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Genetic evidence for limited dispersal in the coastal California killifish, *Fundulus parvipinnis*

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Abstract

The California killifish, *Fundulus parvipinnis*, is a marine species that lives in salt marshes, estuaries and wetlands along the California and Baja California coasts. In order to estimate levels of dispersal between different coastal habitats over its range, we have studied six populations using morphological and genetic markers. Lateral line scale and vertebrae counts showed significant differences between individuals collected north of Punta Eugenia and south of Punta Eugenia. Morphological differences across Punta Eugenia were accompanied by large genetic differences at the mitochondrial control region (5.8%). Gene flow was in general very reduced over the range of the species (pairwise average $F_{st} = 0.70$, $Nm = 0.30$), with a strong break at Punta Eugenia ($F_{st} = 0.95$, $Nm = 0.03$). Such limited interchanges between coastal habitats have important theoretical and conservation implications.

Keywords: *Fundulus parvipinnis*; California killifish; Dispersal; Mitochondrial DNA; Control region

1. Introduction

Coastal wetlands are among the richest marine environments, and are used as spawning grounds and nurseries by a large number of fish species. In order to understand, protect, and enhance these important areas, considerable effort has been placed in trying to determine the geographic origin of juvenile fishes to specific coastal regions. Recently, chemical otolith signatures have proven useful in helping to determine such origins (e.g., Campana et al., 1994; Thorrold et al., 1998; Swearer et al., 1999). In

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addition to chemical signatures, molecular techniques have been added to the ecologist's toolbox to determine geographic origin, dispersal capabilities, and genetic divergence among populations of juvenile fishes. Genetic divergence between coastal populations may be the result of several factors. When migration of individuals between sites is limited, for ecological, physical, or historical reasons, the disruption of gene flow is evidenced by DNA sequence divergence due to random genetic drift. Furthermore, local adaptation to different ecological conditions may also result in genetic differences, due to natural selection (Powers et al., 1993; Burton, 1998).

In the case of the mummichog, *Fundulus heteroclitus*, a coastal killifish that lives along the east coast of north America, Powers and colleagues have shown that populations have diverged due to genetic drift, and also selectively adapted to their local natural environment (reviewed in Powers et al., 1993). Population genetic differences may therefore be used as genetic tags, which could potentially be extremely useful for ecological, management and conservation studies.

In this study, we have focused our analysis on the California killifish *Fundulus parvipinnis*. California killifish live in estuaries and sloughs, between Morro Bay, California, and Almejas Bay, Baja California, Mexico (Miller and Lea, 1972; Fig. 1). *Fundulus parvipinnis* spawns in the intertidal on spring high tides and produces relatively large larvae, presumably capable of maintaining their position within a bay or estuary (Watson, 1996). *Fundulus parvipinnis* is rarely found on the open coast as an adult, and has never been taken as larvae in over 40 years of open-water sampling off the coasts of California and Baja California (Watson, 1996). This suggests that migration between suitable habitats is likely to be limited. California killifish were originally separated into two subspecies based mainly on lateral line scale and vertebrae counts (Osburn and Nichols, 1916; Miller and Hubbs, 1954). *Fundulus parvipinnis parvipinnis* was designated a northern subspecies ranging from Central California to Ensenada, Mexico, and *F. parvipinnis brevis* a southern subspecies from Bahia Magdalena. However, Dumke (1976) argued that these two morphological counts varied clinally and were more likely to be the result of environmental factors rather than genetically based. Dumke therefore suggested that subspecific rankings should be discarded (Dumke, 1976).

In this study, we have selected six populations of *Fundulus parvipinnis* spanning its geographic range, and used the variable mitochondrial control region as a molecular marker. This region has proven useful in other fish populations as an effective molecular marker (Bernardi, 2000). Our goal was to determine the degree of genetic separation between individuals from different coastal wetland sites and estimate the level of dispersal between them.

