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The endangered Sonoran topminnow: Examination of species and ESUs using three mtDNA genes

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Abstract

There has been controversy over the species status of Sonoran topminnows and debate about the presence of ESUs in the Gila topminnow. From examination of sequence variation at 2626 base pairs over three mtDNA genes, we found a 29 (1.1%) nucleotide genetic difference between Gila and Yaqui topminnows. This provides strong support that these two taxa are separate species, *Poeciliopsis occidentalis* (Gila topminnow) and *P. sonoriensis* (Yaqui topminnow) and have been separated for approximately one million years. All the Gila topminnows within Arizona have the same sequence for the three mtDNA genes, that is, there is not reciprocal monophyly for mtDNA sequence data for the two previously designated ESUs. However, evidence of the unique habitat for Monkey Spring, its long-term isolation from other Gila topminnow habitats, and the presence of unique fish and invertebrate taxa in Monkey Spring support the designation of the Monkey Spring topminnows as an ESU. Finally, theoretical considerations using molecular data and estimates of heterozygosity and genetic distance for nuclear genes between populations of the Gila topminnow show that the lack of mtDNA variation is not inconsistent with the level and pattern of nuclear genetic variation observed.

Introduction

Neutral genetic markers, such as mtDNA and microsatellite loci, are generally the most appropriate markers to determine historical distinctiveness of populations and therefore the designation of species, evolutionarily significant units (ESUs), and management units (MUs) (Moritz 1994, 2002; Waples 1995; for a review see Fraser and Bernatchez 2001). However, the designation of these units is not always consistent with the suggested use of these genetic variants and the identity of taxa in the literature. Here we discuss these issues in general and specifically how they relate to the endangered Sonoran topminnow in the United States.

The Sonoran topminnow is a small, live-bearing fish that occurs in Arizona, United States and Sonora, Mexico. Two taxa, the Gila and the Yaqui topminnow, both listed as endangered, exist in Arizona (Minckley 1999). The Gila topminnow was once one of the most abundant fishes in the Gila River drainage, but now exists naturally in the United States in only four isolated Arizona watersheds in eight populations. Other populations exist as a result of extensive restocking efforts. The Yaqui topminnow was never widespread in the United States because the Rio Yaqui drainage includes only a small part of southeastern Arizona, now within the San Bernardino National Wildlife Refuge (SBNWR).

These two taxa were identified as separate species until Minckley (1973) described them as subspecies (see Quattro et al. 1996 for a history of the designations). Later, Minckley (1999) revised his determination and described them as two different species, the Gila topminnow, *Poeciliopsis occidentalis*, and the Yaqui topminnow, *P. sonoriensis*. His determination was based partly on genetic data that showed that the two taxa in the United States had quite divergent mtDNA haplotypes, as determined by restriction fragment analysis (RFLP) (Quattro et al. 1996). In addition, more recent analysis of microsatellite loci showed that the two taxa shared only 3.5% of their alleles and had completely nonoverlapping sets of alleles at a major histocompatibility complex (MHC) locus (Hedrick et al. 2001). However, in spite of these genetic data and Minckley's revision, Mateos et al. (2002) still described these taxa as two subspecies, *P. o. occidentalis* and *P. o. sonoriensis*.

Within the Gila topminnow, Parker et al. (1999) and Hedrick et al. (2001) suggested, based on physical habitat, associated biota, life-history, and nuclear molecular genetic variation that the Monkey Spring (and Cottonwood Spring) population of the Gila topminnow be managed as a separate ESU. Hedrick et al. (2001) suggested that the other natural populations of the Gila topminnow be managed as four different MUs based on nuclear genetic variation, the physical isolation of the populations, and other factors. However, Moritz (1994) recommended that a primary basis for different ESUs is that they should be reciprocally monophyletic for mtDNA data. Quattro et al. (1996) found that for mtDNA RFLPs there was no variation within Gila topminnows. This finding suggests, using Moritz's ESU definition, that all Gila topminnow should be one ESU. In addition, we have also found that, although the Gila topminnow ESUs and MUs were genetically distinctive for nuclear variants, we were able to cross these groups and produce both F_1 and F_2 (first and second generation) "hybrids" (Sheffer et al. 1999).

