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Genetic Evaluation of Captive Populations of Endangered Species and Merging of Populations: Gila Topminnows as an Example

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Abstract

To avoid extinction, captive populations of a number of endangered species have been established. While in captivity, these populations have been managed to retain genetic variation although direct evaluation of this strategy using molecular markers is not common. In addition, when the number of founders for a captive population is small, other founders or populations may be added to increase genetic variation. Here we examined refugial populations of the endangered Gila topminnow (*Poeciliopsis occidentalis*) from 4 locations in the southwestern United States. We found that over 5 years (about 10 generations), genetic variation as measured by 5 microsatellite loci was not lost, presumably because the adult census population size was 500 or greater. In addition, some variation not initially observed was observed later. Some of these variants may have been missed because of sampling but it appears that some may have been contributed by new mutations. In addition, 2 populations of successfully merged ancestry from the 4 source populations were examined. Based on population-specific markers and a quantitative evaluation of ancestry using a likelihood approach, it appears that ancestry from each of the source populations was retained in both populations.

Key words: *effective population size, heterozygosity, microsatellites, mutation, sampling*

Understanding evolutionary genetics in small and isolated natural populations has always been challenging. Specifically, identifying the factors that influence genetic variation is demanding because it is generally difficult to identify, capture, and monitor these populations. For endangered species, because of their importance, both research effort and financial support are often dedicated toward understanding the basis of endangerment of these species and what may prevent their extinction. As a result, genetic study of endangered vertebrates, such as Florida panthers (Johnson et al. 2010) and Swedish wolves (Liberg et al. 2005), provide information that otherwise may not be available for small, isolated populations.

A number of endangered species exist only in a few natural populations in the wild and are therefore in danger of extinction. To reduce this risk, in some cases a sample of individuals from the wild has been captured and used to form a captive population to serve as a source population for reintroduction if the wild populations were to become extinct. While in captivity, these populations have been managed to maintain genetic variation (Ballou et al. 1995) and prevent adaptation to captive environmental conditions (Frankham 2008). Examples of species where captive descendants of wild caught animals that were used to establish wild populations include Mexican and red wolves, black- footed ferrets, and California condors.

In some cases, wild individuals from endangered species have been brought into captivity when environmental conditions in the remaining wild population(s) were potentially detrimental. Such captive populations are maintained at as large an effective population size as possible and/or bred to maintain genetic variation (Ballou et al. 1995). In a few cases when captive breeding is successful, experimental studies in the captive populations have been justified to understand the basis of their endangerment. In this case, maintenance of genetic variation is important so that experiments are a good reflection of results in natural populations.

In some cases where captive populations were established from a small number of founders, individuals from multiple captive populations have been merged to form a population with more founders. For example, in the 1990s the captive population of Mexican wolves (known then as the Certified lineage) was descended from only three founders (Hedrick et al. 1997). Two other captive lineages, each descended from two founders, were examined genetically and also found to be Mexican wolves (Hedrick et al. 1997). As a result, these other two lineages were merged with the Certified lineage and both the captive population of Mexican wolves and the reintroduced population are now descended from seven founder individuals. Such a merger of populations is expected to increase genetic variation and reduce inbreeding depression. This strategy may become more common in the future as wild populations become smaller, more fragmented, and more isolated.

Background on Gila Topminnows

The Gila topminnow (*Poeciliopsis occidentalis*), a small (< 50 mm), live-bearing fish that is a federally listed endangered species in the United States, occurs in the Gila River basin that starts in New Mexico and flows west into the Colorado River. It was once considered among the most abundant fishes in the lower Gila River basin in Arizona but it now persists in only a few watersheds in southeastern Arizona. This endangerment has been primarily caused by loss and fragmentation of adequate shallow-water habitat and the widespread introduction of another livebearer, the nonnative western mosquito fish (*Gambusia affinis*) (Minckley 1999).

In June, 1994, we were permitted to capture 20 Gila topminnows (pregnant females) from each of the four major known remaining watersheds in Arizona (Bylas Spring, Cienega Creek, Monkey Spring, and Sharp Spring) (see Figure 1) bring them into the laboratory, increase their numbers, and maintain them as captive populations. Studies on the descendants of these fish documented some differences in fitness-related characters between these natural populations (for a summary, see Hedrick and Hurt 2012; for earlier related research, see Quattro and Vrijenhoek 1989). Although initially these populations were not designed to provide stock for reintroductions, when the population at one site (Bylas Spring) went extinct in the wild in the mid-1990s, the captive population from Bylas Spring was used as a source to reestablish the wild population.

In an experiment to examine outbreeding depression, topminnows from the 4 major watersheds were crossed for 2 generations (Sheffer et al. 1999). Even though the other experiments cited above showed some differences in fitness-related traits, there was no evidence of outbreeding depression for body size, survival, bilateral asymmetry, fecundity, and sex ratio in these crosses. Although the population from Monkey Spring is thought to have been isolated from the other natural populations in the United States for approximately 10,000 years by a natural travertine dam and has been suggested as a separate ESU by Parker et al. (1999),

