a higher proportion of deformed and still-born offspring (Madsen et al. 1996).

No specific data are available concerning the demographic or genetic situation for the wild populations, beyond the observation that populations have not responded to significant habitat improvements over the last 20 years. However, there are disturbing indications in the captive population that are possibly indicative of genetic issues. A high percentage of individuals are not producing fertile eggs. Based on incomplete data, hatching success is less than 30%, and may be significantly lower. Data are not available for proportion of infertile eggs, developmental abnormalities, etc., but there are indications that they may be significant.

Little was known about levels of inbreeding and genetic diversity in the Louisiana Pine Snake, but it was clear that wild populations are extremely small and fragmented. Trapping surveys indicate that wild populations are declining rapidly (LPS Stakeholders Meeting 2014; Rudolph et al., in prep), suggesting the population is experiencing a bottleneck. Concerns about the status of Louisiana Pine Snake populations led to the establishment of a captive breeding program. The captive population has been segregated into three groups based on geographic regions delineated by major rivers, presumed to be important dispersal barriers. One group, managed by Dr. Steve Reichling of the Memphis Zoo, originated from 18 wild-caught founders mostly from Bienville Parish, Louisiana. The second group, managed by David Heckard at the Audubon Zoo, originated from two wild-caught founders from Fort Polk, Louisiana. The third group, managed by Gordon Henley at the Ellen Trout Zoo, consists of 6 founders from Texas. With one exception, these three captive breeding populations are not being outcrossed to each other; i.e., the three regional captive populations remain genetically isolated from each other. Furthermore, the logistical difficulty in moving snakes has resulted in cases of inbreeding, such as full sibling crosses and parent-offspring crosses (S. Reichling and C. Rudolph, personal communication).

The lack of data on genetic diversity in wild populations leads to a concern about breeding efforts within the captive population. By definition, using only a subset of the wild population as founders of the captive population represents a bottleneck event. This bottleneck event follows a likely bottleneck event experienced by wild populations. Hence, the captive-bred population is the product of two events that

may have reduced genetic diversity, or could lead to loss of genetic diversity within a few generations (Frankham 2005). This loss of genetic diversity may be exacerbated by inbreeding in the captive populations (Frankham 2005; Hedrick and Fredrickson 2010). While the effects of bottlenecks and inbreeding on the fitness of the captive offspring, as well as the general captive population, remain unknown, studies on numerous vertebrates send an ominous warning. Examples of the negative effects of inbreeding on fitness include Mexican wolves (Fredrickson et al. 2007); Florida panthers (Johnson et al. 2010), anurans (Andersen et al. 2004), birds (Heber et al. 2014), and other snakes (Madsen et al. 1996; Gibbs and Chiucchi 2012). Furthermore, captive-bred Louisiana Pine Snakes are being released as part of a reintroduction program. As such, these individuals represent a founder population and it is important to maximize their genetic diversity (Miller et al. 2009; Hedrick and Fredrickson 2010). Accordingly, it is important to quantify the genetic diversity of both wild and captive Louisiana Pine Snake populations so that future management decisions can take this variable under consideration.

The objectives of this study are:

1. Determine the level of genetic structuring among natural populations of Louisiana Pine Snake. These data will help determine whether the natural populations exhibit considerable isolation from each other and, therefore, are unique. If there is not dramatic genetic structure among populations, then there is little reason to be concerned that the populations are genetically unique and crossing of captive snakes from different regions may be a good strategy to maintain as much genetic diversity as possible.

2. Identify the most likely number of genetic groups or "clusters" in the absence of any geographical information and identify individuals that do not strongly assign to any population. This will be especially useful in case there are any captive individuals that have questionable parentage. This will also be informative for out-breeding strategies. If these data suggest Louisiana Pine Snakes do not form genetic groups that are associated with current regional divisions (i.e., Bienville Parish, Fort Polk, and Texas), then it will suggest the different regions are not genetically unique and individuals from those different regions can be crossed with each other.

3. Quantify genetic diversity within and among wild and captive populations of Louisiana Pine Snakes. As discussed, loss of genetic diversity has been associated with reduced fitness and population size in numerous vertebrates. Hence, quantifying genetic diversity is critical for future management strategies regarding a species as rare and facing as many threats as the Louisiana Pine Snake. At the very least, if we find that genetic diversity is reasonably high in Louisiana Pine Snakes, it can be considered a lower priority problem. On the other hand, if we find genetic diversity is low, management to increase genetic diversity may help to ultimately raise population fitness (Madsen et al. 1999; Madsen et al. 2004).

4. Genotype captive snakes to maximize out-crossing. By genotyping individuals for microsatellite loci, we will able to make informed decisions about how to cross individuals to maximize genetic diversity, should that become a concern in Louisiana Pine Snakes. If outcrossing is adopted as a management strategy for captive individuals, efforts to achieve out-crossing should be as stream-lined as possible given that time may be a critical concern for this species. Louisiana Pine Snakes exhibit relatively low reproductive rates and only a few pairs are available for breeding. Hence, achieving a large number of captive-bred individuals can take considerable time. If breeding individuals are paired to maximize outcrossing, a genetically diverse population will be achieved sooner.

TECHNIQUES

SAMPLE COLLECTION

Wild Samples. Tissue samples from wild-caught snakes were grouped into one of three geographic regions: northern Louisiana (NLA), southern Louisiana (SLA), and Texas (TX). This a priori grouping was based on the hypothetical boundaries created by two major rivers: the Red River and the Sabine River. The Red River forms part of the northern state boundary of Texas and then bisects Louisiana in a northwest-southeasterly direction. Hence, the Red River acts as a potential barrier between NLA-TX and NLA-SLA. The Sabine River forms part of the state boundary between Louisiana and Texas, acting as a potential SLA-TX barrier. Given the much larger size of the Red River, it may act as a more effective

barrier than the Sabine. Wild-caught samples include many collected recently and used immediately for this study, but also some caught years ago that were kept frozen. A small number of the wild-caught snakes were kept in captivity and are currently part of the captive breeding program.

