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Population Status and Population Genetics of Northern Leopard Frogs in Arizona

Open-File Report 2011–1186

U.S. Department of the Interior
U.S. Geological Survey

Heritage Fund

NORTHERN ARIZONA UNIVERSITY
College of Engineering, Forestry & Natural Sciences

Cover photograph: Last two northern leopard frogs recorded from the now-extirpated population at Horseshoe Bend on the Colorado River, Coconino County, Arizona (photo by Norm van Vliet, April 13, 2003).

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Prepared in cooperation with Arizona Game and Fish Department and Northern Arizona University

Final report for Arizona Game and Fish Department Heritage Grant "Population Status and Population Genetics of Leopard Frogs in Northern Arizona" (I08002)

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U.S. Department of the Interior
U.S. Geological Survey

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Multiply	By	To obtain
	Length	
kilometer (km)	0.5400	mile (mi)

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°F=(1.8×°C)+32
Vertical coordinate information is referenced to North American Vertical Datum of 1988 (NAVD 88)
Horizontal coordinate information is referenced to North American Datum of 1983 (NAD 83)
Other units used are milliliter (mL), micromoles per liter (mmol/L), and nanogram (ng).

Abbreviations and Acronyms

AGFD	Arizona Game and Fish Department
EDTA	Ethylenediaminetetraacetic acid
F_{IS}	One of the F-statistics, used to describe population genetic structure
GPS	Global Positioning System
PCR	polymerase chain reaction
TFPGA	Tools for Population Genetic Analysis
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

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Population Status and Population Genetics of Northern Leopard Frogs in Arizona

By Charles A. Drost,[1] Ryan P. O'Donnell,[2] Karen E. Mock[2] and Tad C. Theimer[3]

Executive Summary

Increasing isolation of populations by habitat fragmentation threatens the persistence of many species, both from stochastic loss of small isolated populations, and from inbreeding effects in populations that have become genetically isolated. In the southwestern United States, amphibian habitat is naturally patchy in occurrence because of the prevailing aridity of the region. Streams, rivers, and other wetlands are important both as habitat and as corridors that connect populations. However, populations of some species have become more fragmented and isolated by habitat degradation and loss. Northern leopard frogs (*Rana pipiens*) have experienced serious declines in the Southwest. We conducted an extensive survey across the known range of northern leopard frogs in Arizona to determine the current distribution and abundance of the species. From a range that once spanned much of the northern and central part of the State, northern leopard frogs have been reduced to three or four widely separated populations, near Lyman Lake in east-central Arizona, in the Stoneman Lake area south of Flagstaff, along Truxton Wash near Peach Springs, and a population of uncertain extent on Navajo Nation lands. The Lyman Lake and Truxton Wash populations are small and extremely isolated. The Stoneman Lake population, however, is an extensive metapopulation spread across several stream drainages, including numerous ponds, wetlands, and artificial tanks. This is the only population in Arizona that is increasing in extent and numbers, but there is concern about the apparent introduction of nonnative genetic stock from eastern North America into this area.

We analyzed genetic diversity within and genetic divergence among populations of northern leopard frogs, across both extant and recently extirpated populations in Arizona. We also analyzed mitochondrial DNA to place these populations into a larger phylogenetic framework and to determine whether any populations contained genetic material not native to the region. We found a high level of genetic divergence among the population centers (Lyman Lake, Stoneman Lake, Truxton Wash), and low genetic diversity in the small populations at Lyman Lake and Truxton. The extensive population in the Stoneman Lake area had high genetic diversity and relatively high gene flow among ponds and tanks across the entire extent of the area. However, this population also contained a mitochondrial haplotype from northern leopard frogs from the northeastern United States or southeastern Canada, probably representing the introduction of released pets or laboratory animals. These eastern frogs were extensively distributed through this population, and probably contributed to its high genetic diversity.

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Genetic diversity in the outlying populations such as Truxton Wash, East Buckskin Tank, and Hess Tank was low and showed signs of recent bottlenecks. However, supplementing genetic diversity in these native populations with artificial gene flow from the Stoneman Lake area may only be advisable in extreme situations for which there are no other alternatives. Until the nature and effects of genetic mixing of eastern and western genetic stocks of northern leopard frogs are better understood, the long-term persistence of the species in the Southwest may be best served by retaining as much genetic integrity of remaining native populations as possible.

Introduction

The loss of connectivity among habitat patches and the resulting fragmentation of plant and animal populations are important concerns for the long-term preservation of natural populations. Metapopulation studies of some anuran amphibians have demonstrated loss of connectivity among formerly extensive populations and have noted the potential contributing role of such fragmentation to amphibian declines (Mann and others, 1991; Sjögren, 1991; Lehtinen and others, 1999). A variety of causes have been implicated in fragmentation of amphibian habitat, including clearing of forest lands (Gibbs, 1998), road-building (Vos and Chardon, 1998), and introduction of nonnative species, particularly predatory fishes (Bradford and others, 1993). An important additional source of population fragmentation in the southwestern United States is diversion and withdrawal of water, leading to surface drying. Isolation of subpopulations within habitat fragments, and gradual loss of these subpopulations over time, may be an important cause of regional declines of amphibians (for example, in the mountain yellow-legged frog, *Rana muscosa*,* in the Sierra Nevada of California; Bradford and others, 1993).

In the southwestern United States, there is considerable natural fragmentation of amphibian populations by broad stretches of hot, dry, inhospitable habitat separating seasonal and permanent wetlands. In this arid region, the riparian areas along rivers and streams are vital habitat for many amphibians and provide extensive corridors linking populations. Over the last 150 years, however, there has been considerable degradation and loss of such habitats (Dahl, 1990; Bogan and others, 1998), with associated declines of species that depend on them. Some studies have assessed direct effects of such habitat loss and degradation on species persistence (for example, Krueper, 1993), but few have considered secondary effects such as isolation of tributary streams, side canyons, and headwater springs, and the resulting population fragmentation this may cause. Because of their limited dispersal abilities and susceptibility to desiccation in arid habitats away from water, amphibians appear to be particularly vulnerable to this kind of habitat fragmentation.

Northern leopard frogs (*Rana pipiens*) formerly occurred in a variety of stream, pond, and marsh habitats throughout the highland areas of northern and central Arizona and neighboring States. Over the last 40 years, however, northern leopard frogs and their relatives have experienced marked declines throughout the Southwest (Corn and Fogleman, 1984; Clarkson and Rorabaugh, 1989; Sredl, 1998). Northern leopard frogs are currently listed as a species of conservation concern by State and Federal agencies, including the Arizona Game and Fish Department (AGFD; “Species of Special Concern”), the State of Colorado (“Special Concern Species”), the U.S. Forest Service (“Sensitive,” Regions 2 and 3 [Colorado, New Mexico, and Arizona]), and the Navajo Nation (“Threatened”). The species is currently under

* Scientific and common names follow "A field guide to western reptiles and amphibians" (Stebbins, 2003)

consideration for listing under the Endangered Species Act as a Threatened species in the western portion of its range in the United States (Fish and Wildlife Service, 2009).

Northern leopard frogs occur (or previously occurred) in four general areas in Arizona: (1) the Arizona Strip area north of the Colorado River, including Kanab Creek and other large tributary drainages (based on recent historical records); (2) drainages on the south side of the Colorado River in northwestern Arizona (Truxton Wash drainage; Wallace, 1995); (3) the Little Colorado River basin and other streams flowing into Lake Powell and the San Juan River in northeastern Arizona, primarily on Navajo Nation lands (Drost, 2005; surveys by Navajo Fish and Wildlife biologists); and (4) lakes and ponds of the Mogollon Rim highlands in east-central Arizona (Clarkson and Rorabaugh, 1989). There are likely to be multiple levels of genetic differentiation and isolation among populations within these different areas, which will be important in assessing conservation and management needs of the species. An important concern for extant northern leopard frog populations in Arizona is that most or all of them now appear to be isolated from each other.

The work described in this report extends earlier surveys to the remainder of the northern Arizona area that comprises the historical range of northern leopard frogs in Arizona. This includes all of the areas noted above: the Arizona Strip; streams and ponds south of the Colorado River; stream habitats south of Lake Powell; and ponds and tanks in the Mogollon Rim highlands. In addition to increasing understanding of the current regional distribution and population status of northern leopard frogs, analysis of genetic data from these areas allows us to assess patterns of genetic diversity, genetic isolation and recent population bottlenecks in remaining northern leopard frog populations in Arizona. In conjunction with field survey data, this analysis may be crucial in understanding population trends and long-term viability of remaining populations in the region.

Recent rangewide genetic studies on the northern leopard frog (Hoffman and Blouin, 2004a; Hoffman and others, 2003) provide a valuable framework for genetic analyses of northern leopard frogs in Arizona, and allow us to better interpret our results in a regional context. One noteworthy finding of Hoffman and Blouin (2004a) was the existence of two deeply divergent eastern and western clades in the continental distribution of the species. Hoffman and Blouin (2004a) identified a single northern leopard frog with an eastern mitochondrial haplotype from samples collected in the Stoneman Lake area in southern Coconino County. This was the only "eastern" frog that these authors found west of the Great Lakes and Mississippi River, and they assumed that the odd eastern haplotype individual from the Stoneman area represented a released pet or laboratory frog. In the absence of more extensive genetic surveys, the continued occurrence, prevalence, and distribution of "eastern" northern leopard frogs in Arizona has remained unknown. For this reason, we analyzed mitochondrial DNA samples from leopard frog populations throughout their northern Arizona range to determine the occurrence and distribution of eastern and western mitochondrial haplotypes. In management efforts to establish refuge populations and restore native populations, it may be important to identify and avoid areas that contain introduced eastern frogs.

The objectives of this project were to:

1. Determine the current distribution of northern leopard frogs throughout their former known range in northern Arizona;
2. Estimate relative population sizes of all extant populations in the survey area;

3. Assess the degree and pattern of geographic separation and genetic divergence among populations;
4. Evaluate the presence and geographic occurrence of mitochondrial haplotypes from the eastern United States;
5. Identify isolated, relict populations that may require direct management intervention for their persistence;
6. Characterize habitats used by leopard frogs in the region, for use in developing management actions; and
7. Discuss potential management implications and conservation considerations for northern leopard frogs in the region.

Methods

Field Surveys

Field surveys were conducted in areas of suitable aquatic habitat throughout the known historical range of northern leopard frogs in Arizona. Field surveys for this study were conducted between March 2007 and September 2009, but we also include analyses of earlier surveys conducted from 2003 through 2006. The surveys included sites of known present occurrence, sites where northern leopard frogs were found in the past, based on literature and museum collection records ("historical sites"), and other areas of suitable habitat, where northern leopard frogs have not previously been recorded ("potential sites"). The Mogollon Rim highlands, from the Flagstaff area south and east into the White Mountains, have long been a stronghold for northern leopard frogs in Arizona, but surveys indicate major declines even in this area. AGFD personnel assisted with surveying these sites and collecting genetic samples. In northeastern Arizona, including the Little Colorado River basin and the large area of Navajo Nation lands south of Lake Powell, surveys by Navajo Fish and Wildlife Service biologists have documented one area where leopard frogs persist in the Navajo Creek drainage. We worked with the Navajo Fish and Wildlife Service in surveying this area and other potential sites on Navajo Nation lands.

In the remote Arizona Strip area, historical and recent surveys found northern leopard frogs primarily in the Kanab Creek drainage. Grand Canyon National Park and Bureau of Land Management managers provided permits for work in the Arizona Strip, and also provided logistical support and advice on accessing sites to adequately cover the large, remote areas of this region. South of the Colorado River in northwestern Arizona, northern leopard frogs are known from Truxton Wash, in the Peach Springs area. Both AGFD and the Hualapai Tribe assisted in surveying this region.