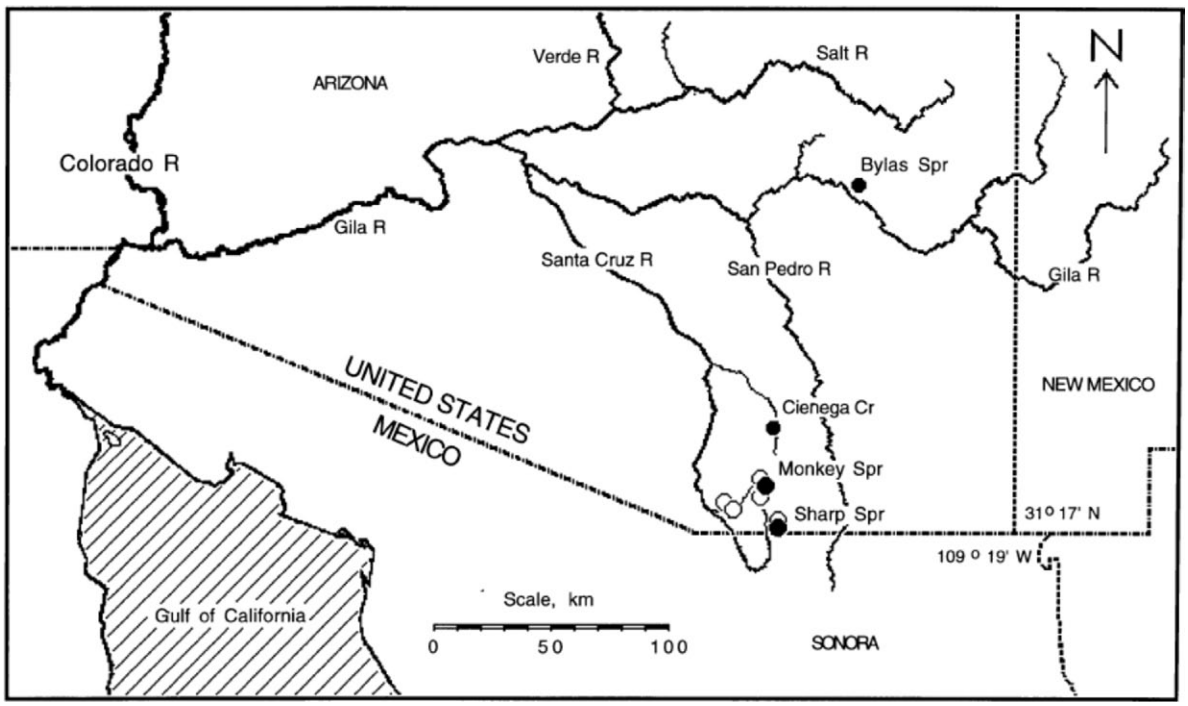

Figure 1. The locations of the four watersheds (Bylas Spring, Cienega Creek, Monkey Spring, and Sharp Spring) in Arizona, USA used in this study (closed circles) and other natural populations (open circles) of the endangered Gila topminnow.

even crosses between Monkey Spring and the other populations showed no outbreeding depression (Sheffer et al. 1999). Although there is substantial genetic difference for both microsatellite loci (Parker et al. 1999) and a major histocompatibility complex (MHC) locus (Hedrick and Parker 1998) between the populations, all the populations (including Monkey Spring) share the same mtDNA sequence (Hedrick et al. 2006).

There have been questions about the appropriate topminnow source for reintroductions and whether the source is an important factor in reintroduction success. For example, many of the original reintroductions were from the Monkey Spring (or the related Boyce–Thompson population, Hedrick et al. 2001), which had very low success. Specifically, Sheller et al. (2006) documented that only 9 of 130 (7.0%) stockings of Monkey Spring fish were successful (only one of these is known to still exist in 2011, Ross Timmons, personal communication) while 6 of 12 (50%) stockings from other sources were successful. An important related question is the fate of the contribution from Monkey Spring and contribution from other sources when they are combined. This should give insight into the relative fitness or suitability of fish from different sources for reintroductions.

There have also been suggestions that it would be useful to have a general topminnow population that could be distributed to schools or other public places in metropolitan areas for use in public education. It has also been suggested that general populations of topminnows be used for mosquito control replacing *G. affinis* (Childs 2006). In addition, the formation of 20 populations with mixed ancestry has now become a criterion of downlisting for Gila topminnows. Because of these various reasons, we formed merged populations with ancestry from the four main watersheds, to evaluate over time the ancestral contribution from the populations.

Our first purpose here is to examine neutral microsatellite markers developed for topminnows from the time when samples were brought into captive populations from the four watersheds in 1994 (and first sampled in June, 1995) until 2000. Such direct evaluations of captive breeding in endangered species over many generations using molecular markers is uncommon. Further, we used these same microsatellite loci to evaluate the ancestry from the four populations that were used in merged populations.

Methods

Parker et al. (1998) identified 10 microsatellite loci, five of which were polymorphic in Gila topminnows. Two of these loci (G49 and OO56) are complicated repeats and three (6-10, C15, and LL53) are dinucleotide repeats. Variation in these five polymorphic loci from samples from the captive populations from Bylas Spring, Cienega Creek, Monkey Spring, and Sharp Spring was examined initially in samples from June 1995 (Parker et al. 1999). This was as soon after collection from the wild populations in June 1994, that there was enough time for them to establish populations in captivity. In 2000, the same loci were examined from samples collected from the captive populations that were used as female parents for establishing merged populations, and in 2001 and 2002 from samples of progeny in the merged populations. To examine whether the populations diverged further over 5 years, the differentiation measure G_{ST} of Nei (1987) and the standardized differentiation measure G_{ST}' of Hedrick (2005) were calculated. The captive populations were maintained in the Animal Resource Center (ARC) at Arizona State University (ASU) in circular 400-gallon raceways with approximately 600 liters of water volume. Since 1994, these populations have been maintained in standardized conditions known for successful topminnow survival and reproduction (25.5°C, 14-h light, and 10-h dark, appropriate levels of dissolved gases, solids, and wastes, and a diet of high protein fish food, spinach, and brine shrimp, see Sheffer et al. 1997).

