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RESEARCH ARTICLE

Rangewide microsatellite phylogeography of the endangered tidewater goby, *Eucyclogobius newberryi* (Teleostei: Gobiidae), a genetically subdivided coastal fish with limited marine dispersal

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Abstract The federally endangered tidewater goby, *Eucyclogobius newberryi*, is the most locally differentiated vertebrate with marine dispersal on the California Coast. It inhabits seasonally closed estuaries along the California coast; a habitat heavily impacted by anthropogenic filling and artificial opening, and exhibits varied metapopulation behavior as a consequence of hydrologic variation and anthropogenic impact. We describe 19 taxon-specific microsatellite loci, and assess genetic variation across the taxon range relative to genetic subdivision. A highly divergent southern clade, with reduced genetic variation, now confined to Northern San Diego County, appears to merit status as a separate species. The mid-coast is subdivided into regional groups with overall similarity to, and minor differences from previous mitochondrial sequence based clades. The northernmost region, although locally differentiated, forms a star phylogeny with limited geographic structure which we attribute to dispersal during Pleistocene/Holocene sea-level rise followed by increasing isolation during the Holocene. Bottleneck/founder events are evident in some habitats thought to have experienced (anthropogenic) extirpation. Further work with more, and larger, samples will be required to assess local and regional differences. Analytical methods employed include Analysis of Molecular Variance (AMOVA), Neighbor-Joining, Bayesian/STRUCTURE analysis and Principle Components Analysis (PCA).

Keywords Endangered · Estuary · Metapopulation · Phylogeography · Tidewater goby

Kristina D. Louie—deceased.

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Introduction

The tidewater goby is intriguing due to its high degree of mitochondrial subdivision (Dawson et al. 2001) despite evidence for marine dispersal (Lafferty et al. 1999a, b). It occupies seasonally closing lagoons, a highly impacted, poorly understood, and potentially critically important coastal habitat. Flood-flow and desiccation extirpate tidewater goby habitats imparting an extirpation/recolonization metapopulation dynamic (Lafferty et al. 1999a, b), and extirpation appears to be accentuated by anthropogenic habitat modification and introduction of exotics (Lafferty and Page 1997; Swift et al. 1989; Rathbun 1991; Jacobs et al. 2005). Deep mitochondrial subdivision (Dawson et al. 2001) and morphologic differences (Ahnelt et al. 2004) raise the possibility of two, currently unrecognized, species rank taxa within this federally endangered fish. Thus study of the tidewater goby, addresses questions regarding the limits of genetic isolation and speciation in coastal estuarine taxa and the role of habitat specialization in limiting dispersal. The tidewater goby also has the potential to serve as a model for the genetic study of metapopulation process in evolution and conservation.

The tidewater goby lives exclusively along the coast of California, primarily in high intertidal habitat seasonally

isolated from the full effect of the tide; most but not all of these habitats are stream mouth estuaries and lagoons that close off from the sea during the low stream-flow "summer" months (Swift et al. 1989, 1993; USFWS 2005). Work to date shows phylogeographic differentiation on a smaller spatial scale in the tidewater goby than in other marine and estuarine vertebrates on the California coast (Dawson et al. 2001, 2002; Barlow 2002; Jacobs et al. 2004). Among invertebrates only the intertidal copepod *Tigriopus californicus* is more locally differentiated (Burton 1998; Edmands 2001; Jacobs et al. 2004). Tidewater goby life history, combined with the seasonal dynamics of its habitat, contribute to this differentiation. Separation of estuaries from the sea during the summer reproductive period appears to limit marine dispersal of larvae (Lafferty et al. 1999a, b; Dawson et al. 2001; Barlow 2002; USFWS 2005; Jacobs et al. 2005). Estuaries frequently connect to the sea in the winter (following rain events that wash out beach berms at estuary mouths) when reproduction is minimal and larvae are few. These high stream flow events have been associated with adult dispersal (Lafferty et al. 1999b). Headlands, hard-rock substrate, as well as stretches of coast lacking estuarine habitat form local and regional phylogeographic breaks (Dawson et al. 2001; Barlow 2002), consistent with nearshore dispersal of weak-swimming (Swenson 1999) benthic adults over soft substrate, rather than of larvae in the water column.

Tidewater goby populations experience extirpation, and recolonize over short distances (usually less than 15 km). Desiccation and flood scouring of stream-mouth lagoonal habitat extirpate sites, and extirpation appears to be an inverse function of habitat size (Lafferty et al. 1999a, b). Proximity of adjacent populations, and intervening substrate, appear to influence recolonization. Thus the available information suggests that, tidewater gobies form a suite of isolated regional metapopulations with local properties that depend on local coastal morphology and process. In addition, it is thought that human activities influences local metapopulation dynamics: by increasing the likelihood of extirpation through, filling, channelization, and jettying open of stream mouths (USFWS 2005), and by introduction of exotic predators, such as Xenopus (Lafferty and Page 1997) and eastern North American centrarchid fishes such as the green sunfish, *Lepomis cyanellus* (Swift et al. 1989; Rathbun 1991). Further, the ability to recolonize appears to decrease through construction of intervening hard substrate such as jetties and marinas. The recovery plan (USFWS 2005) recognizes the importance of augmenting natural metapopulation dynamics to effect recovery, but these plans have yet to be implemented.

Dawson et al. (2001) described mitochondrial sequence-based tidewater goby phylogeography and defined 6 regional clades. These include a larger than 4% sequence divergent San Diego County clade with reduced sequence divergence within it. These six clades then defined the six recovery units (USFWS 2005) described in more detail below. Barlow (2002) documented a close relationship between Ventura County samples and Malibu and Topanga creek in Los Angeles County as would be expected given the artificial reintroduction of the Malibu lagoon from the Ventura River in 1991 coupled with subsequent natural dispersal and colonization from Malibu southward to Topanga in about 1998 (Lafferty et al. 1999a, b; Barlow 2002; USFWS 2005). Barlow's (2002) analysis of more northerly samples also documented dramatic local differentiation between sites separated by rocky shores confirming arguments that adult dispersal over sandy substrate was the primary mode of dispersal and that rocky substrate acted as a barrier as discussed below (see supporting documentation).

Based upon regional clades (Dawson et al. 2001) and geomorphic constraints on dispersal the U.S. Fish and Wildlife Service (USFWS 2005) designated six tidewater goby recovery units: (1) North Coast Unit (NCU)—from Smith River near the Oregon Border to Brush Creek near Point Arena; (2) Greater Bay Area Unit (GBAU)—from Salmon creek north of Bodega Head to the Salinas River; (3) Central Coast Unit (CCU)—from Arroyo de La Cruz south of the Big Sur Coast to Los Osos Creek and Morro Bay; (4) Conception Unit (CU)—from San Luis Obispo Creek to Rincon Creek; (5) the Los Angeles/Ventura Unit (LAVU)—from the Ventura River to Ballona Creek in Los Angeles; and (6) the South Coast Unit (SCU)—from San Pedro to Los Peñasquitos Lagoon near La Jolla. The recovery plan further subdivides five of these managements units into subunits numbering from two (SCU) to as many as 11 (GBAU).

