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ORIGINAL PAPER

Genetic isolation and evolutionary history of oases populations of the Baja California killifish, Fundulus lima

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Abstract The Baja California killifish, *Fundulus lima*, is found in six desert oases of the southern Baja California Peninsula, Mexico. The recent introduction of exotic fishes, particularly redbelly tilapia, have impacted the ecology of *Fundulus lima* such that it is now endangered. Plans of relocating *F. lima* to bodies of freshwater that are free of exotics have been proposed, however little is know about the genetic identity of the current populations. In this study, we examined the mitochondrial control region of *F. lima* samples from 4 oases, and in addition, compared these samples to their sister species, the California killifish *F. parvipinnis*. Using a combination of phylogenetic and coalescent approaches, we were able to determine that the two subspecies of the California killifish, *F. p. brevis*, and *F. p. parvipinnis*, and *F. lima* form an unresolved trichotomy that diverged between 200,000 years and 400,000 years ago. The one *F. lima* individual that we were able to collect in the southernmost oasis grouped with the southern subspecies of the California killifish, *F. parvipinnis brevis*. In contrast, we found that the 3 northern oases grouped together in a ''*Fundulus lima*'' clade. Each oasis is genetically distinct, yet there is no evidence of a marked genetic bottleneck in any populations (Haplotype diversity between 0.5 and 0.8). Future relocation plans will therefore need to be done cautiously to preserve the genetic identity of the original populations.

Keywords *Fundulus lima* · *Fundulus parvipinnis* · Baja California · Desert fishes · Control region

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Introduction

The vulnerability of desert habitats is exacerbated in their aquatic microcosms. Besides the inherently precarious nature of the environment, human overuse of water, and exotic introductions have created a challenge to native species. Indeed, many, if not most native desert fish species are endangered or threatened (Fagan et al. 2005). The Baja California Peninsula, Mexico, is mostly an arid desert, with few natural oases where freshwater is scarce. Because of the paucity of permanent freshwater, and possibly also due to a complex and fairly recent geological history (Bernardi et al. 2003), only three endemic freshwater fishes are found in the peninsula, the San Pedro Mártir rainbow trout, *Oncorhynchus mykiss nelsoni*, the Baja freshwater clingfish, *Gobiesox juniperoserrai*, and the Baja California killifish, *Fundulus lima* (Ruiz-Campos 2000; Ruiz-Campos et al. 2002). The introduced poeciliids, *Poecilia reticulata, Xiphophorus helleri, X. maculatus*, and a cichlid, the red-belly tilapia, *Tilapia zillii*, with their steadily increasing abundance, have had profound negative effects on the freshwater ecosystem of the region (Ruiz-Campos 2000). The introduction of exotic fishes, particularly of the tilapia, is currently menacing the survival of *F. lima* in most of its distributional range (Ruiz-Campos 2000; Ruiz-Campos et al. 2002). Thus, a

plan to relocate *F. lima* in oases that are free of any fishes has been proposed (Ruiz-Campos 2000). Before proceeding in this direction, however, a clear understanding of the genetic history and background of the current populations is needed.

The Baja California killifish, *Fundulus lima* is restricted to oases of the Pacific drainage of the southern Baja California Peninsula (Ruiz-Campos 2000). The species has been recorded only in six oases systems, San Ignacio, La Purísima, Bramonas, San Luis Gonzaga, San Pedro, and Las Pocitas (El Caracol) (Ruiz-Campos et al. 2002). Its closest relative, the California killifish *Fundulus parvipinnis* (Bernardi and Powers 1995; Bernardi 1997) is found in coastal lagoons from Morro Bay, California, to Almejas Bay, Baja California Sur (Miller and Lea 1972; Fig. 1). *Fundulus parvipinnis* has been proposed to be a complex of two subspecies, a northern *F. parvipinnis parvipinnis*, ranging from Morro Bay to Punta Eugenia, and a southern *F. parvipinnis brevis*, ranging from Punta Eugenia to Almejas Bay (Fig. 1) (Miller and Hubbs 1954; Bernardi and Talley 2000; Camarena-Rosales et al. 2001). Studies have suggested that *F. lima* derived from the southern *F. p. brevis*, by invading freshwater streams and later becoming landlocked (Durnke 1976; Camarena-Rosales et al. 2001). However, this hypothesis remains controversial (Camarena Rosales et al. 2001).