The merged populations were established in October, 2000 using 10 adult females from each of the 4 refugial populations. These females all appeared to be pregnant so that the initial number of founders for each population in a genetic sense was at least 80 individuals (assuming at least one male fertilized each female). Female topminnows are known to store sperm from multiple males so the actual number of founding genotypes is probably larger.

Three replicate populations with 25% ancestry from each population were established in October, 2000, one in captivity at the ASU (ARC), one in a pond on the ASU campus near A Mountain (A Mountain), and one in a pond on the campus of Scottsdale Community College (SCC), about 15 km north of ASU. Fin clips were taken for genotyping before the fish were released into the three sites. After the initial stocking, no topminnows were ever seen in the A Mountain pond, either by thoroughly searching in the spring of 2001 or from attempts to capture topminnows by seining. The A Mountain pond was subsequently drained due to contamination with nonnative/exotic competing fish. The captive merged population in the ARC reproduced successfully. The ARC population was sampled 1 year later in October, 2001, and again 2 years later in October, 2002, and both samples genotyped for the polymorphic microsatellite loci. There were no visible topminnows in the Scottsdale Community College pond from surveys in the spring and summer of 2001. In a subsequent visit to the Scottsdale Community College pond (June 2002), topminnows were found and 42 offspring were sampled and subsequently genotyped.

DNA was extracted from fin clips using either the PureGENE extraction method or the Chelex extraction method. Polymerase Chain Reaction (PCR) amplification was performed using primers and protocols described in Parker et al. (1998, 1999). Microsatellite genotypes were sized by comparison to a known ladder on a denaturing sequencing polyacrylamide gel (see Parker et al. 1998, 1999).

To determine quantitatively the extent of changes from the different ancestral populations in the merged populations, we used the following approach. In general, the frequency of allele A_i in the offspring generations is

$$p_i' = 0.25(p_{iB}w_B + p_{iC}w_C + p_{iM}w_M + p_{iS}w_S)/w_i \quad (1)$$

where

$$w_i = 0.25(w_B + w_C + w_M + w_S).$$

Here p_{iB}, p_{iC}, p_{iM}, and p_{iS} are the frequencies of allele A_i in the founders from Bylas Spring, Cienega Creek, Monkey Spring, and Sharp Spring, respectively, and w_B, w_C, w_M, and w_S are the relative contributions from four populations in the merged population. The expression

$$L = C\sum_i \left(p_i'\right)^{N_i} \quad (2)$$

gives the likelihood of the observed numbers of alleles at a given locus given the allele frequencies in the four populations in the female parents and relative contributions from each population. N_i is the number of copies of allele A_i observed and C is the multinomial coefficient.

In this approach, the likelihood for the null hypothesis that $w_B = w_C = w_M = w_S = 1$, equal contributions from the four populations, is first calculated. Then a preliminary search determined if there is a set of values for w_B, w_C, w_M, and w_S that results in an L value that is at least 10^3 times this value or a likelihood ratio

$$\mathrm{LR} = \frac{L(w_B, w_C, w_M, w_S)}{L(w_B = w_C = w_M = w_S = 1)} \quad (3)$$

that is equal to or greater than 10^3.

In this case, the statistic G (Sokal and Rohlf 1995) is approximately $G = 2ln$ (LR) = 13.8, which results in significance at the $p < 0.001$ level, a conservative level of significance that we will use because other conditions of this test may not be met. If there is not this level of significance, then the test is considered nonsignificant and it is assumed that the observations are consistent with $w_B = w_C = w_M = w_S = 1$. If there is a value that results in LR at least 10^3 greater than the null hypothesis, a further systematic search was undertaken to determine the set of w_B, w_C, w_M, and w_S values, to the nearest 0.1 for each w value, that results in the maximum value of $L(w_B, w_C, w_M, w_S)$.

Results

Comparison of Captive Populations from 1995 and 2000

The frequencies of the alleles for the five polymorphic loci obtained from our initial survey in June, 1995, of the four stocks are given in Table 1 (Parker et al. 1999). Note that the stock from Bylas Spring has low genetic variation, and is only polymorphic for one of the loci. For another locus of important adaptive significance (MHC), there was also no variation in the Bylas Spring stock (Parker and Hedrick 1998). All three of the other stocks, Cienega Creek, Monkey Spring, and Sharp Spring, have substantial variation for the microsatellite loci (Parker et al. 1999) and the MHC locus (Parker and Hedrick 1998). Note that the Monkey Spring stock appears to be the most differentiated from the other stocks (Parker et al. 1999; Hedrick et al. 2001) for these loci (and the MHC locus as well). In addition, there are population-specific alleles, or alleles that are in much higher frequency than in other stocks, for each of the stocks (indicated in boldface in Table 1). These alleles will allow a general evaluation of the contribution of ancestry of each of the stocks to the merged topminnow populations (see below).

First, the allele frequencies observed in 2000 were generally a good reflection of the mean observed in the initial sample. In other words, this suggests that there has not been a severe bottleneck in the captive populations that could have greatly reduced the extent of genetic variation. We estimate that 5 years is about 10 generations in the captive populations based on the information that topminnows become reproductive at about 3 months and can remain reproductive for about 1 year. As a result, we assumed the average age at average reproduction is about 6 months, making 2 generations per year a reasonable estimate.