To detect the presence of frogs and estimate population size, we used a combination of diurnal visual surveys for adult frogs, eggs, and larvae, and spotlight surveys at night. To the extent possible, we searched the entire accessible area of perennial water at each site, including the perimeter and shallows of pond and wetland sites, and the perennial length of stream sites. We recorded beginning and ending points of areas surveyed with global positioning system (GPS) receivers, and also recorded total survey time for each area. This enabled calculation of survey effort in relation to both time and area covered, and allowed for estimation of relative population size in different areas (Crump and Scott, 1994). In stream and canyon sites with narrow, linear areas of suitable habitat, and at smaller pond, wetland, and tank sites, these visual surveys provide a repeatable index of population size.

We collected habitat data at each site, including hydrologic measurements of perennial portions of streams and pools (water body type, length, width, depth, and water velocity, if applicable) and characterization of stream substrate and aquatic and streamside vegetation (McDiarmid, 1994). Water quality parameters were measured at most sites, including water temperature, pH, conductivity, turbidity, and dissolved oxygen. We also recorded information on other biological factors that may be important to occurrence of leopard frogs, including presence and relative abundance of native fish species, nonnative fish species, crayfish (*Orconectes virilis*; nonnative) and bullfrogs (*Rana catesbeiana*; nonnative), and signs of beaver (*Castor canadensis*) activity. From previous surveys, we knew that breeding habitat is often localized in relation to overall local distribution, so we also separately recorded habitat information for known or potential breeding sites.

Field surveys focused on the northern leopard frog. However, we encountered many other species in the course of surveying for leopard frogs. We recorded data on all amphibians and reptiles encountered, so the surveys also provide information on the status of other species in the survey areas. Other species encountered included tiger salamander (*Ambystoma tigrinum*), Mexican spadefoot (*Spea multiplicata*), Arizona toad (*Bufo microscaphus*), red-spotted toad (*B. punctatus*), Woodhouse's toad (*B. woodhousii*), canyon treefrog (*Hyla arenicolor*), and terrestrial garter snake (*Thamnophis elegans*).

All fieldwork was carried out under permits from AGFD and the land management agencies for the areas surveyed, where applicable (Bureau of Land Management, Coconino National Forest, Grand Canyon National Park, Navajo Fish and Wildlife Service). Capture, handling, and collection of genetic samples were conducted under an approval from the Northern Arizona University Institutional Animal Care and Use Committee.

Genetic Analyses

Samples for genetic analyses were collected across the known extant range of northern leopard frogs in Arizona (fig. 1). Tissue samples for genetic analyses were collected from leopard frogs by clipping the tip of the third hind toe directly into a microvial containing 95 percent ethanol. To minimize risk of infection, we applied an antibiotic / anesthetic solution to the cut toes of sampled frogs before releasing them at their point of capture (Green, 2001). Surgical instruments were sterilized between each frog to eliminate the risk of sample contamination and to reduce the risk of spreading diseases among frogs. We targeted collection of a minimum of 20 samples per population.

DNA was extracted from toe clips using either a standard chloroform extraction (Müllenbach and others, 1989) or a salting-out extraction method (Sunnucks and Hales, 1996). The purified DNA was resuspended in a Tris-EDTA buffer (0.1M Tris, 0.1M EDTA, pH 9.0) and stored until use at -80°C. Sequence data were obtained for 786 base pairs of the mitochondrial ND1 gene (Hoffman and Blouin, 2004a) for 5 to 11 individuals from each population sampled, using the primers RpND1F and RpND1R (Wilson and others, 2008). These sequences allowed the detection of eastern northern leopard frog haplotypes (Hoffman and Blouin, 2004a). Each 25 mL reaction contained 0.4 mmol/L of each primer, 160 mmol/L dNTPs, 1× polymerase chain reaction (PCR) buffer, 2.5 mmol/L magnesium chloride, 1 unit of Taq polymerase, and ~50 ng of genomic DNA. PCR conditions consisted of 5 minutes of initial denaturation at 95°C; followed by 35 cycles of 94°C for 60 seconds, 54°C for 60 seconds, and 72°C for 90 seconds; followed by a 5-minute final extension at 72°C. The PCR product was visualized on a 0.7 percent agarose gel to check for product quantity and size. PCR products

were purified with QIAquick PCR purification kit (Qiagen) and sequenced with BigDye chemistry (Applied Biosystems) on an ABI 3730 sequencer. Sequences were edited and aligned with SeqMan II software. Sequences were generated in the forward direction first and in the reverse direction if the sequence could not be confidently read throughout the entire 786 base pair amplicon in the forward direction alone. TCS version 1.21 was used to search for unique mitochondrial haplotypes and to examine the relationships among those haplotypes using statistical parsimony (Clement and others, 2000).

To further screen the sampled frogs for eastern haplotypes, we developed an economical restriction assay that could distinguish eastern and western haplotypes without sequencing each individual. An approximately 800-base pair region of the ND1 gene was amplified as described above for the sequencing. This amplified fragment was then digested for 6 hours at 37°C using the restriction enzyme StyI. This enzyme digests eastern fragments but not western fragments at a site that has been shown to be diagnostic for the eastern and western clades in over 500 samples collected from throughout the range of the species (Hoffman and Blouin, 2004a, data from this study). Digestion was arrested by incubating at 70°C for 20 minutes and digested fragments were then visualized on a 1.0 percent agarose gel.

Microsatellite markers developed by Hoffman and others (2003; Rpi100, Rpi101, Rpi102, Rpi103, Rpi104, Rpi107, and Rpi108) and Hoffman and Blouin (2004b; RP193) were used to amplify eight microsatellite loci. PCR conditions included 2 minutes of initial denaturation at 95°C, followed by 30 cycles of the following steps: 95°C for 30 seconds, annealing temperature for 30 seconds, and 72°C for 1 minute; followed by a 10 minute final extension at 72°C. Locus-specific annealing temperatures were as follows: Rpi100, 52°C; Rpi101, 62°C; Rpi102, 50°C; Rpi103, 55°C; Rpi104, 56°C; Rpi107, 52°C; Rpi108, 52°C; and RP193, 56°C. The PCR product was visualized on a 0.7 percent agarose gel to check for product quantity and size. PCR products were then analyzed on an ABI 3100 or 3730 sequencer. Population-level analyses using microsatellite data were conducted only on populations with nine or more individuals.

We used GenePop software (Raymond and Rousset, 1995) to test whether population genotypic proportions deviated from expectations under assumptions of Hardy-Weinberg equilibrium, given observed allele frequencies. An exact test over all loci was performed using the Markov Chain method with 1,000 dememorization steps, 100 batches per locus, and 1,000 iterations per batch. We also used GenePop to determine whether deviations could be attributed to heterozygote excesses or deficits. Probabilities were interpreted using a Bonferroni correction for multiple population-by-locus combinations for all such combinations that were polymorphic (n=85).

Relationships among populations, based on allele frequency differences as measured by Nei's (1972) genetic distance, were summarized by construction of an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram using Tools for Population Genetic Analysis (TFPGA) software, and nodal strength was assessed via bootstrapping over loci using 1,000 replicates. Pairwise population differentiation was assessed using an exact test of each population with a sample size of at least 15 individuals using TFPGA software with 1,000 dememorization steps and 10 batches of 2,000 permutations per batch. Overall genetic structuring among populations was assessed using Wier and Cockerham's θ_{ST} (Wier and Cockerham, 1984; Wier, 1996), an estimator of F_{ST}, using TFPGA version 1.3 (Miller, 1997). Inbreeding within populations was assessed in TFPGA by calculating f, an estimate of Wright's

F_{IS} (Wier and Cockerham, 1984; Wier, 1996). The 95-percent confidence intervals for estimates of θ_{ST} and f were estimated by bootstrapping over loci with 1,000 replicates.

Within-population genetic diversity was measured in three ways: (1) average gene diversity per locus, (2) mean number of observed alleles per locus, and (3) allelic richness (with sample size rarified to n=9 to allow comparability among populations with different sample size numbers). All diversity analyses were performed using FSTAT software (Goudet, 2001).

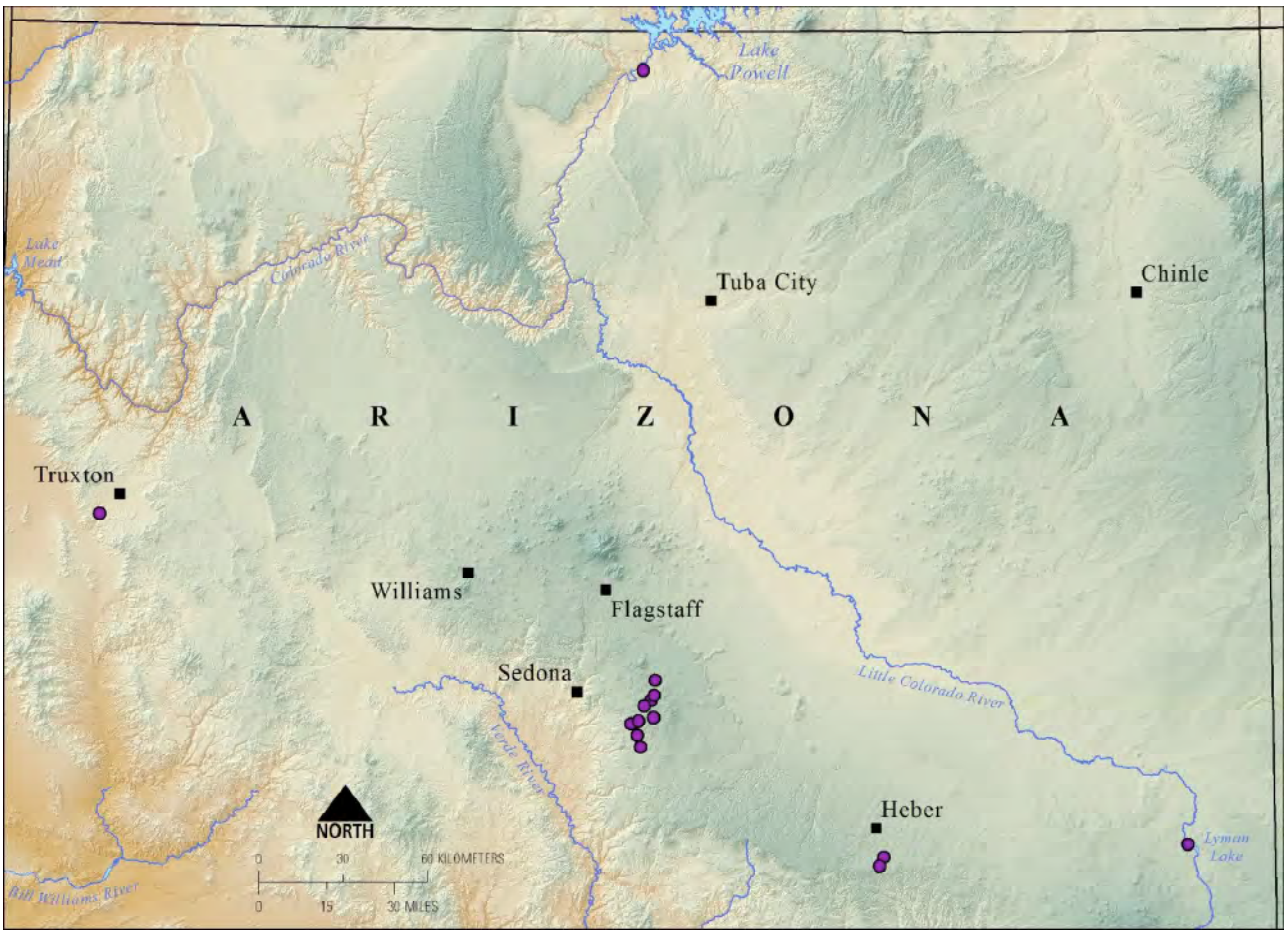


Figure 1. Map showing sites where northern leopard frog populations in northern Arizona were sampled and analyzed for population diversity, genetic distance, and mitochondrial haplotype. Distance from Truxton (westernmost site) to Lyman Lake (eastern site) is 405 km. The cluster of populations around Stoneman Lake (lower center of map) is enlarged in figure 2.

