

Limited Genetic Heterozygosity and Status of Two Populations of the Ramsey Canyon Leopard Frog: *Rana subaquavocalis*

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ABSTRACT.—Using starch gel electrophoresis, we examined proteins specified by 41 presumptive loci representing two breeding populations of *Rana subaquavocalis*, a recently described species of leopard frog from Arizona. Individual and population levels of heterozygosity were low. Mean number of alleles detected (1.2) and mean levels of individual heterozygosity were higher at the Barchas Ranch location (0.042) compared with those from Ramsey Canyon (0.029). Nine loci among the 41 surveyed were polymorphic. Four of these were common to both populations. Each population was polymorphic for the remaining three loci, and each possessed one unique allele. Both populations went extinct by 1996.

Rana subaquavocalis is a recently described species of leopard frog and member of the *Rana pipiens* complex from southeastern Arizona (Platz, 1993). In North America, there are currently seven additional species of leopard frogs, two widely distributed within the eastern half of the United States, including *R. pipiens* and *Rana sphenocephala*. Six species, *R. pipiens*, *Rana berlandieri*, *Rana blairi*, *Rana chiricahuensis*, *Rana yavapaiensis*, and *Rana onca* occur in the western half of the United States, and of these, the last five have more restricted distributions. *Rana subaquavocalis* was originally described (Platz, 1993) from a single locality in the Huachuca Mountains of southeastern Arizona. Subsequently, four additional populations were documented with breeding known to occur at two of these. No population estimate for any of the sites exceeds 50 adults, and the localities are geographically restricted to a 10 km radius within the Huachuca Mountains. As a result, the U.S. Fish and Wildlife Service (15 November 1994, Federal Register Notice) designated *R. subaquavocalis* as a category 1 candidate for federal listing as endangered or threatened. Collection and possession has been prohibited under Arizona Game and Fish Commission Order 41. Populations containing 50 or fewer individuals generally warrant concern regarding the long-term potential for loss of genetic variability (Shaffer, 1981). In this paper, we report the status of genetic variability in the two known breeding populations of *R. subaquavocalis*, identify factors that explain the low levels of heterozygosity and document the eventual fate of each population of this rare species.

MATERIALS AND METHODS

A sample of 15 large tadpoles from the concrete lined pond (type locality) in Ramsey Canyon (elevation 1622 m), 7 km southwest of Sierra Vista; 31°26'59"N, 110°18'13"W, Cochise County, Arizona, were collected in July 1992 and flash frozen in liquid nitrogen, as were 17 newly metamorphosed juvenile leopard frogs from the Barchas Ranch stock tank (elevation 1528 m);

31°28'28"N, 110°17'53"W, Cochise County, Arizona. Specimens were shipped on dry ice to the laboratory for tissue preparation. Muscle, liver, and heart tissues were dissected from juveniles and homogenized using a grinding buffer (0.1 M Tris, 1mM EDTA, 0.1mM NAD, 0.1mM NADP, 0.25% v/v B Mercaptoethanol, pH 7.0). Gut contents were removed from tadpoles and entire individuals homogenized in grinding buffer. Tissue homogenates were stored at -70°C, thawed, and centrifuged at 13,000 rpm for 10 min prior to electrophoresis in 12% starch gels (60% Starch-Art starch, 40% U.S. Biochemicals starch). Two buffer systems were used: TAG 7 (Wright and White, 1992) and LiOH (Turner, 1983). The products of 41 presumptive protein-coding loci (Table 1) were resolved and genotype counts were analyzed using the BIOSYS-1 program (Swofford and Selander, 1981).

One of us (JEP) kept in close contact with personnel at the Ramsey Canyon Mile High Preserve since 1990 and resided on site in Ramsey Canyon from May 1 to 26 June of 1995 to monitor population composition and breeding activity at the type locality. Adult counts were made three to five nights per week. Counts made in 1990, 1991, and 1992 were total counts of transformed individuals. Those made after this time only included adults. Ramsey Creek was surveyed once per week during the 1995 season from the visitors center to 700 m upstream of the concrete lined pond.