The first goal of this study was to evaluate more precisely the differences between the Gila and Yaqui topminnows by examination of sequence variation for three different mtDNA genes in samples of both Gila and Yaqui topminnows. This will allow high resolution of the extent of mtDNA divergence between Gila and Yaqui topminnows, estimation of their divergence time, and a more definitive statement about the status of these taxa as species or subspecies. The second goal was to determine the amount of mtDNA variation within Gila topminnows and between the designated Gila topminnow ESUs by examining sequence variation from the same three mtDNA genes.

Methods and materials

Fin clips were taken from fish from four populations of Gila topminnows (*P. occidentalis*): Bylas Springs, Cienega Creek, Monkey Spring, and Sharp Springs (Figure 1). Ten wild caught individuals from each population were examined for mtDNA variation. These populations represent samples from the main watersheds in which Gila topminnows are extant in the United States (Parker et al. 1999; Hedrick et al. 2001). Fin clips from individuals were also taken from two Yaqui topminnow populations, three from North Pond and two from Tule Spring, from the San Bernardino National Wildlife Refuge (SBNWR) in extreme southeast Arizona.

DNA was isolated from the fin clips using a PureGene kit (Gentra Systems) and used in PCR amplification with mtDNA primer sets. Three mitochondrial genes were sequenced: cytochrome *b* (Cyt *b*), NADH subunit 2 (ND2), and the control region (d-loop). Primers for Cyt *b*, LA (gtgacttgaaaaaccaccgttg) and HA (caacgatctccggtttacaagac) (Dowling et al. 2002), amplified 1140 bp of sequence. Primers for ND2, ILE-1 (gcttccactacaccacttcc) and ASN (cgcgtttagctgttaactaa) (G. J. P. Naylor, pers. comm.) amplified 1047 bp of sequence. Primers for the control region, ProL19 (ccactagctcccaaagcta) and TDKD (cctgaagtaggaaccagatg) (Hughes et al. 1999) amplified 439 bp of sequence. Overall, 2626 bp were sequenced, about 16% of the mitochondrial genome (Broughton et al. 2001). Mitochondrial sequences were analyzed on both strands using an ABI 377 automated sequence (Perkin–Elmer, Foster City, CA). Sequences were compared using MEGA 2.1 (Kumar et al. 2001) and BioEdit Sequence alignment Editor 6.0.5 (Hall 1991–2004). The standard PCR profile used was initial denaturing at 94 °C for 3 min, then 35 cycles of 94 °C for 30 s, annealing temperature for 30 s, 72 °C for 30 s, and then 72 °C for 7 min. An annealing temperature of 50 °C was used for internal primers HA-LE (see

Figure 1. Map showing the four sites of the Gila topminnow samples (dark shading) in Arizona and the two sample sites from Mateos et al. (2002) in Mexico, Rio Altar and Rio Magdalena, and the three sites of the Yaqui topminnow samples (light shading) in Arizona and the two sites of samples from Mateos et al. (2002) in Mexico, Rio Matape and Rio Mayo.

below), midNADH-midNADHR (see below), and ProL-19-TDKD while for HA-LA and ILE-ASN, an annealing temperature of 48 °C was used and 1 min, instead of 30 s, was used. The sequences found have been deposited into GenBank as accessions (+++ to +++).

One individual from each of the Gila topminnow populations was examined by direct sequencing and variation within the Gila populations was examined by using Single Stranded Conformational Polymorphism (SSCP) analysis using standard PCR conditions and included 1 μ Ci of ^{32}P labeled dATP in each reaction. Internal primers for both Cyt *b*, HA and LE (cccaccacacattcaacc) (Dowling et al. 2002), and ND2, mid-NADHF (cactagatctacgcaccgca) and mid-NADHR (agggctagaccggatttcat), were used to amplify an approximately 400 bp PCR product for the SSCP. The internal primers midNADHF and midNADHR were synthesized/created from full-length sequence from the Gila topminnows and used to screen the samples. Alleles were separated by electrophoresis at 4 °C on a 6% polyacrylamide gel. The gel was then transferred to Whatman paper, dried and exposed to X-ray film (Kodak XOMAT AR) overnight. SSCP is a highly efficient technique for rapid screening of DNA polymorphisms from PCR products. Under optimal

conditions, this method has been shown to identify single point mutations with up to 100% sensitivity (Glavac and Dean 1993). Variation in the five Yaqui topminnows from North Pond and Tule Spring was examined by direct sequencing.