With the exception of limited nuclear-intron sequencing (Dawson et al. 2001), description of four microsatellites (Mendonca et al. 2001) and a modest regional microsatellite-based study of the Santa Barbara area (Jacobs et al. 2005) the primary molecular data used to assess the genetic structure of the tidewater goby has been mtDNA sequence. Here our analyses utilize a panel of novel microsatellite loci assessed in a first order survey across the range of *E. newberryi*. The survey determines: (1) if nuclear markers are consistent with previous mitochondrial results documenting deep divergence of the SCU; (2) how a nuclear based range-wide phylogeny compares with previous mitochondrial phylogeography; (3) whether nuclear assessment validates previously established management units; and (4) the utility of these markers for future work on the unusual and variable regional metapopulation processes evident in tidewater gobies (Lafferty et al. 1999a; Dawson et al. 2001; Barlow 2002; Jacobs et al. 2005).

We report microsatellite markers and assess them over the entire range of the tidewater goby with modest sampling. As such the questions addressed here are of larger scale, and include the degree of subdivision and the

validity of species level units. This work is intended to set the table for, but cannot address directly, questions on a fine geographic scale. Regional studies in progress in multiple labs will address, metapopulation processes and recovery status of individual clades and management units.

Materials and methods

Microsatellite discovery and screening

Genetic Identification Services (Chatsworth, CA) constructed genomic libraries enriched for the motifs $(CA)_n$, $(ATG)_n$, $(CATG)_n$ or $(TAGA)_n$ as in Jones et al. (2002). Plasmid DNA prepared from clonal isolates was restricted with Hind III and size fractionated on a 2% agarose/TBE gel. Genomic inserts excised from the gels were purified (UltraClean kit-MoBio, Carlsbad, CA) and sequenced using ABI PRISM BigDye Ready mix (Applied BioSystems, Foster City, CA), an M13 (-20) forward primer (23 bp) and an ABI Prism 377 automated DNA Sequencer.

PCR primers to the flanking sequences of repeats were designed, and loci were screened for polymorphism and heterozygosity with a panel of 55 individuals from 28 locations across the entire range of the species. PCR conditions were optimized for each primer set (Table 1; and supporting documentation).

Sample collection, genotyping

Seining was employed to collect samples, and sampling following listing was conducted under permits from California Department of Fish and Game, No. 002679 (Swift), and from the U. S. Fish and Wildlife Service, TE-793644-5, 6 (Swift). Fish were frozen on dry ice in the field and maintained at −80°C in the Jacobs lab. In an analysis separate from the panel used to assess initial variation of microsatellites described above, a panel of 95 *E. newberryi* DNAs including individuals from 16 sites, encompassing each of the six management units was established. These samples were extracted (as described above) from material collected between 1990 and 2001, flash frozen in the field and stored at −80°C. These 95 individuals were genotyped for 18 microsatellite markers (*ENE2* through *ENE19*). We used *ENE2*, *ENE3* and *ENE4* from Mendonca et al. (2001) and omitted *ENE1* as Mendonca reported this marker to be fixed in a panel of Bay Area populations. Genotypes were established using QIAGEN Multiplex PCR kits with a fluorescent dye-labeled M13F (-20) primer (Boutin-Ganache et al. 2001). Forward primers included M13 "tails" for labeling and reverse primers were unlabeled. Reactions were performed in 10 µl volumes containing 1.5 µl DNA, 2.1 µl molecular grade water, 0.4 µg/µl BSA, 5.0 µl Qiagen multiplex master mix, and 1.0 µl primer mix prepared according to the manufacturer's guidelines. PCR was performed on an MJ-Research PTC-200 programmable thermal controller using the following profile: 95°C for 15 min; 25 cycles of 94°C for 30 s, 55°C for 90 s, 72° for 60 s; then 21 cycles of 94°C for 30 s, 50°C for 90 s, 72°C for 60 s with a final extension at 60°C for 30 min. PCR products were analyzed on an ABI 3730KL capillary sequencer and alleles were analyzed using ABI GENEMAPPER version 3.0 (Applied Biosystems).

Population genetic analyses

Observed and expected heterozygosity were calculated with GENEPOP (Raymond and Rousset 1995) and ARLEQUIN version 3.11 (Excoffier et al. 2005). SCU populations exhibited nonspecific amplification for marker *ENE2*, suggesting a shared mutation; *ENE2* was excluded from further analyses specific to populations in the SCU, and SCU populations were dropped from subsequent *ENE2* locus-specific statistics. The data set contained no other missing genotypes.

Phylogeny was reconstructed using POPULATIONS 1.2.30 (Langella 1999) with the Neighbor-Joining method and Nei's D_A distance (Nei et al. 2003). Bootstrapping was performed on the loci using the grouped populations option and 10,000 replicates.

For principal components analysis (PCA) on microsatellite data we converted the matrix of individuals' genotypes into an allele presence/absence matrix and then used MATLAB (MathWorks 2008) to perform PCA on the new matrix to visualize the results.

ARLEQUIN version 3.11 was used to test Hardy–Weinberg equilibrium, linkage-disequilibrium, analysis of molecular variance (AMOVA) and pairwise F_{ST}. Hardy–Weinberg equilibrium was assessed using 100,000 Markov chain and 1,000 dememorization steps. Locus *ENE2* was excluded from calculations of linkage-disequilibrium due to missing data. Linkage-disequilibrium was calculated using 10,000 permutations within populations. AMOVA testing used 10,100 permutations and employed the "several groups of populations, within-individual level" option using two different groupings of the data set. The first grouping used a single division that separated the SCU from the rest of the data. The second grouping used the following four partitions: the NCU + the GBAU; the CCU; the CU + LAVU; and the SCU. Pairwise F_{ST} calculations excluded locus *ENE2* due to missing data and were performed using the distances computed from the number of alleles and 10,000 permutations.

