Molecular Ecology 1996, 5, 521–529

RAPD marker estimation of genetic structure among isolated northern leopard frog populations in the south-western USA

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Abstract

Amphibians in the south-western United States are currently experiencing population declines. Causal explanations for these population changes as well as the implementation of sound management practices requires an understanding of the genetic structure of natural amphibian populations. To this end, we estimated genetic differences within and among seven isolated populations of northern leopard frogs, *Rana pipiens*, from Arizona and southern Utah using random amplified polymorphic DNA (RAPD) analyses. Fourteen arbitrarily designed primers detected 38 polymorphic loci in 85 individual frogs. Three types of population structure were observed in this study. (i) Two populations showed low genetic diversity (D = 0.10 and 0.04) and may have been established by relatively recent events. (ii) Two were not genetically distinct and exhibited a high degree of within-population diversity (D = 0.35). The possibility of gene flow between these populations is high due to their geographical proximity and their shared genetic structure. (iii) Three populations were genetically distinct from each other and the other populations, and exhibited intermediate within-population variation (D = 0.19, 0.17, 0.14). Genetic distances among the seven populations ranged from 0.00 to 0.20, suggesting that some of these leopard frog populations are genetically distinct. Although based on relatively small samples, these data suggest that leopard frog populations in the south-west are likely to represent unique genetic entities worthy of conservation. The management implications of these results are that isolated leopard frog populations should be evaluated on an individual basis to best preserve them.

Keywords: *Rana*, northern leopard frog, population genetic structure, RAPD

Received 10 November 1995; revision accepted 19 March 1996

Introduction

The recent, widespread decline of native amphibian populations has received much attention and has been attributed to a variety of biotic and abiotic causes. These include habitat destruction, acid rain, pollution, predation, and competition with exotic species (Hammerson 1982; Jennings & Hayes 1985; Hayes & Jennings 1986; Clarkson & Rorabaugh 1989; Barinaga 1990; Blaustein & Wake 1990; Griffiths & Beebee 1992; Hovingh 1993; Corn 1994). Most species of amphibians are known to be particularly sensitive to environmental changes due to their aquatic life stages and permeable skin, and the relationship between these animals and their immediate environment is often stronger than that of other major phyla, such as birds, mammals and reptiles. This sensitivity to the surrounding environment is important because amphibians can become indicators of such conditions as water and air pollution.

There is presently little documentation for true cause and effect between environmental pollution and the demise of amphibian populations and the lack of long-term studies has made it difficult to distinguish human-caused declines from natural population fluctuations which may be extremely variable (Berven & Grudzien 1990; Pechmann *et al.* 1991; Weitzel & Panik 1993). However, conservation of these species in the face of their perceived decline represents a major challenge to slow dwindling world biological diversity.

Historically, the leopard frog (*Rana pipiens* complex) has been one of the most common amphibians in North America. However, populations of these species appear to be declining in numbers over much of their range (Hammerson 1982; Roberts 1986; Corn 1994). Field surveys