We determined whether populations bore signatures of recent bottlenecks by employing the mode shift test of Luikart and others (1998) and the sign test in BOTTLENECK version 1.2.02 (Cornuet and Luikart, 1997). In stable populations at mutation-drift equilibrium, most alleles are expected to be at low frequencies. When a population experiences a demographic bottleneck, low frequency alleles are preferentially lost and the most common (modal) allele frequency categories are expected to shift so that the lowest frequency category is no longer the most commonly observed. The mode shift test is a qualitative assessment of distribution of allele frequencies. The sign test is a quantitative analysis of the number of loci with either an excess or

a deficiency of genetic diversity compared to predicted diversity based on the number of alleles detected. A population bottleneck typically results in an excess of diversity for a given number of alleles. Only populations with sample sizes greater than 11 were used for this analysis.

In the Stoneman Lake area populations (fig. 2), additional analyses were performed to assess genetic structuring and correspondence between mitochondrial and microsatellite data. We used STRUCTURE software version 2.2 (Pritchard and others, 2000) to assess these patterns. STRUCTURE uses a Bayesian approach to determine the likelihood that each individual is a member of particular groups. We used STRUCTURE in two ways: (1) to determine whether microsatellite genotypes were clustered according to mitochondrial haplotype origin (eastern versus western); and (2) to assess overall spatial structuring in the Stoneman Lake complex. For the first analysis, we specified that the number of groups be set at two (k=2), forcing STRUCTURE to assign individuals to one of two groups based on the minimization of Hardy-Weinberg disequilibrium. We then asked whether individuals with eastern versus western mitochondrial haplotypes tended to be assigned to different groupings based on this STRUCTURE analysis. For this analysis, we used an admixture model and assumed correlated allele frequencies but did not use haplotype grouping as prior information. We used a burn-in period of 10,000, with a run length of 100,000 iterations. The run was repeated 20 times for k=2. Five individuals whose mitochondrial haplotypes were not known due to failure of the mitochondrial sequence to amplify in PCR were designated as being of unknown haplotype. If structuring of nuclear genes is concordant with structuring of mitochondrial genes, haplotype group membership based on microsatellite data will be strongly bimodal, and we can infer that the groups of individuals defined by mitochondrial haplotypes are not interbreeding. Alternatively, if structuring among nuclear genes is not concordant with structuring among mitochondrial genes at the individual level, we can infer that interbreeding is occurring between individuals with distinct mitochondrial haplotypes.

STRUCTURE software was also used to determine the most likely number of clusters (k) in the Stoneman Lake area populations, without pre-assignment to mitochondrial groups or populations. Parameters were as described for the k=2 analyses, above, with 10 replicates per hypothesized k, and k varying from 1 to 12. We used the approach of Evanno and others (2005) to determine k optimality.

In addition, we used GenAlEx software (Peakall and Smouse, 2006) to perform a principal coordinates analysis of individuals from the Stoneman Lake area population, based on a matrix of inter-individual distances without preassignment to a particular population. In this analysis, clustering of individuals within populations suggests that genetic distances between different populations are due to limited gene flow between those populations. Clustering without regard to population membership suggests that some other mechanism of differentiation among individuals is operating (for example, hybridization).

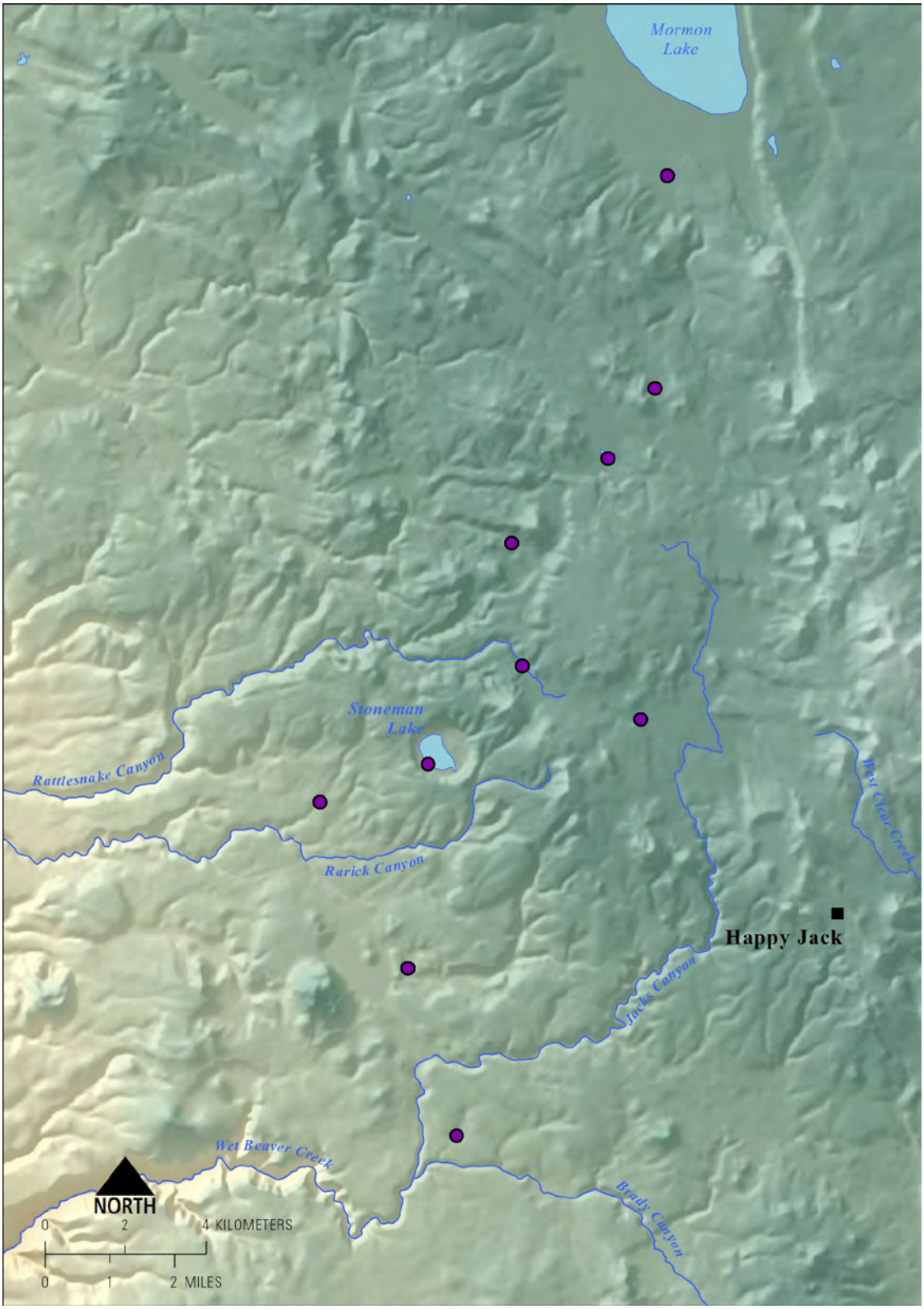


Figure 2. Map showing locations of northern leopard frog populations in the Stoneman Lake area of northern Arizona, sampled for genetic analyses in 2007–9. Distance from Little Mormon Lake (northernmost population) to Brady Tank (southernmost population) is 24.4 km straight-line distance.

Results

Populations—Distribution, Numbers, and Trends

We conducted a total of 114 field surveys from 2007 through 2009. These surveys were distributed across the four broad regions that cover the overall range of northern leopard frogs in Arizona: (1) the central / Mogollon highlands of the State, extending from the Flagstaff area southeast along the Mogollon Rim to the White Mountains; (2) the Little Colorado River basin, including the northeastern part of the State, from the edge of the Mogollon highlands north to the Utah border and the Colorado River; (3) northwestern Arizona, west of Oak Creek in Coconino County and south of the Colorado River; and (4) the Arizona Strip, covering the area of northwestern Arizona that is north and west of the Colorado River (including the river mainstem and tributary canyons on the north side of the Colorado River). Broken down by these broad survey areas, the numbers of surveys ranged from six in the Little Colorado River basin, to 56 in the Mogollon highlands (table 1).

We also incorporated data from earlier surveys throughout the study area, dating back to 2003 (table 1, "Total"). The majority of these surveys were of tributary canyons of the Colorado River, from Glen Canyon Dam near Page, Arizona, to the Grand Wash Cliffs marking the end of the western Grand Canyon. These earlier surveys covered most of the perennial streams flowing into the Colorado River, on both the north and south sides of Grand Canyon and Marble Canyon. Including these other data, there were nearly 400 surveys, with number of surveys in different regions ranging from 56 in the central highlands to 138 in the Arizona Strip region.

Table 1. Field surveys for the northern leopard frogs, by broad area in northern Arizona.

[Project period, number of surveys from 2007 through August 2009; Total, total number of surveys, including previous work by the authors from 2003–7]

Area	Project period	Total
Central / Mogollon highlands	56	56
Little Colorado River basin	6	88
Northwestern Arizona, south of Colorado River	37	116
Arizona Strip	15	138
Total	114	398

Our field surveys found frogs in three local areas, widely separated in three of the four broad regions of the study area. These included: (1) Lyman Lake in the Little Colorado River region; (2) a relatively extensive series of tanks, ponds and springs in the Stoneman Lake area of the Mogollon highlands region; and (3) Truxton Wash in northwestern Arizona, south of the Colorado River (fig. 3, bottom). In addition, separate surveys by David Mikesic of the Navajo Fish and Wildlife Service found northern leopard frogs at one other site in northeastern Arizona, in the Inscription House / Navajo Creek drainage on the Navajo Nation. This site is also widely separated from the other areas where northern leopard frogs are now known to persist in northern Arizona. We found no northern leopard frogs in the Arizona Strip region during the survey period and as far as we can determine, the species no longer occurs in this region. Northern leopard frogs declined to local extinction at one site—Horseshoe Bend in Glen Canyon below Glen Canyon Dam—during the last 10 years. Northern leopard frogs were formerly common at

this small site immediately along the Colorado River between Glen Canyon Dam and Lees Ferry (Drost and Sogge, 1995). We last saw a single northern leopard frog at Horseshoe Bend in 2004, and repeated surveys since then have failed to find any leopard frogs. Current status of the known remaining populations is as follows:

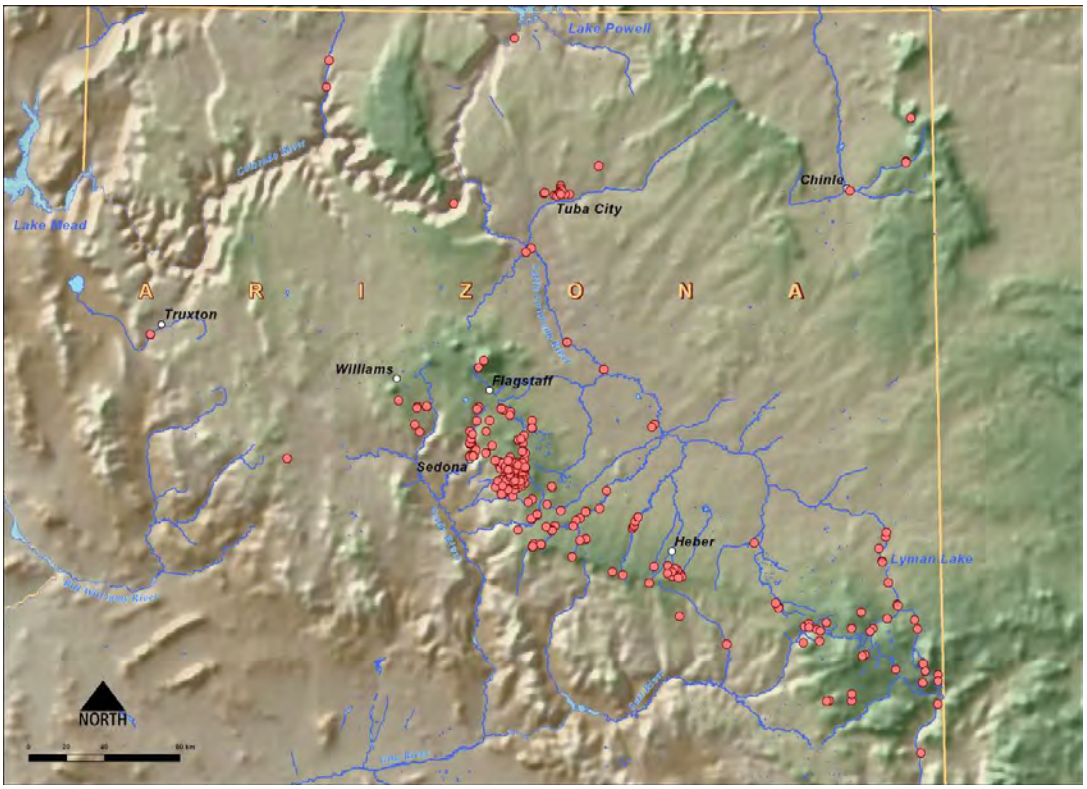
Central Highlands / Mogollon Rim

Natural and manmade ponds, stock tanks, lakes and wetlands in the Stoneman Lake vicinity comprise the last extensive area where northern leopard frogs persist in Arizona. Historical locations of northern leopard frogs in the Mogollon highlands area extended from the San Francisco Mountains and tributaries of San Francisco Wash in the Flagstaff area, south and west to the White Mountains, including headwater streams flowing north into the Little Colorado River, as well as a few populations in streams flowing west and south into the Verde / Gila River system.