MEGA 2.1 (Kumar et al. 2001) was used in calculating all standard summary statistics including Kimura-2-parameter (K2P) genetic distances and Nei-Gojobori (Jukes-Cantor) non-synonymous to synonymous substitution ratio (dN/dS). A relative rate test, using K2P genetic distance estimates, was used to test rate constancy and determine confidence intervals for the divergence time between Gila and Yaqui topminnows (Phyltest version 2.0) (Takezaki et al. 1995; Kumar 1996). This method uses the two-tailed normal deviate test to determine if two lineages are evolving at significantly different rates given a third, closely related, outgroup lineage. Here *P. lucida* was used as an outgroup in both ND2 and Cyt *b* analyses.

Mateos et al. (2002), in a biogeographic study of the genus *Poeciliopisis* in Mexico, determined sequences for Cyt *b* in two Gila topminnows, one each from Rio Altar and Rio Magdalena, Sonora and ND2 for the Gila topminnow from Rio Altar (Figure 1). They also determined sequences for Cyt *b* in three Yaqui topminnows, one from Black Draw on the SBNWR, one from Rio Matape, Sonora, and one from Rio Mayo, Sonora and ND2 for the Yaqui topminnows from SBNWR and Rio Matape (Figure 1). We use these sequences to provide a context for understanding the variation within and between Gila and Yaqui topminnows in the United States.

Results

Cyt b

All the Gila topminnows from the four different Arizona populations were identical for the 1140 bp Cyt *b* sequence. Similarly, Yaqui topminnows from the two Arizona populations were identical for this region (the SBNWR sample of Mateos et al. 2002 was also identical). The sequences for the Arizona Gila and Yaqui topminnow samples were different at 12 bp, spread throughout the gene, out of 1140 bp (1.05%) and the K2P genetic distance between the taxa was 0.011 (Table 1). Using a Cyt *b* sequence from *P. lucida* as a outgroup (Mateos et al. 2002), rate constancy was not rejected using the relative rate test as implemented in PHYLTEST. Therefore, assuming a mutation rate of 5×10^{-9} (Stepine et al. 1997), the estimated divergence time between Gila and Yaqui topminnows is 1.06 million years with a 95% confidence interval of 0.45–1.67 million years.

All 12 of the differences between Gila and Yaqui topminnows were transitions. Only two out of the 12 nucleotide differences resulted in non-synonymous amino acid changes and the estimated ratio of non-synonymous to synonymous substitutions was 0.056. Figure 2a gives a phylogenetic tree for these sequences and the four other sequences from Mateos et al. (2002).

ND2

All the Gila topminnows from the four different Arizona populations were also identical for the 1047 bp ND2 sequence. Similarly, Yaqui topminnows from the two Arizona populations were identical for this gene (the SBNWR sample of Mateos et al. 2002 was also identical). The sequences for the Arizona Gila and Yaqui topminnow samples are different at 12 bp, spread throughout the gene, out of the 1047 bp (1.15%) and the K2P genetic distance was 0.012 (Table 1). Using a ND2 sequence from *P. lucida* as a outgroup (Mateos et al. 2002), rate constancy was not rejected. Therefore, assuming a mutation rate of

Table 1. Summary of the data for the differences for three mtDNA genes between Gila and Yaqui topminnows including the corrected genetic distance K2P, the ratio of transitions to transversions, and the estimated proportions of non-synonymous to synonymous substitutions and the ratio dN/dS

Gene	Nucleotides	Differences	K2P	Transitions/transversions	dN/dS
Cyt *b*	1140	12	0.011	$12/0 = \infty$	$0.002/0.035 = 0.056$
ND2	1047	12	0.012	$9/3 = 3$	$0/0.046 = 0.0$
Control	439	5	0.012	$4/1 = 4$	–
Total	2626	29	0.011	$25/4 = 6.25$	$0.001/0.041 = 0.024$