Samples were obtained through both governmental and non-governmental sources that are part of the Louisiana Pine Snake Stakeholders group. Most samples from northern Louisiana were from snakes originating in Bienville Parish, Louisiana. Most samples from southern Louisiana were from snakes captured on Fort Polk, a United States Army base. Samples from Texas originated from a broad geographic area throughout eastern Texas, including Angelina National Forest, Sabine National Forest, and private land. The acquisition of samples was dictated by availability rather than a rigorous sampling regime given the extremely limited number of individuals that are captured in the wild. Sample collection also spanned a broad time period, with some samples collected over 20 years ago. Hence, it is important to note that results presented here do not necessarily represent the current situation in wild populations. Rather, results may be biased towards a situation when Louisiana Pine Snake populations were larger, given the continuing evidence of population decline over the years (Rudolph et al., in prep).

Captive Samples. Captive Louisiana Pine Snakes are currently housed in zoos all over the United States. Tissue samples from many of these captive snakes were collected at the zoos and sent to the authors as part of this study. Most of these snakes are descendants from a group of snakes collected from northern Louisiana in the 1990s. These samples form the bulk of captive tissue used for this study, with most individuals being held at the Audubon Zoo and the Memphis Zoo. However, snakes from Texas and southern Louisiana are also in captivity, albeit relatively few. Additional tissue samples have been obtained from offspring from some captive snakes that are part of the reintroduction program. DNA from these samples has been extracted and they are currently being amplified and genotyped. However, they are not included in these analyses as 1) they are still being genotyped and 2) they are not part of the actual captive population and are not part of the potential breeding population. Zoos contributing tissue include: Audubon Zoo, New Orleans, LA; Dallas Zoo, Dallas, TX; Ellen Trout Zoo, Lufkin, TX; Gladys Porter Zoo, Brownsville, TX; Jacksonville Zoo, Jacksonville, FL; Knoxville Zoo, Knoxville, TN; Little Rock

Zoo, Little Rock, AR; Memphis Zoo, Memphis, TN; North Carolina Zoo, Asheboro, NC; Orianne Society, Athens, GA; Tulsa Zoo, Tulsa, OK; Woodland Park Zoo, Seattle, WA.

DNA Extraction, Amplification, and Genotyping

Whole genomic DNA was extracted from blood, muscle, or shed skin samples originating from wild and captive Louisiana Pine Snakes. Muscle tissue was only collected from deceased snakes that had been frozen, often for years. Tissue from live snakes consisted of either shed skin or blood collected from the caudal vein and then stored in pure, non-denatured ethanol. Using modified protocol techniques, DNA was extracted using Gentra Puregene Tissue Kits or Gentra Puregene Mousetail Kits (Qaigen, Inc., Valencia, CA, USA). Fifteen microsatellite loci (Kwiatkowski et al. 2010) were amplified using M13(-21) labeled primers and modified techniques from Schuelke (2000). This process uses three primers in the initial amplification reaction: a sequence specific forward primer with a M13(-21) tail at the 5' end, a sequence-specific reverse primer, and the universal fluorescent-labeled M13(-21) primer (Integrated DNA Technologies, Coralville, Iowa, USA). This technique entails two sets of thermocycling; the first incorporates the forward primer as with any PCR and once the forward primer is used up, a second set incorporates the universal M13(-21) primer with the dye. The advantage of this system is it is much less expensive than typical fluorescently labeled primers. PCR conditions were optimized for each locus with annealing temperature and number of cycles varying among loci (Table 1). PCR was done using GoTaq® G2 Flexi DNA Polymerase kits from Promega (Madison, Wisconsin, USA), with reaction volumes of 25 µL including 50-100 ng of DNA, 5 µL of 5X buffer, 2.0 mM $MgCl_2$, 0.14 µM M13-labeled forward primer, 0.25 µM reverse primer, 0.012 µM M13 forward (-29) 800 or 700 primer, 0.02 units/µL of taq polymerase, and water to bring to volume. Reagent amounts may have varied somewhat depending on the locus. PCR cycles varied among loci (Table 1), but followed the general format of:

1. Initial denaturing period (1 cycle):	94°C, 5 min
2. Amplification cycles (variable; see Table XX):	94°C, 30 sec Variable annealing temperature, 30 sec 72°C, 25 sec
3. M13(-21) cycles (8 cycles):	94°C, 30 sec 53°C, 30 sec 72°C, 25 sec
4. Final extension period:	72°C, 7 min
5. Holding:	4°C

Table 1. Annealing temperatures and number of cycles used during the amplification phase of PCR for each locus. Primer names follow (Kwiatkowski et al. 2010).

Primer	Annealing Temperature	Number of Amplification Cycles
Piru5	60.0°C	27
Piru8	60.3°C	28
Piru9	60.5°C	26
Piru12	59.5°C	26
Piru13	61.1°C	26
Piru15	61.1°C	26
Piru16	61.6°C	27
Piru25	60.6°C	27
Piru27	61.7°C	25
Piru31	59.6°C	26
Piru33	60.1°C	27
Piru34	59.4°C	26
Piru35	62.6°C	25
Piru42	61.8°C	25
Piru48	59.8°C	26

PCR products were genotyped using a LI-COR 4300 DNA Analyzer (LI-COR, Lincoln, Nebraska, USA) at Stephen F. Austin State University. Samples were loaded on a 6.5% polyacrylamide gel (KB^{Plus} Gel Matrix, LI-COR) after a denaturation period of 95°C for 3 min with 2 mL of added loading buffer. Gels were run in 1X TBE buffer under recommended conditions with a 25 min pre-run (before loading samples) followed by a 3 hr run (1500 V,40 mA, 45°C) using either the 700 nm or 800 nm channels. Samples were genotyped by comparison to DNA fragments of standard size (50-700 bp IR Dye ®, LI-COR) using SAGA^{GT} software (LI-COR) integrated with the LI-COR 4300. Before final genotyping, the automated

assignment of gel lanes and DNA standards by SAGA2 was visually inspected. Scored alleles were also visually inspected before final genotyping.