2. Materials and methods

2.1. Collections, morphological study and DNA extraction

Ten individuals of the California killifish, *Fundulus parvipinnis*, were collected for each of the following six localities: Carpinteria Slough (CAS, Santa Barbara area),

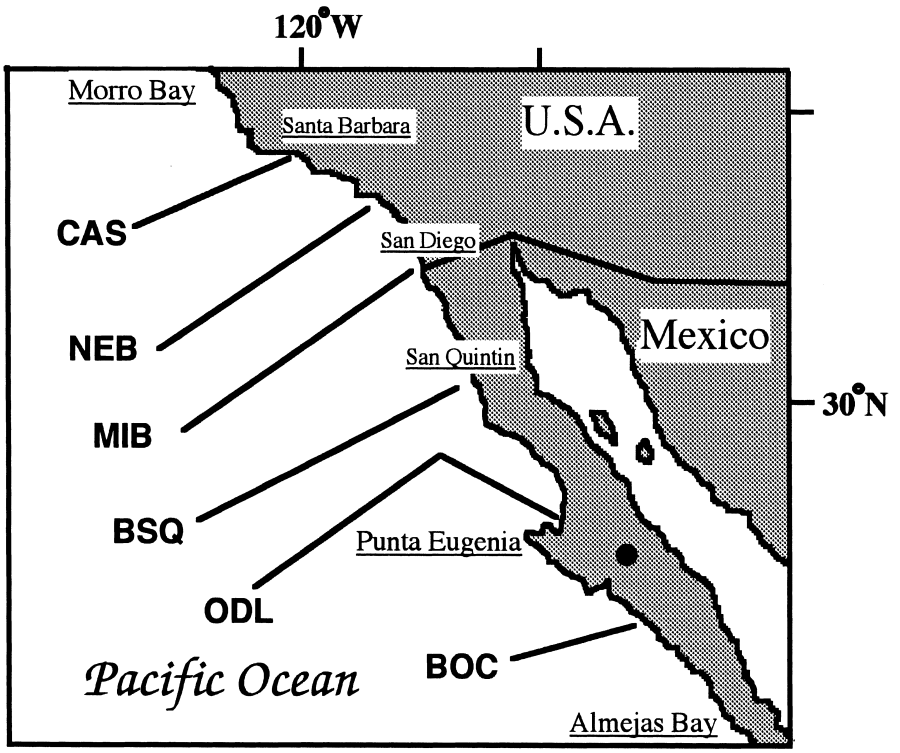

Fig. 1. *Fundulus parvipinnis* sampling locations. Sampling sites are shown on a map of the California, and Baja California Coast. Samples were collected at the following localities: Carpinteria Slough (CAS), Newport Bay (NEB), Mission Bay (MIS), in California, and Bahia San Quintin (BSQ), Ojo de Liebre lagoon (ODL), and La Bocana (BOC) along the Baja California coast. The sampling location of *Fundulus lima*, San Ignacio Oasis, is marked as a black solid circle.

Newport Bay (NEB, Los Angeles area), Mission Bay (MIB, San Diego area), in California, and San Quintin Bay (BSQ, northern Baja California area), Ojo de Liebre Lagoon (ODL, central Baja California area), and La Bocana (BOC, southern Baja California area) in Baja California, Mexico (Fig. 1). The closest relative to *Fundulus parvipinnis*, *F. lima*, was used as an outgroup (Bernardi and Powers, 1995; Bernardi, 1997). *Fundulus lima* is a freshwater species endemic to the oases of the Pacific drainage of Baja California Sur, Mexico, from San Ignacio to Arroyo La Pocitas (Ruiz-Campos, 2000). It is found in two localities, the Oasis of San Ignacio and possibly in the Oasis of San Luis Gonzaga. Two individuals from the Oasis of San Ignacio (type locality), were used in this study (Fig. 1).

Individuals were preserved whole at ambient temperature in ethanol until DNA extraction. Lateral line counts of all individuals were performed following Dumke's method (Dumke, 1976). Vertebral counts, after X-rays, were performed on four populations. 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).

2.2. Polymerase chain reaction (PCR) amplification

Amplification of the mitochondrial control region (D-loop) was accomplished using universal primers from the literature (Meyer, 1993). The amplifications (25 μl) contained 10 to 100 ng of DNA, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 units of Taq DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA), 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, USA).