Pairwise isolation by distance was assessed via Mantel test between the log of the genetic distance (both Nei's

Table 1 Microsatellite loci for *Eucyclogobius newberryi,* including primer sequence (forward primer listed above reverse), length, cloned sequence repeat motif, annealing temperature (Ta) of the pair °C, size range of alleles (base pairs), number of alleles (*k*) observed

Locus	Primer sequence (5′–3′)	Length	Motif	Ta (°C)	Fragment size (bp)	*k*
ENE5	GCTTGTGCAGTATGGGATCTC[a]	21	$(GT)_4(AT)(GT)_9$	61	306–326	7
	CTCGGAGCGTTCATTTATCTC	21				
ENE6	TCAGGTTTGTGCTAAAATGATG[a]	22	$(CA)_{11}(CATACA)_5(CA)_4$	60	241–257	8
	TCCGATGACCACTTGTCC	18				
ENE7	TCACATGAATCGGAGACAGT[a]	20	$(CAT)_7(CAC)(CAT)$	60	135–159	7
	CAGAGAGGGCACTTTTTCAG	20				
ENE8	GAGGAAGGCGAGCTGATTA[a]	19	$(ACCATCATC)_4(ACC)(ATC)_8$	62	101–204	19
	CGGAGAGAAGGTGTTGAGAG	20				
ENE9	CCTTCATTTTTCCATCAGAAGCG[a]	23	$(ATG)_{28}$	59	131–209	24
	CCTTATTTACATCTTCCCTCCA	22				
ENE10	AAACAAGGGGAAAGGAAAAAGCC[a]	23	$(CCT)(CTT)_3(CCT)(CAT)_5$…	60	168, 171	2
	GAAACAACTGCTGGAGGACT	20	…$(CAC)_2(CCC)(TCA)(TCC)_4$			
ENE11	CAGGGCATCTGAGTGAAATA[a]	20	$(CAT)(CTT)(CAT)_4(CTT)(CAT)_3$	57	180–219	4
	GATTAAAACAGTCCCAAAAACAC	23				
ENE12	CTGGGATTGTCTTGGAACAG[a]	20	$(GAT)_9$	65	183–240	15
	GGGTGTGTGTGAGAGAGTGG	20				
ENE13	TGAAGCATCTTTGGGTGTC[a]	19	$(GAT)_3(GAC)(GAT)_{13}$	59	266–278	3
	GTTTGAAATGGTCACTGTGTG	21				
ENE14	TCTGGCAGCTCTAGTGAATCAC[a]	22	$(CTG)_{11}$	59	245–275	6
	CCGAAAGTGAATTGTAATGTGG	22				
ENE15	CCCGGAGGAGTTAGAGGAA[a]	19	$(TGGA)_7$	62	281–293	4
	GAGCCTGTGGTTTGTCGAG	19				
ENE16	GTCGCCTTGATTTTATTGTGA[a]	21	$(TGGA)_6$	56	138–216	10
	CTCAGCGTGGTTTCATTAT	19				
ENE17	CAGAGGTAGATCAGAAGAAC[a]	20	$(ATCC)_6$	56	165–173	3
	CCGGATAAAGTGCAGAAAAT	20				
ENE18	GGAGAACGAGAGAGAAAGA[a]	19	$(GA)_4(AC)(GA)_{25}$	58	132–152	10
	GGCTGGTGTTTGATACATC	19				
ENE19	CGCGTCAGTTTTCACCTTTA[a]	20	$(TCTA)_{11}$	58	110–134	7
	GAGAATGCCCAAAATCACC	19				
ENE20[b]	GAAAAAGTTCCTACAAGTCCAAA[a]	20	$(CA)_8(TA)(CA)_5(GA)(CA)_5$	63	116–128	2
	GACACTTCTTCCGTCTCC	18				
ENE21[b]	TGCAGAGAAAGAGACAGGTATT[a]	22	$(CATCAA)_2(CAT)_7(CTG)_2(CAT)_3$	53	154–168	4
	ATTGAGGTGCTGACACTGAG	20				
ENE22[b]	TGTTGGTGTTGTTGATGTTAGG[a]	22	$(TCCA)_6$	60	121–125	2
	GAGATAGGCTCCAGTCAC	18				
ENE23[b]	AAGAAGGAAAAGAACAAGCAAAGAG[a]	25	$(GTGA)_5$	57	105–111	3
	CTTCCCTCCACTACTCCT	18				

[a] 23 nucleotide M13 sequence (5′-AGGGTTTTCCCAGTCACGACGTT-3′) added to the 5′ end of the forward primer to allow annealing of the dye-labeled M13 primer to the PCR product

[b] Locus was discovered and assessed in the initial survey panel. But not further examined

distance (D_A) of microsatellite genotypes and F_{ST} were assessed) and the log of the geographic distance along the coast in the program IBDWS 3.15 (Jensen et al. 2005) using 30,000 randomizations.

STRUCTURE 2.2.3 (Pritchard et al. 2000) was used to infer population subdivision in: (1) the full data set, (2) the data excluding the SCU samples, and (3) the NCU + G-BAU samples only. We employed the admixture model with ten replicates for each number of partitions/clusters permitted in the analysis (K) between 2 and 24 using a burn-in of 50,000 iterations followed by 500,000 Markov-Chain Monte-Carlo (MCMC) steps. Each replicate

produces a "Q-matrix" of coefficients that relates probability of individual assignment to a particular cluster and estimates the log likelihood of the data, log Pr($X|K$). Due to the stochastic elements of MCMC, replicates can produce distinctly different clustering results for the same K.

We employed CLUMPP version 1.1.1 (Jakobsson and Rosenberg 2007) to amalgamate each K value's ten Q-matrices and to illuminate any "non-symmetric modes", or "multimodality", that lay in the STRUCTURE output (see supporting documentation). We then used the method of Evanno et al. (2005) and calculated ΔK, an ad hoc statistic that peaks where the estimated log likelihood begins to plateau, thought to be indicative of the appropriate number of genetic clusters, K.

Results

Microsatellite discovery

Nineteen polymorphic loci were discovered with a variable number of alleles per locus ($k = 2$–24, Table 1). These markers are named *ENE5* through *ENE23* following Mendonca et al.'s (2001) description of *ENE1* through *ENE4*. In the initial screen, observed heterozygosity of the loci varied ($H_o = 0.00$–0.66), 3 of the 19 discovered loci (*ENE20*, *ENE21* and *ENE22*) deviated from Hardy–Weinberg equilibrium ($P < 0.01$), and 4 of the 19 discovered loci (*ENE20* through *ENE23*) were excluded from the subsequent analyses due to limited allelic variation.

Descriptive statistics

The 95 individual multilocus range-wide survey data were largely complete (99.36%); missing data originated from nonspecific amplification of locus *ENE2* for SCU populations ($N = 11$). We determined the number of alleles (k), as well as observed and expected heterozygosity (H_o and H_e, respectively) for all loci. This was done for both management units and individual locality samples (Table 2 and Table S1, respectively). Loci were polymorphic across all six Management Units (mean $k = 7.95$), with relatively low levels of observed heterozygosities within Management Units ($H_o = 0.123$–0.333; mean $H_o = 0.239$, SD $= 0.181$; Table 2) and reduced levels of variation at the locality level ($H_o = 0.07$–0.37; mean $H_o = 0.240$; Table S1). Furthermore the distinction between expected and observed heterozygosity across the data set and within management units (Table 2) indicates substantial regional/phylogeographic and local genetic (Wahlund effect) subdivision.