The goal of this study was 1. to determine the degree of genetic isolation between different oases populations of *F. lima*, and 2. to determine the evolutionary relationships between *F. lima, F. p. parvipinnis*, and *F. p. brevis*. To address these questions, we used a phylogeographic approach based on mitochondrial control region sequences using samples from seven populations of *F. parvipinnis* spanning its entire range, and from four (out of six) oases where *F. lima* is present. Such genetic data, together with ecological

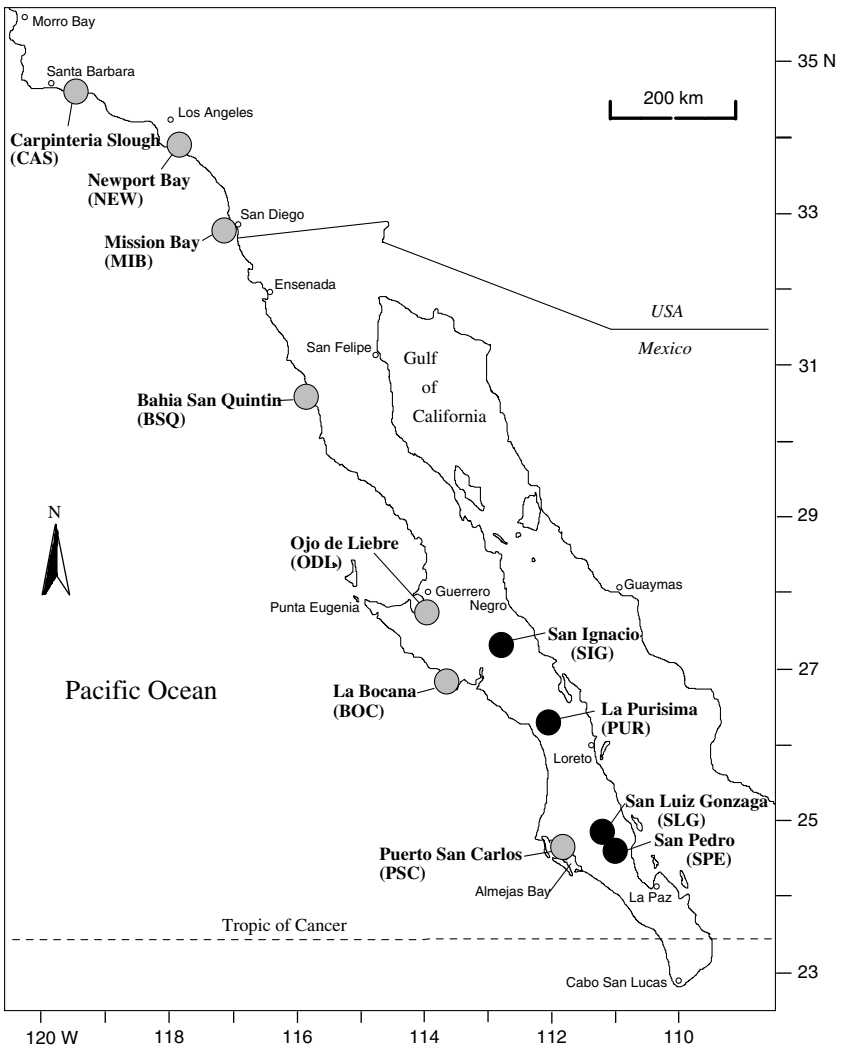

Fig. 1 *Fundulus lima* and *F. parvipinnis* sampling locations. Sampling sites are shown on a map of the California, and Baja California Coast. Samples of *F. parvipinnis* are shown in grey. *F. parvipinnis* were collected at the following localities: Carpinteria Slough, Newport Bay, Mission Bay, in California, and Bahia San Quintín, Ojo de Liebre lagoon, La Bocana, and Puerto San Carlos along the cost of Baja California peninsula. The sampling locations of *Fundulus lima*, San Ignacio, La Purísima, San Luis Gonzaga, and San Pedro, are shown in black

surveys, may help for a coordinated effort in managing this endangered species.