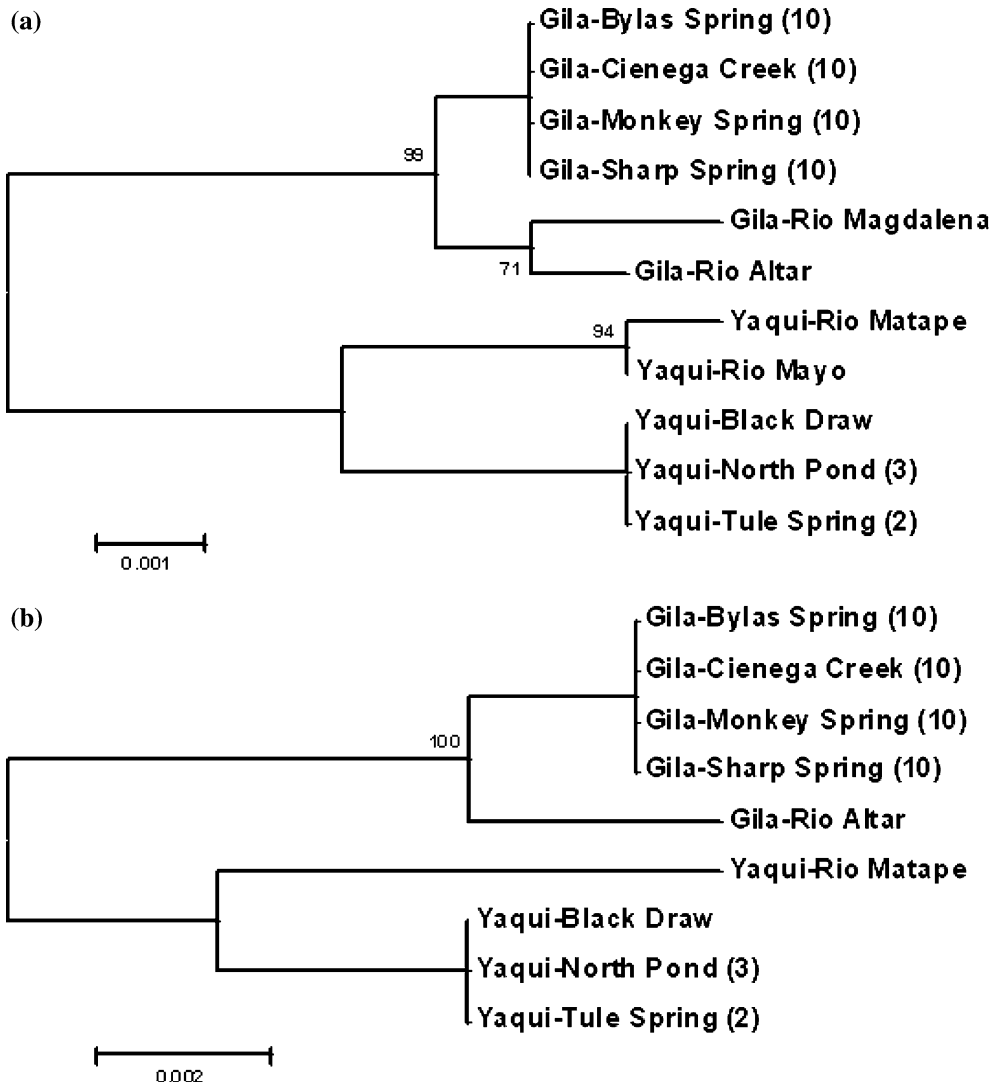

Figure 2. A neighbor-joining phylogenetic tree for the sequence from the (a) Cyt *b* and (b) ND2 genes illustrating that sequences from the four Gila topminnow populations were identical (Bylas Spring, Cienega Creek, Monkey Spring, and Sharp Spring), the two populations of Yaqui topminnows were identical and the other sequences from Mexico from Mateos et al. (2002). The numbers indicate bootstrap support values for the corresponding nodes in the tree.

6.5×10^{-9} (Bermingham et al. 1997), the estimated divergence time between Gila and Yaqui topminnows is 0.89 million years with a 95% confidence interval of 0.38–1.41 million years.

Nine of the 12 differences between Gila and Yaqui topminnows were transitions (transition/tranversion ratio was 3) and none of the 12 nucleotide differences resulted in non-synonymous amino acid changes. Figure 2b gives a phylogenetic tree for these sequences and the two other sequences from Mateos et al. (2002).

Control region

All the Gila topminnows from the four different Arizona populations were also identical for the 439 bp control region sequence. Similarly, Yaqui topminnows from the two Arizona populations were identical for the region. The sequences for the Arizona Gila and Yaqui topminnow samples are different at 5 bp, spread throughout the sequence, out of the 439 bp (1.14%) and the K2P genetic distance was 0.012 (Table 1). Divergence time

between Gila and Yaqui topminnows was not estimated from the control region because a reliable estimation of the mutation rate was not available. Four of the five differences are transitions (transition/tranversion ratio was 4).

