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Population subdivision in marine environments: the contributions of biogeography, geographical distance and discontinuous habitat to genetic differentiation in a blennioid fish, *Axoclinus nigricaudus*

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Abstract

The relative importance of factors that may promote genetic differentiation in marine organisms is largely unknown. Here, contributions to population structure from a biogeographic boundary, geographical distance and the distribution of suitable habitat were investigated in *Axoclinus nigricaudus*, a small subtidal rock-reef fish, throughout its range in the Gulf of California. A 408-bp fragment of the mitochondrial control region was sequenced from 105 individuals. Variation was significantly partitioned between 28 of 36 possible combinations of population pairs. Phylogenetic analyses, hierarchical analyses of variance and a modified Mantel test substantiated a major break between two putative biogeographic regions. This genetic discontinuity coincides with an abrupt change in ecological characteristics, including temperature and salinity, but does not coincide with known oceanographic circulation patterns or any known historic barriers. There was an overall relationship of increasing genetic distance with increasing geographical distance between population pairs, in a manner consistent with isolation-by-distance. A significant habitat-by-geographical-distance interaction term indicated that, for a given geographical distance, populations separated by discontinuous habitat (sand) are more distinct genetically than are populations separated by continuous habitat (rock). In addition, populations separated by deep open waters were more genetically distinct than populations separated by continuous habitat (rock). These results indicate that levels of genetic differentiation among populations of *A. nigricaudus* cannot be explained by a single factor, but are due to the combined influences of biogeography, geographical distance and availability of suitable habitat.

Keywords: gene flow, isolation-by-distance, mitochondrial DNA, phylogeny, phylogeography, population structure

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Introduction

A continuing challenge in evolutionary biology is to understand the processes by which populations become genetically distinct. In general, genetic drift and local adaptation are counteracted by the unifying effects of gene flow. In many marine organisms, gene flow is high due to a planktonic life stage that can result in movement over large distances (reviewed in Palumbi 1994; Shulman 1998). The combination of high dispersal and few barriers to larval movement presents a challenge for understanding how divergence occurs in marine environments. Although the predominant mechanisms leading to population differentiation are not always clear (Palumbi 1994), several factors may be important either singly or in combination, including limited dispersal ability (Waples 1987; Duffy 1993; Hunt 1993; Doherty *et al*. 1995), local adaptation (Koehn *et al*. 1980; Powers *et al*. 1991; Schmidt & Rand 1999), oceanographic currents (Shulman & Bermingham 1995; Benzie & Williams 1997; Palumbi *et al*. 1997; Rocha-Olivares & Vetter 1999; Stepien 1999), habitat discontinuities (Winans 1980; Burton & Feldman 1981; Bell *et al*. 1982; Stepien & Rosenblatt

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1991; Doherty *et al*. 1995; Johnson & Black 1995), isolation-by-distance (Hellberg 1996; Lavery *et al*. 1996; Palumbi *et al*. 1997) and historic vicariance (Avise 1992; McMillan & Palumbi 1995; Lavery *et al*. 1996). Here, we focus on the impact of biogeographic barriers, geographical distance and habitat discontinuities on genetic differentiation in marine organisms.

Biogeographic regions are often described based on the overlapping ranges of many species, and boundaries between these regions may derive from historical discontinuities or from present-day environmental differences, such as differences in temperature or salinity. Although the underlying causes of such interspecific boundaries are not always well understood, these boundaries represent natural places to look for genetic discontinuities within species as well. For example, deep intraspecific divisions and the presence of many sister species pairs on either side of Cape Canaveral, Florida (separating Atlantic and Gulf of Mexico regions) point to historic vicariance in this region (reviewed in Avise 1992). Similarly, several Indo-Pacific taxa show increased divergence between Pacific and Indian ocean populations (Lavery *et al*. 1995, 1996; McMillan & Palumbi 1995; Chenoweth *et al*. 1998b; Duda & Palumbi 1999). However, not all biogeographic divisions correspond to intraspecific division, for example, intraspecific divergences are negligible across Point Conception, California where Californian and Oregonian regions meet (reviewed by Burton 1998).