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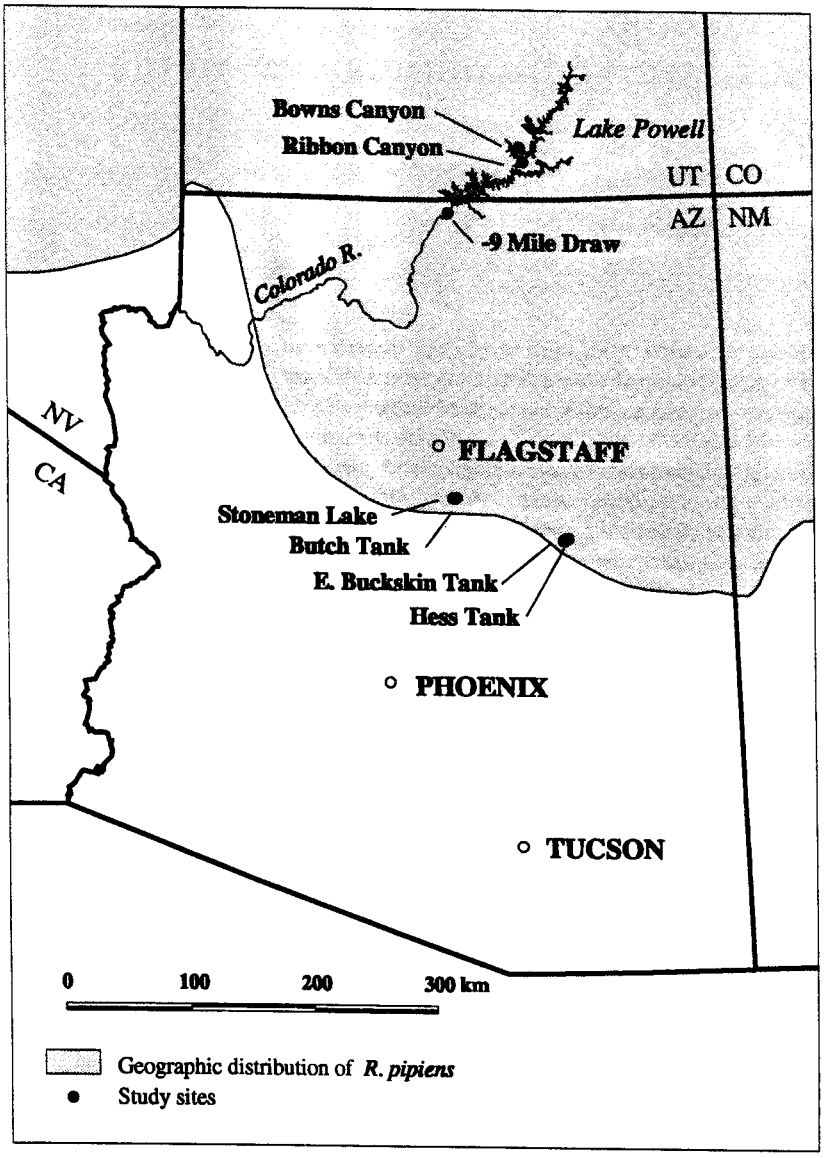

Fig. 1 Map of Arizona and Lake Powell showing sampling sites for seven *Rana pipiens* populations. Also shown is general distribution range for the species (Stebbins 1985) which consists of discrete isolated aquatic systems.

conducted in the south west between 1983 and 1987 indicate that *Rana* populations have been reduced or lost from historical sites (Clarkson & Rorabaugh 1989). Additionally, they are often small and susceptible to local extinction events which may be caused by drought or reproductive failure (Corn & Fogelman 1984).

Much research has been devoted to the systematics of the leopard frog complex (Dickerson 1913; Brown 1973; Moore 1975, 1944; Platz 1976; Hillis *et al.* 1983; Hillis 1988). In Arizona, there are currently six recognized species (Platz 1993, 1976). These are *R. berlandieri, R. blairi, R. chiricahuensis, R. yavapaiensis, R. pipiens* and *R. subaquavocalis*. This study examines the genetic structure of northern leopard frog (*R. pipiens*) populations in northern Arizona and southern Utah.

The northern leopard frog is currently a candidate for threatened wildlife status in Arizona (Arizona Game & Fish Department 1988). Genetic data are needed to understand the structure of populations so that appropriate decisions can be made towards conservation of the species. Hillis & Moritz (1990) emphasized that an understanding of population genetic structure must underlie sound species-management decisions.

We used random amplified polymorphic DNA (RAPD) analyses to examine population genetic structure of *R. pipiens*. This recently developed method (Williams *et al.* 1990)

because populations were, in general, small (estimated 50–100 metamorphosed individuals per population at the time of sampling) and we wanted to have the least impact to a potentially threatened species. Also, small sample sizes do not have as adverse effect in estimating diversity and genetic distances when a large number of loci are examined rather than a small number (Nei 1978; Gorman & Renzi 1979).