GENETIC DATA

Wild Samples. A total of 69 wild-caught samples were used for the analyses from northern Louisiana (N = 26), southern Louisiana (N = 29), and Texas (N = 14). Deviations from Hardy-Weinberg equilibrium (HWE) among loci were investigated using GENEPOP v4.2 (Raymond and Rousset 1995) with critical P-values adjusted using Bonferroni corrections (Rice 1989). The proportion of null alleles among loci was examined using CERVUS 3.0.6 (Kalinowski et al. 2007).

Captive Samples. Analyses of captive samples were more limited given the low number of samples, especially from Texas and southern Louisiana. Hence, most analyses focused on the northern Louisiana samples (N = 42). Some analyses of captive TX and SLA populations may be included, but they should be interpreted with caution. The SLA samples comprise only a single adult pair and their offspring for a total of six samples. The captive TX samples are almost all wild-caught adults now in captivity as part of the breeding program, with the exception of one sample from a recent offspring of a pair of the adults, for a total of seven samples. Deviations from HWE were investigated as described above for the wild samples, as were null alleles.

GENETIC STRUCTURE AND CLUSTERING

Genetic structure among the three wild geographic populations was investigated with pairwise F_{ST} (a measure of genetic variability among populations) values using FSTAT version 2.9.3.2 (Goudet 1995). F_{ST} values were not calculated for the captive SLA and TX populations given that so few samples are available for each of them and those from SLA are from a single parent pair and their offspring.

Although the current captive population of Louisiana Pine Snakes is segregated based on geographic region, this does not necessarily accurately represent genetic clustering since little is known about isolating mechanisms acting during the evolutionary history of this species. The number of genetic clusters, K, was estimated using STRUCTURE 2.3.4 (Pritchard et al. 2000). STRUCTURE uses Markov Chain Monte Carlo (MCMC) simulations to estimate the log likelihood of a designated number of

populations. A burn-in of 75,000 iterations was followed by 200,000 iterations using the admixture model with correlated allele frequencies. We conducted 10 independent runs for each value of K ranging from 1 to 8. Results from these simulations can be difficult to interpret as the posterior probability estimates for the ideal K do not necessarily give a straight forward indication of which value of K accurately represents the data; rather, the posterior probability estimate, L(K), is considered an *ad hoc* guide (Pritchard et al. 2000; Evanno et al. 2005). However, a more reliable method of detecting the true value of K relies on the second order rate of change of the likelihood function (ΔK). This procedure compares the change in the posterior probability value from one value of K to the next, using the fact that once the correct number of clusters is reached, the mean of L(K) tends to peak or become asymptotic and its variance tends to increase (Evanno et al. 2005). Hence, plots comparing K with L(K) and ΔK reveal the probable number of clusters. These plots were created in STRUCTURE HARVESTER v0.6.94 (Earl and vonHoldt 2012). After identifying the ideal K, the proportion of each cluster (Q) comprising the different populations was visualized using the STRUCTURE run with the highest likelihood for that K.

HETEROZYGOSITY AND INBREEDING

Allele frequencies, observed heterozygosity, and expected heterozygosity were determined using CERVUS 3.0 (Kalinowski et al. 2007) for all microsatellite loci in the three geographic populations. Genetic diversity was also estimated with mean number of alleles for each locus and allelic richness (the number of alleles controlling for sample size) using FSTAT version 2.9.3.2 (Goudet 1995). Inbreeding levels were estimated using F_{IS} , a measure of inbreeding or heterozygote deficit within a population. F_{IS} ranges from -1 for a completely outbred population to +1 for a completely inbred population. A random mating within populations is expected to have an F_{IS} value of zero. F_{IS} was calculated in FSTAT 2.9.3.2. A second estimate of inbreeding, the average multilocus heterozygosity (MLH) was also calculated for each individual as the total number of heterozygous loci divided by the total number of genotyped loci (Gibbs and Chiucchi 2012). Mean MLH was calculated for comparing populations. There are so few captive TX individuals that they are not included in the analyses, and because the captive SLA samples are all from one family group (one set of parents and their offspring), their data should be interpreted with caution.

GENETIC BOTTLENECK

Evidence for recent genetic bottlenecks was tested using BOTTLENECK 1.2.02 (Piry et al. 1999). BOTTLENECK 1.2.02 uses two tests for detecting bottlenecks. The first looks for the hetereozygosity excess that can result from reduced population size (Cornuet and Luikart 1996; Piry et al. 1999). Testing for heterozygosity excess can be done using three models that differ in how mutations occur. Generally, a stepwise mutation model (SMM) or two-phase model (TPM), rather than the infinite alleles model (IAM), is considered more representative of the mutation process for short tandem repeating sequences such as microsatellites, especially those with longer repeat segments (e.g. Cornuet and Luikart 1996; Piry et al. 1999). However, the SMM and TPM tests are more conservative and can have less power for detecting heterozygosity excess, making them more prone to false negatives (Cornuet and Luikart 1996; Cristescu et al. 2010). Furthermore, populations that have undergone severe size reduction, which may be the case for Louisiana Pine Snakes, may have reduced polymorphism among loci making bottleneck detection under SMM difficult (Cornuet and Luikart 1996). Finally, recent tests suggest that IAM best detects known bottlenecks (Cristescu et al. 2010). Considering all of these concerns, data here were tested under both IAM and SMM, as well as under TPM with a variance of 15% and SMM contributing 50%. We have selected 50% given the conflicting recommendations of different studies which either used a large contribution of SMM (Chiucchi and Gibbs 2010) or a small contribution (Cristescu et al. 2010). For all three models, tests were run using 5,000 iterations. Results of Wilcoxon tests are reported.

The second test, the mode-shift test, examines the frequency distribution of alleles. When populations are in equilibrium and have not gone through a bottleneck, a large proportion of alleles should be at low frequency and a smaller proportion of alleles at intermediate frequencies, giving an L-shaped frequency distribution (Luikart et al. 1998). In bottlenecked populations, low frequency alleles are most likely lost, shifting the frequency distribution away from an L-shape. Mode-shift tests were done using BOTTLENECK 1.2.02.