Despite the generally high levels of gene flow in marine organisms, isolation-by-distance (Wright 1943) has been reported for a number of fishes and invertebrates (e.g. Johnson & Black 1995, 1998a,b; Pogson *et al*. 1995; Chenoweth *et al*. 1998a; Gold & Richardson 1998; Benzie 1999; Mamuris *et al*. 1999; Huang *et al*. 2000; Nesbø *et al*. 2000). There is some evidence that relationships between geographical and genetic distances depend on sampling scale. For example, high levels of genetic divergence are observed in Pacific urchins (Palumbi *et al*. 1997) and coconut crabs (Lavery *et al*. 1995, 1996) but only over large geographical scales (> 5000 km). In surgeonfish, in contrast, genetic and geographical distance are correlated within, but not among, archipelagos (Planes *et al*. 1996). Comparisons between low and high dispersal solitary corals (Hellberg 1996) and mud snails (Wilke & Davis 2000) reveal isolation-by-distance only for the low dispersal species but not for the high dispersal species.

Finally, discontinuity in suitable habitat may reduce gene flow among populations of marine organisms. For example, several studies have found that island populations isolated by open water are genetically divergent from mainland populations (Winans 1980; Bell *et al*. 1982; Stepien & Rosenblatt 1991; Doherty *et al*. 1995; Johnson & Black 1995), although divergence is minimal in a coastal urchin between western and eastern Pacific populations spanning 5400 km of open water (Lessios *et al*. 1998). Along coastlines, populations of tidepool copepods from rocky outcrops separated by sandy beach are more divergent than populations from the same outcrop (Burton & Feldman 1981), and estruarine populations of an atherinid fish (Johnson *et al*. 1994), a catfish (Ayvazian *et al*. 1994) and a littorinid snail (Johnson & Black 1998b) are more divergent than shoreline (continuous habitat) populations. Among pelagic organisms, deep oceanic channels are associated with genetic divergence in both cod (Bentzen *et al*. 1996; Ruzzante *et al*. 1998) and squid (Shaw *et al*. 1999). Clearly, habitat requirements differ among species but, where discontinuities in habitat have been identified, they often appear to contribute to genetic divergence.

Although individual studies point to the importance of biogeographic boundaries, geographical distance and habitat discontinuities for genetic differentiation, relatively few studies have considered these factors simultaneously (Johnson & Black 1995, 1998a,b; Lavery *et al*. 1995, 1996; Benzie 1999). Here, we test the hypothesis that these three factors in combination are important for generating population differentiation in the Cortez triplefin, *Axoclinus nigricaudus* (Allen & Robertson 1991).

A. nigricaudus is a small (25–40 mm standard length) subtidal fish with demersal (benthic) eggs and planktonic larvae. *A. nigricaudus* is found only on rocky shores in the Gulf of California, Mexico. On the western coast of the Gulf (Baja) rocky shores are almost continuous (Fig. 1). In contrast, rocky regions on the eastern coastline of the Gulf (Sonora) are few and are surrounded by extensive sandy shores (beaches and estuaries). Thus, populations are separated by either continuous suitable habitat (rocky coast) or by unsuitable habitat (sandy shores or open water). The Gulf of California contains at least two distinct biogeographic regions (northern and central, Fig. 1), based on range distributions, community composition of fishes and differences in salinity, temperature and tidal regimes (Walker 1960; Thomson & Gilligan 1983). There are no reported examples of sister taxa between these two regions. Thus, *A. nigricaudus* presents an opportunity to simultaneously evaluate the importance of a biogeographic boundary, geographical distance and discontinuities in suitable habitat for population differentiation. We sampled *A. nigricaudus* throughout its range and found that all three factors in combination contribute to patterns of mitochondrial DNA (mtDNA) differentiation among populations.