We toe-clipped three *R. yavapaiensis* individuals from central Arizona and three individuals (*R. pipiens*) from a biological supply source (Charles D. Sullivan Co., Inc., Nashville, TN, USA) to obtain extreme intra- and interspecific differences. These last three frogs originated in New England, but exact collection sites were not known by the company.

DNA techniques

For DNA extraction, each toe was macerated with a razor blade on a glass plate and put into a 1.5-mL microcentrifuge tube with 200 μL extraction buffer (from Mullenbach *et al.* 1989), 2 μL proteinase K, and 2 μL dithiothreitol. Tubes were incubated on an orbit shaker at 37 °C. for ≈ 16 h. After digestion, we followed the salt-chloroform protocol of Mullenbach *et al.* (1989). Samples were stored at –80 °C. DNA was quantified by comparison with electrophoresis standards and using a TKO 100 DNA fluorometer (Hoefer Scientific Instruments, San Francisco, CA, USA).

We used polymerase chain reactions (PCR) to randomly amplify polymorphic DNA (RAPD) after the methods of Williams *et al.* (1990). The RAPD technique involved using 10-base oligonucleotides (obtained from J.E. Karlsom, Biotechnology Labs, University of British Columbia, BC, Canada) to prime PCR for DNA from each frog. We used 4.0 mM $MgCl_2$, 0.2 mM dNTP, 1× Stoeffel buffer (Roche Molecular Systems, Branchburg, NJ, USA), 1× primer, 5 units Stoeffel fragment, and a DNA template of 2.5 ng for each 25 μL PCR reaction. Thermal cycling (PTC-100, MJ Research, Cambridge, MA, USA) consisted of 44 repetitions of 94 °C for 1 min, 36 °C for 1 min, and 72 °C for 2 min. The 72 °C step was held for 10 min in the final cycle. Reactions were then held at 1 °C for a maximum of 16 h. Amplified DNA was electrophoresed on Agarose-Synergels [0.7% Agarose, 0.46% Synergel (Diversified Biotech, Inc., Boston, MA, USA)] and stained with ethidium bromide. Gels were photographed using a MP-4 polaroid land camera and UV transilluminator.

We screened 167 primers (primer data set available upon request) across two *R. pipiens* and one *R. yavapaiensis* to identify robust primers. The two *R. pipiens* individuals represented the two most geographically distant Arizona–Utah populations (Butch Tank and Bowns Canyon). We then selected 14 primers for RAPD analyses of all 91 individuals in the study based on band resolution and repeatability of markers. We obtained 83 polymorphic fragments from these primers in the final electrophoretic data set.

RAPD analyses

Fragments (or markers) for each polymorphic locus were scored as present (1) or absent (0) for each primer. We investigated genetic relationships within and among populations using cluster and principal coordinate (PCO) analyses with the software program NTSYS-pc (Rohlf 1993). Estimates of genetic distance (Nei 1972) between all pairs of frogs in this study were obtained using 83 polymorphic markers. These estimates were higher than if monomorphic markers (80) were included in the analyses, but the relative distances between the populations remained the same. Genetic distances were analysed using cluster analysis (UPGMA) to reveal the genetic affinities among all frogs. A correlation matrix was calculated to perform principal coordinates analysis (PCO) to reveal the genetic affinities among the seven Arizona and Utah *R. pipiens* populations.