RESULTS

GENETIC DATA

Wild Samples. Out of 45 loci/population tests, six (13%) deviated from HWE. Of these six, the SLA and TEX populations only deviated from HWE once each (loci Piru9 and Piru15, respectively). When considering each of the 15 loci, there was never more than one population out of the three that deviated from HWE. One locus, Piru31, had high null allele frequency in all three populations, so it was eliminated from analyses. For the captive populations, only two loci deviated from HWE, both in NLA. Locus Piru31 once again had a high proportion of null alleles and was eliminated from the analysis. All analyses of captive SLA and TX populations should be interpreted with caution as they both contain very few samples (N = 6) and SLA consists of a single family group (one pair with their four offspring).

GENETIC STRUCTURE AND CLUSTERING

There was only modest structuring among the three wild populations with F_{ST} values below 0.1 for all pairwise comparisons (Table 2). The largest difference was between the two Louisiana populations, followed by the NLA-TX comparison. F_{ST} between TX and SLA was the smallest. F_{ST} values between the captive NLA population and the wild populations were also small, indicating little genetic structuring among them. Not surprisingly, the captive NLA-wild NLA pairwise value was smallest (Table 2).

Table 2. F_{ST} values for pairwise comparisons among the three wild populations and the captive NLA population.

	NLA	SLA	TEX	Captive NLA
NLA	*	0.060	0.048	0.021
SLA		*	0.026	0.067
TEX			*	0.061
Captive NLA				*

Plots based on ΔK indicate there are four genetic clusters in wild Louisiana Pine Snakes (Figures 1 and 2). The allelic contributions of these four genetic groups to individuals in the three geographic populations can be seen in Figure 3, and the proportion of genetic clusters to each population in Figure 4. One set of

alleles is unique primarily to the NLA population (yellow in Figures 3 and 4). Another set of alleles is unique primarily to the SLA population, although a small proportion of these alleles are found in the TX population and an even smaller proportion in NLA (blue in Figures 3 and 4). A third set of alleles is shared primarily between NLA and TX, with a small proportion also being found in SLA (green in Figures 3 and 4). Finally, the fourth set of alleles is found in all three populations, but is shared primarily between the SLA and TX populations (red in Figure 3).

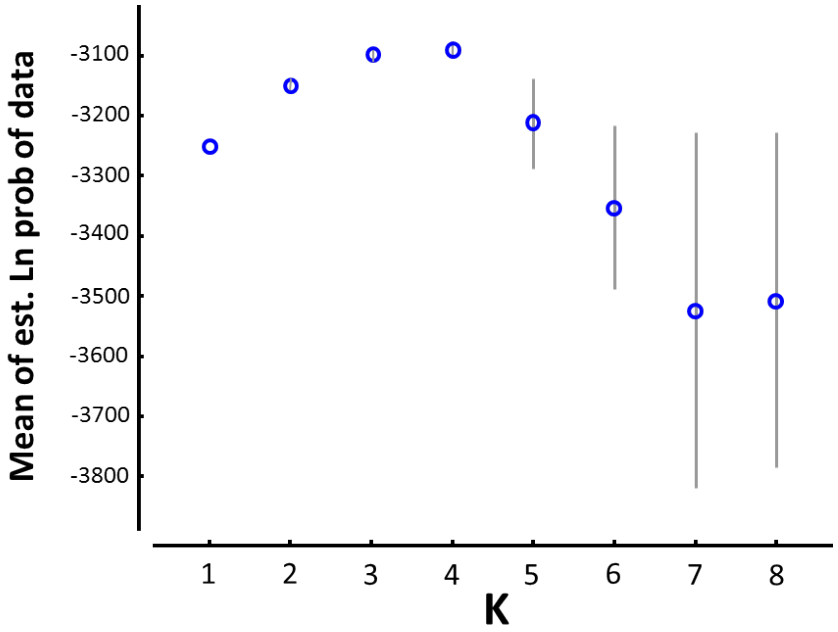

Figure 1. Output plot of K, number of populations, and the mean and variance of 10 posterior probabilities at each K from STRUCTURE HARVESTER.

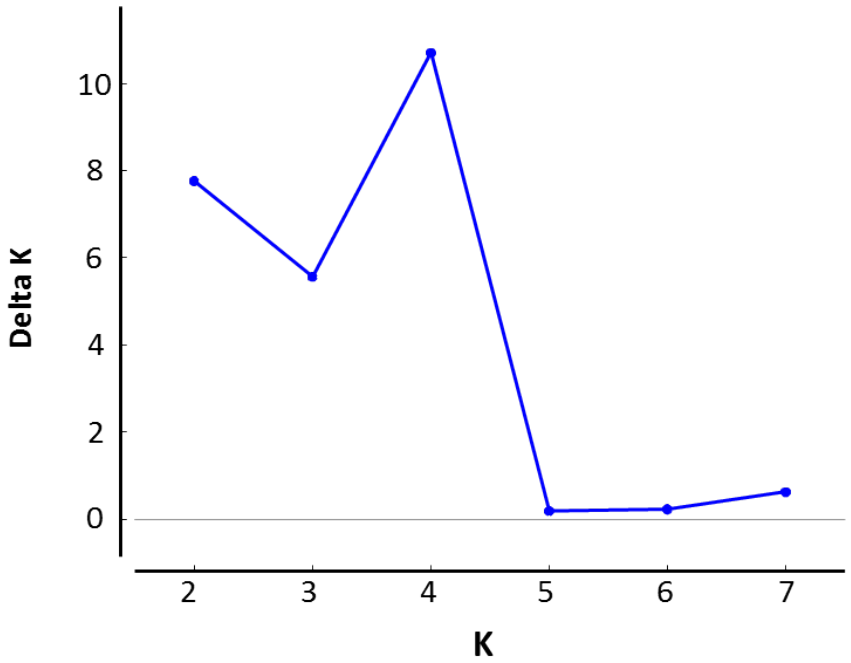

Figure 2. Output plot of K, number of populations, and ΔK, the second order rate of change of the likelihood function from STRUCTURE HARVESTER. The sudden jump at K = 4 indicates the most likely number of genetic clusters.