Materials and methods

Populations sampled

In total 105 *Axoclinus nigricaudus* individuals were collected from nine geographical locations in the Gulf of California (Fig. 1, Table 1). The study sites were selected to include

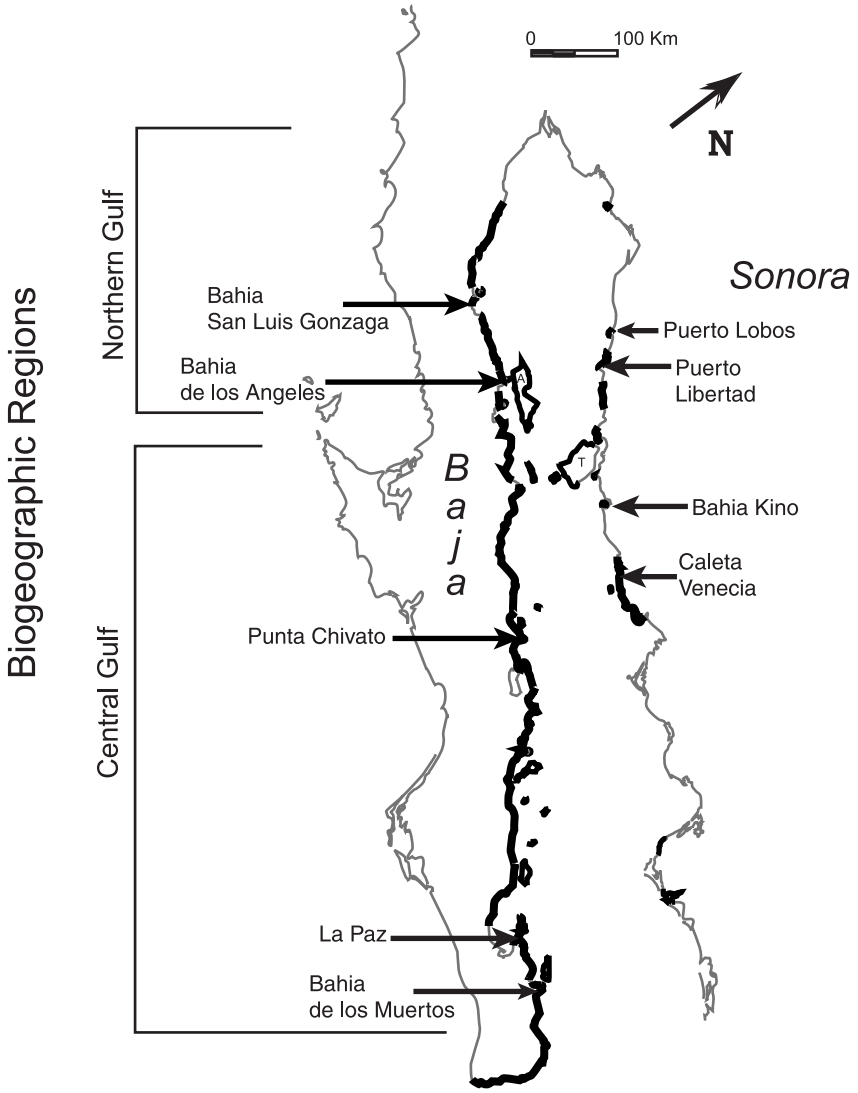

Fig. 1 The Gulf of California. Rocky shorelines are indicated by bold lines. Arrows point to individual collecting locations. The ranges of the biogeographic regions are shown on the left. The boundary between the northern biogeographic region and central biogeographic region falls between Isla Angel de la Guarda (A) and Isla Tiburon (T).

Table 1 Study sites

Coastline	Study site	Biogeographic region	Specific collecting location	*n*	Latitude	Longitude
Baja	Bahia San Luis Gonzaga	N	Punta Willard, north of bay	10	29°49′64″	114°23′80″
	Bahia de los Angeles	N	Punta Gringo, north of bay	10	29°2′21″	113°32′14″
	Punta Chivato	C	at the point	9	27°4′53″	111°56′88″
	La Paz	C	Tecolote Beach, north of La Paz	6	24°20′17″	110°19′43″
	Bahia de los Muertos	C	north-east point of bay	10	23°59′30″	109°49′31″
Sonora	Puerto Lobos	N	south of lighthouse	10	30°15′55″	112°51′28″
	Puerto Libertad	N	Bahia Santa Margarita, ≈ 20 km N. of town	10	30°1′47″	112°44′30″
	Bahia Kino	C	off Roca Alcatraz	10	28°48′37″	111°58′22″
	Caleta Venecia	C	in bay	30	28°7′87″	111°17′60″