Two assumptions of RAPD analyses are that each marker represents a Mendelian locus in which the visible 'dominant' marker allele is in Hardy–Weinberg equilibrium with a null 'recessive' allele and marker alleles from different loci do not comigrate to the same position on a gel (Lynch & Milligan 1994). Although heterozygotes cannot be detected directly with RAPD, we can estimate genetic diversity. However, the statistical power of these estimates is reduced relative to a codominant system (Fritsch & Rieseberg, in press). Despite the possibility of underestimating genetic diversity (when heterozygotes are undetected in visible dominant markers) or overestimating genetic diversity (when band frequencies are below 50%), RAPD provides useful estimates of genetic variation when the interpretation includes an awareness of the assumptions. We calculated estimates of population diversity for each population to obtain relative indices (Weir 1990). As an additional diversity estimator, we calculated the proportion of polymorphic markers for each population by subtracting the percentage of fixed alleles from 100 (Weir 1990).

Results

Ambiguous markers were not included in the analyses or were scored as missing data on a case-by-case basis. We used a blind scoring method in which the scorer was unaware of the identity of the individuals. Less than 1.0% of the final results were missing data (n = 3458). Primers used, sequences, and number of polymorphic markers observed for each are presented in Table 2. Thirty-eight

Table 2 List of UBC primers, sequences, and polymorphic markers observed

UBC Primer	Sequence (5′ to 3′)	Polymorphic markers (by molecular weight)
15	CCT GGG TTT G	400, 530, 650, 700, 750, 1000, 1150, 1200
42	TTA ACC CGG C	600, 650, 690, 710, 720, 850, 870, 950, 1100
78	GAG CAC TAG C	300, 650, 700, 800, 850
203	CAC GGC GAG T	250, 650
206	GAG GAC GTC C	450
211	GAA GCG CGA T	300, 500, 630, 650, 690, 950, 1000, 1050, 1350
213	CAG CGA ACT A	80, 350, 400, 420, 520, 680, 820, 830, 1400
217	ACA GGT AGA C	250, 920, 1100
501	CGG ATA TAC C	400, 500, 1050, 1100
503	ATC GTC CAA C	530, 580, 850
504	ACC GTG CGT C	600, 700, 750, 850, 880, 950, 970, 1000, 1400, 1600
510	CGC ATC TCT T	300, 400, 750, 800, 1000
780	CCT CTT CCT C	480, 550, 750, 1100, 1200, 1500
786	TCC CTT CCT C	500, 950, 1000, 1050, 1200, 1300, 1400, 1500, 1600

markers differentiated the Arizona *R. pipiens* populations, and 45 additional markers detected differences between species (*R. pipiens* and *R. yavapaiensis*). There were 80 monomorphic markers in the data set.

The cluster analysis revealed clear differentiation of all populations except Stoneman Lake and Butch Tank individuals (Fig. 2). The three individuals from Stoneman Lake were not genetically distinct from the Butch Tank population. Hess Tank and E. Buckskin Tank populations showed a close affinity with a genetic distance (GD) of ≈ 0.7. The greatest GD within *R. pipiens* was between the northernmost populations (Ribbon Canyon, Bowns Canyon, and –9 Mile Draw) and the other four Arizona populations (GD = 0.2). The Ribbon Canyon, Bowns Canyon, and –9 Mile Draw populations were related and formed a tritomy of essentially equal genetic distances (GD = 0.15). The interspecific GD between Arizona *R. pipiens* populations and the *R. yavapaiensis* individuals was 0.6. The largest intraspecific GD (0.31) was between the New England *R. pipiens* and the Arizona populations. Because of their commercial source, however, we cannot verify species purity or exact collection sites of the New England individuals.

Analysis of genetic correlations among populations using PCO revealed multidimensional relationships among populations (Fig. 3). Although Fig. 3 shows only