RESULTS

Seven of 41 loci were polymorphic (polymorphic criterion: more than one allele detected) in one or both of the populations (Table 2). Three loci were polymorphic in both populations: EST-1, SOD, and PK. Four were polymorphic in only one or the other of the populations: Barchas Ranch was polymorphic at the EST-3 and ACO-2 loci, and Ramsey Canyon was polymorphic at MDH-1 and ME-1 (Table 2). Significant differences in allele frequency occurred between populations at the MDH-1 locus, but none of the others was statistically different using the sequential Bonferonni correction for simultaneous tests (Rice, 1989). Despite allele frequency differences at MDH-1, the overall contingency Chi-square value was not statistically

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TABLE 1. Locus Nomenclature, E.C. numbers, and electrophoretic conditions.

Locus	Enzyme	E.C. #	Tissue and buffer system
ACO-1,-2	Aconitase	4.2.1.3	Liver, TC7
ADH-1,-2	Alcohol Dehydrogenase	1.1.1.1	Liver, TC7
AGP	Alphaglycerophosphate Dehydrogenase	1.1.1.8	Liver, TC7
ALD	Aldolase	4.1.2.13	Liver, TC7
ALP	Alkaline Phosphatase	3.1.3.1	Liver, TC7
AP	Acid Phosphatase	3.1.3.2	Muscle, TC7
CK-1,-2	Creatine Kinase	2.7.3.2	Muscle, TC7
DLR	Dihydroliipoamide Reductase	1.6.4.3	Muscle, TC7
DIP	Dipeptidase	3.4.13.11	Liver, LiOH
EST-1,-2,-3	Esterase (alpha naphthyl acetate substrate)	3.1.1.1	Liver, LiOH
FBP	Fructose biphosphatase	3.1.3.11	Liver, TC7
FUM	Fumarase	4.2.1.2	Liver, TC7
G3PD	Glyceraldehyde-3-phosphate Dehydrogenase	1.2.1.12	Liver, TC7
G6PD-1,-2	Glucose-6-phosphate Dehydrogenase	1.1.1.49	Liver, TC7
GOT	Glutamate Oxaloacetate Transaminase	2.6.1.1	Liver, LiOH
HEX	Hexokinase	2.7.1.1	Muscle, LiOH
HBDH	Hydroxybuterate Dehydrogenase	1.1.1.30	Liver, LiOH
IDDH	Iditol Dehydrogenase	1.1.1.14	Liver, TC7
IDH-1,-2	Isocitrate Dehydrogenase	1.1.1.42	Liver, TC7
LDH-1,-2	Lactate Dehydrogenase	1.1.1.27	Liver, LiOH
MDH-1,-2	Malate Dehydrogenase	1.1.1.37	Liver, TC7
ME-1,-2	Malic Enzyme	1.1.1.40	Liver, TC7
MPI	Mannose Phosphate Dehydrogenase	5.3.1.8	Liver, LiOH
PGI	Phosphoglucose Isomerase	5.3.1.9	Muscle, TC7
PGM	Phosphoglucose Mutase	2.7.5.1	Muscle, TC7
6PGD	6-Phosphogluconate Dehydrogenase	1.1.1.44	Muscle, TC7
PNP	Purine-nucleoside Phosphorylase	2.4.2.1	Liver, TC7
PK	Pyruvate Kinase	2.7.1.40	Liver, TC7
SOD	Superoxide Dismutase	1.15.1.1	Liver, TC7
TPI	Triose Phosphate Isomerase	5.3.1.1	Liver, TC7
XDH	Xanthine Dehydrogenase	1.2.1.37	Liver, LiOH

greater than expected from sampling error ($F = \chi^2_{\text{observed}}/\chi^2_{\text{error}} = 26.560/82 = 0.324$, $df = 10$, n.s.). The standardized genetic variance ($F_{ST} = 0.056$) was not significantly different from zero ($F_{ST} [2N_T] = \chi^2 = 3.584$, $df = 1$, $0.05 < P < 0.10$); (Workman and Niswander, 1970). Average individual heterozygosities were $2.9 \pm 1.3\%$ for Ramsey Canyon and $4.2 \pm 1.7\%$ for Barchas Ranch. Neither of the populations deviated from Hardy-Weinberg equilibrium expectations (Fisher's exact test, $P > 0.177$). Genetic distance estimates between the two populations were low: Nei unbiased genetic distance = 0.003; Modified Rogers' distance = 0.063; Cavalli-Sforza and Edwards chord distance = 0.079.