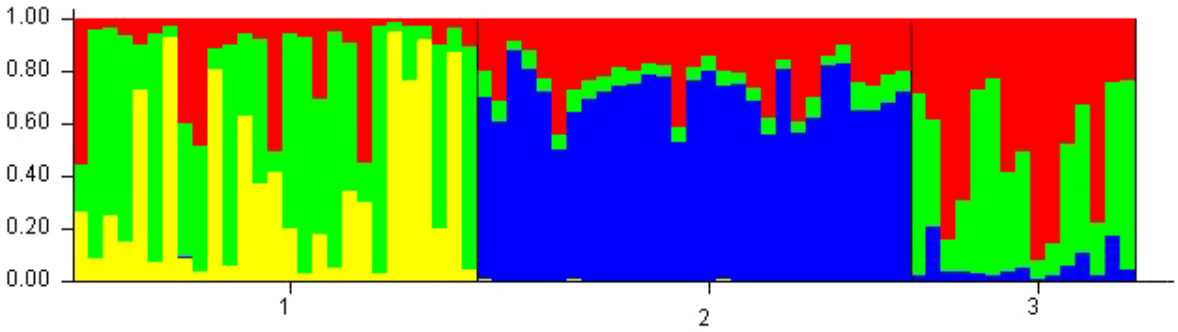

Figure 3. Genetic clustering plot from STRUCTURE analysis for K = 4. Each vertical line represents an individual with the proportion of the four genetic clusters represented by different colors. The narrow black vertical lines divided the different geographic populations: 1 = NLA, 2 = SLA, and 3 = TX.

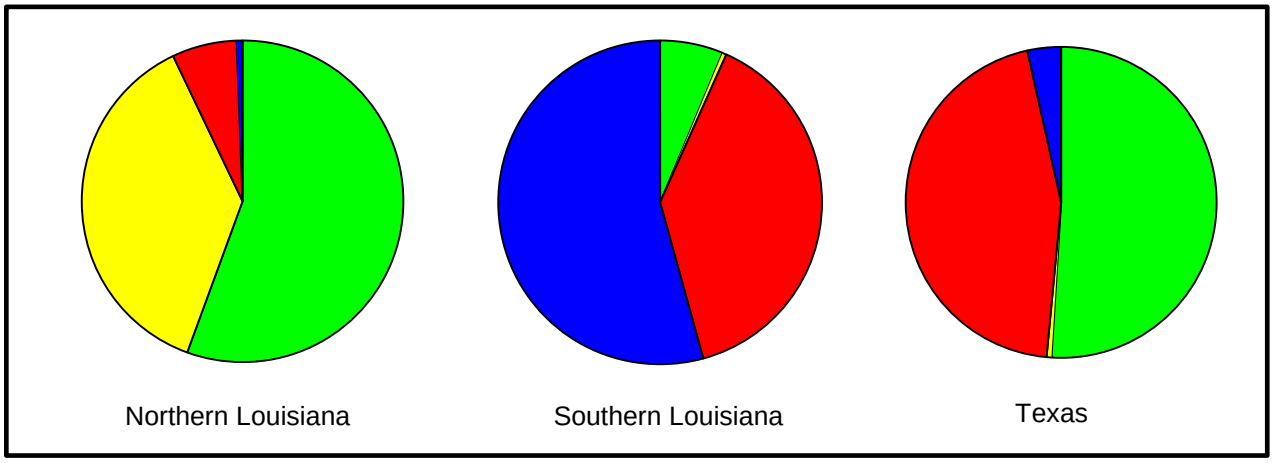

Figure 4. Proportion of each genetic cluster (Q) comprising each geographic population.

HETEROZYGOSITY AND INBREEDING

In wild-caught samples, observed heterozygosity was lower than expected in 34 out of 42 loci comparisons considering all three wild populations (Table 3). Considering each population, similar patterns occurred among the three, with H_O lower than H_E in 12 of 14 loci for NLA, 11 of 14 loci for SLA, and 11 of 14 loci in TX. Loss of heterozygosity was less of a problem in the captive populations as a whole, with 18 of 28 loci having lower observed heterozygosity than expected, but especially in the NLA population. In that population, H_O was lower than H_E in 7 of 14 loci (Table 4). Comparisons with the captive SLA and TX populations should done with caution given the low sample sizes for these populations. Allelic richness was also considerable lower in wild populations compared to the captive NLA. Allelic richness was low in the captive SLA and TX populations, but, again, sample sizes were so low in these populations for this estimate that we are considering these values for comparison with other populations; rather, we report these values here for future comparisons once more data is collected.

Table 3. Genetic diversity data from wild-caught samples. K = number of alleles, N = number sampled, H_O = observed heterozygosity, H_E = expected heterozygosity.

Population	Locus	K	N	H_O	H_E	Allelic Richness
Northern LA	Piru5	7	23	0.391	0.635	5.931
	Piru8	9	25	0.800	0.853	7.442
	Piru9	4	24	0.375	0.586	3.673
	Piru12	4	23	0.391	0.554	3.212

	Piru13	8	24	0.750	0.759	6.283
	Piru15	8	26	0.654	0.827	7.133
	Piru16	9	26	0.846	0.877	8.156
	Piru25	8	25	0.640	0.820	6.859
	Piru27	7	26	0.885	0.721	5.568
	Piru33	5	26	0.346	0.563	4.216
	Piru34	7	24	0.667	0.798	5.871
	Piru35	3	25	0.520	0.672	3.000
	Piru42	6	25	0.680	0.599	4.482
	Piru48	17	26	0.654	0.879	11.097
	Mean	7.3		0.614	0.725	5.923
Southern LA	Piru5	4	28	0.321	0.559	3.621
	Piru8	6	28	0.571	0.732	4.812
	Piru9	5	28	0.393	0.729	4.266
	Piru12	5	25	0.520	0.672	4.120
	Piru13	7	26	0.923	0.784	6.136
	Piru15	8	29	0.621	0.762	6.525
	Piru16	8	28	0.679	0.779	6.771
	Piru25	6	26	0.692	0.719	5.371
	Piru27	11	29	0.793	0.802	7.082
	Piru33	6	27	0.741	0.709	4.222
	Piru34	8	28	0.679	0.699	6.321
	Piru35	4	27	0.296	0.480	3.726
	Piru42	3	26	0.538	0.411	2.423
	Piru48	16	27	0.741	0.906	11.147
	Mean	6.9		0.608	0.696	5.467
Texas	Piru5	6	11	0.364	0.680	6.000
	Piru8	7	14	0.714	0.796	6.351
	Piru9	4	13	0.462	0.505	3.963
	Piru12	4	12	0.583	0.572	3.917
	Piru13	7	14	0.714	0.799	6.492
	Piru15	6	13	0.769	0.794	5.692
	Piru16	7	13	0.538	0.766	6.923
	Piru25	9	13	0.846	0.862	8.365
	Piru27	5	14	1.000	0.770	4.921
	Piru33	4	13	0.538	0.625	3.845
	Piru34	6	13	0.692	0.742	5.692
	Piru35	4	13	0.692	0.751	4.000
	Piru42	2	12	0.250	0.228	2.000
	Piru48	12	14	0.714	0.876	10.415
	Mean	5.9		0.634	0.700	5.613