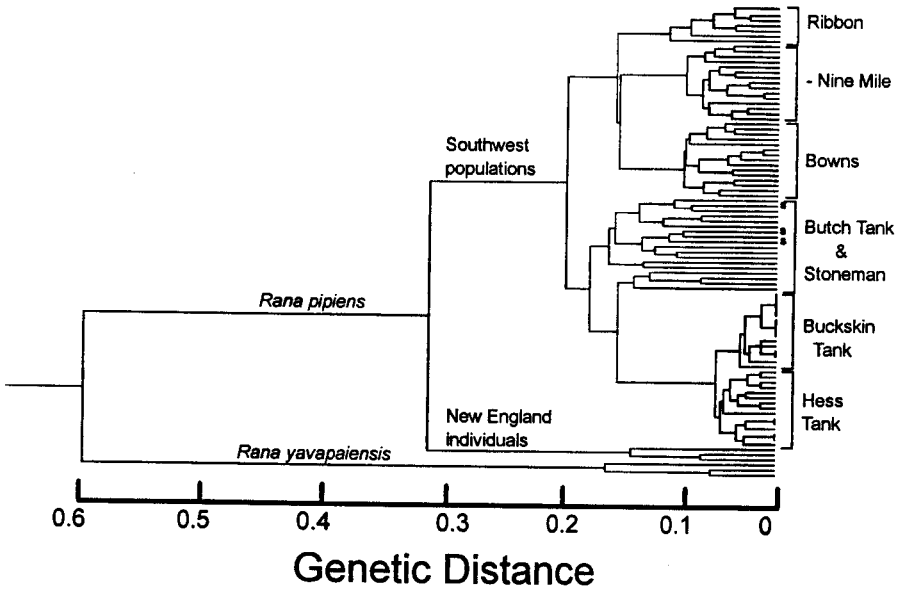

Fig. 2 Cluster analysis of *Rana pipiens* populations, including three individuals collected in New England and three *R. yavapaiensis* individuals. All individuals are included. Longer vertical bars at the origin represent more than one individual (in Buckskin and Hess populations). Stoneman individuals are designated by an **s** in the cluster.

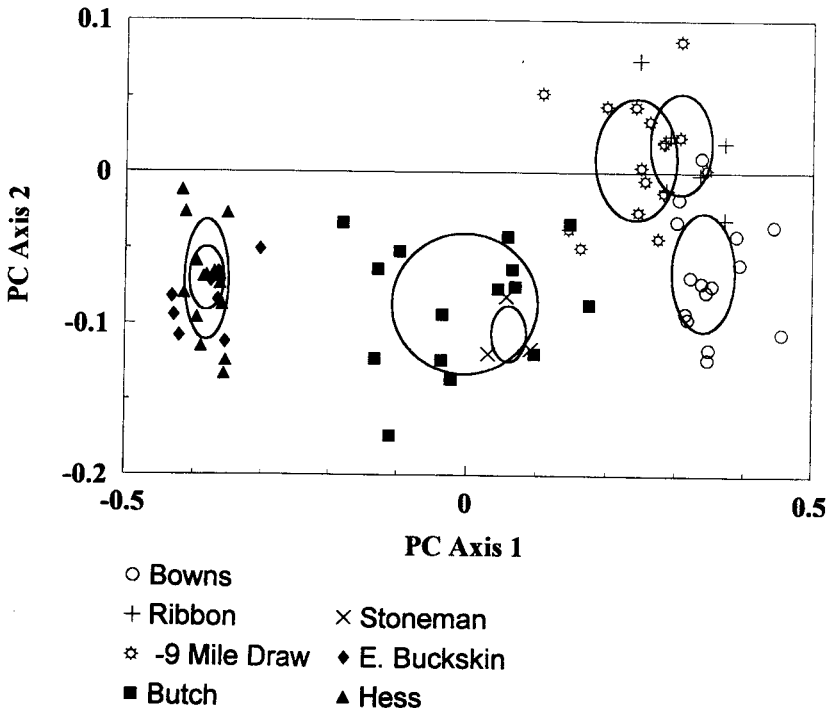

Fig. 3 Principal coordinate analysis of *Rana pipiens* populations. Ellipses are drawn to show standard deviations around the mean eigenvalues for each Arizona and Utah *R. pipiens* population. The first two principal coordinates explain 69.2% and 9.5% of the variation, respectively. The information on the *y*-axis is over-represented for reader clarity. Some symbols represent more than one individual in cases where *x* and *y* coordinates are identical.