2.3. 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 implemented by the Software package PAUP (version 4.0, Swofford, 1998). Maximum Parsimony using common algorithms could not be used due to prohibitive computer time. Thus, topological confidence was evaluated with 1000 bootstrap replicates (Felsenstein, 1985) for Neighbor-Joining and also with 1000 replicates using the Fast-Step method for Maximum Parsimony (only one tree kept at each replicate). In both Neighbor-Joining and Maximum Parsimony, bootstrapping analyses were performed with equal weighting of transitions and transversions, as no evidence of saturation effects was observed. Gene flow (F_{st}), and migration rate (Nm) were calculated using the software package DNAsp (Rozas and Rozas, 1997) following Hudson et al. (1992). An Analysis of Molecular Variance (AMOVA) was further performed using the Arlequin (ver. 2000) software package (Schneider et al., 2000).

3. Results

3.1. Morphology

Average lateral line scale counts and vertebral counts for all sampled individuals are shown in Table 1. As mentioned by Dumke (1976), lateral line counts varied more (33–39) than vertebral counts (33–37). Lateral line and vertebral counts within a population were homogeneous, with standard deviations less than 1 in all cases but for CAS vertebral counts (1.13). Lateral line count distributions showed two clear breaks. One between the Newport Bay and Mission Bay populations, and one between populations north and south of Punta Eugenia. Individuals collected south of Punta Eugenia (La Bocana) were on average lower (by 3.9 counts) and did not overlap with counts from individuals collected north of Punta Eugenia (all the remaining populations).

Table 1
Number of lateral line scales and vertebrae in *Fundulus parvipinnis*. Standard deviations are given between parentheses

	Counts							Average (±S.D.)
	33	34	35	36	37	38	39	
Lateral line								
Carpinteria Slough				2	8			38.8 (±0.42)
Newport Bay							10	39.0 (±0.00)
Mission Bay				2	8			36.8 (±0.42)
San Quintin					1	9		37.9 (±0.31)
Ojo De Liebre				1	8	1		37.0 (±0.47)
La Bocana	1	8	1					34.0 (±0.47)
Vertebral								
Carpinteria Slough	1	1	4	3	1			35.2 (±1.13)
Newport Bay		8	2					34.2 (±0.42)
San Quintin		4	6					34.6 (±0.52)
La Bocana	8	1	1					33.3 (±0.67)

In contrast to lateral line counts, vertebral counts of all populations did overlap. However, individuals collected south of Punta Eugenia also exhibited, on average, lower counts than individuals from north of Punta Eugenia (average difference 1.36). Differences in lateral line and vertebral counts between regions south and north of Punta Eugenia were statistically significant (*t*-test, $P < 0.01$). Our data were very similar to the original counts used by Miller and Hubbs (1954) to distinguish the two subspecies of *Fundulus parvipinnis*. Indeed, Miller and Hubbs (1954) found that both counts were lower for individuals collected south of Punta Eugenia than for individuals collected north of Punta Eugenia, and that the average difference in lateral line and vertebral counts was 2.4 and 2, respectively.

3.2. Phylogenetic analysis

A portion of the mitochondrial control region was sequenced for all *Fundulus parvipinnis* individuals sampled and for two individuals from the outgroup *Fundulus lima* (GenBank Accession numbers AF297395–AF297456). Out of the 396 aligned base pairs, 46 were variable, and 36 were phylogenetically informative. A one nucleotide indel at position 131 was observed in the La Bocana population. No other insertions or deletions were found. The Neighbor-Joining reconstruction obtained with our samples is shown in Fig. 2. Several weighting schemes (transitions vs. tranversions) were also used to evaluate alternative topological hypotheses. The topology of the tree shown in Fig. 2 remained unchanged when using any of the weighting schemes (5:1 to 1:5), thus saturation effects were assumed not to be playing a major role in shaping the topology of the phylogenetic tree presented here. Results from 1000 bootstrap replicates using both Neighbor-Joining and Maximum Parsimony methods are also shown in Fig. 2.