Also meaningful is that some alleles were observed in the 2000 samples that were not seen in 1995 (indicated by an * in Table 1). For example, nine alleles at the most polymorphic locus (C-15) were found in low frequencies in the 2000 samples of Cienega Creek, Monkey Spring, and Sharp Spring that were not seen in the 1995 samples from these sites. Similarly, single alleles at locus OO56 were found in the Cienega Creek and Monkey Spring samples in 2000 not found in the 1995 samples, and at locus LL53 three alleles were observed in the 2000 Monkey Spring sample not seen in the 1995 sample and two alleles were observed in the 2000 Sharp Spring sample not seen in the 1995 sample.

Reflecting the general stability of genetic variation over this time period in the captive populations is the observation that averaged over the four populations, the mean expected heterozygosity and mean number of alleles in 1995 are 0.206 and 2.45, respectively (bottom of Table 2) and the expected heterozygosity and mean number of alleles in 2000 are similar (slightly higher) at 0.246 and 2.75, respectively. The largest change in expected heterozygosity between 1995 and 2000 is an increase for the Monkey Spring population (from 0.195 to 0.335), mainly due to locus LL53. The largest change in the number of alleles between 1995 and 2000 is an increase for Cienega Creek population (from 2.0 to 2.8), mainly due to locus C-15. Both loci OO56 and LL53 increased in expected heterozygosity and number of alleles from 1995 to 2000. For all populations and loci, the observed and expected heterozygosities are not significantly different, as expected if mating were at random within populations.

In June, 1995, the numbers of adult fish in the four captive populations were estimated by sampling given proportions of the raceways and taking into account the distribution of fish within the raceways. These estimated numbers were 354 for Bylas, 676 for Cienega, 496 for Monkey, and 841 for Sharp (for a mean of 592). To obtain the expected impact of genetic drift from these population sizes, let us assume that the effective population size (N_e), taking into account all factors, is about 25% of the adult numbers (Nunney 1993) or $N_e = 150$. Given 10 generations, the expected loss in genetic variation from 1995 to 2000 with this N_e is only $1 - (1 - 1/300)^{10} = 3.3\%$ (Hedrick 2011), consistent with the similarity in genetic variation in the samples from 1995 and 2000.

Table 1 Allele frequencies in sample from the four watersheds shortly after collection in 1995 (Parker et al. 1999) and the mean allele frequencies from the four different watersheds in 2000 after 6 years in captivity (these are allele frequencies in the female parents of the two successful merged topminnow populations)

		Bylas Spring		Cienega Creek		Monkey Spring		Sharp Spring	
Locus	Allele	1995	2000	1995	2000	1995	2000	1995	2000
G49	149	—	—	—	—	—	—	0.038	0.053
	157	—	—	—	—	—	—	—	0.026*
	159	0.250	0.921	1.000	1.000	1.000	1.000	0.962	0.921
	161 (B)	**0.750**	**0.079**	**—**	**—**	**—**	**—**	**—**	**—**
6-10	**287 (M)**	**—**	**—**	**—**	**—**	**1.000**	**1.000**	**—**	**—**
	297	1.000	1.000	1.000	1.000	—	—	1.000	1.000
C-15	202	—	—	—	—	—	—	0.050	0.000
	204	—	—	—	—	0.025	0.000	0.200	0.000
	208	—	—	—	—	0.088	0.050	0.012	0.525
	210	—	—	—	0.025*	0.012	0.075	—	—
	214 (M)	**—**	**—**	**—**	**—**	**0.612**	**0.125**	**—**	**—**
	216 (M)	**—**	**—**	**—**	**—**	**0.225**	**0.600**	**—**	**—**
	218	—	—	0.013	0.000	—	0.100*	—	—
	220	—	—	—	—	—	—	—	0.050*
	222	—	—	—	—	0.012	0.000	—	—
	224	—	—	—	—	0.012	0.000	—	0.100*
	226	—	—	—	—	—	—	0.012	0.000
	228	—	—	—	0.050*	—	—	0.100	0.050
	230	—	—	—	—	—	—	0.012	0.000
	232	—	—	0.362	0.075	0.012	0.000	0.025	0.025
	234	—	—	—	—	—	—	0.100	0.000
	236 (S)	**—**	**—**	**—**	**0.075***	**—**	**—**	**0.338**	**0.100**
	238 (C)	**—**	**—**	**0.612**	**0.075**	**—**	**0.025***	**0.112**	**0.050**
	240 (B)	**1.000**	**1.000**	**0.013**	**0.675**	**—**	**0.025***	**0.025**	**0.100**
	242	—	—	—	0.025*	—	—	—	—
	246	—	—	—	—	—	—	0.012	0.000
OO56	**143 (C)**	**—**	**—**	**0.200**	**0.350**	**—**	**0.118***	**—**	**—**
	145	1.000	1.000	0.800	0.625	0.762	0.676	1.000	1.000
	149 (M)	**—**	**—**	**—**	**0.025***	**0.238**	**0.206**	**—**	**—**
LL53	**142 (M)**	**—**	**—**	**—**	**—**	**0.988**	**0.650**	**—**	**—**
	144 (C)	**—**	**—**	**0.488**	**0.325**	**—**	**0.125***	**—**	**—**
	146	1.000	1.000	0.512	0.675	—	—	—	0.025*
	150	—	—	—	—	0.012	0.100	0.425	0.275
	152	—	—	—	—	—	0.025*	—	0.225*
	154 (S)	**—**	**—**	**—**	**—**	**—**	**0.100***	**0.550**	**0.425**
	164	—	—	—	—	—	—	0.025	0.050

Abbreviations: B, Bylas Spring; C, Cienega Creek; M, Monkey Spring; S, Sharp Spring. The alleles with an * indicate ones observed in samples from 2000 but not observed in the 1995 samples. Population-specific alleles, or alleles that are in much higher frequency than in other stocks, for each of the stocks are indicated in boldface and by population.