the first two dominant coordinates, it is important to remember that 85 eigenvalues contribute to the total variation in the system. E. Buckskin and Hess Tank populations exhibited a high degree of overlap. Similarly, the Butch Tank population and the Stoneman Lake individuals were overlapping. The northernmost populations were distinct from the others and overlapped with each other. PCO analysis was consistent with the deep branches of the cluster analysis. For example, Butch Tank and –9 Mile Draw populations were distinct, but certain individuals overlapped in the PCO analysis. The multilocus distribution was not apparent from the cluster analysis.

Genetic diversity estimates revealed large differences among populations (Table 3). We included the three individuals from Stoneman Lake in the Butch Tank population because this was the only case in which individuals from one population were positioned in the same cluster with individuals from another population. This combined population had the highest within-population diversity (D = 0.35). This estimate was very high and equal to the diversity estimate for all populations combined. Hess Tank and E. Buckskin Tank had the lowest within-population diversity (D = 0.10 and 0.04, respectively). Ribbon Canyon, Bowns Canyon, and –9 Mile Draw populations had intermediate within-population diversity estimates (D = 0.19, 0.17, and 0.14). The rank correlation between diversity values and percentage polymorphic loci was over 0.9. The Stoneman-Butch Tank populations had the highest percentage polymorphic loci (82%) and the E. Buckskin–Hess Tank populations had the smallest percentage polymorphic markers (11 and 21%, respectively).

Discussion

Patterns of genetic diversity in Rana pipiens

In the south-western USA there are many isolated aquatic ecosystems surrounded by a predominantly xeric landscape. These disjunct habitats affect a species' ability to survive and disperse (Lande 1991; Wilcove *et al.* 1986; Thomas *et al.* 1994). When species are divided into populations with no gene flow between them, different alleles may become fixed in each population. This can lead to greater genetic heterogeneity among populations (Wright 1943; Kimura & Weiss 1964; Endler 1977). Spatially isolated populations may diverge rapidly through random genetic changes if the populations are small.

There is a wide range of within-population diversity among the *R. pipiens* populations in this study. This genetic variability among populations may be due to a number of random genetic changes or different ecological selection pressures. The types of habitats in this study vary greatly. The tanks (stock ponds) in central Arizona are relatively recent artificial habitats. Hess Tank was constructed prior to 1939 and E. Buckskin Tank was constructed prior to 1955. The first populations of leopard frogs were not documented until 1990. The relatively permanent water in

Table 3 Estimates of genetic variation

Population	Diversity (D)	Polymorphic markers (%)	Number of polymorphic markers
Bowns	0.19	39	15
Ribbon	0.14	32	12
–9 Mile Draw	0.17	42	16
Butch-Stoneman	0.35	82	31
E. Buckskin	0.04	11	4
Hess	0.10	21	8
All populations	0.35	100	38

these tanks has created an attractive breeding area and, at the same time, reduced the possibility of gene flow across drainages that often dry up during parts of the early summer and fall. If we assume the leopard frog populations in Hess and E. Buckskin tanks are relatively recent (as compared with the other natural habitats), then the low diversity we observed may be due to founder effects or a bottleneck event. The close genetic relationship between the two populations is also understandable, given the geographical distance between them (3.5 km). The Hess Tank population has a greater within-population diversity estimate and it is possible that a few frogs from this tank (which was constructed earlier) colonized E. Buckskin Tank during seasonal floods when drainages or streams were flowing. It is also possible that frogs were artificially introduced to both stock ponds for bait.

Stoneman Lake is the oldest and largest contiguous habitat (61 ha surface area) in the study (McCabe 1971). Gene flow among leopard frog populations may be enhanced throughout the area by the large watershed and many drainages. Butch Tank, constructed in 1952, is 3 km north east of Stoneman Lake and has a surface area of 0.3 ha. Of all the populations in the study, these are geographically the closest and have the highest probability for gene flow.