representative sites in the northern and central Gulf biogeographic regions and on both coastlines. In addition, a single congener, *A. carminalis*, was also collected to provide an outgroup for genealogical analyses. Fish were frozen whole in liquid nitrogen. Subsequently, a piece of muscle tissue was removed for DNA extraction. Each individual was deposited as a voucher specimen in the University of Arizona Fish Collection (UAZ).

mtDNA amplification and sequencing

Genomic DNA was prepared from muscle tissue following Sambrook *et al*. (1989). Universal fish primers A and E (Lee *et al*. 1995), which target a portion of transfer RNA (tRNA)-pro and the central conserved region of the mitochondrial control region, were used in a polymerase chain reaction (PCR) to amplify the first hypervariable region with 0.5 units of *Taq* DNA polymerase (Amersham) in 10-μL reactions. The final concentrations were 100 nM forward and reverse primers, 200 μM dNTPs, 10 mM Tris pH 8.3, 50 mM KCl and 2.5 mM $MgCl_2$. Reactions were optimized at 40 cycles of 95°C for 30 s, 52°C for 30 s and 72°C for 45 s. PCR products were sequenced using a chain termination protocol in which each of the four dideoxynucleotide terminators (ddNTPs) was labelled with ^{33}P following the manufacturer's directions (USB Thermosequenase radiolabelled terminator cycle sequencing kit). Both PCR primers and internal sequencing primers (AN.F3: 5′-AGCGATACACCAACTAACAAT-3′, AN.R4: 5′-TGGTCGGTTCTTACTACATTA-3′) were used to generate overlapping contigs such that ≈ 90% of each reported fragment was sequenced in both forward and reverse directions. Sequencing products were electrophoresed on an 8% acrylimide gel and exposed to film overnight.

Descriptive statistics

All *A. nigricaudus* sequences were aligned manually. The number of unique haplotypes and number of transitions and transversions were counted. The average number of pairwise differences, $\hat{\pi}$ (Nei & Li 1979), diversity based on the number of segregating sites, $\hat{\theta}$ (Watterson 1975), and their standard errors were calculated using ARLEQUIN (Schneider *et al*. 2000). Both $\hat{\pi}$ and $\hat{\theta}$ estimate the neutral parameter $\theta = 2N\mu$ for mtDNA, where N is the effective population size for females and μ is the mitochondrial neutral mutation rate. To check for deviations from neutral expectations for the frequency spectrum of polymorphisms, significance values were calculated for Tajima's D (Tajima 1989), Fu and Li's D (Fu & Li 1993), and Fu's F (Fu 1996) using a program made available by Fu (http://hgc.sph.uth.tmc.edu/fu/). These statistics compare values for $\hat{\theta}$ and $\hat{\pi}$, the distribution of mutations on internal branches to external branches on a gene tree, and the number of haplotypes observed to a given value of $\hat{\pi}$, respectively.

Genealogical estimations

To estimate genealogical relationships among haplotypes and among populations, trees were constructed using maximum parsimony and neighbour-joining algorithms. For rooted parsimony analyses, *A. nigricaudus* haplotypes were aligned with a congener, *A. carminalis*, using PILEUP in GCG. Parsimony analyses were conducted in PAUP 4.0b2a (Swofford 2000), with transitions and transversions weighted equally and also with transversions given a weight six times that of transitions (observed number of transitions to transversions in aligned sequences was 69 : 11). Heuristic searches were performed using stepwise addition for initial trees and the tree-bisection-reconnection method of branch swapping. For each search, the maximum limit of trees was set at 1000 and a strict consensus tree was generated. These conditions were repeated 10 times under both transition : transversion weighting schemes. Haplotype trees were also constructed in PAUP 4.0b2a using neighbour-joining (Saitou & Nei 1987) with distances between sequences estimated under a Kimura (1980) two-parameter model and no among-site rate variation. A neighbour-joining tree based on genetic divergence, K_{ST} (Hudson *et al*. 1992a), between populations was estimated, in which each of the nine populations sampled was an operational taxonomic unit. This tree was bootstrapped 1000 times by resampling haplotypes with replacement within each population, recalculating K_{ST}, and generating a neighbour-joining tree for each bootstrap replicate. Randomization and K_{ST} calculations were performed using a program kindly provided by P. J. Palsbøll and neighbour-joining trees from each bootstrap were constructed using PHYLIP (Felsenstein 1993).