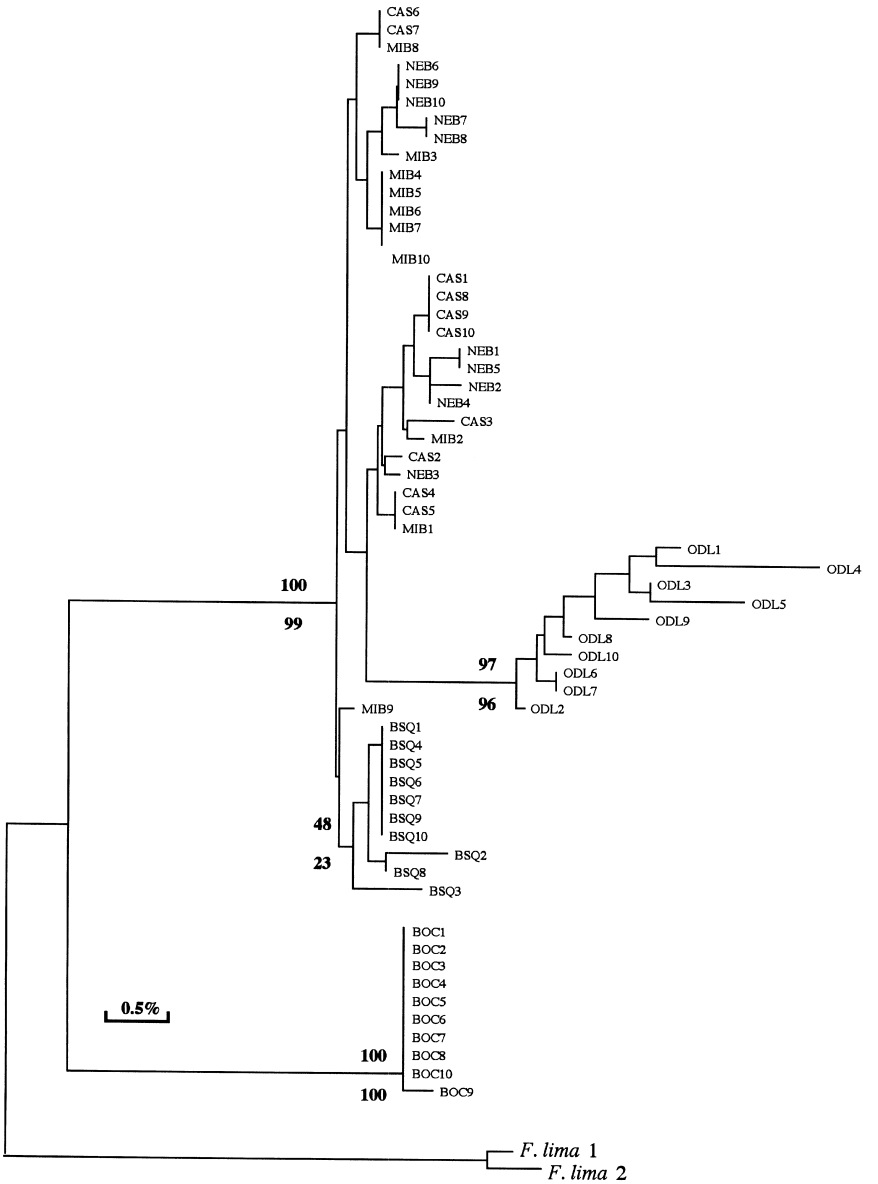

Fig. 2. Phylogenetic relationships between California killifish. The phylogenetic tree, based on mitochondrial control region (D-loop) sequences, was obtained using the Neighbor-Joining method implemented by the software package PAUP (version 4.0). Labels correspond to sampling localities as follows (from north to south): Carpinteria Slough (CAS), Newport Bay (NEB), Mission Bay (MIS), Bahia San Quintin (BSQ), Ojo de Liebre lagoon (ODL), and La Bocana (BOC). See Fig. 1 for geographic locations. The length of each branch is proportional to the number of nucleotide substitutions. Scale bar represents 0.50% sequence divergence. The tree is rooted using *Fundulus lima* as an outgroup. Bootstrap support higher than 70% is shown above the nodes (for the Neighbor-Joining method) and below the nodes (for the Maximum Parsimony method).