The populations in the canyons around Lake Powell may have been established at their sites for a long time because these habitats are natural (even if Lake Powell is not). At the time of breeding (April to June) the tributaries are flowing for several kilometers (The River Forecast Centre, personal communication) and frogs can migrate up and down for long distances.

Ribbon and Bowns Canyon populations and the population below Glen Canyon Dam at –9 Mile Draw have been artificially separated since the construction of Glen Canyon Dam, that was started in 1956 and completed in 1963 (Martin 1989). This is perhaps the most dramatic artificial habitat fragmentation for northern leopard frogs. The potential for gene flow between frog populations has been disrupted. The –9 mile population below the dam is totally isolated by at least 79 km from any other known population. In this case, habitat fragmentation has played a major role in preventing dispersal and gene flow.

We found that the greatest genetic distance exists between populations that are also the most geographically distant. The three northernmost populations had a GD of 0.2 from the southern populations and also had the greatest geographical distance from them (Fig. 2). Hess Tank and E. Buckskin Tank populations had a GD of only 0.07 and were geographically close, isolated habitats. These results support the hypothesis that geographical distance is an important factor influencing the genetic relatedness of populations (Wright 1943).

Implications for conservation management of R. pipiens

A major priority in conservation biology is that genetic variation must be preserved in order to promote species survival (Schonewald-Cox *et al.* 1983; Mitton & Grant 1984; Allendorf & Leary 1986). This does not necessarily mean that causal links between population fitness and heterozygosity exist (Hedrick & Miller 1992; Avise 1994). The debate about heterozygosity and its relationship to population fitness continues.

It is important to maintain levels of heterozygosity or diversity found in natural conditions (Hedrick *et al.* 1986). This study indicates that some populations of *R. pipiens* have high levels of within-population diversity and others have fairly low levels. Conservation management strategies must consider whether there is more genetic variation within populations or between populations (Hedrick *et al.* 1986). In a captive-breeding programme, an appropriate strategy would be to maintain similar levels of heterozygosity within each population. If genetic variation was distributed across populations, then it would be appropriate to promote a similar population structure even if within-population diversity was relatively low. A captive-breeding programme should consider the genetic structure of each population.

Programmes that involve facilitated dispersal of natural populations must consider the relatedness of populations, geographical distance and within-population genetic diversity. Our study shows that populations in relatively close proximity have a closer affinity than distant populations. These results support the concept of relocating individuals within close geographical ranges in facilitated dispersal programmes.

Management decisions concerning the conservation of *R. pipiens* populations in the south-western USA should consider the results of this study which show that:

1 populations of *R. pipiens* in Arizona and Utah in relatively close proximity have a closer affinity than distant populations and that there are large differences in within-population diversity;

2 the concept of relocating individuals within close geographical ranges is supported if facilitated dispersal or reintroduction is a management option.

3 management of isolated leopard frog populations should be evaluated on an individual basis to best preserve the genetic structure of populations.

This study provides evidence for variation in population genetic structure in northern leopard frog populations and contributes to the conservation of the species.

Acknowledgements

This research was funded primarily by the Arizona Game and Fish Heritage Fund (Project I94001) and secondarily by DOE Program for Ecosystem Research Grant No. 94ER6189 and NSF Ecological Research Grant No. DEB-9408009. Glen Canyon Environmental Studies and Glen Canyon National Recreation Area provided logistical support to some of the sites. The study could not have been completed without the frog-catching abilities of L. Harvey. M. Sredl of Arizona Game and Fish gave much appreciated advice on the initial grant proposal, as well as provided information about leopard frog localities. H. Mayes, M. Kearsley, L. Kearsley, and G. Bennett provided graphical assistance. Additional information and permits were provided by the United States Forest Service. S. Corn and two anonymous referees provided critical reviews of the manuscript.