Table 4. Genetic diversity data from captive samples. K = number of alleles, N = number sampled, H_O = observed heterozygosity, H_E = expected heterozygosity. Values for captive SLA and TX should be interpreted with caution because of low samples sizes for each and the SLA samples comprise a family of two adults and their offspring.

Population	Locus	K	N	H_O	H_E	Allelic Richness
Northern LA	Piru5	6	52	0.442	0.540	5.475
	Piru8	7	55	0.873	0.808	5.000
	Piru9	5	57	0.489	0.636	4.825
	Piru12	4	57	0.684	0.599	3.786
	Piru13	8	48	0.786	0.735	8.000
	Piru15	11	48	0.579	0.824	10.535
	Piru16	11	54	0.917	0.870	10.909
	Piru25	7	57	0.684	0.745	6.571
	Piru27	8	53	0.953	0.813	6.991
	Piru33	9	56	0.717	0.669	8.417
	Piru34	8	52	0.781	0.799	7.824
	Piru35	4	57	0.340	0.721	4.000
	Piru42	6	50	0.592	0.490	5.972
	Piru48	17	54	0.889	0.908	16.332
	Mean	7.9		0.695	0.726	7.474
Southern LA	Piru5	2	4	0.250	0.250	2.000
	Piru8	3	9	0.889	0.680	2.919
	Piru9	4	9	0.333	0.739	3.636
	Piru12	3	9	0.889	0.569	2.441
	Piru13	4	7	1.000	0.780	3.762
	Piru15	2	8	0.625	0.525	1.999
	Piru16	4	9	1.000	0.725	3.604
	Piru25	3	9	0.333	0.451	2.376
	Piru27	3	8	0.875	0.575	2.497
	Piru33	3	9	0.667	0.569	2.441
	Piru34	3	9	1.000	0.699	2.956
	Piru35	2	7	0.146	0.143	1.571
	Piru42	3	8	0.250	0.242	2.000
	Piru48	4	9	0.889	0.778	3.744
	Mean	6.9		0.653	0.552	2.710
Texas	Piru5	4	6	0.667	0.712	3.818
	Piru8	6	7	0.571	0.824	5.121
	Piru9	3	7	0.286	0.275	2.429
	Piru12	4	6	0.667	0.561	3.652
	Piru13	5	7	0.714	0.791	4.417
	Piru15	4	7	0.714	0.758	3.712
	Piru16	5	6	0.333	0.788	4.652
	Piru25	7	7	0.857	0.901	6.209

Piru27	5	7	1.000	0.769	4.363
Piru33	4	7	0.571	0.747	3.703
Piru34	5	7	0.857	0.791	4.417
Piru35	4	6	0.600	0.733	4.000
Piru42	2	6	0.500	0.409	2.000
Piru48	7	7	0.857	0.846	5.780
Mean	5.3		0.657	0.708	4.162

F_{IS} values in all wild populations indicated a level of inbreeding above that expected in a randomly breeding population (Table 5). Inbreeding was highest in the NLA population, followed by SLA and TX (Table 5). F_{IS} values were considerably lower in the captive populations with significant outbreeding in SLA, although this is based on a single family. MLH values were significantly lower in wild populations than captive ($P < 0.0001$ for all wild samples vs all captive samples). When considering individuals with low MLH values, potentially problematic crosses in captive snakes were revealed. For example, the two snakes at the Knoxville Zoo were crossed and produced two offspring whose MLH values were some of the lowest (0.42 and 0.50). A similar situation occurred with another pair that had offspring with values of 0.42 and 0.42, although they also had offspring with values of 0.60 and 0.69.

Table 5. Estimates of inbreeding among wild and captive populations, FIS and multilocus heterozygosity (*P = 0.01, **P = 0.005, ***P = 0.001). Captive SLA and TX populations should be interpreted with caution; both include limited number of samples and SLA is a single family group.

Population	F_{IS}	Mean MLH
Wild NLA	0.17***	0.58
Wild SLA	0.15***	0.60
Wild TX	0.12*	0.62
Wild All		0.60
Captive NLA	0.05	0.68
Captive SLA	-0.19**	0.67
Captive TX	0.07	0.65
Captive Overall		0.67

GENETIC BOTTLENECK

Using an infinite alleles model (IAM) for microsatellite evolution, bottlenecks were detected in all three geographic populations (Table 6). Using a SMM or TPM analysis, no genetic bottlenecks were detected in any of the populations. Allele frequency distributions in all three wild populations exhibited an L-shape,

indicating a bottleneck was not detected. For the captive populations, a bottleneck was detected in the NLA population with the IAM procedure. A bottleneck based on allele frequency distribution was detected in the SLA population, although this group consists of a single family and not a larger group.

Table 6. Results of tests for genetic bottlenecks among wild and captive Louisiana Pine Snakes using BOTTLENECK 1.2.02, including P values of Wilcoxon tests for heterozygosity excess and mode shift test of allele frequency distribution. Two phase models were run with 15% variance and 50% contribution of SMM.