Four markers (*ENE2* in 8-Baldwin, *ENE3* in 14-Ventura, *ENE16* in 9-Corral, and *ENE18* in both 4-Ten Mile and 9-Corral) were found to deviate significantly from Hardy–Weinberg equilibrium ($P \leq 0.05$) prior to Bonferroni correction for multiple comparison. After correcting for multiple comparisons, no markers were found to deviate significantly from Hardy–Weinberg equilibrium. Similarly, 26 pairs of loci in individual populations showed significant linkage ($P \leq 0.05$), however only one pair of loci appeared linked in two populations (*ENE7* and *ENE8* showed linkage in both 10-Villa and 14-Ventura). Thus, departure from Hardy-Weinburg and effects of linkage are presumed to be minimal.

In six loci all alleles in the SCU were private: *ENE2* consistently produced specific PCR products in other samples, but amplified non-specifically in the SCU samples; of the two alleles recovered for *ENE 10,* one (168 nt) was fixed in the SCU and the other (171 nt) was fixed everywhere else; all alleles recovered from four loci (*ENE8*, *ENE12, ENE13*, *ENE16*) were private in the SCU, and in three of these (*ENE8*, *ENE13*, *ENE16*) the range of allele sizes in the SCU did not overlap the allele size range recovered elsewhere. Thus, given current sampling and amplification protocols, six of the 18 loci were diagnostic for the SCU while other management units are not diagnosed by any single locus. In addition, the SCU consistently differed in allele size from the rest of the state (Fig. 1).

Pairwise fixation index

Our pairwise F_{ST} calculations exclude locus *ENE2* due to nonspecific amplification for SCU samples. F_{ST} values were calculated using the distances computed from the number of alleles and 10,000 permutations (Table S2). All pairwise F_{ST} values were significant before Bonferroni correction ($P < 0.05$), except for comparison between the two samples localities in the SCU (15-San Mateo and 16-Hidden; $P = 0.11$). After correcting for multiple comparisons, no results were significant. However, the Bonferroni correction is known to be conservative when considering large numbers of tests; in our case of 120 tests, the threshold for significance became $P < 0.0004$. Interpretation of these pairwise F_{ST} (Table S2) results is necessarily tentative and will require confirmation with larger samples. However, it is noteworthy that: the lowest pair-wise F_{ST} value was between 15-San Mateo and 16-Hidden ($F_{ST} = 0.093$) and conversely, the highest pair-wise F_{ST} values occur in comparisons of these SCU samples with samples from the rest of the state (mean $F_{ST} = 0.72$); relatively low levels of differentiation ($F_{ST} < 0.15$) are observed between some adjacent population pairs within management units; suggesting connectivity; high average of pair-wise F_{ST} of 3-Arcata (0.51) with the other NCU sites and of 7-Frijoles (0.452) with other GBAU sites may be due to reduced genetic variation (Table S1) resulting from bottlenecks of these populations, and higher values of pair-wise F_{ST} in the

Table 2 Observed number of alleles (k) for Management Units under study with observed and expected heterozygosities in parentheses (H_o; H_E)

Locus	k	N. Coast $N = 24$	k	G. Bay Area $N = 24$	k	C. Coast $N = 12$	k	Conception $N = 18$	k	Ventura/ LA $N = 6$	k	S. Coast $N = 11$	k	Total $N = 95$
ENE2	3	(0.208; 0.660)	3	(0.083; 0.260)	2	(0.250; 0.413)	5	(0.333; 0.566)	3	(0.500; 0.704)	NA[a]	NA[a]	7[a]	(0.226; 0.731)[a]
ENE3	1	(0; 0)	4	(0.292; 0.567)	2	(0.333; 0.444)	2	(0.056; 0.054)	2	(0; 0.408)	1	(0; 0)	5	(0.126; 0.382)
ENE4	1	(0; 0)	2	(0.167; 0.375)	2	(0.083; 0.080)	1	(0; 0)	1	(0; 0)	2	(0.091; 0.087)	2	(0.063; 0.440)
ENE5	5	(0.417; 0.517)	3	(0.125; 0.190)	3	(0.750; 0.635)	1	(0; 0)	1	(0; 0)	2	(0.364; 0.496)	7	(0.274; 0.695)
ENE6	5	(0.375; 0.722)	6	(0.333; 0.460)	5	(0.583; 0.517)	2	(0.167; 0.239)	1	(0; 0)	1	(0; 0)	8	(0.284; 0.767)
ENE7	2	(0.125; 0.117)	5	(0.333; 0.545)	4	(0.417; 0.406)	2	(0.444; 0.401)	3	(0.500; 0.500)	1	(0; 0)	7	(0.284; 0.622)
ENE8	4	(0.375; 0.605)	6	(0.250; 0.361)	7	(0.750; 0.750)	6	(0.722; 0.795)	4	(1.000; 0.663)	3	(0.091; 0.169)	19	(0.421; 0.842)
ENE9	11	(0.500; 0.856)	16	(0.750; 0.913)	8	(0.667; 0.836)	8	(0.722; 0.824)	5	(0.833; 0.725)	3	(0.636; 0.612)	24	(0.663; 0.935)
ENE10	1	(0; 0)	1	(0; 0)	1	(0; 0)	1	(0; 0)	1	(0; 0)	1	(0; 0)	2	(0; 0.205)
ENE11	1	(0; 0)	2	(0; 0.080)	1	(0; 0)	2	(0.111; 0.279)	2	(0.333; 0.337)	2	(0.091; 0.087)	4	(0.053; 0.111)
ENE12	7	(0.333; 0.771)	7	(0.500; 0.747)	7	(0.750; 0.802)	6	(0.389; 0.590)	4	(0.500; 0.459)	2	(0.091; 0.087)	15	(0.421; 0.859)
ENE13	1	(0; 0)	2	(0.208; 0.187)	1	(0; 0)	1	(0; 0)	1	(0; 0)	1	(0; 0)	3	(0.053; 0.250)
ENE14	3	(0.042; 0.119)	2	(0.083; 0.278)	1	(0; 0)	2	(0.056; 0.054)	3	(0.500; 0.439)	3	(0.273; 0.244)	6	(0.105; 0.186)
ENE15	1	(0; 0)	2	(0.042; 0.353)	3	(0.167; 0.226)	1	(0; 0.054)	1	(0; 0)	1	(0; 0)	4	(0.053; 0.333)
ENE16	5	(0.667; 0.683)	8	(0.542; 0.726)	3	(0.333; 0.573)	5	(0.500; 0.679)	3	(0.833; 0.622)	1	(0; 0)	10	(0.495; 0.833)
ENE17	2	(0.167; 0.219)	2	(0.250; 0.219)	2	(0.250; 0.413)	2	(0.222; 0.278)	1	(0; 0)	1	(0; 0)	3	(0.179; 0.235)
ENE18	2	(0; 0.219)	6	(0.333; 0.733)	4	(0.417; 0.715)	4	(0.611; 0.602)	1	(0; 0.133)	1	(0; 0)	10	(0.253; 0.791)
ENE19	4	(0.458; 0.522)	5	(0.208; 0.264)	3	(0.250; 0.226)	3	(0.444; 0.495)	2	(0.167; 0.337)	2	(0.455; 0.500)	7	(0.347; 0.600)
Ave.	3.28	0.204; 0.334	4.56	0.250; 0.403	3.28	0.333; 0.391	3	0.265; 0.328	2.17	0.287; 0.296	1.65	0.123; 0.134	7.95	0.239; 0.546