Materials and methods

Collections, and DNA extraction

Fundulus lima and *F. parvipinnis* individuals were collected from different sites throughout their respective distributional ranges in the Baja California Peninsula (Table 1, Fig. 1) using minnow traps and seines. In addition to those samples, we used 60 *F. parvipinnis* and 2 *F. lima* sequences from Bernardi and Talley (2000) (Table 1), and one *F. heteroclitus* sequence from GenBank (U06066) that was used as an outgroup.

After collection, individuals were preserved whole at ambient temperature in ethanol until DNA extraction. Muscle tissue was removed from the preserved fish and digested overnight at 55°C in 500 µl of extraction buffer (Tris 10 mM, NaCl 400 mM, EDTA 2 mM, SDS 2%, Proteinase K). DNA was then extracted by standard chloroform (no phenol) protocol and isopropanol precipitation (Sambrook et al. 1989).

Polymerase chain reaction (PCR) amplification

Amplification of the mitochondrial control region (D-loop) was accomplished using universal primers from the literature, CR-A and CR-E (Lee et al. 1995). The amplifications (25 µl) contained 10 to 100 ng of DNA, 10 mM Tris–HCL (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 2.5 units of Taq DNA Polymerase (Perkin–Elmer, Norwalk, Connecticut), 150 µM of each dNTP, and 0.3 mM of each primer, and used a cycling profile of 45 s at 94°C, 45 s at 48°C, 1 min at 72°C, for 35 cycles. Automated sequencing was performed in both directions with the amplification primers using an ABI 373 automated sequencer (Applied Biosystems, Foster City, CA).

Sequence analysis

Sequences were aligned with the aid of the computer program Clustal V in Sequence Navigator (Applied Biosystems). Phylogenetic relationships were assessed using the Neighbor-Joining (NJ) method (Kimura 2 distance) and Maximum Parsimony implemented by the Software package PAUP (version 4.0, Swofford 2003). Topological confidence was evaluated with 2000 bootstrap replicates (Felsenstein 1985) for Neighbor-Joining and also with 2000 replicates using the Fast-Step method for Maximum Parsimony (only one tree kept at each replicate). Haplotype numbers (Hn), and Haplotype diversity (Hd) were calculated using the software package DNAsp 4.0.6 (Rozas et al. 2003).

Genetic divergence

The time of divergence between species or populations can be estimated using genetic divergence, or an estimate of coalescence time (Edwards and Beerli 2000). In both cases, mutations are assumed to

Table 1 Collection localities for *Fundulus* spp. Columns represent the number of individuals included in the study, and the abbreviations used in Figs. 1 and 2

Locality	*n*	Label	Latitude	Longitude	Altitude	Origin
Species						
Fundulus lima						
San Ignacio	26	SIG1,2	27 N 16′ 26.1	112 W 54′ 46.5	143 m	This study, 4/13/02
	2	SIG				B,T
La Purísima	14	PUR	26 N 14′ 19.8	112 W 00′ 03.6	160 m	This study, 4/16/02
San Luis Gonzaga	2	SLG	24 N 53′ 59.8	111 W 14′ 56.4	190 m	This study, 4/17/02
San Pedro	1	SPE	24 N 50′ 43.2	110 W 59′ 32.3	233 m	This study, 4/18/02
Fundulus parvipinnis parvipinnis						
Carpinteria Slough	10	CAS				B,T
Newport Bay	10	NEW				B,T
Mission Bay 10	MIB				B,T	
Bahía San Quintín	10	BSQ				B,T
Ojo De Liebre Lagoon	10	ODL				B,T
Fundulus parvipinnis brevis						
La Bocana	10	BOC				B,T
Puerto San Carlos	4	PSC				This study, 4/12/02