Regular monitoring of the Ramsey Canyon population and Ramsey Creek from May 1 to 26 June 1995 revealed a fixed number of adults which could all be accounted for at each census. Of the total of 19, 16 were in residence at each survey of the concrete lined pond. Of these, 11 were adult females and five were adult males. Weekly daytime surveys of Ramsey Creek, which is the water source for the concrete pond, revealed two adult females and a subadult of unknown sex. These three were seen consistently at the same location between the visitor center and the concrete lined pond. No adult individuals were seen above the pond, and no juveniles were seen anywhere. The Duck pond on the Barchas Ranch was surveyed four times during the May/June 1995 season. Thirty eight of an estimated 50 juveniles and subadults were toe clipped.

One of us (JEP) continued to monitor the two populations after the completion of the genetic assessment presented here with the following results. In early summer of 1996, a severe drought caused the Barchas Ranch Duck pond to dry out eliminating it as suitable habitat and reducing the known number of breeding populations to one (E. Wallace, pers. comm.). Through September 1995, there were 19 individuals in the concrete pond in Ramsey Canyon (H. Riley, pers. comm.). In summer of 1996, there were no sightings of frogs at the Ramsey Canyon concrete pond. From that time until the present, no breeding activity has been documented at the type locality despite reintroduction efforts by Arizona Game and Fish Non-Game Division. In 2000, a dead frog was documented to have a chytrid fungal infection (M. Sredl, pers. comm.).

DISCUSSION

Early efforts to quantify individual heterozygosity levels in amphibians (Selander and Johnson, 1973; Nevo et al., 1984) based on databases of 11 and 61 species, respectively, produced mean H -values of 0.082 and 0.067, respectively. These were the highest among major vertebrate groups. More comprehensive reports based on data representing 188 species of amphibians (Nevo and Beiles, 1991) and the work of Ward et al. (1992) involving data for 116 species of amphibians, reaffirm that amphibians as vertebrates, have among the highest mean levels of heterozygosity (H).

TABLE 2. Genetic variability assessment of two populations of the Ramsey Canyon Leopard Frog based on 41 electrophoretic loci. Standard errors are given in parentheses.

Population	Sample size	Mean no. of alleles per locus	Percentage of loci polymorphic	Mean heterozygosity (direct count) ^a	Mean heterozygosity Hardy-Weinberg expected ^b
Barchas Ranch	15	1.2 (0.1)	17.2	0.042 (0.017)	0.039 (0.015)
Ramsey Canyon	17	1.2 (0.1)	17.1	0.029 (0.013)	0.030 (0.013)

^a A locus was considered polymorphic if more than one allele was detected.

^b Unbiased estimate based on Nei, 1978.

Two studies, which are of comparative value with our results, involve several ranid species that live in the less mesic western half of the United States. A third involves a mesic situation where rates of gene flow between populations is likely to be substantially higher. Both Case (1978) and Green (1988) surveyed several Pacific Coast ranid frogs including *Rana aurora*, *Rana cascadae*, *Rana pretiosa*, *Rana boylei*, and *Rana muscosa*. Green (1988) is more comparable to our work in that he examined 31 loci, whereas Case (1978) surveyed 15 loci. Mean levels of heterozygosity were variable ranging from 0.046 in one population of *Rana aurora aurora* to 0.08 in a sample of *R. pretiosa*. Case (1978) reported a similar but somewhat lower range of values (0.025–0.06). In a more mesic location, one of us (TG) examined multiple populations of *R. sylvatica* for 50 loci, in the Shenandoah Mountains of Virginia and found mean *H*-values from 0.094–0.189 (unpubl.). Higher levels seen in *R. sylvatica* are undoubtedly influenced by the mesic nature of the area and the fact that this species is an explosive breeder, often congregating in numbers approaching 4000 individuals in parts of the Shenandoah Mountains of Virginia, according to Berven (1981). Among the studies cited here, both Ramsey Canyon (*H* = 0.042) and Barchas Ranch (*H* = 0.029) are comparable to the lower values reported by Green (1988) and Case (1978) for western ranids.