Clarkson and Rorabaugh (1989) surveyed a sample of 14 historical sites for northern leopard frogs in the central highlands area, but failed to find frogs at any of those sites. They did, however, find a previously unreported site with northern leopard frogs at Viet Spring (called also "Veit Spring") on the south side of the San Francisco Mountains. We re-surveyed the Viet Spring site and the surrounding drainage in July 2008. The pond was completely dry, and we found no water either upstream or downstream, including at the spring area. The earthen dam at the pond site was still intact, but judging by the vegetation growing in the former pond area, the area had not held water for any length of time for several years.

Populations in the Stoneman Lake area have, in contrast to the other northern leopard frog sites in the State, expanded substantially in the last 7–10 years (S. MacVean, AGFD, oral commun.; see fig. 4). The expansion of northern leopard frogs in this area has been largely northward, with the known range expanding 14 km (8.7 mi) straight-line distance from Roundup Park to the northwest margin of Mormon Lake. As perspective on the magnitude of this increase, it nearly doubles the north-south extent of occupied sites in the area (the distance from Roundup Park south to Brady Tank in the Stoneman Lake core area is 16.5 km/10.3 mi).

A



B

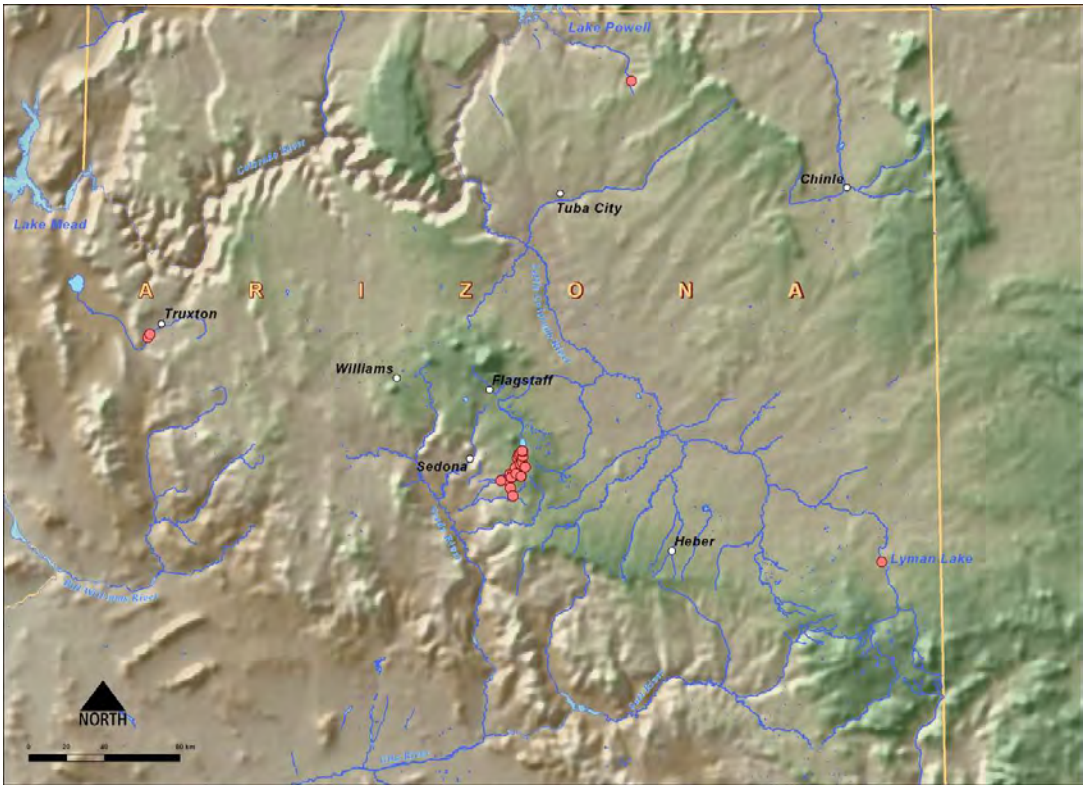


Figure 3. Map showing historical (*A*) and current (*B*) known distribution of northern leopard frogs in Arizona. Historical distribution is from museum specimen records and published reports. Extant distribution is from field surveys conducted since 2003.

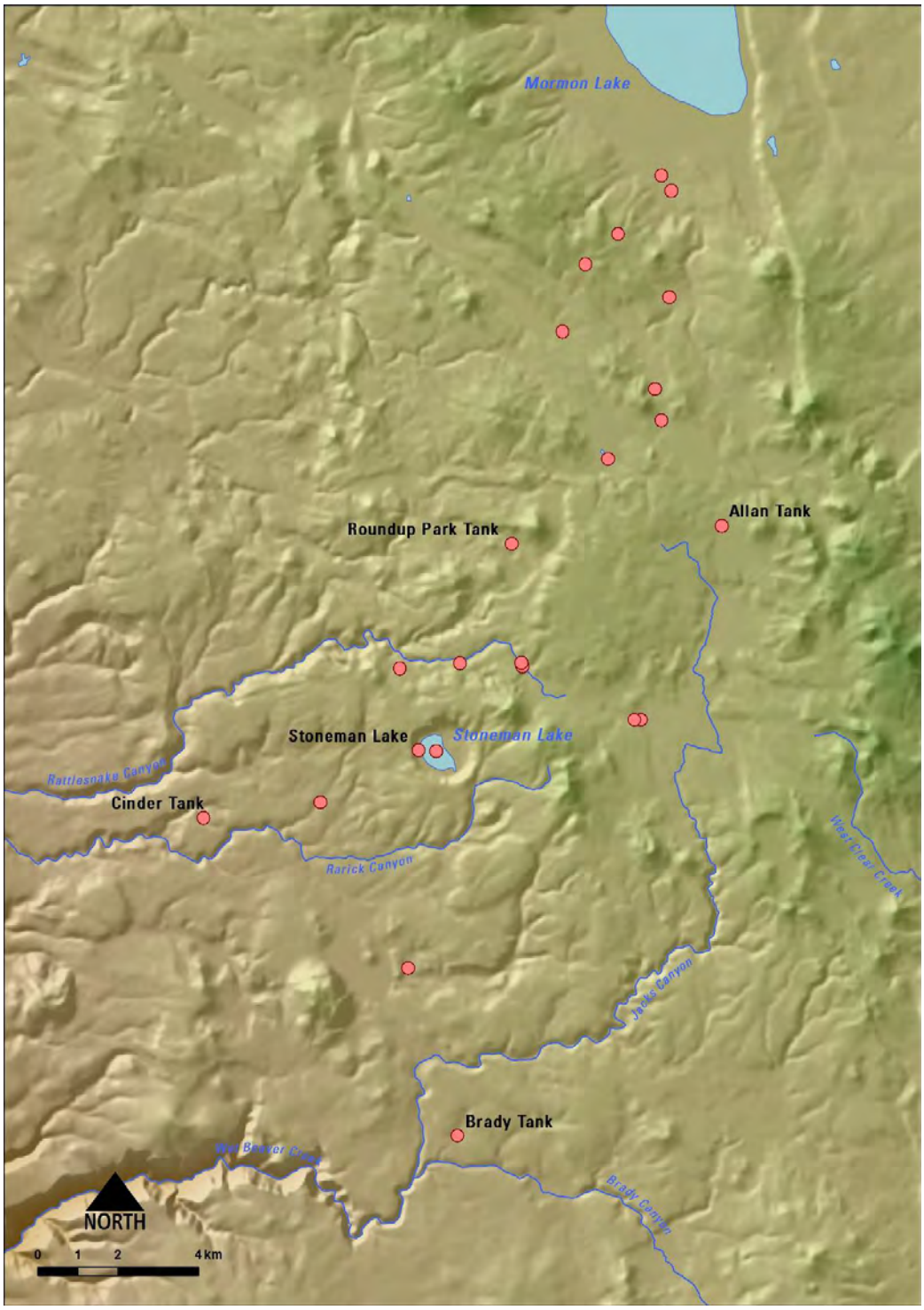


Figure 4. Map showing northern leopard frog distribution in the Stoneman Lake area. Labeled sites represent the approximate north–south and east–west extent of the Stoneman Lake metapopulation.

Little Colorado River Basin

Extant northern leopard frog populations in the Little Colorado River basin are found at Lyman Lake, south of St. Johns in Apache County, and the Inscription House area of the Navajo Creek drainage, on Navajo Tribal lands (fig. 3, bottom).

Our surveys in the Tsegi and Navajo Creek drainages on the Navajo Nation have been limited to the vicinity of National Park Service lands comprising Navajo National Monument, plus surveys of the lower ends of streams where they flow into the San Juan River or Lake Powell. The only leopard frogs found recently in these areas were near the Inscription House unit of Navajo National Monument, in Inscription House Canyon. Inscription House Canyon contains a small, west-flowing tributary of the large northerly flowing Navajo Creek. The canyon has small seep springs in its upper reaches, and seasonal puddles along its length, but most of the canyon is dry in summer and fall. The Navajo Creek drainage has more extensive water, and we suspect that it is the source of frogs seen in Inscription House Canyon. We did not search along the main length of Navajo Creek, and additional surveys would be needed to determine the population status of leopard frogs in this area.

Other areas surveyed on Navajo Tribal lands included Pasture Canyon and parts of Moenkopi Wash in and near Tuba City, Tsaile Creek and Tsaile Lake, east of Canyon de Chelly National Monument, lower Chinle Wash, and parts of Lukachukai Creek and ponds in the Big Lake area of the Chuska Mountains, where northern leopard frogs had been recorded historically. No leopard frogs were found in any of these areas, and some of these sites were dry (for example, Big Lake and most surrounding ponds) at the time of the surveys.

Lyman Lake supports the last known extant population of northern leopard frogs in the upper Little Colorado River drainage. The area that supports leopard frogs consists of a series of ponds and marshy meadows along the outflow from the Lyman Lake dam. The population here appears to be declining, and has had only minimal successful reproduction during our survey period. Crayfish occur in high numbers throughout the area where frogs have been found at this site. We also surveyed the Little Colorado River and its tributaries downstream of the Lyman Lake outflow. The main stream is fast-flowing and incised, does not appear suitable for leopard frogs, and we did not find them there. There are several broad, open, marshy tributaries that flow into the mainstream. These appear to offer potential leopard frog habitat, but we have not located frogs in these areas to date. [An additional noteworthy aspect of the Lyman Lake site is the presence of an evidently healthy population of painted turtles (*Chrysemys picta*). This is one of the few sites within Arizona where this species has been found and during our surveys we saw several individuals of different sizes.]