MtDNA haplotypes

All Gila topminnows from the four different Arizona populations had identical haplotypes for 2626 bp at the three mtDNA genes. Similarly, Yaqui topminnows from the two Arizona populations had identical haplotypes for these three genes. Overall, the sequences for the Gila and Yaqui topminnow samples are different at 29 bp out of the 2626 bp (1.10%) and K2P genetic distance was 0.011 (the percentage of differences and the K2P distance for the three genes were nearly identical and were not significantly statistically heterogeneous, $\chi^2 = 0.058$, df = 2).

Transitions outnumbered transversions; the average transition/transversion ratio across all three loci was 6.25. Only two out of 24 nucleotide differences at the Cyt *b* and ND2 loci resulted in non-synonymous amino acid changes and both substitutions were found in the Cyt *b* gene. The overall dN/dS ratio was 0.024, much less than unity and consistent with strong purifying selection, as commonly found for mitochondrial coding genes. Nucleotide composition was A–T biased and the average base pair frequencies for the three loci were 30.0% T, 28.9% C, 27.8% A, and 13.3% G, similar to nucleotide composition frequencies found for other teleost fishes (Broughton et al. 2001).

Discussion

In the samples that we examined from the United States from both the Gila and Yaqui topminnows, there was no variation within either species for 2626 bp of mtDNA sequence from three genes and there were 29 nucleotide differences between the species for a K2P genetic distance of 0.011 between species. How do these findings apply to species designations of the Gila and Yaqui topminnows and to the determination of two ESUs within the Gila topminnow discussed in the introduction?

Species designation

Clearly there is extensive and consistent divergence in three different mtDNA genes between the Gila and Yaqui topminnows. Using mutation rates for Cyt *b* (Stepine et al. 1997) and ND2 (Bermingham et al. 1997) estimated for fishes, these two taxa are estimated to have been separated for 0.89 and 1.06 million years, respectively, or around one million years on average. This divergence time is consistent with earlier suggestions based on allozyme variation (Vrijenhoek et al. 1985) and geological evidence suggesting that the Gila and Yaqui drainages were separated during the early Pleistocene (Melton 1960).

The study of Quattro et al. (1996) examined mtDNA variation in Gila and Yaqui topminnows with 13 six-cutter enzymes and 3 five-cutter enzymes and they found 71 restriction sites. Assuming that all sites were found by six-cutter enzymes, then their survey covered approximately 426 bp. Here we examined 2626 bp, at least 6.2 times as many bp. In addition, the location of the restriction sites of Quattro et al. (1996) are not known while we know specific DNA sequences for the three genes that we examined. In addition, we specifically examined the control region, which is generally the most variable mtDNA region. In other words, we feel that our study makes a significant contribution in documenting the differences between these two species by providing both higher resolution and higher quality data than the earlier survey of Quattro et al. (1996).

We have recently examined both pre-mating and post-mating reproductive isolation between these two species of topminnows. Behavioral observations provided strong evidence of assortative mating when males were given a choice of conspecific or heterospecific females (Hurt et al. 2004). This mate preference was asymmetric in that Gila males were more discriminating than were Yaqui males. Evidence for post-mating barriers was less pronounced, as both interspecific crosses and backcrosses produced viable offspring (Hurt and Hedrick 2003). There was, however, evidence of incompatible gene complexes; F_1 offspring from Gila male $\times$ Yaqui female crosses were highly male biased, averaging only 8.3% female offspring and F_1 females had smaller brood sizes than did pure species females.

Recently we have used cytonuclear genotype frequencies of first and second generation offspring from mixed Gila/Yaqui populations to measure the total strength of reproductive barriers (Hurt et al. 2005). Resulting frequencies were used to calculate an isolation index analogous to that used by Coyne and Orr (1989) to measure reproductive barriers in biologically isolated sympatric species pairs of *Drosophila*, where the measure $I = 1 -$ (frequency of hybrid offspring/frequency of homospecific offspring). Values of this index can range from $-\infty$ (complete disassortative mating) to 1 (complete assortative mating) and a value of 0 indicates random mating. The isolation index for Gila/Yaqui calculated from first generation genotype frequencies was 0.872, falling within the range of what Coyne and Orr required for "species status" of allopatric taxonomic pairs.