[a] All South Coast samples had non-specific amplification for locus ENE2

GBAU could reflect local isolation within this unit as noted in Barlow's (2002) mitochondrial data.

Isolation by distance was assessed using the logs of genetic and geographic distance, with both F_{ST} and Nei's D_A as metrics of genetic distance, and isolation appears significantly influenced by phylogenetic partition and isolation by distance is not further considered.

Phylogeography

In a phylogeny reconstructed with Nei's distance D_A and Neighbor-Joining (Fig. 2), 11 of the 16 locality samples in the analysis form "monophyletic" clusters of genotypes, and 2 sites have single individuals that fall outside of "their" cluster. Only 15-San Mateo and 16-Hidden in the SCU and 1-Smith in the NCU lack this local coherence. Rooting as in Dawson et al. (2001) yields basal branching in geographic order from south to north. The SCU, the longest and presumptive basal branch is supported in 100% of bootstrap (of loci) replicates, consistent with the fixed differences and dramatic divergence in allele size (Fig. 1) noted above. The single LAVU sampling site, (14-Ventura) falls within, rather than sister to, the CU. The CU + LAVU cluster has 92% support and separates into two well-supported clusters in the

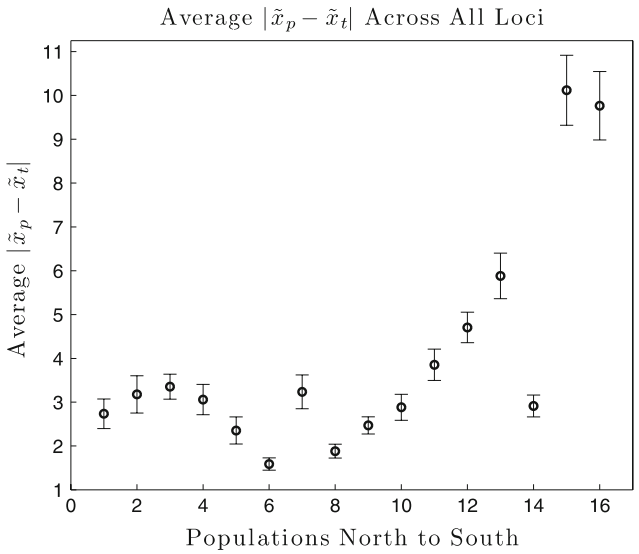

Fig. 1 Absolute difference between population median allele size ($\sim x_p$) and total median allele size ($\sim x_t$) averaged across all loci (except ENE2 which had nonspecific amplification for all South Coast Unit individuals). The *x*-axis is population order from north to south, with the same coding as in Fig. 2. Note that the southernmost SCU populations 15 and16 have distinctly different in allele sizes

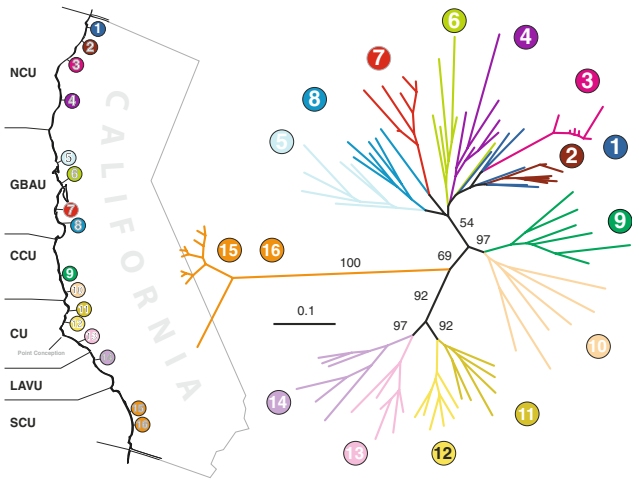

Fig. 2 Neighbor-Joining network of Nei et al.'s D_A distance reconstructed in POPULATIONS 1.2.30. Numbers next to branches represent locus bootstrapping values for 10,000 replicates. Each locality, with the exception of the South Coast Unit samples, has a unique color. Localities are coded from north to south: 1-Smith River, 2-Stone Lagoon, 3-Arcata Bay, 4-Ten Mile River, 5-Estero Americano, 6-Rodeo Lagoon, 7-Arroyo de los Frijoles, 8-Baldwin Creek, 9-Arroyo del Corral, 10-Villa Creek, 11-San Luis Obispo Creek, 12-Santa Ynez River Lagoon, 13-Arroyo Burro, 14-Ventura River Lagoon, 15-San Mateo Creek, and 16-Hidden Lagoon. They are referred to in the text without geographic descriptors (i.e. creek). Approximate boundaries between management units are shown as thin horizontal lines. Management units are labeled: North Coast Unit (NCU), Greater Bay Area Unit (GBAU), Central Coast Unit (CCU), Conception Unit (CU), Los Angeles & Ventura Unit (LAVU), and South Coast Unit (South Coast Unit)

general area of Point Conception. All samples from north of the CU form a modestly supported cluster (69%). Within this, the CCU forms a well-supported cluster (97%) and all GBAU + NCU samples form a minimally supported cluster (54%). This minimal internal support, with substantial within-sample grouping of genotypes suggests a "star phylogeny," although there is also some association between the 3 northernmost samples. All samples in the LAVU, CU and CCU are reconstructed as isolated clusters, and these multilocus microsatellite data provide local resolution comparable to or greater than that suggested by mitochondrial sequence data (Dawson et al. 2001; Jacobs et al. 2005).