Altitude of freshwater habitats, dates of collection, and GPS coordinates when available are given. Samples from Bernardi and Talley (2000) are indicated as B,T

proceed randomly and uniformly (molecular clock). Molecular clock enforcements (Maximum Likelihood trees with, or without, an enforced molecular clock) were tested using a Shimodaira and Hasegawa test (Shimodaira and Hasegawa 1999) implemented by PAUP. Genetic divergence was estimated using the Kimura 2-parameter substitution model. In order to account for polymorphism in each population (or species), divergence was estimated as the average pairwise distance between populations (or species) minus the average pairwise distance within a population (or species). Time of divergence was estimated by using a calibrated molecular clock based on the genetic divergence of the mitochondrial control region in trans-isthmian geminate species of *Chromis* damselfishes (6.93% and 7.83% per million year, Domingues et al. 2005).

Historical demography

Historical demography (population fluctuations based on coalescent models) was evaluated using population parameters Θ Theta, $\Theta = 2N_e\mu$, where μ is the mutation rate for mitochondrial DNA) and g (the exponential growth parameter in units of μ^{-1}) that were estimated using a coalescent approach with FLUCTUATE 1.4 (Kuhner et al. 1998. The parameter Θ was estimated with population growth (parameters are estimated jointly) or with growth kept constant ($g = 0$). Both estimates were obtained by running ten replicates, which generated a mean value and its associated standard deviation. Analysis of each dataset was done with 10 short Monte Carlo chains of 4000 steps each and 5 long chains of length 20,000, with a sampling increment of 20. FLUCTUATE generated a random topology for initial searching.

Fundulus coalescence times were also determined. When population growth is zero, mitochondrial coalescence is reached in N_e generations, and N_e is calculated with the equation given above ($\Theta = 2N_e\mu$) (Avise 1994, 2000). When population growth is different than zero, the time of coalescence (t_0) was estimated by assuming that coalescence was reached when the population size was reduced to 1% of its present day value using the equation ($N_t = \Theta e^{-(g\mu)t}$), following Wares and Cunningham (2001). In order to estimate coalescence time, we used a mutation rate (μ = substitutions per site per generation) calculated for trans-isthmian geminate species of *Chromis* damselfishes for the same molecular marker ($\mu = 8.24 \times 10^{-8} - 9.30 \times 10^{-8}$, Domingues et al. 2005).

Results

Sequences

A total of 110 sequences were compared. Alignments resulted in 396 base pairs (bp) that required 6 one base pair gaps, which were encoded as a single mutation each (although removing those gaps did not change the results). Out of 396 aligned base pairs, 70 bp were variable and 61 bp were phylogenetically informative (the outgroup was removed from this analysis). Sequences from this study were deposited in GenBank (Accession numbers XXXX).

Phylogenetic analysis

Phylogenetic relationships obtained with Maximum Parsimony and Neighbor-Joining methods were identical. The phylogenetic analysis resulted in three well-supported clades that formed an unresolved trichotomy. The first clade includes all the *F. lima* samples from the oases of San Ignacio, La Purísima, and San Luis Gonzaga. Within this clade, samples from each of the three oases formed three well-supported sub-clades, with samples from La Purísima and San Luis Gonzaga grouping together (with weaker bootstrap support, 74% and 65% for NJ and MP methods). A second clade comprised all the samples collected north of Punta Eugenia, corresponding to *F. p. parvipinnis*. This result was consistent with Bernardi and Talley (2000), where population structure within this clade was analyzed and therefore will only briefly be described later. A third clade included all the *F. parvipinnis* samples collected south of Punta Eugenia (La Bocana and Puerto San Carlos) as well as the *F. lima* individual collected at the southernmost oasis of San Pedro (Fig. 1, 2). This clade corresponds to the southern form *F. p. brevis* (see discussion). Within this clade, La Bocana and Puerto San Carlos samples grouped as two sister sub-clades (84% and 69% bootstrap support with NJ and MP methods).