Northwestern Arizona, South of Colorado River

Northern leopard frogs in the Truxton Spring and Truxton Wash area seem to be maintaining consistent numbers, though the area occupied is restricted and extremely isolated. There are several large perennial streams to the north and northeast of the Truxton site that flow north into the Grand Canyon. These include Diamond Creek, Spencer Creek, and Clay Tank Canyon (Lost Creek). We surveyed almost the entire length of these drainages, and found no ranid frogs. Unexpectedly, however, we discovered an isolated population of lowland leopard frogs (*Rana yavapaiensis*) in the large, perennial stream in Surprise Canyon, on the north side of the Grand Canyon in this area (Oláh-Hemmings and others, 2010).

Arizona Strip

Our surveys in the Arizona Strip area have covered the Colorado River mainstem and tributary streams in the Marble Canyon / Grand Canyon / Lake Mead area with perennial flow. We also surveyed portions of the Virgin River and its tributary streams in the northwestern corner of Arizona. Besides the Horseshoe Bend site between Lees Ferry and Glen Canyon Dam, northern leopard frogs were previously known from downstream along the Colorado River at Cardenas Marsh, below the confluence of the Colorado River and Little Colorado River (Tomko, 1975; Tomko took several photos of the leopard frogs found at this site, and the photos were examined by the authors and determined to be *Rana pipiens*). We surveyed the area of the former Cardenas Marsh (which is now completely dry), including side canyons and small patches of riverside marsh along this stretch of the Colorado River. No leopard frogs have been reported from this area since Tomko's time, and we did not find any frogs, nor any suitable habitat. The remaining riverside marshes in this area are small, narrow, and densely grown up with common reed (*Phragmites australis*). The little water in these riverside marshes is cold river overflow from daily fluctuating regulated flows in the Colorado River, and is probably too cold for northern leopard frogs.

We did not find leopard frogs along Kanab Creek or its tributaries in Arizona, although the species occurred here historically, and may persist along portions of Kanab Creek in southern Utah. To our knowledge, the only other site where northern leopard frogs have been reported in the region north and west of the Colorado River in Arizona is along Bright Angel Creek in Grand Canyon National Park (Mike Sredl, AGFD, unpublished data). We surveyed extensively along Bright Angel Creek and its tributaries, from the north rim of Grand Canyon to the Colorado River, and did not find any leopard frogs.

Genetic Analyses

We analyzed genetic samples from 259 frogs from 21 populations in the study region (table 2). Samples from Horseshoe Bend, Hess Tank, and East Buckskin Tank were collected in 1994 by Diana Kimberling and associates (Kimberling and others, 1996). These populations are now extirpated, so we could not collect more recent samples. We also incorporated in our analyses samples collected by Kimberling and associates from the Stoneman Lake area, though the great majority of Stoneman Lake-area samples are new material collected during this study. For all other populations, we collected samples in 2007 through 2009. Sample sizes ranged from 1 to 31 per population. Our target sample size was 20 samples per site, but small numbers of frogs at many locations precluded reaching this target.

Populations included in the genetic analyses were distributed across northern Arizona, but were clustered in five general areas (fig. 1). The Horseshoe Bend population (also referred to as "-9 Mile Draw," and now extirpated) was located along the Colorado River near the northern border of Arizona. Also isolated in eastern Arizona were the Hess Tank and East Buckskin Tank populations, which were very near each other. These two sites were part of a complex of ponds and streams spread over several square km in the Black Canyon drainage south of Heber and Overgaard. The Truxton Wash population is similarly isolated from all other populations, in western Arizona southwest of Peach Springs and northeast of Kingman. The remaining 16 populations were spatially clustered in the Stoneman Lake area (fig. 2).

Three mitochondrial haplotypes were found in these populations. Two haplotypes were closely related and nested with Hoffman and Blouin's (2004a) western clade. One of these western haplotypes was found in all five population groups in our study. The second western haplotype was restricted to the Stoneman Lake area populations, but was widespread among the populations in the southern 80 percent of this area (fig. 4). The third haplotype was included in Hoffman and Blouin's (2004a) eastern clade, a clade otherwise limited to populations in the northeastern United States and adjacent southeastern Canada. The eastern haplotype was found only in the Stoneman Lake area populations, and was the most common haplotype in the northern half of the area, from Roundup Park north (fig. 4 and fig. 5*A*). Restriction assays (the alternative method of discriminating eastern versus western frogs) indicated similar patterns of mitochondrial haplotypes.

Table 2. Sample sites and genetic sample data for northern leopard frogs in northern Arizona.

[Samples sequenced, number of samples for which we obtained 786 bp of sequence data from the mitochondrial ND1 gene; Percent eastern, percentage of samples that matched eastern U.S. mitochondrial haplotypes, as assessed either by sequencing or restriction assay; mtDNA, mitochondrial DNA; msats, microsatellites. Samples marked "*" are from 1994; leopard frog populations at these sites are now thought to be extirpated]

Locality name and location	Total samples	Samples sequenced mtDNA	Percent eastern haplotype
Colorado River; northern Arizona			
Horseshoe Bend (-9 Mile Draw)*	14	5	0
Heber / Black Canyon area			
East Buckskin Tank*	15	6	0
Hess Tank*	15	5	0
Eastern Arizona			
Lyman Lake	2	2	0
Northwestern Arizona			
Truxton Wash	25	5	0
Stoneman Lake area			
Site 1	1	1	100
Site 2	2	2	100
Site 3	3	3	100
Site 4	23	9	83
Site 5	4	4	75
Site 6	3	3	67
Site 7	31	15	55
Site 8	25	10	43
Site 9	16	6	13
Site 10	19	5	11
Site 11	24 (11 msats)	5	4
Site 12	2	2	0
Site 13	1	1	0
Site 14	9	5	0
Site 15	23	5	0
Site 16	2	2	0

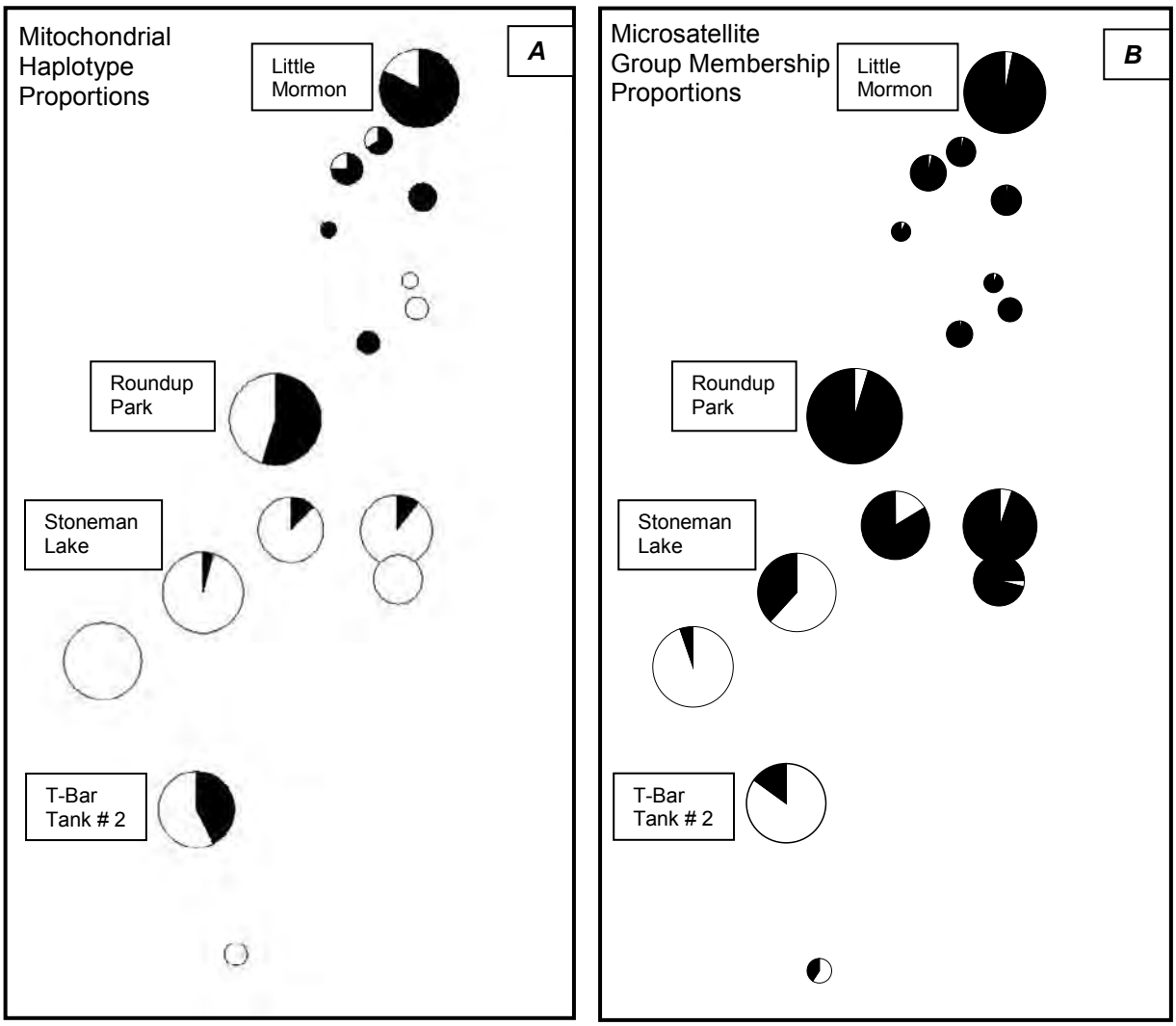


Figure 5. *A*, Distribution of eastern and western mitochondrial haplotypes of northern leopard frogs in the Stoneman Lake area. Black indicates relative proportion of individuals with the eastern haplotype, and white indicates proportion of western haplotypes. Results of sequencing and restriction assays are combined here. *B*, Distribution of microsatellite group membership based on STRUCTURE analysis using k=2 without mitochondrial group pre-assignment. Black represents proportion of STRUCTURE group 1 membership ("eastern"), and white represents STRUCTURE group 2 membership ("western"), averaged across individuals. In both *A* and *B*, circles represent sampled populations with size of circle proportional to sample sizes from the populations (n=1 to n=31).

Genetic Diversity

Most of the microsatellite loci that we analyzed were highly polymorphic, ranging from 5 to 11 alleles in our samples (table 3). Genetic diversity was high across the populations in the Stoneman Lake area, intermediate at Horseshoe Bend and low in the Truxton Wash population and in the Hess Tank / East Buckskin Tank area by all three measures of diversity (table 4). Sample size for Lyman Lake was too small to assess genetic diversity in that population. Deviations from genotypic frequencies expected under Hardy-Weinberg equilibrium, using a Bonferroni correction for multiple tests, were found in only two of the population by locus combinations. Both of these were at Rarick Tank, for locus Rpi101 and locus Rpi107. The disequilibrium at Rpi101 was due to a heterozygosity excess (P=0.0022).

Clustering of populations assessed from microsatellite-based genetic distances among populations generally reflected broad spatial relationships (fig. 6). East Buckskin and Hess Tank were very similar, but taken together were very distinct from the other populations. Truxton Wash and Horseshoe Bend were likewise unique when compared to the other populations, although they tended to cluster more closely with the Stoneman Lake populations than did Stoneman Lake with the East Buckskin / Hess Tank cluster, contrary to geographic expectations. The Stoneman Lake populations formed a well-supported cluster, as would be expected given their hydrologic connectivity and geographic proximity.

Table 3. Total number of alleles found for each microsatellite locus in populations of northern leopard frogs at sites across northern Arizona.

Microsatellite locus	Total alleles
Rpi100	8
Rpi101	8
Rpi102	8
Rpi103	11
Rpi104	5
Rpi107	11
Rpi108	6
RP193	9

Table 4. Estimates of genetic diversity for populations of northern leopard frogs in Arizona, based on microsatellite analyses.