If the population size in the separate, isolated populations was small enough, then genetic drift would be expected to result in an increase in differentiation. In the initial survey in 1995, $G_{ST} = 0.587$ and $G_{ST}' = 0.791$ and in the 2000 sample, $G_{ST} = 0.513$ and $G_{ST}' = 0.730$. In other words, four populations were quite divergent in 1995 as discussed by Parker and Hedrick (1999) and in 2000 they are still divergent at nearly the same level. The small decline in differentiation, contrary to expectations that completely isolated populations should increase in differentiation over time, appears to be primarily the result of the near loss in the Bylas samples of allele 161 at locus G49 dropping in frequency from 0.750 to 0.079, resulting in the average frequency of allele 159 becoming 0.98 over the four populations in 2000 and much lower differentiation for this locus.

Ancestry in Merged Populations

The allele frequencies in the female parents of the two merged populations and their progeny are given in Table 3. The allele frequencies in the parents for both the ARC and SCC populations were generally a good reflection of the mean observed in the 1995 sample (given in the first column). In addition, population-specific (or high frequency) alleles have generally been retained in the progeny, suggesting that all the four stocks have all contributed to the progeny.

More specifically, there are two alleles, G49-161 and C-15-240, found only in Bylas Spring or only in high frequency in Bylas Spring. G49-161 is still found in the offspring in the ARC, but in lower than initial frequency and this allele was not observed in the offspring from SCC. However, allele

Table 2 The observed (H_O) and expected (H_E) heterozygosities (with small sample size correction) and number of alleles (n) estimated in samples from the four watersheds in 1995 (Parker et al. 1999) and in 2000 after 6 years in captive refugia

		Bylas Spring		Cienega Creek		Monkey Spring		Sharp Spring		Mean	
Locus	Measure	1995	2000	1995	2000	1995	2000	1995	2000	1995	2000
G49	H_O	0.355	0.150	0.000	0.000	0.000	0.000	0.076	0.054	0.106	0.050
	H_E	0.385	0.162	0.000	0.000	0.000	0.000	0.074	0.052	0.113	0.054
	N	2	2	1	1	1	1	2	2	1.5	1.5
6-10	H_O	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	H_E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	N	1	1	1	1	1	1	1	1	1	1
C-15	H_O	0.000	0.000	0.608	0.410	0.608	0.770	0.810	0.564	0.500	0.425
	H_E	0.000	0.000	0.505	0.538	0.580	0.621	0.820	0.703	0.470	0.454
	N	1	1	4	7	8	7	12	8	6.25	5.75
OO56	H_O	0.000	0.000	0.304	0.540	0.329	0.664	0.000	0.193	0.156	0.340
	H_E	0.000	0.000	0.328	0.473	0.372	0.500	0.000	0.174	0.173	0.280
	N	1	1	2	3	2	3	1	2	1.5	2.25
LL53	H_O	0.000	0.000	0.633	0.564	0.025	0.410	0.506	0.718	0.288	0.412
	H_E	0.000	0.000	0.513	0.450	0.025	0.555	0.530	0.707	0.264	0.417
	N	1	1	2	2	2	5	3	5	2.0	3.25
Mean	H_O	0.071	0.030	0.309	0.303	0.192	0.369	0.278	0.306	0.212	0.252
	H_E	0.077	0.032	0.269	0.292	0.195	0.335	0.285	0.327	0.206	0.246
	N	1.2	1.2	2.0	2.8	2.8	3.4	3.8	3.6	2.45	2.75

H_E here is not based on the mean allele frequencies over populations but is the mean value of H_E averaged over populations.

C-15-240 is in higher frequency in the offspring of both the ARC and SCC than it was in the initial survey. There are three population-specific alleles, C-15-238, OO56-143, and LL53-144, for Cienega Creek. Two of these, OO56-143 and LL53-144, are highly represented in the ARC and SCC offspring. Allele C-15-238 was not observed in the ARC population in 2001 but was at higher frequency in 2002 and declined in the SCC population. There are five population-specific alleles, 6-10-287, C-15-214 and 216, OO56-149, and LL53-142 for Monkey Spring, and all of these are represented in the offspring except for C-15-216, which is not represented in the ARC progeny; OO56-149, which was not observed in the ARC progeny in 2002; and LL53-142. which was not observed in the SCC progeny. There are two population-specific alleles for Sharp Spring, C-15-236 and LL53-154. C15-236 appears to have been retained at similar to original frequencies, whereas LL53-154 was not observed in the most recent samples from both populations.

Also reflecting this general stability is the observation that in 1995 the mean expected heterozygosity (using the mean allele frequency from the four populations) is 0.500 (bottom of Table 4) and the expected heterozygosity in the parents of the ARC and the SCC populations are 0.479 and 0.459, respectively, virtually the same. However, the overall number of alleles remaining in the parents is slightly less than that found initially (5.2 for ARC and 4.8 for SCC vs. 6.4 in 1995). This reduction is entirely from locus C-15, which had 12 and 10 alleles in the ARC and SCC parents compared to the 18 observed in the 1995 survey. Because the sample size of the 1995 survey is 160 individuals compared to 40 each for ARC and SCC in 2000, this reduction in the number of alleles is not unexpected. Further, the observed heterozygosity in the parents for both the ARC and SCC populations is less than the expected heterozygosity (bottom of Table 4), consistent with Wahlund effect expectations when the parents from different populations are combined.