Population structure

Most populations of *F. p. parvipinnis* did not partition into distinct clades (except ODL which grouped in a well-supported clade), yet gene flow between these regions was very low as described in Bernardi and Talley (2000). In contrast, each population of either *F. p. brevis* and *F. lima* grouped in a monophyletic assemblage, thus showing evidence of no, or very limited, levels of gene flow between populations.

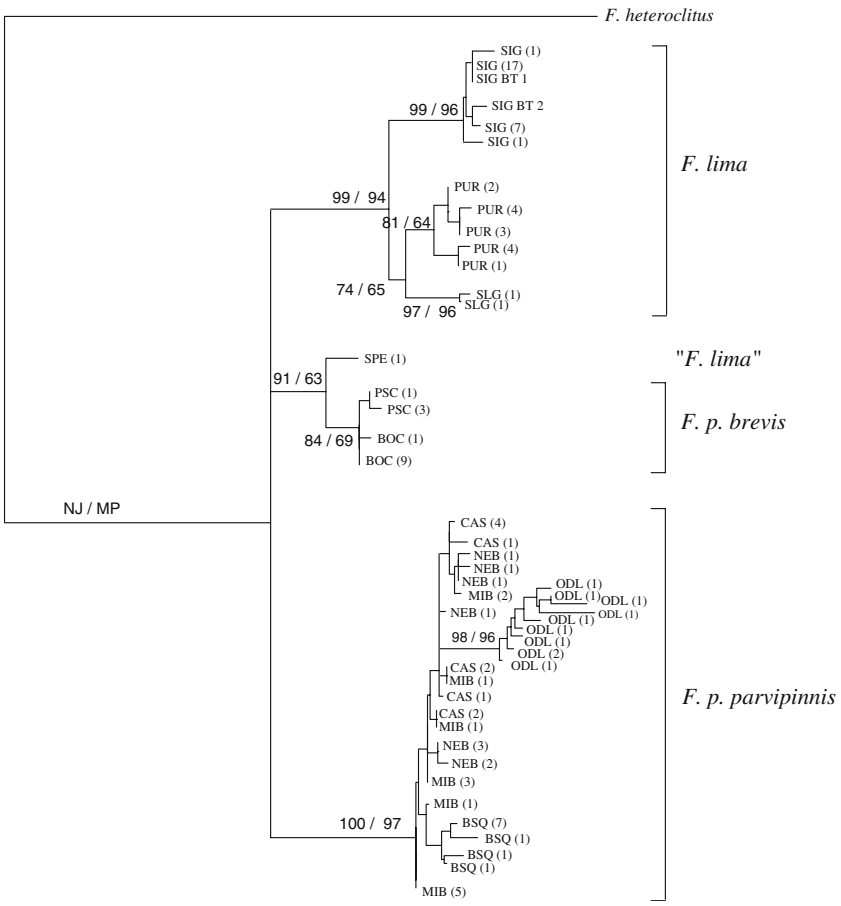

Fig. 2 Phylogenetic relationships between California and Baja California killifish. The phylogenetic tree, based on mitochondrial control region (D-loop) sequences, was obtained using the Neighbor-Joining Method implemented by the software package PAUP (version 4.0). Labels correspond to sampling localities following Table 1. See Fig. 1 for geographic locations. The length of each branch is proportional to the number of nucleotide substitutions. The tree is rooted using *F. heteroclitus* as an outgroup. Bootstrap support higher than 60% is shown at the nodes for the Neighbor-Joining and the Maximum Parsimony methods

Genetic divergence and divergence times

Genetic divergence within species varied (Table 2), *F. lima* exhibiting the highest pairwise sequence divergence (1.94%) and *F. p. brevis* the lowest (0.41%). Divergence within populations was also different with *F. p. parvipinnis* showing the highest levels (average 0.59%), and *F. p. brevis* the lowest (average 0.09%) (Table 2). This was also mirrored by similar trends in haplotype diversity (Table 3). Genetic divergence between species was similar in all three pairwise comparisons, ranging between 4.23% and 5.61% (Table 2), again reflecting the trichotomy obtained in the phylogenetic analysis.