Principle components analysis

Principle Components Analysis of all samples (Fig. 3) strongly separates the SCU genotypes from all other samples on Principle Component (PC) 1. PC2 ordinates the remaining recovery units from south to north separating out the CU + LAVU, while the CCU is further separated on PC3. The GBAU + NCU are on the negative end of all axes. This ordination replicates the order of branching of major groups observed in the neighbor-joining tree.

AMOVA

A single division separating the SCU from all other localities partitioned the variation 43.7% between the groups, 25.2% within the groups, 28.5% within individuals and 2.6% among individuals. Use of the four major clusters from the Neighbor-Joining tree (Fig. 2) as groups partitioned the variation 64.1% among groups, 17.7% within groups, 17.7% within individuals and 0.5% among individuals within samples.

Bayesian clustering analysis and K cluster estimation

We used the methodology of Evanno et al. (2005) to estimate the plateaus of log likelihood, suggestive of an appropriate *K* (number of partitions/groups to consider in the analysis) for STRUCTURE analysis (see ESM for additional discussion of multimodality). We found the maximum value of ΔK at $K = 4$ with a secondary peak at $K = 8$ (Fig. S1). In addition to the second peak at $K = 8$, a plot of the mean log likelihood against *K* shows a plateau at $K = 8$ (Fig. S2) and the variance of the log likelihood score also increases. Although solutions with smaller *K* are often preferred (Pritchard et al. 2000; Pritchard and Wen 2004), the large number of partitions evident in the Neighbor-Joining reconstruction (Fig. 2) supports consideration of both $K = 4$ and 8.

In plots of the arithmetic mean clustering configuration for $K = 4$ (Fig. 4), SCU and CU + LAVU genotypes form

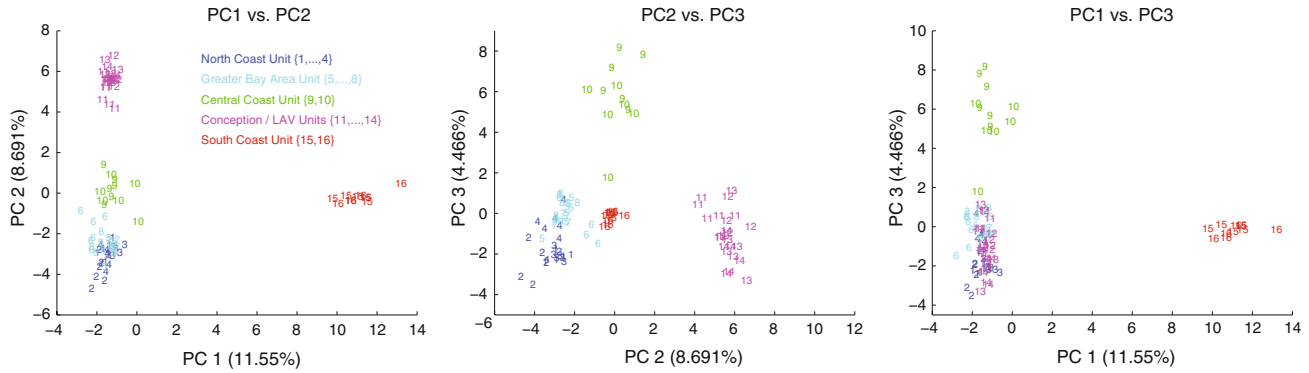

Fig. 3 Scatter plots of principal components analysis: Principal component 1, subsuming 11.55% of the data variation, separates the SCU from the rest of the data; Principal component 2, subsuming 8.69% of the variation, ordinates the remaining groups in geographic order with CU + LAVU well separated at the positive end of the axis; Principal component 3 subsuming 4.466% of the variation, serves to separate the CCU from the other samples

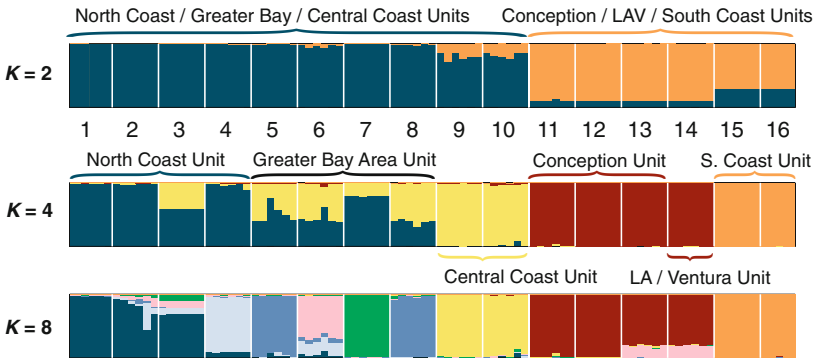

Fig. 4 Genetic structure among tidewater goby populations obtained using STRUCTURE on a data set of 95 individuals with 18 microsatellite loci, with replicates averaged by CLUMPP. The number of clusters in each plot is indicated by the value of K on the left side of the figure. Vertical bars represent individuals (listed left to right in north–south order in each plot), and are made up of stacked columns proportional in height to the average membership to clusters. Localities are separated by vertical white lines and are coded as in Fig. 1. We varied K from 1 through 24 for the full data set, though we only show $K = 2$, 4 and 8

two of the four groups. Genotypes from CCU, and the two northernmost sites 1-Smith + 2-Stone, are efficiently assigned to the remaining 2 clusters. Genotypes from intervening sites (3-Arcata to 8-Baldwin) are assigned to both of these clusters with varying probability, but with consistent proportion across some localities. This stratification results from the calculation of the arithmetic mean of the ten $K = 4$ Q-matrices, combined with non-synonymous modes. Such stratification is not present in the $K = 4$ Q-matrix with the highest log likelihood, which instead shows four clear clusters (NCU; GBAU + CCU; CU + LAVU; SCU).

Plots of the arithmetic mean clustering configuration for $K = 8$ (Fig. 4) assigns genotypes to the following 8 clusters: SCU; CCU; CU + LAVU (with some multimodality in 13-Burro + 14-Ventura); 4-Ten Mile; 7-Frijoles; 6-Rodeo (with some multimodal behavior); and 5-Americano + 8-Baldwin, from opposite ends of the GBAU. In addition, assignment to a unique cluster declines from the north end of the range 1-Smith > 2-Stone > 3-Arcata, with 3-Arcata showing stratification of assignment to other clusters. The individual Q-matrix with the largest value of log Pr ($X|K$) for $K = 8$ shows no within-sample stratification and instead clusters: SCU; 13-Burro + 14-Ventura; 11-San Luis Obispo + 12-Santa Ynez; the CCU; 8-Baldwin + 5-Americano; 7-Frijoles; 6-Rodeo + 4-Ten Mile; and 1-Smith + 2-Stone + 3-Arcata.