[n, number of samples genotyped at microsatellite loci. Populations with sample sizes less than nine are not shown]

Population	n	Average gene diversity per locus	Total observed number of alleles	Allelic richness (rarified to n=9)
Horseshoe Bend	14	0.508	23	21.7
East Buckskin Tank	15	0.219	15	14.5
Hess Tank	15	0.194	12	11.8
Truxton Spring / Wash	25	0.298	14	13.4
Stoneman Lake populations				
Site 1	16	0.643	38	34.3
Site 2	22	0.570	28	24.4
Site 3	19	0.619	33	29.1
Site 4	9	0.586	29	29.0
Site 5	23	0.551	28	25.2
Site 6	31	0.613	33	29.2
Site 7	11	0.603	37	35.0
Site 8	25	0.587	24	23.2

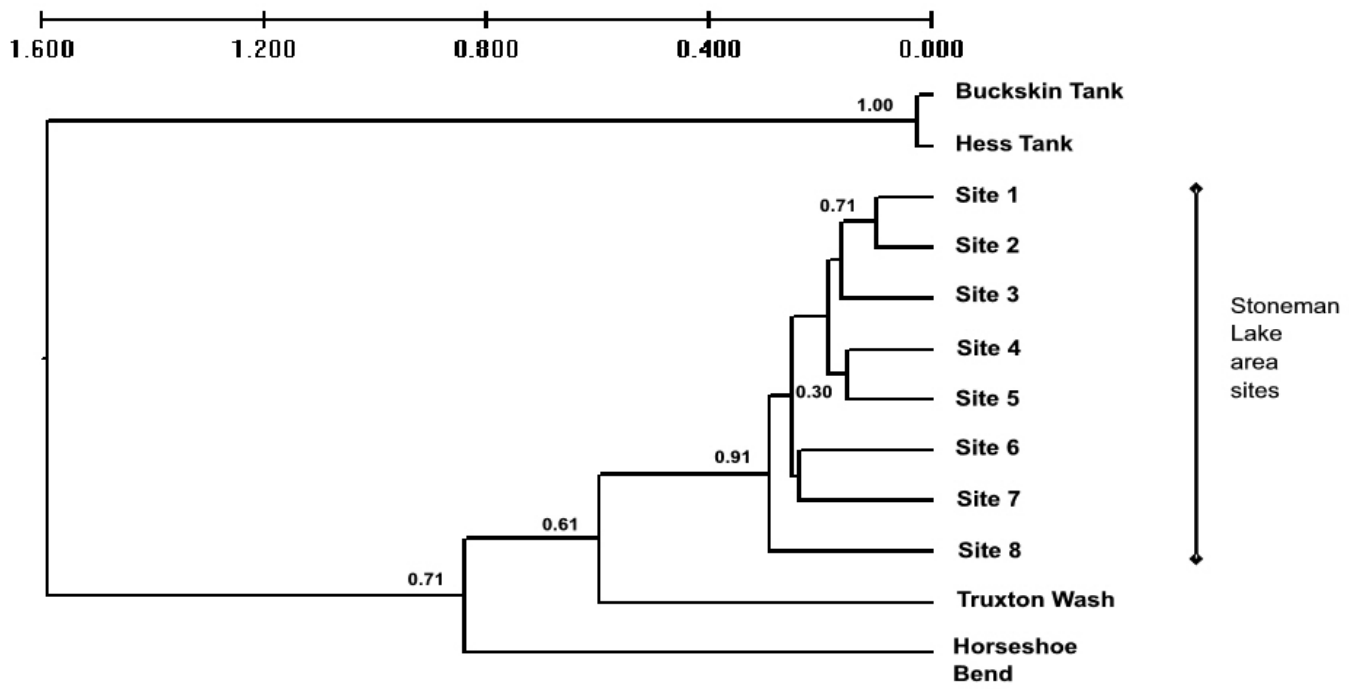


Figure 6. Diagram showing UPGMA dendrogram of Nei's (1972) distances based on microsatellite allele frequencies. Proportion of bootstrap replicates regenerating each node with greater than 30 percent frequency is noted at each node. Bootstrap values less than 50 percent should be interpreted with caution.

Microsatellite Analysis—Eastern Versus Western Genetic Pattern

In addition to comparing mitochondrial haplotypes, we also used an analysis of microsatellite allele frequencies to evaluate eastern versus western genetic influence among the Stoneman Lake populations. Lacking a reference population for the source of eastern frogs, we used a Bayesian algorithm implemented in STRUCTURE software to explore clustering patterns based on microsatellite genotypes and compare them with haplotype grouping and geographic structure. Frequencies of individual assignments to the two microsatellite groups identified by STRUCTURE did not correspond perfectly with eastern versus western mitochondrial haplotypes (figs. 5 and 7), but populations did display different proportions of these markers (fig. 5*B*). These frequency differences were used to designate the microsatellite groups as "eastern" and "western." The distinct origins and current separation of these groups was indicated by a microsatellite-based θ_{ST} of 0.273 between frogs with eastern and western mitochondrial haplotypes. However, the program STRUCTURE assigned many individual frogs to eastern or western groups in contrast to their mitochondrial haplotypes, suggesting that gene flow has occurred between these two groups and mitochondrial haplotypes are not a reliable indicator of the majority of the genetic heritage of any given frog in this region (fig. 7). If individuals with a particular haplotype (eastern or western) also represented a distinct group with respect to nuclear alleles, we would expect very high assignment fidelity within mitochondrial groups; in other words, individuals with western mitochondrial haplotypes would appear as mostly red lines and individuals with eastern mitochondrial haplotypes would appear as mostly green lines. STRUCTURE analysis with k=2 suggested a gradient of group membership (fig. 5*B*) generally concordant with the gradient of mitochondrial types (fig. 5*A*). Both measures show a higher proportion of "eastern" frogs in northern populations, and a higher proportion of "western" frogs in southern populations.

STRUCTURE analysis for k optimality (k=1 to 12) indicated that the most likely population structure in the Stoneman Lake complex of populations is k=6. However, the individuals assigned to these six hypothesized groups were not concordant with sampling locations, and the probability of membership in each of the six groups was mixed for most individuals, suggesting that the structure detected using this approach was an artifact. The failure of STRUCTURE to resolve biologically plausible groups may be due to the existence of multiple, poorly sampled subpopulations.

Hoffman and Blouin (2004a) originally identified one eastern individual among a sample of ten frogs collected from Roundup Park Tank and adjacent small tanks in 2001 (these samples were collected by S. MacVean, Arizona Game and Fish Department, who shared the collection data with us). We intensively sampled the Roundup Park Tank area in 2007 and 2008, and collected tissue from 31 adult and subadult frogs. Of these 31 frogs, 17 had the eastern haplotype. This is a statistically significant increase in the proportion of frogs with eastern mitochondrial haplotypes in this area, the only area for which we have adequate sample sizes from two samples separated in time (Fisher's Exact Test, p=0.025).

Principal coordinates analysis (PCoA) of the microsatellite data from the Stoneman Lake–area populations shows three marginally distinct clusters, arrayed from left to right along coordinate 1 (fig. 8). However, most sites for which we had large sample sizes spanned all three of these clusters (for example, T-Bar Tank #2, Stoneman Lake, Roundup Park), suggesting that the implied PCoA structure may not be biologically relevant.

Figure 7. Graph showing probability of individual membership in one of two clusters of northern leopard frogs in the Stoneman Lake area, detected by STRUCTURE analysis (k=2) based on microsatellite allele frequencies. Individuals are represented as vertical bars. The two clusters defined by STRUCTURE analysis are shown as red (associated with western haplotypes) and green (associated with eastern haplotypes). An individual with a 90 percent red bar has a 90 percent probability of being assigned to the red / western STRUCTURE cluster and a 10 percent probability of being assigned to the green / eastern STRUCTURE cluster. Individuals are grouped into those with western mitochondrial haplotypes (left of the vertical black line) and those with eastern mitochondrial haplotypes (right of the vertical line). “Unknown” (extreme left of figure) represents five frogs whose mitochondrial DNA failed to amplify with PCR.

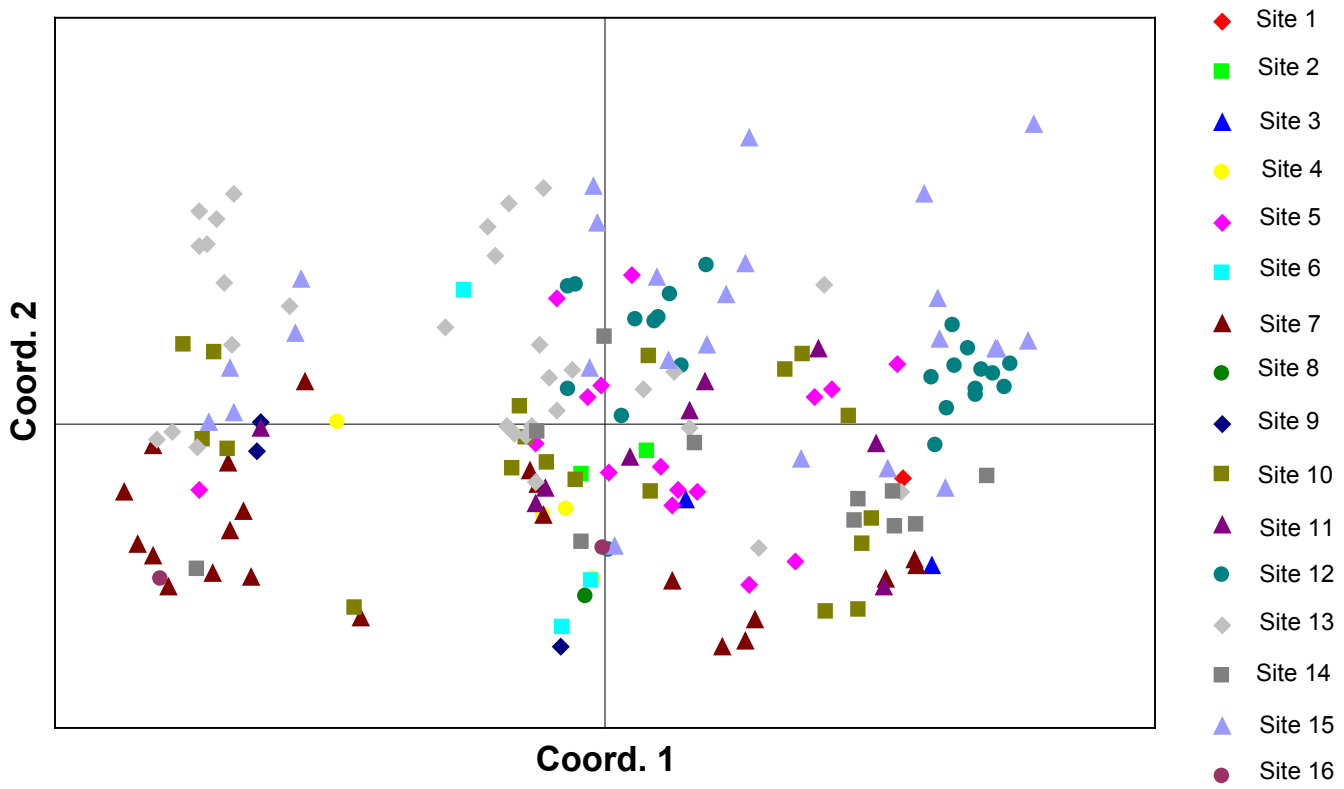


Figure 8. Principal coordinates analysis diagram, based on a matrix of microsatellite genetic distances between all individuals in Stoneman Lake area populations. Some clustering is evident, but clusters do not correspond strongly with population membership.

Population Structure and Small Population Effects

Overall, population-level structuring was very pronounced (θ_{ST}=0.315; 95 percent confidence interval=0.261–0.374). Across populations, inbreeding was not significant (f= -0.035; 95 percent confidence interval = -0.081–0.023). Consistent with the θ_{ST} results, pairwise exact testing of all 11 populations for which at least 15 individuals were sampled indicated that each population was distinct from every other ($P<0.05$) except for East Buckskin and Hess Tanks ($P=0.32$).