Our data did not show any departure from a molecular clock (Shimodaira and Hasegawa test $p < 0.05$) indicating that a molecular clock could not be rejected. When using a molecular clock calibrated for mitochondrial control regions (6.93% and 7.83% per million year, Domingues et al. 2005), the divergence between the three clades ranged between 405,000 years and 270,000 years ago.

Historical demography

Population sizes (related to Θ values), were shown to be highest in *F. p. parvipinnis* compared to the two other species (Table 3). Growth was also high for *F. p. parvipinnis* ($g = 269$) and nil for the two other species. Relative historical population size was determined, allowing us to estimate the coalescence time for *Fundulus* species. Considering a mutation rate calibrated for *Chromis* ($\mu = 8.24 \times 10^{-8} - 9.30 \times 10^{-8}$, Domingues et al. 2005), the *F. p. parvipinnis* population was 1% of its present size approximately 640,000 years to 567,000 years ago, *F. lima* 103,000 years to 91,400 years ago, and *F. p. brevis* 30,000 years to 27,000 years ago.

Discussion

Evolution of F. parvipinnis and F. lima

Within the genus *Fundulus*, which is predominantly an eastern north American freshwater genus, the evolution

Table 2 Genetic divergences in *Fundulus* species and populations

Panel a: *Fundulus lima*	SIG	PUR	SLG		
San Ignacio	0.22				
La Purísima	2.79	0.58			
San Luis Gonzaga	3.43	1.75	0.25		
Panel b: *Fundulus p. brevis*	SPE	PSC	BOC		
San Pedro	N/A				
Puerto San Carlos	1.34	0.13			
La Bocana	1.51	0.29	0.05		
Panel c: *Fundulus p. parvipinnis*	CAS	NEB	MIB	BSQ	ODL
Carpinteria Slough	0.44				
Newport Bay	0.00	0.52			
Mission Bay	0.00	0.00	0.46		
Bahía San Quintín	0.72	0.57	0.29	0.34	
Ojo de Liebre	1.08	1.03	1.24	1.41	1.21
Panel d: *Fundulus* spp.	*F. l.*	*F. p. b.*	*F. p. p.*		
F. lima	1.94				
F. p. brevis	4.23	0.41			
F. p. parvipinnis	*5.61*	*4.82*	*1.55*		

Pairwise comparisons between and within populations of *F. lima, F. p. parvipinnis*, and *F. p. brevis*, and between and within these species. Sequence divergences are based on control regions using Kimura 2 parameter distances. Labels are from Table 1

Table 3 Historical demography of *F. lima, F. p. brevis*, and *F. p. parvipinnis*

	n	nH	Hd	Θ *c*	Θ *v*	*g*
Fundulus lima	44	12	0.80	0.017 (±0.001)	0.018 (±0.003)	2.15 (±25.8)
F. p. brevis	15	05	0.63	0.005 (±0.000)	0.006 (±0.001)	0.00 (±23.2)
F. p. parvipinnis	50	27	0.96	0.044 (±0.002)	0.085 (±0.007)	261.9 (±61.3)

Columns represent the species investigated, sample numbers (*n*), number of Haplotypes (nH), Haplotype diversity (Hd), Theta with no growth (Θ *c*), Theta with growth (Θ *v*), and population growth (*g*). The latter three columns are averages of 10 replicates, between parentheses are their standard deviations

of the western species *F. parvipinnis* and *F. lima* has presented some problems, as exemplified by their occasional subgeneric status (Farris 1968, Wiley 1986, Bernardi 1997). Here we show that the nominal species, and the two subspecies within *F. parvipinnis, F. p. brevis* and *F. p. parvipinnis* form a trichotomy. Specifically, the two *F. parvipinnis* subspecies are neither sister clades, nor closer to each other than they are to *F. lima*. The approximate time of cladogenesis between the three clades being approximately 300,000 years to 400,000 years ago. Our study did not have a taxonomic purpose, however, our data suggest that these three clades may possibly be regarded as three different species, or alternatively three very distinct populations (McCune and Lovejoy 1998).