If random mating occurs among the parents founding the populations, the genotypes in their progeny should be in Hardy-Weinberg proportions. This expectation is met here with the overall observed and expected heterozygosities in the ARC populations of 0.450 and 0.467, respectively, and the observed and expected heterozygosities in the SCC population of 0.346 and 0.335, respectively. Note that the mean for the ARC sample in 10/2002 did not include data for locus G49.

The level of expected heterozygosity (and number of alleles) declined between the parents and the 2001 sample of the progeny in the ARC by 2.5%, whereas it declined by 27.0% between the parents in the SCC and the progeny sampled in 6/2002, an order of magnitude more. If we assume that in the ARC, the parents and progeny were two generations apart and in the SCC, the parents and progeny were three generations apart, then the effective population sizes that are consistent with these reductions are 41.7 and 5.0 for the ARC and SCC, respectively (using the expression $[1 - 1/(2N_e)]^t$ where t is the number of generations, Hedrick 2011). In other words, during the period in 2001 in the SCC population when no topminnows were observed, there appears to have been a population bottleneck consistent with three generations of an effective population size of 5. The expected heterozygosities for the four loci surveyed in both 2000 and 2002 in the ARC population were 0.576 and 0.503, for a reduction of 12.7%. Assuming that these samples were taken four generations apart, then the effective population size is 15.2 over this period, suggesting that the average effective population size in the ARC population was not large over this

Table 3 The allele frequencies estimated in female parents used to start the two merged populations at the Animal Resource Center (ARC) and Scottsdale Community College (SCC) and subsequent estimates in their progeny

			ARC			SCC	
			Parents	Progeny	Progeny	Parents	Progeny
Locus	Allele	1995	10/2000	10/2001	10/2002	10/2000	6/2002
G49	149	0.010	0.025	0.026	nd	—	0.000
	157	**—**	**—**	**—**	**nd**	**0.007**	**0.000**
	159	0.803	0.925	0.947	nd	0.980	1.000
	161 (B)	**0.188**	**0.050**	**0.026**	**nd**	**0.014**	**0.000**
6-10	**287 (M)**	**0.250**	**0.250**	**0.190**	**0.136**	**0.250**	**0.214**
	297	0.750	0.750	0.810	0.864	0.750	0.786
C-15	202	0.012	—	—	—	—	—
	204	0.056	—	—	0.023*	—	—
	208	0.025	0.150	0.131	0.045	0.138	0.000
	210	0.003	0.012	0.024	0.000	0.038	0.073
	212	—	—	0.024**	0.023**	—	—
	214 (M)	**0.153**	**0.062**	**0.107**	**0.068**	**—**	**0.037***
	216 (M)	**0.056**	**0.125**	**0.000**	**0.000**	**0.175**	**0.073**
	218	0.003	0.025	0.000	0.000	0.025	0.000
	220	—	0.025	0.000	0.000	—	—
	222	0.003	—	—	—	—	—
	224	0.003	0.025	0.000	0.000	0.025	0.000
	226	0.003	—	—	—	—	—
	228	0.025	0.062	0.048	0.000	—	—
	230	0.003	—	—	—	—	—
	232	0.100	0.038	0.167	0.114	0.012	0.049
	234	0.025	—	0.071*	—	—	—
	236 (S)	**0.084**	**0.050**	**0.119**	**0.114**	**0.088**	**0.049**
	238 (C)	**0.181**	**0.012**	**0.000**	**0.273**	**0.125**	**0.012**
	240 (B)	**0.260**	**0.425**	**0.310**	**0.341**	**0.362**	**0.683**
	242	—	—	—	—	0.012	0.000
	244	—	—	—	—	—	0.024**
	246	0.003	—	—	—	—	—
OO56	**143 (C)**	**0.050**	**0.212**	**0.204**	**0.238**	**0.079**	**0.158**
	145	0.890	0.738	0.738	0.762	0.712	0.817
	149 (M)	**0.060**	**0.050**	**0.060**	**0.000**	**0.209**	**0.024**
LL53	136	—	—	—	—	—	0.024**
	142 (M)	**0.247**	**0.200**	**0.107**	**0.533**	**0.125**	**0.000**
	144 (C)	**0.122**	**0.125**	**0.476**	**0.200**	**0.183**	**0.548**
	146	0.378	0.388	0.310	0.100	0.379	0.429
	150	0.109	0.188	0.036	0.167	—	—
	152	—	—	0.024*	—	0.125	0.000
	154 (S)	**0.138**	**0.088**	**0.048**	**0.000**	**0.175**	**0.000**
	164	0.006	0.012	0.000	0.000	0.012	0.000

Abbreviations: B, Bylas Spring; C, Cienega Creek; M, Monkey Spring; S, Sharp Spring. Population-specific alleles, or alleles that are in much higher frequency than in other stocks, for each of the stocks are indicated in boldface and by population. Given for comparison, the value in the left-hand column is the original mean from the 1995 sample (Parker et al. 1999).

* indicate alleles seen in a progeny sample but not in the 2000 sample of parents from that population, ** indicates alleles seen in a progeny sample but not seen previously, and nd indicates no data.

period. Consistent with this low effective population size, the average number of alleles for these four loci declined from 5.75 in 2000 to 4.0 in 2002.

Four alleles, indicated by * in Table 3, were observed in a progeny sample but not seen in the parents of that population. These alleles were C-15-204, C-15-234, and LL53-152 in the ARC population and C-15-214 in the SCC population. Three new alleles, indicated by ** in Table 3, were observed in the ARC and SCC progeny that were not seen either in the parents in 2000 or in the general survey in 1995. These alleles were C-15-212 in the ARC progeny and C-15-244 and LL53-136 in the SCC progeny. All of these alleles were seen in quite low frequencies.