3.3. Phylogenetic relationships

Out of the six sampled populations, the three southern ones (La Bocana, Ojo De Liebre, Bahia San Quintin) formed distinct clades (Fig. 2). Clades of La Bocana (BOC) and Ojo de Liebre (ODL) were well supported (99 to 96% of bootstrap replicates) while individuals from Bahia San Quintin formed a monophyletic assemblage in only 23 to 48% of the bootstrap replicates. In contrast, northern populations from Carpinteria Slough, Newport Beach and Mission Bay did not partition into distinct clades.

At a higher level, samples partitioned in two major clades. One clade comprised all the samples collected at La Bocana (BOC, south of Punta Eugenia), the other clade included all the individuals collected north of Punta Eugenia. These two clades were very robust (99 to 100% of the bootstrap replicates) and were genetically distant (average sequence divergence 5.8%).

3.4. Population structure, gene flow, and genetic divergence

As expected from the phylogenetic relationships described above, gene flow between sampling localities was found to be very reduced (average $F_{st} = 0.70$, $Nm = 0.30$). While the Mission Bay (MIB), Newport Bay (NEB), and Carpinteria Slough (CAS) populations did not partition in distinct clades, gene flow between these regions was still very low as shown in Table 2 (average $F_{st} = 0.38$, $Nm = 0.86$). Furthermore, gene flow levels between adjacent populations showed a geographic trend, with high gene flow levels in the north, and very low levels in the south, as shown in Fig. 3. The lowest gene flow levels were obtained across the Punta Eugenia region (average $F_{st} = 0.95$, $Nm = 0.03$). This result was further confirmed by an analysis of molecular variance (AMOVA), where 74.8% of the total genetic variance in the samples could be attributed to the divergence between north of Punta Eugenia and south of Punta Eugenia populations.

Samples from Ojo de Liebre and Bahia San Quintin, which represent monophyletic assemblages, also showed low levels of gene flow with other samples collected north of Punta Eugenia ($F_{st} = 0.70$, $Nm = 0.21$ for ODL; $F_{st} = 0.64$, $Nm = 0.29$ for BSQ).

Average sequence divergence within each population also showed interesting patterns. Average sequence divergence within clades was 0.51% with the highest divergence found in the ODL population (1.21%) and the lowest divergence observed in the BOC

Table 2
Gene flow among *Fundulus parvipinnis* populations. Pairwise comparisons of geographic regions are represented by values of F_{st} (below diagonal) and *Nm* (above diagonal). Region labels are described in Fig. 1. Values were calculated using DNAsp (Rozas and Rozas, 1997)

	CAS	NEB	MIB	BSQ	ODL	BOC
CAS		0.72	1.19	0.22	0.22	0.02
NEB	0.41		0.68	0.29	0.23	0.03
MIB	0.30	0.42		0.46	0.20	0.03
BSQ	0.69	0.63	0.52		0.20	0.02
ODL	0.70	0.68	0.71	0.71		0.05
BOC	0.96	0.95	0.95	0.96	0.92	