Discussion

Marker discovery and assessment

We identified 19 novel microsatellite markers for *E. newberryi* that combine with three variable markers from Mendonca et al. (2001) to form a panel of 22 species-specific markers. We employ these here in a statewide survey, however they should also prove effective for prospective sample intensive studies of regional population genetics and metapopulation dynamics.

Genetic structure—North Coast and Greater Bay Area Units

In the microsatellite data the localities in GBAU and NCU form a group in the neighbor-joining tree (Fig. 2). However, with the exception of the grouping of the northernmost 2 or 3 sites, this region lacks broad geographic structure. Nevertheless the samples localities are quite distinct: genotypes cluster in a site specific manner (Fig. 2); substantial differences between expected and observed heterozygosity suggest genetic partitioning/Wahlund effect (Table 2); the STRUCTURE analysis resolves sites rather than groups of sites (Fig. 4) and pairwise F_{ST} values tend to be large. Bottlenecking of some sites, such as observed in 7-Frijoles and 3-Arcata, may accentuate this pattern of site-specific isolation.

Barlow's (2002) mitochondrial control region sequence data densely sampled the GBAU (16 sites) including the 4 GBAU sites considered here. In the Barlow mitochondrial results, proximal sites separated by sandy coasts (e.g. Estero Americano and Estero San Antonio; Estero San Gregorio and Arroyo de las Frijoles) share haplotypes. Comparisons around rocky promontories (e.g. Bodega Head, Point Reyes, Pigeon Point) don't share haplotypes and generate highly divergent pair-wise Φ_{ST} values ($\Phi_{ST} = 0.5$–0.8). Despite this differentiation, and clades of related mitochondrial haplotypes endemic to one or a few communicating sites, these genetically isolated sites were not consistently monophyletic indicating a history of in situ evolution, but not complete coalescence.

We interpret patterns in both the mitochondrial (Barlow 2002) and microsatellite data to a phase of inheritance of ancestral variation followed by subsequent local evolution in isolation. In this scenario, substantial habitat formation occurred during the Pleistocene/Holocene sea level rise, which flooded stream valleys generating greater numbers of more closely spaced estuarine habitats. Subsequent endemic evolution and partial coalescence would then follow reduction of habitat number and isolation of habitats resulting from coastal retreat as the Holocene progressed (Barlow 2002). NCU habitats may also have been colonized from the populations to the south in the GBAU during the Pleistocene/Holocene transition as suggested by similarity of northernmost pair of samples. Alternatively these similarities in the northernmost sites could be due to dominance of recent migrants from the large population at Lake Earl.

Genetic structure in Central Coast Unit

Neighbor-joining (Fig. 2) supports a separate CCU group, as was the case with mtDNA sequence results (Dawson et al. (2001). However, with mtDNA the CCU was the most basal of the three clades north of the SCU, which is not the case here. Given the modest branch support for the respective topologies in the two analyses, this conflict is minor. Within the CCU the two sampled sites have low pairwise F_{ST} and each forms a monophyletic group of genotypes. Comparable separation between sites to the north and south of Estero Point in the CCU is also suggested in the mitochondrial haplotypes (Dawson et al. 2001), and may result from this stretch of rocky coast dividing the CCU. This requires further assessment with more samples.

Genetic structure—Los Angeles and Ventura Management Units

The Dawson et al. (2001) mitochondrial maximum likelihood phylogeny shows 94% bootstrap support for a clade that was designated the Los Angeles/Ventura Unit by the USFWS (2005). We also recover this cluster in our neighbor-joining tree (Fig. 2); however, it falls within, rather than as a sister to, a well-supported (92% bootstrap) CU cluster. This CU cluster is subdivided near Pt. Conception, a partition not evident in the mitochondrial data (Dawson et al. 2001).

The above observation would tend to undercut the independent status of the LAVU. However, 200 control region sequences from 10 sites on the Santa Barbara Coast (CU) recovered 10 unique haplotypes (Jacobs et al. 2005) and 150 control region sequences from five sites in the LAVU recovered 6 unique haplotypes (Barlow 2002), yet no haplotypes are identical between these adjacent regions of the management units. In addition, a modest microsatellite examination of 10 sites on the Santa Barbara coast suggests complex local geographic structure (Jacobs et al. 2005). Thus the nature of management boundaries across the CU + LAVU will have to be reconsidered with more detailed sampling of microsatellites.

Genetic structure—South Coast Unit

These microsatellite data document the dramatic distinction of the SCU from all other recovery units with all analytical approaches. Evidence of this distinction includes: 6 of 18 loci show diagnostic (fixed) differences; average alleles size differs dramatically (Fig. 1); mean pair-wise $F_{ST} = 0.72$; Neighbor-joining clusters are 100% bootstrap supported (Fig. 2); Principle Components Analysis ordinates the SCU at the end of axis 1 far removed from all other samples (Fig. 3); this partition explains 43% of the variation in AMOVA; and STRUCTURE consistently documents a distinct SCU group at K of 3 and above (e.g. Fig. 4).

The SCU contains fewer alleles and heterozygosity is lower than in any other unit (Table 2) or pair of adjacent

samples (Table S1), and pair-wise F_{ST} (0.093) between the two sites 15-San Onofre, and 16-Hidden is the lowest for any two sites in the analysis (Table S2). This lack of genetic variation accords with the history of extirpation and recolonization of all or nearly all sites in the SCU (Lafferty et al. 1999a, b; USFWS 2005). Thus, this unit appears significantly bottlenecked in association with extinction-recolonization dynamic and historical loss of many of the prime habitats in the region.

The mtDNA sequence data (Dawson et al. 2001) showed an excess of 4% sequence divergence interpreted as 2–4 million years of separation, placing the isolation of the SCU late in the Pliocene. Although we do not attempt a branch time calculation with our microsatellite data, our findings, including multiple fixed differences and dramatic differences in allele size range, support deep divergence. Limited mtDNA haplotype diversity within the SCU was also suggested by the Dawson et al. (2001) result, consistent with our inferences of population bottleneck in this unit. In San Diego and Orange counties estuarine habitat restoration plans have not considered the tidewater goby and have largely been directed at opening estuaries to the ocean, which increases tidal flow and eliminates seasonal closure dynamics (e.g. Zedler 1996; Coats et al. 1989).

Ahnelt et al. (2004) studied ontogenetic reduction of the superoccipital canal systems defining 9 character states, and scoring them in 546 *E. newberryi* museum specimens from 26 localities spread across the 6 currently recognized management units. Clustering and principle components assessments show the SCU to be the most distinct, based on nearly complete reduction of these canals. Some morphologic differentiation parallels the other major clades resolved in this study. In addition, Ahnelt et al. (2004) supports the location of the boundary of the SCU with the LAVU as it documents the association of museum preserved material from extirpated localities with genetic studies (e.g. Dawson et al. 2001 and this research).