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The research reported in this paper was carried out in the laboratory of Dr Paul Keim, professor at Northern Arizona University. His laboratory studies patterns of molecular genetic variation in diverse organisms. Dr Diana Kimberling, Post-doctoral research associate conducted the study with Dr Stephen Shuster, associate professor of biological sciences. Arnaldo Ferreira, master's degree candidate, provided valuable assistance in the RAPD technique. The research is part of an endeavor by the Arizona Game and Fish Department to gain genetic information on state threatened and endangered populations.

is advantageous over isozyme electrophoresis because it generates much greater numbers of loci required for genetic analyses. The method is also less complex and requires much smaller amounts of DNA than restriction fragment length polymorphism (RFLP; Fritsch & Rieseberg, in press). The advantages of RAPD make it well suited to the genetic study of rare and endangered organisms, where concerns about small sample sizes and obtaining adequate information (i.e. detecting polymorphic loci) are often paramount.

We asked the following questions in this study:

1 to what degree are geographically distant and isolated *R. pipiens* populations genetically distinct in Arizona and southern Utah?

2 what patterns of genetic diversity exist within and among populations?

3 what are the implications of these patterns of genetic diversity for conservation management of this species?

Materials and methods

Habitats sampled

Records from the Arizona Game and Fish Department and Glen Canyon National Recreation Area were used to locate sites for sampling populations. Seven populations were sampled for this study in summer 1994: five from northern Arizona and two from southern Utah (Fig. 1). The habitats labelled as 'tanks' in Fig. 1 are artificial stock ponds. Locations, habitat area and type and distance to nearest known conspecific population are presented in Table 1. Stoneman Lake and Butch Tank are 100 km distant from Hess Tank and E. Buckskin Tank populations. Stoneman Lake is ≈ 235 km south of –9 Mile Draw on the Colorado River. Ribbon Canyon and Bowns Canyon are part of the Lake Powell system in southern Utah.

Tissue collection

Frogs were caught from around the perimeter of tanks, as well as in the middle using a fishing tube and aquatic net. This method was used to increase the probability of random sampling in the population. Both adults and juveniles were sampled, but the possible relatedness of these individuals was less than using tadpoles for the study. An aquatic net was used for sampling the stream/riverine populations. The most distal hind toe from each transformed frog was removed using scissors rinsed with 95% ethanol and wiped dry. Clipped toes were immediately placed in cryotubes in a liquid nitrogen dewar or packed in dry ice.

Frogs were monitored for 15 min after the toe clip to ensure no immediate adverse effects and then released back into their habitat.

Sample sizes

Fifteen individuals were toe-clipped from each population, except for populations from Stoneman Lake (n = 3) and Ribbon Canyon (n = 7). Dense *Scirpus* and *Typha* stands at these sites precluded effective use of nets, and the few frogs present at Stoneman Lake during three sampling trips (20 working-hours) greatly inhibited capture success. We used modest sample sizes in this study

Table 1 Location, habitat size, habitat type, dominant vegetation and distance to nearest known conspecific population

Population	n	Longitude	Latitude	Surface area (ha)	Habitat type	Dominant vegetation	Distance to nearest population (km)
Bowns	15	110.5205	37.2200	0.75	Stream	*Typha, Equisetum*	12
Ribbon	07	110.5015	37.1520	0.58	Pond/ Stream	*Typha, Equisteum, Juncus*	12
–9 Mile Draw	15	111.5203	36.8863	0.40	Marsh/ Riverine	*Typha, Scirpus, Juncus, Carex*	79
Butch	15	111.4946	34.7987	0.30	Pond	*Myriophyllum*	3
Stoneman	03	111.5174	34.7754	60.73	Lake	*Myriophyllum, Scirpus*	3
E. Buckskin	15	110.5649	34.3391	0.30	Pond	*Lemna*	3.5
Hess	15	110.5820	34.3111	0.10	Pond	*Lemna*	3.5