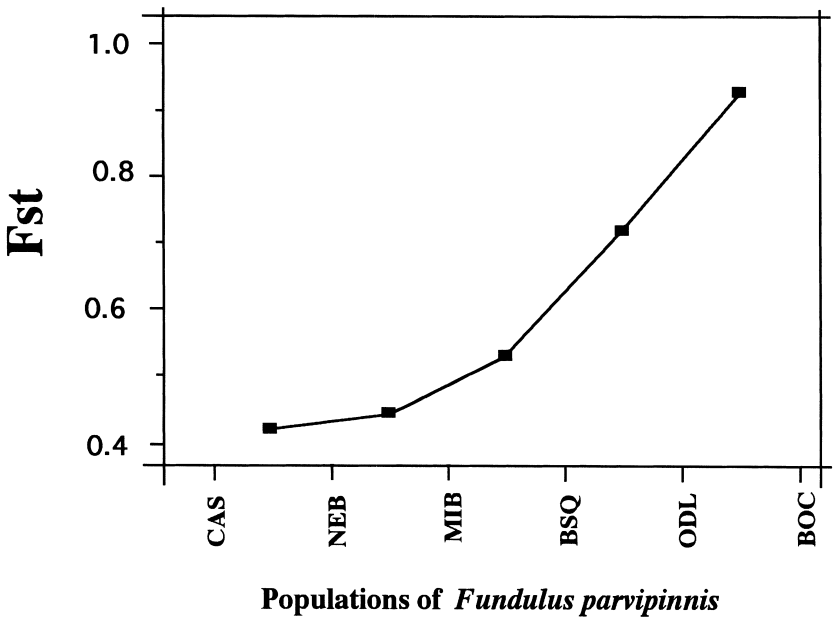

Fig. 3. Gene flow levels between neighboring populations. F_{st} values are from Table 2. Left to right population positions on the horizontal axis follow a north to south geographic location (see Fig. 1 for locality abbreviations).

population (0.05%). No obvious geographical trend was found, indicating that these values are more likely to be the result of population sizes and population size fluctuations, such as bottleneck events, than due to recent migration events.

4. Discussion

4.1. Habitat connectivity and gene flow

The estuarine habitat is, and has historically been, more isolated in the Baja California region than along the California coast. Small estuaries and wetlands suitable for *Fundulus* are far more closely spaced in southern California than are similar estuaries in Baja California. Thus there is likely to be much more connectivity across the northern region than the southern one. This important structural difference in habitats along the California and Baja California coastlines is likely to be the primary cause for the gene flow gradient that we observed (Fig. 3). While overall gene flow levels are low, higher habitat connectivity in the north is likely to result in higher levels of gene flow than in the south. The large distance between isolated habitats in the south is probably sufficient to prevent significant levels of gene flow in *Fundulus parvipinnis*.

4.2. Phylogeography of Fundulus parvipinnis

Biogeographic breaks (species level) have been shown to coincide in some cases with phylogeographic breaks (population level). Such is the case in southern Florida (Avise, 1992). This observation, however, has been shown not to be a general rule. For example, the major biogeographic break on the west coast of north America, Point Conception, has been shown not to be an effective barrier to gene flow in several marine organisms (Burton, 1998). Our data suggest that Punta Eugenia may be an important barrier to dispersal for *Fundulus parvipinnis*. Punta Eugenia has traditionally been considered a biogeographic boundary (Briggs, 1974) but little work has been done there. Our previous work on three other fish species, *Girella nigricans* (Terry et al., 2000), *Embiotoca jacksoni* (Bernardi, 2000), and *Gillichthys mirabilis* (Huang and Bernardi, 2000), evidenced that gene flow across Punta Eugenia was reduced. It is likely that local current patterns and physical forcing combined with the short larval stage of *Fundulus parvipinnis* are responsible for the limited dispersal observed across Punta Eugenia. Punta Eugenia may be an important species and population boundary, similar to the major biogeographic and phylogeographic break in Florida. Further sampling will be necessary to determine precisely the area where the phylogeographic break occurs.

4.3. Populations, subspecies and species

Fundulus parvipinnis populations were found to be extremely divergent. Specifically, individuals from La Bocana showed a sequence divergence of over 5% from the remaining populations. This is among the highest intra-specific control region sequence divergences observed within a fish species (Table 3). Previous studies have proposed that *Fundulus parvipinnis* should be split into two separate subspecies, or may be two distinct species, *F. parvipinnis parvipinnis* in the north, and *F. parvipinnis brevis* in the south. This taxonomic split was a reflection of morphological differences, lateral line scales and vertebral counts, for populations sampled across Punta Eugenia. These

Table 3
Average sequence divergence between populations and between species of representative fish species. Data for *Fundulus parvipinnis* populations north and south of Punta Eugenia are compared with values found in the literature for the same mitochondrial region (D-loop)