ESUs

The lack of mtDNA variation within the Gila topminnow is consistent with the lack of RFLP mtDNA variation observed by Quattro et al. (1996). However, the substantial variation found for nuclear markers by Hedrick and Parker (1998), Parker et al. (1999), and Hedrick et al. (2001) suggested that with a higher resolution mtDNA assay, such as sequence analysis of multiple genes that we have reported here, that variation within Gila topminnows might be found. The Yaqui topminnow has also shown high variation for nuclear markers (Hedrick et al. 2001) but no variation for RFLP mtDNA variation in a United States sample (Quattro et al. 1996). As in the Gila topminnow, there was no mtDNA sequence variation within the United States Yaqui topminnow sample, even though there was extensive nuclear variation in these samples in earlier studies.

Hedrick et al. (1999, 2001) concluded that Gila topminnows should be divided into two ESUs, one composed of Monkey Spring (and the related nearby Cottonwood Spring), and the other ESU composed of the remaining natural populations (Bylas Spring, Sharp Spring, Cienega Creek, and the three other Sonoita Creek populations). This was based on a number of factors pointing to the long isolation and differential adaptation of the Monkey Spring population from the other populations. Using both microsatellite and MHC loci, the Monkey Spring was the most differentiated population from the other Gila topminnow populations.

In addition, Monkey Spring is the only warm spring site (28 °C) and does not have the extreme seasonal temperature variation experienced by the other habitats. It appears to have been isolated from Sonoita Creek by a 10 m high natural travertine dam for around 10,000 years. Laboratory experiments examining fitness components in Gila topminnow populations demonstrated an adaptive difference in the Monkey Spring population, specifically the development time of Monkey Spring males was twice that of other populations (Cardwell et al. 1998). Further, an endemic species of pupfish (*Cyprinodon arcuatus*) and distinct form of chub (*Gila intermedia*) occupied Monkey Spring until they became extinct in the 1960s due to introduced non-native fishes. Finally, recent examination of springsnails (*Pyrgolopsis thompsoni*) from Monkey Spring and all other known springsnail populations in the area found that the two Monkey Spring springsnail mtDNA haplotypes are substantially divergent from the haplotypes found in other *P. thompsoni* populations (Hurt 2004). These factors all point to strong isolation of Monkey Spring organisms and influence of its unique environment on important adaptive change in the organisms inhabiting it. In other words, there is strong genetic, ecological, and life history data as well as the presence of other distinct taxa that support Monkey Spring as a separate ESU (Minckley 1999; Hedrick et al. 2001).

Moritz (1994) suggested that reciprocal monophyly for mtDNA sequences be used as a basis of designating ESUs. In this instance, we found no mtDNA variation within or between these populations, much less reciprocal monophyly. How can we resolve the difference between Moritz's suggestion and our observation? First, as we will show below it is not surprising that we did not observe mtDNA variation in Gila topminnows based on the apparent effects of genetic drift reducing mtDNA variation. If we assume that there was no initial mtDNA difference between Monkey Spring and the other populations 10,000 years ago and complete isolation between them since (no gene flow into Monkey Spring), the expected divergence per site is $2ut$ where u is the mutation rate per year and t is the number of

years since isolation. Assuming that $u = 10^{-9}$ and $t = 10^4$, then the expected divergence for the whole mtDNA region we examined is only $2(10^{-9})(10^4)(2626) = 0.053$. In other words, for this amount of time the expected divergence is small and it is not unlikely that it would not be observed. However, as we detailed above, this isolation of Monkey Spring has been long enough for changes in the life history of the Gila topminnow and the evolution of a species of pupfish and distinct forms of chub and springsnail.

Second, there has been extensive discussion and criticism on the reliance of reciprocal monophyly of mtDNA as the basis of ESU identity (Waples 1995; Crandall et al. 2000; Fraser and Bernatchez 2001). It is clear that the Gila topminnow Monkey Spring ESU "is a product of past evolutionary events and that represents the reservoir upon which future evolutionary potential depends" (Waples 1995). Although both Crandall et al. (2000) and Fraser and Bernatchez (2001) suggest alternatives to the ESU approach, it is obvious that the Monkey Spring ESU would qualify as distinctive "based on the concepts of ecological and genetic exchangeability" (Crandall et al. 2000) and would qualify for conservation under the "adaptive evolutionary conservation" scheme envisioned by Fraser and Bernatchez (2001).