To determine quantitatively the relative contributions from the four populations in the merged populations, we used the likelihood approach outlined above. First, for three loci, G49, 6-10, and OO56, for most locus–progeny sample combinations, equal contributions from the four populations was an explanation consistent with the data (Table 5). The exception was for OO56 in the SCC 6/2002 sample where no contribution from Monkey Spring ($m_M = 0$) gave the highest value. In this instance, the Monkey Spring high-frequency

Table 4 The observed (H_O) and expected (H_E) heterozygosities and number of alleles (n) estimated in the 1995 sample when equal numbers from each of the four populations are combined and for the female parents used to start the merged population at the Animal Resource Center (ARC) and the progeny at 10/2001 and 10/2002 and the merged population at the Scottsdale Community College (SCC) and the progeny at 6/2002

			ARC			SCC	
			Parents	Progeny	Progeny	Parents	Progeny
Locus	Measure	1995	10/2000	10/2001	10/2002	10/2000	6/2002
G49	H_O	0.106	0.071	0.105	nd	0.087	0.000
	H_E	0.320	0.091	0.102	nd	0.123	0.000
	n	3	3	3	nd	3	1
6-10	H_O	0.000	0.000	0.190	0.182	0.000	0.190
	H_E	0.375	0.375	0.308	0.235	0.375	0.336
	n	2	2	2	2	2	2
C-15	H_O	0.500	0.450	0.667	0.591	0.400	0.366
	H_E	0.751	0.771	0.852	0.775	0.790	0.516
	n	18	12	9	8	10	8
OO56	H_O	0.156	0.425	0.524	0.286	0.273	0.366
	H_E	0.202	0.408	0.410	0.363	0.244	0.307
	n	3	3	3	2	3	3
LL53	H_O	0.288	0.550	0.762	0.600	0.308	0.810
	H_E	0.750	0.751	0.662	0.638	0.763	0.515
	n	6	6	6	4	6	3
Mean	H_O	0.174	0.299	0.450	0.415*	0.214	0.346
	H_E	0.500	0.479	0.467	0.502*	0.459	0.335
	n	6.4	5.2	4.6	4.0*	4.8	3.4

The mean values for ARC on 10/2002 indicated by an * are the averages for the loci excluding locus G49, and nd indicates no data.

Table 5 Estimates of the relative contributions, w_B, w_C, w_M, and w_S, from the four ancestral populations, Bylas Spring, Cienega Creek, Monkey Spring, and Sharp Spring, respectively, in progeny samples from the Animal Resource Center (ARC) in 10/2001 and 10/2002 and Scottsdale Community College (SCC) in 6/2002

	ARC 10/2001					ARC 10/2002					SCC 6/2002				
Locus	w_B	w_C	w_M	w_S	LR	w_B	w_C	w_M	w_S	LR	w_B	w_C	w_M	w_S	LR
G49	1	1	1	1	ns	nd	Nd	nd	nd	nd	1	1	1	1	ns
6-10	1	1	1	1	ns	1	1	1	1	ns	1	1	1	1	ns
C-15	0.4	1	0.4	1	$>10^5$	1	1	0.3	1	$>10^3$	1	1	0.4	0.0	$>10^{18}$
OO56	1	1	1	1	ns	1	1	1	1	ns	1	1	0.0	1	$>10^{12}$
LL53	0.0	1	0.1	0.1	$>10^{30}$	0.0	0.4	1	0.0	$>10^{12}$	1	0.0	0.0	0.0	$>10^{46}$

LR is the ratio of the likelihood of the given set of contributions divided by the likelihood for equal contributions, ns indicates not significant ($< 10^3$), and nd indicates no data.

allele OO56-149 is greatly decreased in the progeny. However, the SCC population has been through an extreme bottleneck and these effects may be the result of genetic drift rather than any differential selective effects.

For locus LL53, the highest contribution for Cienega Creek in the first sample is caused by similarity of the frequencies of alleles 144 and 146 in Cienega Creek and the progeny and the highest contribution of Monkey Spring in the second sample is caused by the high frequency of allele 142 in the progeny. Although more complicated because of more alleles at locus C-15, the differential estimated contributions from the populations are also consistent with lower or higher frequencies in the populations and the progeny samples.

Overall, the message from the population-specific alleles and this quantitative analysis is that all four populations appear to be represented in the progeny. For the eight locus–progeny sample combinations that have differential estimated contributions, the mean relative contributions were 0.55, 0.80, 0.40, and 0.51 for Bylas, Cienega, Monkey, and Sharp, respectively. That is, the highest relative contribution is from Cienega Creek and the lowest relative contribution is from Monkey Spring. Leaving out the four SCC samples that were strongly influenced by the population bottleneck, for the remaining four locus–progeny sample combinations from the ARC samples, the mean relative contributions were 0.35, 0.85, 0.45, and 0.52, again with Cienega Creek the highest but the order of the other three changed. Overall from these quantitative estimates, it appears that the Cienega Creek population is making higher genetic contributions than the other three populations.

Discussion

One of the main concerns about maintaining captive populations of endangered species is the loss of genetic variation. As a result, the primary recommendation for captive populations is that mean kinship be minimized (Ballou et al. 1995), which generally results in high maintenance of genetic variation, an approach that is widely used in captive populations. However, in some captive populations it is not possible to make specific matings to minimize mean kinship and loss of genetic variation. In these cases, recommendations are that the effective population size be maximized so that the loss of genetic variation is minimized. In the example evaluated here of four captive populations of the endangered Gila topminnow, the maintenance of captive populations for 5 years (about 10 generations) at high census numbers (generally > 500 adults) did not result in a loss of genetic variation at microsatellite loci. In fact, some new alleles were observed in the samples from 2000 that were not seen in 1995 and the heterozygosity and number of alleles were actually somewhat higher in 2000 than 1995.