Consideration of species status for the southern tidewater goby

The question arises as to whether the differences noted above merit consideration of the southern (SCU) tidewater goby as a distinct species, or as an Evolutionarily Significant Unit (ESU, Ryder 1986) under the Endangered Species Act. Reproductive isolation (Mayr 1995) and diagnosability are critical components to establishing species under most speciation concepts, and degree of divergence is also important. Tidewater gobies in the SCU have been reproductively isolated from all others for what is inferred to be in excess of 2 million years (Dawson et al. 2001), and are additionally diagnosable at 6 of the 18 microsatellite loci considered here. The Ahnelt et al. (2004) result also suggests that average morphology allows the diagnosis of SCU adults on a locality or sample, if not an individual, basis.

Ecological distinction plays an important role in species assessment under the Endangered Species Act (e.g. Waples 1991), where differences in morphology, range or habitat are often used to infer distinct status (Coyne and Orr 2004). In this regard the reduction in the lateral-line canals of the head (Ahnelt et al. 2004) likely reflects differential reception of vibrations associated with flow, or movement of predators or prey. Thus, these changes in the SCU suggest ecologic/adaptive distinction.

We compare treatment of the southern (SCU) tidewater goby to the ESU partition of federally endangered steelhead, *Oncorhynchous mykiss*, and to the federally listed endangered Devil's hole pupfish, *Cyprinodon diabolis,* in the region. Steelhead occupy [or historically occupied] many of the same drainages that tidewater gobies occur in and also depend on lagoonal resources (Hayes et al. 2008). Six ESUs subdivide the steelhead range in California, largely along the coast. Several of these are considered endangered, threatened or are under review (e.g. Federal Register/Vol. 71, No. 3/Thursday, January 5, 2006). The southernmost Southern California Steelhead Unit south of the Santa Maria River is federally listed as endangered. However, this unit is gradationally distinguished in terms of F_{ST} values and proportional differences in the alleles present, for both mitochondrial control region and microsatellites (Nielsen et al. 1994; Clemento et al. 2008). Examination of any single genetic locus does not appear to permit the diagnosis of individual southern steelhead. The steelhead ESUs were also partly defined by their migratory (anadromous) habit, and freshwater populations had been excluded. However, recent genetic evidence does not distinguish freshwater resident populations from the migratory stocks (Clemento et al. 2008). Thus, the migratory portions are now defined as Distinct Population Segments (DPS) rather than ESUs since they lack a distinct evolutionary trajectory from the freshwater forms (National Marine Fisheries Service 2007). Although other ecological criteria could be important, applying the same genetic differentiation criteria to tidewater gobies would result in a very large number of ESUs—all management units and subunits would likely qualify. Thus, the deeply divergent tidewater gobies of the South Coast Unit *far exceed* these criteria.

The Devil's Hole pupfish, *Cyprinodon diabolis*, occupies a single spring in southern Nevada. It was one of the taxa initially used to justify the Endangered Species Act, and most authors concur that drying from glacial pluvials during the last 20,000 years played a significant role in isolation of *C. diabolis* (e.g., Echelle et al. 2005). Mitochondrial sequence based tree topology indicates that the *C. diabolis* is not reciprocally monophyletic relative to

other species, but falls within the named subspecies *C. nevadensis nevadensis* (Duvernell and Turner 1998). Additionally, the mitochondrial sequences for *C. diabolis* are far less divergent from *C. nevadensis nevadensis* than the South Coast Unit *E. newberryi* are from the rest of the *E. newberryi,* though it should be noted that the *C. diabolis* published mitochondrial sequences are one-fourth the length of sequences recovered by Dawson et al. (2001) and are not strictly comparable.

F_{ST} values based on microsatellites for the bottlenecked Devil's Hole pupfish population have values around 0.5 relative to other adjacent pupfish populations, and none of the 6 microsatellites examined are specifically diagnostic. Thus, by a number of relevant criteria for duration of reproductive isolation the Devil's hole pupfish is substantially and objectively less distinct on a molecular level than the southern SCU tidewater goby. In the pupfish case, justification of species/ESU distinction includes ecological criteria (e.g. Bernatchez 1995), as well as interest in the relative recency of this example of speciation. However, southern (SCU) tidewater gobies also appear to be ecologically and morphologically distinct as discussed above, a topic that merits further study. Thus, the degree of distinction, diagnosability, reciprocal monophyly of mtDNA (Dawson et al. 2001) and microsatellite markers far exceed the above examples of fishes listed as endangered ESUs, and species, under the Endangered Species Act. The southern tidewater goby, in the SCU, clearly merits protection under the Endangered Species Act, and would also appear to merit formal description as a species-level taxon.

Evolutionary scenario and summary

The major distinction between *E. newberryi* in the SCU and elsewhere occurs in a region well south of Point Conception which constitutes a break or transition zone in a number of phylogeographies (Burton 1998; Dawson et al. 2001; Jacobs et al. 2004 Dawson et al. 2006). Uplift of the coast and sedimentary deposition during the Pleistocene eliminated large Pliocene embayment features in the Santa Maria, Santa Ynez and Santa Clara river valleys as well as in the Los Angeles Basin (Hall 2002; Jacobs et al. 2004). Thus at the inferred time of divergence of the SCU, rocky headlands at Point Buchon, Point Sal, Point Conception and Point Mugu/Northern Channel Islands were far more elongated peninsulas, potentially isolating bay or estuarine taxa (Jacobs et al. 2004) and potentially aiding in the separation of tidewater gobies in the SCU to the south. The major northern mtDNA clades likely diverged slightly more than 1 million years ago (Dawson et al. 2001), suggesting that the isolating effects of some of the steeper rocky coastal regions in the Big Sur region and Pt. Buchon persisted through several of the more recent Pleistocene transgressive/regressive sea level cycles. The support for comparable clades in analyses of these microsatellite data is consistent with this history.

The Pleistocene/Holocene transgression appears to have had a major influence in mixing genetic variation, yielding a star phylogeny regionally across the GBAU + NCU. This may entail some northern expansion as well. Holocene isolation by rocky-coast barriers to dispersal in the GBAU + NCU appears to have lead to genetic differentiation of local entities (Figs. 2, 4). Lafferty et al's (1999a, b) observations on extinction and recolonization suggest metapopulations are important and regionally variable. These and other local and regional attributes of these systems will need to be addressed with more sample intensive regional studies. The microsatellites employed here should facilitate this process.

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