Seven populations (Horseshoe Bend, East Buckskin Tank, Hess Tank, Rarick Tank, Roundup Park Tank, T-Bar Tank #2, and Truxton Wash) showed a modal shift towards higher allele frequency categories, which indicates past population bottlenecks (including recent founder events; fig. 9). Sign tests performed using BOTTLENECK version 1.2.02 indicated that Horseshoe Bend, Butch Tank, Little Mormon Lake, North of Pratt Park, Rarick Tank, Roundup Park Tank, and T-Bar Tank #2 showed significant heterozygote excess under the infinite alleles mutation model, but only T-Bar Tank #2 showed excesses under the stepwise alleles model (both of these measures are also indications of significant recent bottlenecks). Several of these populations had sample sizes under 20, which could cause spurious results. Under the two-phase model of mutation, generally considered the most accurate for use with microsatellites (Di Rienzo and others, 1994), Little Mormon Lake, Rarick Tank, and T-Bar Tank #2 showed indications of significant recent bottlenecks in the form of heterozygote excesses.

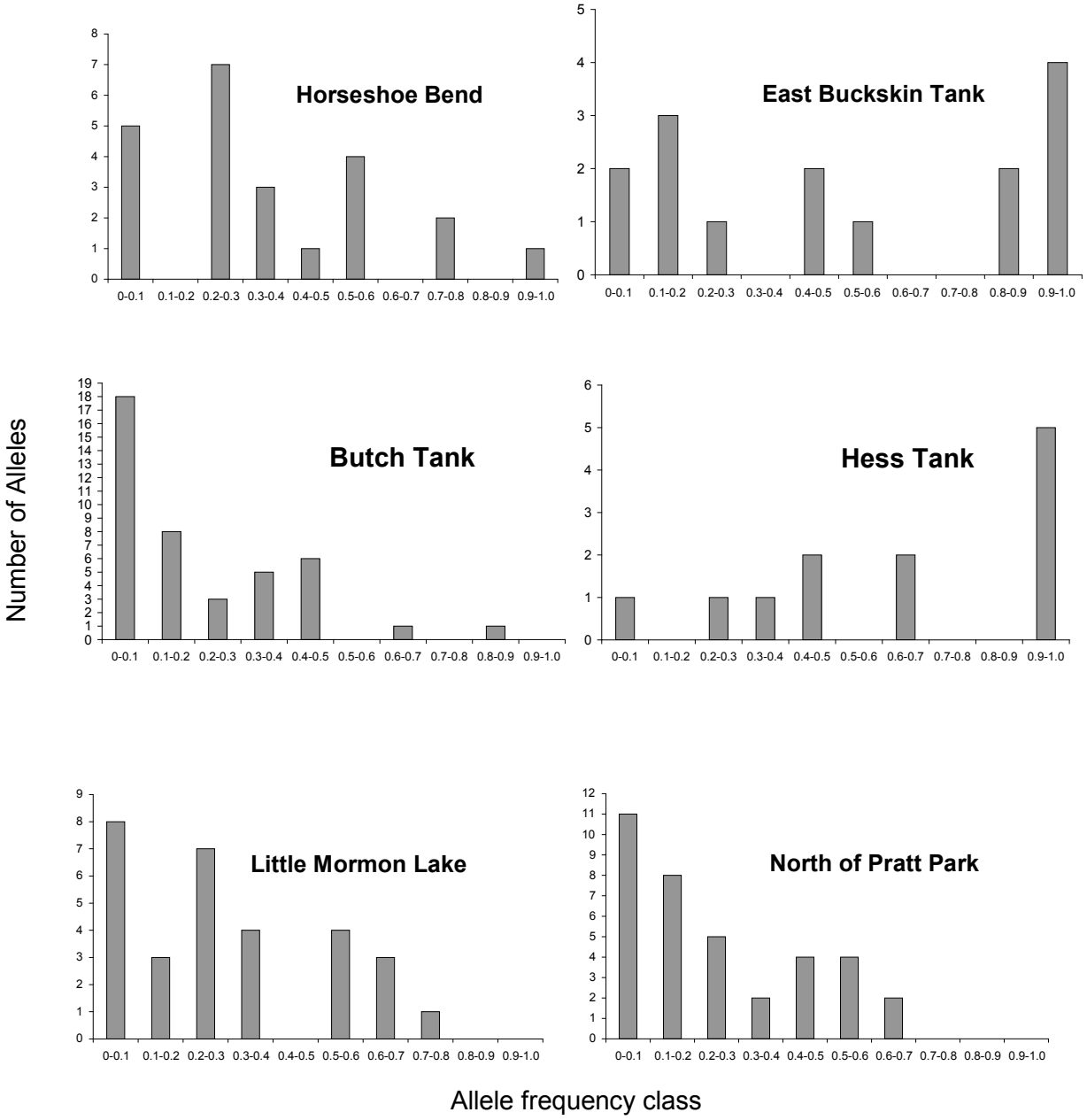
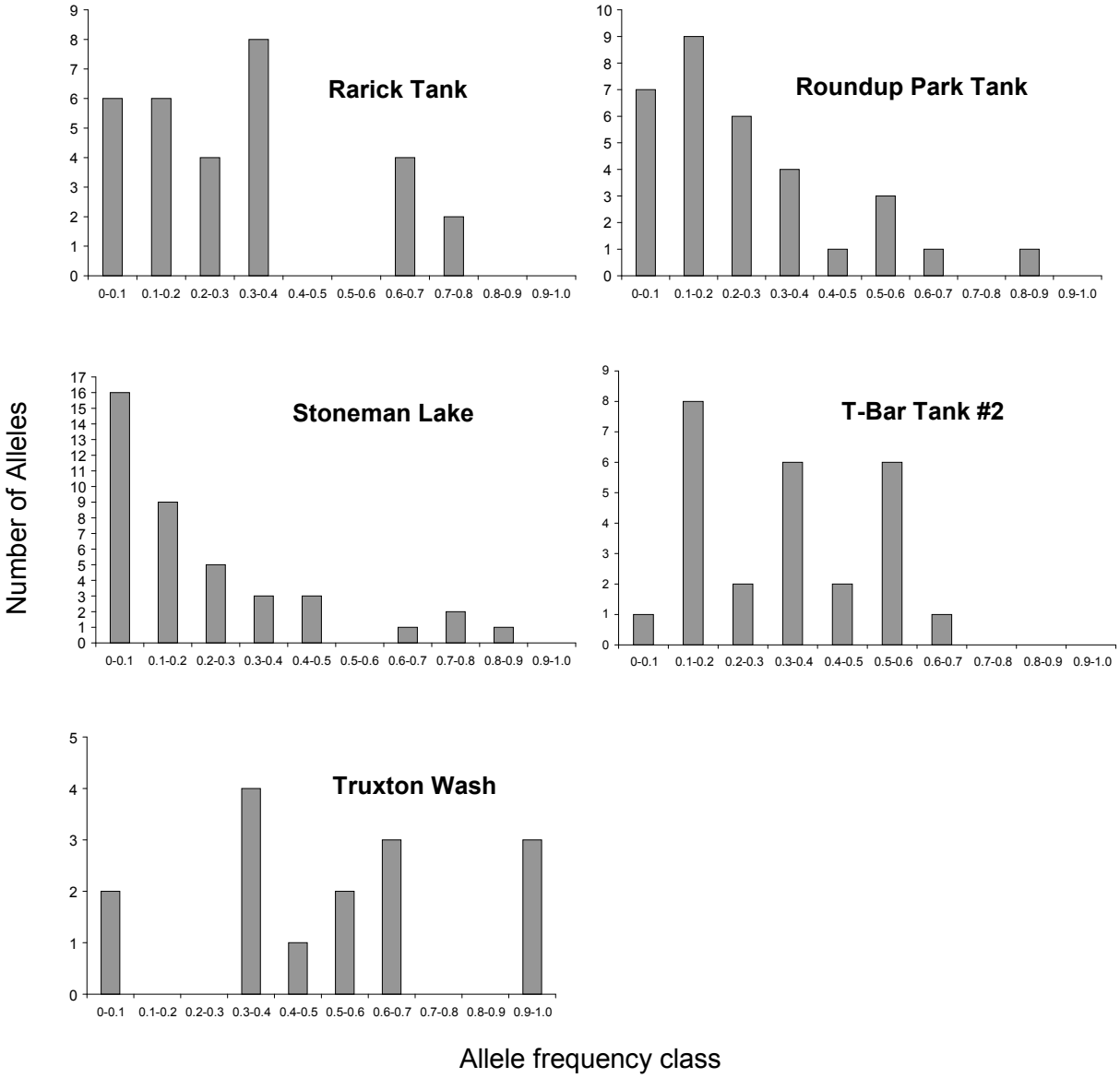


Figure 9. Allele frequency histograms for 11 populations of northern leopard frogs in northern Arizona with at least 15 individuals sampled. Distributions skewed to the left (in other words, with a modal allele frequency of 0.01–0.1) indicate populations without a significant recent bottleneck. Distributions where the mode is not 0.01–0.1 indicate populations that have likely gone through recent bottlenecks.



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Discussion

Northern Leopard Frog Distribution and Numbers

Based on surveys conducted between 1983 and 1987, Clarkson and Rorabaugh (1989) documented a severe decline in northern leopard frogs in Arizona that had occurred over the previous decade. They surveyed 14 sites that previously supported northern leopard frogs, and did not find extant populations at any of the sites (although they did document one new, previously unreported population, at Viet Spring north of Flagstaff). Clarkson and Rorabaugh also found conspicuous declines in other species of leopard frogs in Arizona during the same period, particularly in the Chiricahua leopard frog (*R. chiricahuensis*). Although the surveys of Clarkson and Rorabaugh for northern leopard frog were limited, the pattern and severity of decline in the species is borne out by more recent, extensive surveys by Mike Sredl and associates of the Arizona Game and Fish Department (AGFD; for example, Blomquist and Sredl, 2002).

The historical pattern of occurrence of northern leopard frogs shows the species was formerly distributed primarily in the Little Colorado River drainage (notably through the Mogollon highlands to the White Mountains), with less extensive occurrences in other tributaries of the Colorado River in northern Arizona (fig. 3). In the Little Colorado River headwaters areas of the White Mountains, Mogollon highlands, and Flagstaff area, the species appears to have crossed over into the upper parts of west- and south-flowing drainages. Although the literature and museum record is not exhaustive, the species' distribution evidently included some relatively isolated populations, notably in the Truxton Wash area and in the eastern Grand Canyon. Such a pattern is not unexpected at the edge of a species' range.

However, the declines over the last 35–40 years have resulted in a much more pronounced pattern of fragmentation and isolation of the few populations that are left in Arizona. Except for the Stoneman Lake metapopulation, the remaining northern leopard frogs in Arizona consist of single-point populations that are completely isolated from exchange with other populations. The distances and barriers between Lyman Lake, the Stoneman Lake area, Truxton Wash, and Navajo Creek are so great that there is no longer any possibility for natural genetic exchange. The now-extirpated population at Horseshoe Bend was similarly completely isolated.

Decline of northern leopard frogs appears to be continuing since the surveys of Clarkson and Rorabaugh (1989), and AGFD. The "new" population documented by Clarkson and Rorabaugh at Viet Spring is no longer extant, and the isolated population at Horseshoe Bend disappeared in about 2005. At least for more recent extirpations, there appear to be identifiable causes in terms of habitat loss or degradation. For example, the main breeding pool at the small Horseshoe Bend site gradually became completely filled in with a dense stand of common reed (*Phragmites australis*). The last breeding attempts by northern leopard frogs at the Horseshoe Bend site were in small overflow pools immediately adjacent to the Colorado River (see cover photo), which were subject to flooding with cold water and washout from fluctuating regulated flows of the river. The Viet Spring and pond site, and the associated drainage, were completely dry at the time of our surveys. Growth of small trees and shrubs in the dry pond site indicated that the small tank had not held water for at least several years. Similarly, large wetlands in the vicinity of Big Lake in the Chuska Mountains are now largely dry, and we have been unable to find any northern leopard frogs remaining in this region. Recent extirpations of local populations

in the Glen Canyon area also appear to be attributable to habitat degradation, notably invasion by nonnative crayfish (Drost and others, 2008).