Merging of populations of endangered species may result in more genetic variation and reduction of detrimental variation (inbreeding depression) that has accumulated from genetic drift. Recently, a number of examples of genetic rescue (Tallmon et al. 2004) where the addition of unrelated individuals to an endangered species population with low fitness have been documented (Hedrick and Fredrickson 2010). With the further isolation of populations and reduction of population size in many endangered species, the importance of merging populations or moving individuals between populations will likely increase. In the example of merging four captive populations of the endangered Gila topminnow here, evaluation suggests that ancestry from all populations was retained, both from population-specific markers and a quantitative evaluation of ancestry using a likelihood approach. Although the polymorphic microsatellite loci that were examined here may not be reflective of a large proportion of the ancestral genomes, they may indicate differential contributions to the merged population due to higher or lower fitness.

Observation of New Alleles

In the samples taken from the captive populations in 2000, there were a number of new alleles for the microsatellite loci that were not observed in the samples taken from the captive population in 1995. In addition, there were also new alleles observed in the progeny of the merged populations not observed in the female parents of those populations and there were also new alleles seen in the progeny of the merged populations that had not been observed in the female parents or the 1995 survey. Note that 10 of the 17 new alleles observed in 2000 but not in 1995 were only seen in one or two copies out of a sample (frequencies of 0.025 or 0.05). Similarly, all of the 7 new alleles seen in the progeny of the merged populations were present in low frequency.

There are several potential explanations for the observation of these new alleles. First, in 1995, 40 individuals ($2N$ =80) were sampled from estimated adult census population numbers of 500 or more for each captive population (see above). In other words, low frequency alleles may have been present in the populations at this time and missed because of sampling. Or, allele frequencies that were low in 1995 may have increased by 2000 and were found in that sample of 20 individuals per population. Similarly, the new alleles observed in the progeny of the merged populations may have been in the 1995 captive populations and the male parents of the merged populations but not observed by chance in the 1995 sample or the female parents of the merged populations.

Second, some new alleles may have been generated by mutation. For example, a number of alleles that were seen in one or two copies could have been generated by mutation between 1995 and 2000 or in the merged populations after their founding in 2000. Of the new alleles never seen before 9 of 20 are at the C-15 locus. The mutation rates are not known for this locus but because C-15 has much more variation than the other loci, it may have a higher mutation rate (with more variation, the first explanation, sampling error, may also be more likely). For the 10 locus–population combinations that were monomorphic in 1995, all were still monomorphic in 2000.

Third, although extreme care to not mistakenly move fish between raceways (e.g., different nets were always used for each raceway), at some point such an error could have occurred. For example, C-15 allele 210 found in Cienega Creek in 2000 was in Monkey Spring in 1995 and allele 228 found in Cienega Creek in 2000 was in Sharp Spring in 1995. However, Cienega Creek and Monkey Spring shared no alleles at locus 6-10 in 1995 and Cienega Creek and Sharp Spring shared no alleles at LL53 in 1995, making such a transfer error extremely unlikely to go undetected. In fact, excluding the five examples of never before seen alleles in the 2000 samples, in 11 of the 12 situations of alleles in 2000 samples not in the 1995 samples of the same populations are situations where there are no shared alleles at either 6-10 or LL53 between the putative source and recipient populations, making such an error easily detectable. In other words, the likelihood of inadvertent transfer of topminnows between refugia appears quite low.

Finally, although the same protocol was used to score the alleles for the 1995 and 2000 samples, these were done by different people (K. Parker for the 1995 sample and R. Lee for the 2000 sample). However, the identity of scoring for all other alleles makes such a scoring difference very unlikely.

Overall, there are two likely explanations for the observation of new alleles in later samples. First, they were missed by chance in the earlier sample because of low frequencies and seen later because the frequency had increased or included by chance in the later sample. Second, some of the alleles may have been generated by mutation. Although this possibility seems unlikely in 10 generations in a census population size of around 500 adults, the fact that some new alleles had never been seen in any populations and the overall increase in heterozygosity and number of alleles observed, makes this a real possibility.

Conclusions and Interpretations

The intensive study of some endangered species can provide evolutionary genetic information, not only about the species under examination but about small and isolated populations of other species. For example, in this study of the endangered Gila topminnow, there appears to be a contribution of mutation to genetic variation observed in the captive populations. However, to put these observations in context, it would be important to determine the rate of new mutations for other types of loci and their influence on fitness in such captive situations. Similarly, in the merging of Gila topminnow populations examined here, it would be important to determine the influence on overall fitness of combining source populations. Again other types of loci, such as large numbers of single-nucleotide polymorphisms (SNPs), could provide a more detailed evaluation of ancestry in the merged populations.

Several conclusions specifically about Gila topminnows are relevant. As we stated above, Monkey Spring topminnows were used for many reintroductions, virtually all of which were unsuccessful. Parker et al. (1999) and Hedrick et al. (2001) classified the Gila topminnow into two ESUs and divided one of these ESUs into four different MUs (Moritz 1994). This division for conservation management of Gila topminnows was suggested for several reasons including the substantial molecular genetic differences between the units, geographic isolation of their locations, separation of their locations generally by extensive dry reaches of river, and habitat and life history differences between the groups. At this point, reintroductions use topminnows taken from the watershed being restocked and movement of stocks between watersheds is avoided when possible.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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