Population	Wilcoxon Test			Allele Frequency Distribution
	IAM	SMM	TPM	
Wild NLA	**0.05**	0.96	0.34	L-shaped
Wild SLA	**0.0001**	0.99	0.18	L-shaped
Wild TX	**0.01**	0.98	0.42	L-shaped
Captive NLA	**0.001**	0.92	0.09	L-shaped
Captive SLA	**0.05**	0.07	0.15	Shifted Mode
Captive TX	**0.05**	0.48	0.07	L-shaped

DISCUSSION

GENETIC STRUCTURE AND CLUSTERING

We began the study assuming three *a priori* groups corresponding with geographic areas separated by two rivers: north of the Red River in northern Louisiana, south of the Red River in southern Louisiana, and west of the Sabine River in Texas. Genetic clustering analysis revealed that wild Louisiana Pine Snakes can be grouped into four clusters rather than the three geographic groups. However, there is some concordance with the *a priori* groups. One cluster is a set of alleles almost exclusive to the northern Louisiana population (yellow in Figure 3), suggesting the Red River, which separates northern Louisiana from both southern Louisiana and Texas, may be an effective barrier to gene flow. However, another cluster comes from set of alleles shared by Texas and northern Louisiana but not southern Louisiana. This implies gene flow between the two populations. Additionally, one cluster of alleles is shared among

all three populations, indicating some gene flow (red in Figure 3). Finally, a cluster found primarily in southern Louisiana snakes, although a small proportion of those alleles contribute to the Texas population.

The genetic clustering, along with the F_{ST} values among the three geographic populations, has implications for future conservation concerns. It is clear that there is only modest genetic structuring among the populations, especially between Texas and southern Louisiana. Indeed, when considering both the genetic clustering results and F_{ST} values, one could interpret these results to imply that the Texas and southern Louisiana groups can be considered a single population. As a comparison, F_{ST} values among 16 populations of the adder *Vipera berus* were much higher, typically above 0.2 (Ursenbacher et al. 2009). Given the low F_{ST} values in Louisiana Pine Snakes, concerns about maintaining three distinct captive populations based on geography should be somewhat alleviated, especially given the low numbers of captive snakes from southern Louisiana and Texas (see recommendations below).

Genetic Variation, Inbreeding, and Bottlenecks

Analyses of heterozygosity, inbreeding, and bottlenecks all indicate that concern about the wild populations is warranted. Using an infinite alleles model (IAM) for microsatellite evolution, bottlenecks were detected in all three geographic populations (Table 6). Using a SMM or TPM analysis, there was little evidence that genetic bottlenecks have occurred in wild Louisiana Pine Snake samples. The extreme discrepancies between IAM and SMM are not necessarily surprising as they represent two extreme models of mutation (Piry et al. 1999). However, that can result in confusion as to which model to follow. A recent test of these models using populations that have gone through known bottlenecks found that the IAM procedure was best at detecting them (Cristescu et al. 2010). Similarly, caution should be used when interpreting the mode-shift test, as it may also be weak at detecting a bottleneck (Cristescu et al. 2010).

Regardless of bottleneck outcomes, heterozygosity is low in all three wild populations and inbreeding coefficients in the wild populations were above 0.1, approaching 0.2 in the northern and southern Louisiana populations (Table 5). In some vertebrates, inbreeding at that level can affect fitness, such as

larvae survival in anurans (Andersen et al. 2004) and litter size in Mexican wolves (Fredrickson et al. 2007). However, the association between inbreeding coefficients and fitness can be quite variable, where higher coefficients do not necessarily translate to decreased fitness. Data on fitness variables, such as clutch size, hatching success, and hatchling survival, do not exist for wild Louisiana Pine Snakes, and are limited in captive snakes. Moreover, trapping efforts in all three geographic areas have had limited success and recent models predict extinction of all three populations in the near future (Rudolph et al., in prep). Hence, it is apparent that wild populations continue to dwindle, which will further contribute to the genetic concerns presented here. The declining wild populations and the genetic data emphasize the importance of the current captive breeding program.

The Role of the Captive Breeding Program

The effects of inbreeding depression on populations are well documented in vertebrates (review by Frankham 2005). For example, inbreeding was associated with decreased larval survival in European tree frogs (Andersen et al. 2004), smaller litter size in Mexican wolves (Frederickson et al. 2007), and smaller litter size and neonate deformities in adders (Madsen et al. 1996). Inbreeding can also impact physiological traits such as cold tolerance (Dierks et al. 2012), an important concern for an ectothermic animal such as Louisiana Pine Snakes, and can ultimately influence the decline of populations at a global scale, as is the case in amphibians (Allentoft and O'Brien 2010).

Effects of inbreeding depression and bottlenecks in declining populations can be reduced through a technique called genetic rescue. This entails introducing new individuals and, therefore, new alleles to a population (Hedrick and Fredrickson 2010; Heber et al. 2013; Vander Wal et al. 2013). Genetic rescue has been effective at raising fitness in wild populations, such as greater prairie chickens (Westemeier et al. 1998) and European adders (Madsen et al. 2004). Data presented in this study suggest genetic rescue should be considered for Louisiana Pine Snakes. Admittedly, better data on the association between inbreeding and fitness variables would be desirable (see further discussion below). However, collecting fitness data from Louisiana Pine Snakes in the wild is simply not practical given the extreme difficulty in capturing individuals. Furthermore, models on population status suggest immediate action is necessary to prevent the extinction of populations in the near future (Rudolph et al., in prep). Some captive-bred

Louisiana Pine Snakes, from NLA stock, have been reintroduced to a single site in northern Louisiana (LPS meetings 2014). However, the release site is in an area thought to be previously unoccupied and, hence, these snakes likely will not contribute to a genetic rescue.

Hedrick et al. (2010) presents ten guidelines for deciding if genetic rescue is a good management option and how it should be approached. Some of these guidelines would be difficult to meet in Louisiana Pine Snakes simply because the data is so difficult to collect (e.g., fitness data on wild snakes). However, other guidelines should be carefully considered. For example, Hedrick and Fredrickson (2010) recommend that a closely related donor population be used to minimize effects of outbreeding depression. Given the low F_{ST} values among all populations, they could all be considered closely related. However, the closeness of Texas and southern Louisiana snakes makes those populations ideal as reciprocal donor sources. The importance of good data on captive crosses is also emphasized. Of particular concern is the lack of fitness data from captive snakes that are breeding (see recommendations below).