Taxa	Sequence divergence range (%)		Ref.
	Intraspecific	Interspecific	
Fundulus parvipinnis	5.8	9.7	This study
Girella nigricans	4.9	17.9	Terry et al., 2000
Xiphias gladius	3.4	6.9	Alvarado Bremer et al., 1995
Acipenser spp.	2.3	0–40	Brown et al., 1993, 1996
Rhinogobius spp.	1.8	11.3–11.7	Chen et al., 1998
Embiotoca jacksoni	1.5	13.9	Bernardi, 2000
Melanochromis sp.	1.1	1.1–4.9	Bowers et al., 1994
Anguilla spp.	1.1	21.8	Sang et al., 1994
Merluccius spp.	0.3–1.3	2–20	Quinteiro et al., 2000

differences were also observed in our own samples (Table 1). Thus a potential relationship between genetic and morphological differences was found. However, Dumke (1976) has argued that morphological differences should not be equated to subspecific rankings, but rather to environmental factors (mainly temperature). The argument in favor of the temperature hypothesis is that morphological differences were found to vary clinally, with anomalies in locations where the north–south temperature gradient was disrupted, such as the cold upwelling sites at Punta Banda, which occur within warmer surroundings. At Punta Banda, morphological features matched water temperature and not geographic location. Unfortunately, Dumke did not study the genetic background of his study individuals and a clear interpretation of his results is difficult.

The genetic and morphology-based divergence found across Punta Eugenia may either be the result of a combination of restricted gene flow with morphological differences due to local adaptations, or to historical separation of two taxa that are currently undergoing secondary contact. Able and Felley (1986) showed that northern and southern populations of the east coast *Fundulus heteroclitus* had matching morphological and genetic differences. This implies that secondary contact of *F. heteroclitus* populations occurred along the east coast. Lateral line scale counts were on average 1.85 counts lower in their southernmost population (Florida) than in their northernmost population (Newfoundland), as opposed to 2.4 (to 3.9) for the range of *F. parvipinnis* populations. Furthermore, northern and southern *F. heteroclitus* populations showed a 1.5% cytochrome *b* sequence divergence (Bernardi et al., 1993). Considering that cytochrome *b* varies about four times slower than the control region (Bowen and Grant, 1997), this figure is comparable to the 5.8% sequence divergence that was obtained between northern and southern populations of *F. parvipinnis*. Therefore, the similarity in both morphological and genetic variations between northern and southern populations of *F. heteroclitus* on the east coast, and *F. parvipinnis* on the west coast suggest that similar evolutionary mechanisms could be responsible for the observed results. The subspecific separation of *F. heteroclitus* in the northern *F. heteroclitus macrolepidotus* and the southern *F. heteroclitus heteroclitus* may mirror the separation of *Fundulus parvipinnis* in the northern *F. parvipinnis parvipinnis* and the southern *F. parvipinnis brevis*. Tests for concordance between several characters including morphological and genetic markers (Able and Felley, 1986) will be necessary to determine the taxonomic status of *F. parvipinnis*.

4.4. Isolation and conservation

Although coastal wetlands are recognized as primary ecotopes to a large number of species, they are among the most threatened habitats on earth. It is essential to fully understand the biological webs and linkages between geographically separated wetlands in order to effectively protect these areas. This is particularly urgent in California, where 90% of the historic wetland habitat has been lost due to anthropogenic activity (Schoenherr, 1992), driving many species of plants and animals to the brink of extinction. Habitat fragmentation associated with this loss of wetlands could potentially disrupt natural exchange between populations, reducing within-population diversity for

small isolates. Alternatively, since California and Baja California have historically had relatively small, isolated wetlands (Zedler, 1996), high F_{st} values across populations may signify local adaptation (e.g., see Williams and Davis, 1996). Additional sampling at a variety of spatial scales is necessary to tease apart the mechanisms underlying the genetic structure of *F. parvipinnis* populations.

No matter the mechanisms, however, our data show that exchanges between sampled populations of *Fundulus parvipinnis* are very limited. Therefore, the conservation of California and Baja California coastal wetlands is critically important in order to maintain the genetic diversity of this species. Given its limited dispersal potential, population genetics studies of *F. parvipinnis* may serve as an indicator, providing conservation scientists with an early warning of negative effects of habitat fragmentation.

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