Evolutionary history of F. lima

The Baja California killifish, *F. lima*, has been proposed to be an evolutionary freshwater offshoot of the southern coastal subspecies *F. p. brevis* (Camarena-Rosales et al. 2001). Control region data presented here do not show a closer relationship between *F. lima* and *F. p. brevis*, compared to the northern *F. p. parvipinnis* (Fig. 2). When considering genetic distances alone, *F. lima* is closer to *F. p. brevis* (4.23%) than to *F. p. parvipinnis* (5.61%), however these differences do not translate into a closer phylogenetic relationship between *F. lima* and *F. p. brevis* (differences between pairs are due to apomorphies).

One individual collected in freshwater at San Pedro, assumed to be *F. lima*, exhibited a *F. p. brevis* haplotype. This may have been achieved via two possible scenarios: 1. mtDNA from*F. p. brevis* is introgressing in *F. lima* populations in San Pedro via hybridization, or 2. the invasion of the freshwater system in San Pedro was relatively recently achieved by *F. p. brevis* individuals, which are now landlocked and separated from the marine forms of *F. p. brevis*. Our data are too limited to positively distinguish between these two scenarios, however it is important to note that the San Pedro haplotype is different from the other *F. p. brevis* haplotypes, indicating that the 2nd scenario is a distinct possibility. Further

sampling and nuclear molecular markers will help solve this issue.

Genetic isolation of oases populations of Fundulus lima

San Ignacio, La Purísima, and San Luis Gonzaga populations of *F. lima* showed very low levels of connectivity (no gene flow was detected between them). For *F. lima*, coalescence was reached approximately 100,000 years before present, thus, the genetic isolation between populations is likely to be the result of a long history of separation. Yet this isolation in remote and very small habitats did not result in extreme bottlenecks. Indeed, genetic diversity was high for the species (Hd = 0.80), as well as for each oasis (San Ignacio Hd = 0.5396; La Purísima Hd = 0.82, San Luis Gonzaga Hd = 1). While San Ignacio is geographically closer to La Purísima, samples from La Purísima and San Luis Gonzaga were more closely related to each other than either of them were to the San Ignacio samples. While this result suggests interesting potential scenarios of freshwater captures, further sampling at the additional two localities were *F. lima* is present will be essential to fully understand the historical pathways that have connected freshwater bodies in Baja California.

Conservation status of F. lima

The Baja California killifish, *F. lima*, is a species that has historically been present in fairly low numbers (Ruiz-Campos 2000). The recent arrival of exotic introduced species has dramatically reduced these numbers. Indeed, our study was seriously hampered by the lack of large numbers of individuals. Based on intensive sampling carried out between October 2002 and January 2005, the populations of *F. lima* at the Río San Luis (mission de San Luis Gonzaga and Las Cuedas sites) and Río San Pedro basins (San Pedro de La Presa site) have been virtually extirpated by competitive interaction with redbelly tilapia (Ruiz-Campos, unpublished data). Our results show that the San Pedro sample may be part of an important clue in understanding the relationships between *F. lima* and *F. p. brevis*, and their potential evolutionary relationships, yet, extensive search of this habitat only revealed the single specimen used in this study. In the Río Las Pocitas basin, the Baja California killifish is known to occur in the locality of El Caracol (Ruiz-Campos et al. 2002). However, only one specimen of this taxon was recently taken (Jan. 2005). Fortunately, exotic tilapia is not present in this basin (Ruiz-Campos, unpublished data).

Relocation of *F. lima* individuals in oases that are free of exotic fishes is probably an effective way of protecting the species, however our results show that the genetic identity of the source populations need to be maintained. Indeed, the reciprocal monophyly that was observed in different oases populations of *F. lima* justifies them to be considered Evolutionary Significant Units (ESUs, sensu Moritz 1994), and their conservation should take into account this level of genetic individuality.

Desert habitats are extremely fragile and other desert fishes, including topminnows such as the Death Valley pupfishes, have been shown to be very susceptible to habitat change (Duvernell and Turner 1998; Martin and Wilcox 2004). We find the current situation of the Baja California killifish very unfortunate, yet we are hopeful that proper management will help this unique species to thrive.

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