The northern leopard frogs in the Mogollon highlands that now occur only in the vicinity of Stoneman Lake were formerly much more widely distributed. Based on literature and museum records (figure 3*A*) and available habitat, they evidently had a more or less continuous distribution from streams and ponds in the Flagstaff area south and east to the vicinity of Alpine in the White Mountains, and thence into the highlands of western New Mexico. Although nonnative fish and periodic drought-related drying have likely had adverse effects on the frogs in this area, the cause of the nearly complete loss of northern leopard frogs (and the related Chiricahua leopard frog) through so much of this extensive area is not at all clear.

Against this backdrop of widespread decline, the persistence and expansion of northern leopard frogs in the Stoneman Lake area stands out as one encouraging sign. The overall extent of the area occupied by northern leopard frogs in this vicinity has approximately doubled in the last decade. Although the populations experience some year-to-year variation in abundance, frogs in this area occur in numbers at several good-quality breeding sites, as well as at numerous intervening smaller tanks and ponds (S. MacVean, AGFD, unpublished data). The interconnected metapopulation in the Stoneman Lake area is the only northern leopard frog population in Arizona that is expanding in its extent.

For the other northern leopard frog populations in Arizona that are discussed in this report, current status ranges from marginal to relatively secure. At one extreme, we found only three adult frogs and one metamorph during two surveys of the Lyman Lake population and surrounding areas. Further, the area occupied by the frogs has large numbers of nonnative crayfish. We found no northern leopard frogs in nearby habitats that appeared suitable.

Northern leopard frogs in the Truxton Wash area appear to be maintaining consistent numbers from year to year, at two different sites. These sites are close to each other, and the frogs appear to be successfully reproducing at both sites. Although total numbers of northern leopard frogs in the Truxton Wash area appear to be moderately high at present, our genetic analyses indicate a recent genetic bottleneck in this population (fig. 9). The Truxton site is isolated by over 200 km (125 mi) from the next nearest known population of northern leopard frogs. Geographically, this site is just to the north of a low north-south divide, with streams north of the divide flowing in a northerly or westerly direction, to empty into the Colorado River in the western Grand Canyon. South of the divide, streams flow in a southerly direction into the Big Sandy River and the Bill Williams River. Surveys to the north, south, east, and west of Truxton Spring and Truxton Wash failed to find additional populations of northern leopard frogs. Truxton Wash itself quickly becomes dry both upstream (east) and downstream (west) of the known leopard frog site. To the south, Willow Creek along the south side of Interstate 40 supports lowland leopard frogs. This location is 36 km (20.0 mi) straight-line distance south–southeast of the Truxton Wash site. We extensively surveyed large drainages north of Truxton Wash (for example, Peach Springs Wash, Diamond Creek, Spencer Creek), but found no ranid frogs of any species.

Northern leopard frogs have also been reported in the Inscription House / Navajo Creek drainage on Navajo Nation lands in northeastern Arizona. Except for the initial report of their occurrence, however, we have been unable to gather any additional data on distribution and numbers in this area—hence the current status of the species there remains unknown.

Patterns of Genetic Divergence Among Populations

Throughout the study area, genetic divergence among populations is generally high. This is not surprising given the great distances that separate most of the remaining sites. Several populations, especially those at Truxton Wash, Hess Tank, East Buckskin Tank, and Horseshoe Bend, show indications of both reduced genetic diversity and loss of rare alleles, indicating persistently small population sizes and recent population bottlenecks. Hoffman and Blouin (2004b) measured genetic diversity in peripheral (edge of range) and central (interior) populations of the northern leopard frog. Although the loci we used did not match precisely with the loci they used, there is sufficient overlap that we can compare levels of genetic diversity at a coarse level. Some of the populations in the Stoneman Lake area had levels of diversity that were approximately equal to those of Hoffman and Blouin's "interior" populations. Populations outside of the Stoneman Lake area all had genetic diversity that was roughly equivalent to or lower than Hoffman and Blouin's "peripheral" populations.

Most of the populations with sample sizes greater than 11 showed molecular signatures of recent demographic bottlenecks, despite the low statistical power associated with small sample sizes. These "bottleneck" signatures were somewhat inconsistent across methodological approaches, but were generally congruent with low allelic richness (table 4). This pattern is perhaps not unexpected given the general decline of many southwestern riparian areas and springs during the last century, but it is a cause for concern because of the potentially negative synergistic effects of small population sizes, population isolation, and inbreeding depression.

East Buckskin Tank and Hess Tank were both very similar to one another and very different from the other populations in terms of microsatellite allele frequencies (fig. 6). These sites are close to one another (3.4 km straight-line distance) in the same drainage, and the frog populations at these sites were also very similar to one another for genetic diversity measures. Kimberling and others (1996; see also Miller and others, 1999) suggested that they may both have become established from nearby populations in the 1990s. Mitochondrial haplotypes from these populations match those native to the region, so if these populations are the result of a recent introduction or colonization, then they probably did come from a source population in the same region. Microsatellite allele frequencies have diverged from those of other native populations in the region, possibly due to founder effects or drift, exacerbated by small population size and isolation. Inbreeding in these two populations was so extreme that four pairs of individuals and one triplet of individuals were identical at all eight microsatellite loci, indicating that this population was either founded by very few individuals from another source or had recently gone through an extreme population bottleneck.

Stoneman Lake Area and Possible Hybridization

In contrast to other northern leopard frog populations in Arizona, populations in the Stoneman Lake area showed greater diversity but less genetic divergence. In part, this reflects the large effective population size in the area, which is characterized by a complex of many ponds that are evidently connected by some level of gene flow. Unfortunately, the elevated genetic diversity in the Stoneman Lake complex has been augmented by the introduction of northern leopard frogs from eastern North America. In samples collected in August 2001, Hoffman and Blouin (2004a) found one individual that contained an eastern mitochondrial haplotype, out of ten frogs sampled from the vicinity of Roundup Park Tank. They suggested that this eastern frog may have been a released laboratory animal or pet. Our analyses for this project reveal that eastern haplotypes were present in the Stoneman Lake vicinity at least as early

as 1994, and that they are currently widespread throughout this metapopulation. We reexamined samples collected in 1994 by Diana Kimberling (Kimberling and others, 1996) from Butch Tank and Stoneman Lake. Of 15 samples collected by Kimberling at Butch Tank, two were the eastern haplotype (table 5). Kimberling only collected three samples from Stoneman Lake, and none of those had the eastern haplotype. As discussed earlier, the proportion of eastern haplotype frogs has increased since the earlier samples were collected.

Table 5. Location and numbers of eastern haplotypes of northern leopard frogs in the Stoneman Lake area from earlier studies, compared to recent samples. Data from Kimberling and others (1996; samples collected in 1994), Hoffman and Blouin (2004a; samples collected in 2001), and this study (2007). Eric Tank, Gash Tank, and Roundup Park Tank are in close proximity and are taken to represent the same population.

[Eastern, number of eastern haplotypes from our analyses; n, sample size; %, percentage of eastern mitochondrial haplotypes in each sample]

	1994			2001			2007		
	eastern	n	%	eastern	n	%	eastern	n	%
Eric Tank				0	5	0			
Gash Tank				0	1	0			
Roundup Park Tank				1	4	25	17	31	55
Butch Tank	2	15	13				0	1	0
Stoneman Lake	0	3	0				1	21	5
Total	2	18	11	1	10	10	18	53	34

The eastern haplotype detected in the Stoneman Lake area is a haplotype found in New York, New England, and adjacent areas of Canada in Quebec and Ontario. This is an area that has historically been (Gibbs and others, 1971) and continues to be (Angela White, Biologist / Product Developer, Carolina Biological Supply, oral commun.) a source of northern leopard frogs for the commercial trade. We cannot be sure of the exact timing of the introduction of these eastern genotypes with the information that we currently have. However, our analyses do show that eastern mitochondrial haplotypes are currently widespread in the Stoneman Lake area, and have increased in prevalence. Further, microsatellite data suggest that eastern and western genetic lineages are broadly introgressed in this area.

A possible alternative explanation for the eastern mitochondrial haplotypes in the Stoneman Lake area is that they represent a relict haplotype from the Pliocene when eastern and western haplotype frogs interbred south of their current range. We think this explanation is unlikely for several reasons. First, F_{ST} values between frogs with eastern and western mitochondrial haplotypes indicate that although some introgression has occurred, microsatellite alleles are not randomly assorted among eastern and western mitochondrial haplotypes as would be expected if these haplotypes had been present and interbreeding since the Pliocene. Second, the eastern haplotype that is present in Arizona is from a remote group of populations that is not likely to have been interbreeding naturally with Arizona frogs. Hoffmann and Blouin (2004a) sampled northern leopard frog populations from across North America and did not find this eastern haplotype anywhere west of New York State and adjacent Quebec province. Last, and

most compelling, we did not find eastern genetic types in the other Mogollon highland populations (for example, Hess and Buckskin Tanks, Lyman Lake) which shared a recent genetic connection.

Although it is unlikely, we cannot entirely eliminate the possibility that eastern genotypes in the Stoneman Lake area are relicts of a pre-Pleistocene contact between eastern and western lineages. To more rigorously test this hypothesis, it would be necessary to sequence several nuclear genes in addition to the mitochondrial genes already sequenced in this study. By comparing coalescence times among DNA sequences, estimates of the timing of introduction could be generated and hypotheses of the timing of introduction (for example, pre-Pleistocene or Holocene) could be tested. Assessing the degree of introgression on the basis of allele frequencies, as in the assignment test displayed in fig. 7, would be a powerful approach for evaluating introgression between eastern and western frogs in the Stoneman Lake area if allele frequencies for western frogs and those of eastern frogs are very different. However, we have no reference data on allele frequencies in putatively introduced "eastern" frogs, and this method may not have much power to detect introgression if eastern frogs have allele frequencies similar to native western frogs.

Management Implications

It appears very likely that northern leopard frogs in the Stoneman Lake area have substantial eastern genetic influence from frogs introduced in the recent past. This potentially complicates management of the species in Arizona, and raises some difficult questions. The remote and isolated populations such as Truxton Wash and Hess Tank / East Buckskin Tank have (or had) low genetic diversity and might benefit from the addition of genetic diversity from other populations. All other factors being equal, the obvious choice for a source population to increase genetic diversity would be populations in the Stoneman Lake area. Populations in this area have the highest genetic diversity of any populations in Arizona, and because this is a complex with high population numbers and gene flow among populations, this area is most likely to tolerate the export of individuals for relocation or genetic exchange programs. However, the elevated genetic diversity of this area is likely due to interbreeding with nonnative individuals introduced from the northeastern United States or southeastern Canada. Spreading individuals from this population to other areas would mix nonnative genotypes into pure native populations, potentially leading to outbreeding depression and the swamping of locally adapted genotypes. Further, if such introductions were successful, they would be irreversible.

Maintenance of local adaptation is not the only concern in the management of endangered and threatened species. If remote localized populations are threatened with extinction because of small population sizes and the lack of genetic diversity, it may be more important to maintain some form of northern leopard frog there than to try to preserve genetic integrity at the peril of complete loss of the population. In cases where populations are at immediate risk of extinction, it may be necessary to introduce individuals from diverse areas such as Stoneman Lake. Alternatively, "western" northern leopard frogs from populations in southern Utah (Drost and others, 2008) or New Mexico might be used to supplement threatened populations in Arizona. Demographic studies of population size will be more informative than genetic studies in determining if and when the need for local augmentation of populations is reached.

This study included all of the sites known to still harbor northern leopard frogs in Arizona. However, some historical localities and other areas of potential habitat remain unsampled. Further field survey work of perennial streams and ponds in these areas would be

required to locate other extant populations of this species in Arizona. If additional populations are found, they may provide another source of genetic diversity for translocations, in areas not influenced by the probable introduction of eastern genetic forms.

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