Theoretical examination of nuclear and mtDNA results

To examine the likelihood of the observed differences in nuclear and mtDNA variation, let us first examine the general effect of genetic drift on genetic distance for nuclear variation by assuming than an ancestral population is split into two isolated populations that accumulate chance genetic changes over time. The standard genetic distance of Nei (1987) between two such populations at time t (Chakraborty and Nei 1977; Hedrick 1999) is

$$D_t = -\ln \frac{1 - H_0}{[(1 - H_{xt})(1 - H_{yt})]^{1/2}} \qquad (1a)$$

where H_{xt} and H_{yt} are the heterozygosities for nuclear genes in populations x and y in generation t and H_0 is heterozygosity in the ancestral population, assuming no mutation for the moment. If we assume that $H_{xt} = H_{yt} = H_t$, then

$$D_t = -\ln \frac{1 - H_0}{1 - H_t}. \qquad (1b)$$

Both D_t and H_t can be estimated from contemporary data and we can estimate the ancestral heterozygosity H_0 by solving expression (1b) as

$$H_0 = 1 - (1 - H_t)e^{-D_t}. \qquad (2)$$

For example, using the data from Parker et al. (1999), the average H_t for the four populations of the Gila topminnow for five microsatellite loci is 0.204 and the D_t between the Monkey Spring population and the other three populations is 1.038. Then, using expression (2), $H_0 = 0.718$.

The loss of heterozygosity is a function of the effective population size and the number of generations as

$$H_t = H_0\left(1 - \frac{1}{2N_e}\right)^t$$

(Hedrick 2005) and the ratio of observed to ancestral heterozygosity is approximately

$$\frac{H_t}{H_0} \approx e^{-x}$$

where $x = t/(2N_e)$. This expression can be solved so that

$$x \approx \ln H_0 - \ln H_t. \qquad (3)$$

With $H_t = 0.204$ and $H_0 = 0.718$, then $x = 1.259$.

Let us assume that t is the same for mtDNA and that the effective population size is 1/4 as much. Then, for a mtDNA gene

$$\frac{H_t}{H_0} \approx e^{-4x} = 0.0065. \qquad (4)$$

In other words, the estimated extent of genetic distance generated by genetic drift for nuclear loci would result in only 0.65% of the original mtDNA diversity remaining in the Gila topminnow population. As a result, it does not appear unexpected that there is no mtDNA variation while there is variation for microsatellite and MHC loci.

Now let us assume mutation makes a significant contribution to divergence for the microsatellite loci, then

$$D_t = 2ut - \ln \frac{1 - H_0}{1 - H_t} \qquad (5)$$

where $2ut$ is the divergence generated by mutation (u is the per generation mutation rate here and t is the number of generations) (after Chakraborty and Nei 1977). We can solve this expression for H_0 as

$$H_0 = 1 - (1 - H_t)e^{-D_t + 2ut}. \quad (6)$$

To obtain an idea of the magnitude of $2ut$, let us assume that t is at least 10,000 generations, the approximate number of years that the Monkey Spring population has been isolated, and that $u = 10^{-4}$, a value not unusual for microsatellite loci, making $2u \approx 2$. Extrapolating from the numerical results of Li (1976) comparing the infinite allele model to stepwise-mutation model with $u = 10^{-4}$, it appears that $2ut \approx 0.5$ for the stepwise mutation model, generally thought to be closer to the mutation model appropriate for microsatellite loci. In other words, about half the divergence may be the result of mutation.

To understand the potential impact of mutation, let us assume that a proportion of the total divergence is the result of mutation. Assuming that 0.25, 0.5 and 0.75 of the total divergence is the result of mutation and using expression (6), $H_0 = 0.634$, 0.526, and 0.386, respectively. Using expressions (3) and (4) with these values of H_0, then only 0.0011, 0.0023, and 0.078 of the original mtDNA diversity would be remaining in the Gila topminnow population when 0.25, 0.5 and 0.75 of the total divergence is the result of mutation. This illustrates that the larger the proportion of the divergence generated by mutation, then the expected proportion of original mtDNA remaining is larger. However, even if 75% of the divergence were explained by mutation, then only 7.8% of the mtDNA variation would be expected to be remaining.

The low mtDNA variation within these two species suggests that the ancestral female effective population size for both species may have been small at some point. Although the census numbers in these populations were generally many thousands when they were sampled, a bottleneck of a small number of females in the past may have caused this low variation in mtDNA. In addition, if females are multiply mated (topminnows can store sperm from multiple males) then the overall amount of variation for nuclear genes may be larger than the four times amount expected based on the maternal, haploid inheritance of mtDNA (Hedrick 2005).

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