A number of well-known factors can contribute to low levels of heterozygosity in diploid bisexual species including genetic drift, founder effect, inbreeding, biased sex ratio, and mating system. Nei et al. (1975) showed that organisms with high intrinsic rates of increase rapidly regain heterozygosity if population size is reduced briefly, but if the number of breeding adults is greatly reduced and the population remains small (recruitment is low; *r* is relatively small), then erosion of genetic variability is expected to be severe. Suitable habitat at both of our study sites is quite limited; therefore both populations qualify as small. If no other factors are at work, populations lose variability each generation but allelic variation at all loci should be in Hardy-Weinberg equilibrium. Seven of the polymorphic loci in our study showed allelic frequencies within Hardy-Weinberg expectations, consistent with loss of genetic variability under genetic drift.

Inbreeding in small populations results in over representation of homozygotes at the expense of heterozygotes. The fact that six of seven polymorphic loci in our study contained heterozygotes in expected numbers suggests that losses are not the product of inbreeding. In fact, the overall population average value of *F* (*F*_{IS}) was -0.074, which indicates a slight, but not statistically significant, excess of heterozygotes (*F*_{IS}² [*N*] = $\chi^2 = 0.175$, *df* = 1, *n.s.*; Li, 1965).

Biased sex ratio in which adult males are under represented or a breeding system in which some do not breed, further reduce effective population size (*N*_e). Subsequent to our electrophoretic survey, one of us (JEP) monitored the population at Ramsey Canyon documenting a decline from 56 individuals in 1992 to 19 individuals in 1995. Of 56 individuals in 1992, 42 were used in a skeletochronology study (Platz et al., 1997). Of these, 22 were male and 20 female. By 1995, the only place that egg masses were documented was in the concrete lined pond where the 16 adults were monitored. Assuming that all five males participated in mating, the sex ratio of 1:3.2 would result in an *N*_e of 13.75. Therefore, biased sex ratio came in to play post-1995.

Virtually nothing was known about *R. subaquavocalis* prior to its description (Platz, 1993), but in all likelihood it dispersed into our study sites along Ramsey Creek from larger populations inhabiting the San Pedro River 8 km to the east. This is based on the notion that according to Davis (1986), large numbers of beaver occupied the river. Beaver presence creates ideal habitat for leopard frogs. About 150 years ago, the beaver were trapped out of the San Pedro, and water was subsequently diverted for irrigation, further rendering the river unsuitable for leopard frogs. *Rana subaquavocalis* is now restricted to a few human-made ponds like the ones we examined.

Whether the levels of individual heterozygosity we observed are "abnormally low" is problematic. Case's (1978) findings and those of Green (1988) are similar to or only marginally higher than those of the current study. Nevo and Beiles (1991) reported a value of 0.046 as an average mean *H*-value for 17 species of aquatic amphibians, which include those of Case (1978) and Green (1988). Our results are marginally lower but similar to those of Nevo and Beiles (1991) for aquatic species. They concluded that levels of heterozygosity in amphibians are better explained by ecological factors than demographic ones and that aquatic forms are buffered compared to those species that live in more terrestrial environments.

Some relatively widespread amphibians show low levels of heterozygosity but do not seem to be in immediate jeopardy. *Ambystoma tigrinum stebbinsi* in Arizona possess even lower levels of individual heterozygosity (*H* = 0.005; Jones et al., 1988). Nevo et al. (1984) identified factors that best explained patterns of levels of heterozygosity among the amphibia; often these were ecological. Our findings lie within the lowest 17% among the 188 species they reported on. Demographic factors, chiefly population size, best explain our findings. Low levels of observable protein polymorphisms, an indication of limited genetic variability, are

an important concern if novel, harsh selection pressures arise. Although the focus on ecological (environmental factors) is important in terms of what drives variability in large populations over long time periods, small population size resulting in limited variability in the face of novel environmental influences such as a chytrid fungus may predispose them to extinction.

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