It should be warned that genetic rescue has potential complications. Introduced animals could swamp out local genetic variation and, ultimately, traits that may be adaptive (Hedricks and Fredrickson 2010). To counter this swamping effects, introduction levels should be kept low enough to that local allele frequencies are not dramatically changed and adaptive variation is not eliminated. Given the low numbers of reintroduced Louisiana Pine Snakes so far, this is unlikely to be a problem, but it should be considered in the future if breeding numbers greatly increase. Admittedly, this concern may be moot if wild populations continue to decline to levels near extirpation.

Relying on donors from a limited population can also be problematic because it results in a small effective population size (Hedricks and Fredrickson 2010). To prevent this, as much of the endangered (in this case, wild) population as possible should be included as donor sources. What this means for Louisiana Pine Snakes is that snakes from all three populations (NLA, SLA, and TX), should be included in the breeding program and that wild-caught snakes should be considered as an addition to the breeding program. In Louisiana Pine Snakes, most of the donor snakes have been from northern Louisiana, so a greater contribution should come from southern Louisiana and Texas, if possible.

RECOMMENDATIONS FOR FUTURE MANAGEMENT OF CAPTIVE LOUISIANA PINE SNAKES.

Captive breeding entails difficult considerations; inbreeding should be avoided while simultaneously maintaining potential genetic uniqueness of populations. However, results from this study suggest some options for future actions.

1. Crosses between Texas and Southern Louisiana. Genetic clustering and structuring indicate Texas and southern Louisiana populations are genetically the closest of the three *a priori* populations. Given this, and the low number of Texas and southern Louisiana snakes in the captive program, crossing these individuals should be considered. Currently, Texas captives consist of six (4 males, 2 females) wild caught snakes and their offspring and Southern Louisiana captives consist of two wild caught snakes and a handful of their offspring. The Texas captives could be crossed with each other and with any of the Southern Louisiana snakes. These crosses would help avoid inbreeding without the worry of losing genetically distinct populations. By all means, sibling crosses and sibling/parent crosses in the Southern Louisiana snakes should be avoided (or in any population for that matter).

2. Crosses between Texas and Northern Louisiana. Genetic structure analyses indicate sharing of alleles between these populations, although the genetic distance between them is greater than between Texas and southern Louisiana. However, given the low F_{ST} values between these two populations, crosses between these two populations should be considered a viable option if there are concerns about genetic diversity and inbreeding in captive populations.

3. Crosses between Northern Louisiana and Southern Louisiana. Genetic structure is most pronounced between these two populations based on F_{ST}. However, the F_{ST} value was relatively low compared to other ectotherms (e.g., Arens et al. 2006; Ursenbacher et al. 2009; Ferchaud et al. 2011). We recommend that crosses in 1 and 2 above be considered first, although crossing between NLA and SLA could be considered if there is continued concern about inbreeding in the captive population.

4. Adding wild individuals to the captive program. Removing individuals from the wild and adding them to the captive program is a difficult decision and there is not a "right or wrong" answer to the problem. An argument can be made for keeping potentially breeding individuals in the wild population, especially given the concern about declining numbers. However, there are substantial benefits to adding wild individuals to the captive breeding program. The current captive population is the second and third generation of snakes resulting from relatively few individuals primarily from northern Louisiana. Even with a careful protocol to minimize it, inbreeding may again become a problem in successive generations (Hedrick and Fredrickson 2010). Indeed, the low MLH values in some captive snakes may be indicative of this. However, inbred donors can still relieve the effects of inbreeding depression; translocation of inbred donors helped to increase heterozygosity and allelic diversity in South Island robins (Heber et al. 2013). Of even greater concern are the low numbers of southern Louisiana and Texas snakes in the captive breeding program. Texas populations may be on the brink of extirpation (LPS Stakeholders Meeting 2014; Rudolph et al., in prep) and captive breeding may be the last chance of maintaining those alleles. Given that southern Louisiana and Texas are genetically close and could be considered a single population, adding wild-caught snakes from southern Louisiana to the breeding program may also be beneficial.

5. Fitness data. Detailed fitness data from the captive populations, such as clutch size, hatching success, and hatchling survival, should be collected so it can be compared to inbreeding coefficients. This data would be invaluable for giving a better understanding of how inbreeding is associated with fitness. If this association is weak, inbreeding in Louisiana Pine Snakes may be a variable of less concern compared to declining numbers and habitat loss, especially in wild populations. However, if inbreeding is approaching levels associated with declining fitness, it emphasizes that genetic rescue needs to be considered.

6 Captive population management. The current captive population(s) are small and reproductive rates are low. The reasons for low reproductive output are not well known. This should be rectified to provide additional insight into the genetic situation in the captive population, and to allow for rapid growth

of the captive population, and to provide increased numbers of animals for release. The extent of loss of genetic diversity in population bottlenecks is a consequence of both the magnitude of the bottleneck (minimum number of individuals) and the length of time a population spends in a bottleneck (Frankham 2005; Hederick and Fredrickson 2010). The current small size of the captive population remains a significant bottleneck, keeping the breeding population at a low effective population size. Consequently, rare alleles will continue to be lost, even with ideal population management (Brook et al. 2002; Frankham 2005). Quickly maximizing the captive population will help reduce the loss of rare alleles and maintain genetic diversity within the captive population.

SUMMARY

Genetic data from wild caught Louisiana Pine Snakes indicates concern about loss of genetic diversity and inbreeding is warranted. If genetic problems have begun to affect populations, it will exacerbate the effects of habitat loss and fragmentation, contributing to the on-going decline of wild populations. A better understanding of the association between inbreeding and fitness in wild populations would be desirable, but the decline in wild populations makes this impractical. If genetic effects are influencing population declines, genetic rescue may help alleviate the impact. As such, the continued efforts of the captive breeding program become all the more valuable.

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