Population subdivision

Two analyses of molecular variance (AMOVA: Excoffier *et al*. 1992) were used to test for significant population structure. Both AMOVAs were performed in ARLEQUIN (Schneider *et al*. 2000) using uncorrected pairwise differences between haplotypes and the haplotypes were permuted 1000 times. In the first analysis, a one-factor AMOVA was employed to assess the degree of population structure over all populations. Here, variation within populations was compared with variation between populations. In the second test, sequences from all nine geographical sites were included in a two-factor AMOVA in which variation was partitioned among biogeographic regions, populations and individuals. Populations were assigned to biogeographic regions following Thomson & Gilligan's (1983) biogeographic regions (northern Gulf = Gonzaga + Los Angeles + Lobos + Libertad, central Gulf = Chivato + La Paz + Muertos + Kino + Venecia, Fig. 1).

Genetic distance and F_{ST} (Wright 1951) are often used to measure variation among populations. Because F_{ST} is inflated by anything that reduces within-population variation (Charlesworth 1998), an absolute measure of genetic distance, D_m (Nei 1973), was used to measure divergence among population pairs in this study. The minimum population distance, D_m (Nei 1973), is equivalent to the between-population distance corrected for a within-population

component of diversity (Nei & Li 1979). Average numbers of pairwise nucleotide differences between and within populations were calculated in ARLEQUIN (Schneider *et al*. 2000). Genetic distances (D_m) were estimated from between and within population diversity for the 36 possible population pairs using a weighting scheme described by Charlesworth (1998) to correct for unequal sample sizes. In order to test whether the genetic distances were significantly greater than random, all individual pairs of populations were tested for geographical subdivision using Hudson *et al*.'s (1992a) statistic Ks*, which is a weighted measure of average nucleotide differences between haplotypes from different populations. This statistic was calculated for each population pair and the significance was then assessed by randomly assigning haplotypes to each of the two populations (holding relative population sizes constant) and recalculating the statistic 10 000 times. The overall significance for population pairs was adjusted for multiple tests using a sequential Bonferroni correction. Because species-level F_{ST} values are widely reported in the literature, Hudson *et al*.'s (1992b) F_{ST} was calculated in order to compare overall levels of genetic partitioning in *A. nigricaudus* with published results.

Biogeography, geographical distance and discontinuous habitat

Genetic distances between *A. nigricaudus* populations may result primarily from one factor. Alternatively, several factors in combination might be responsible for the observed patterns of genetic differentiation. Whereas multiple regression analyses allow the contributions of individual parameters to be estimated, associated tests of significance rely on the assumption that all data points are independent. In this study, there are 36 nonindependent pairwise comparisons among nine populations. In order to avoid statistical tests that assume independence of data points, we first explored the data structure using multidimensional scaling. Second, we explicitly tested for contributions of biogeography, geographical distance and discontinuous habitat to genetic structure by comparing observed results against null distributions generated by permutations of the original data.

Multidimensional scaling (Kruskal & Wish 1978) takes the distances between objects and tries to approximate those distances in a reduced number of dimensions through an iterative fitting procedure. A two-dimensional plot fitted to genetic distances (D_m) between populations was created after 100 iterations in VISTA version 5.6 (http://forrest.psych.unc.edu/). The goodness of fit between the fitted and observed distances was measured by a stress test (Kruskal & Wish 1978). In order to assess whether eastern Gulf populations (separated by unsuitable sandy habitat) are, on average, more genetically distant from each other than western Gulf populations (connected by nearly continuous rocky habitat), two polygons were drawn connecting each set of populations and the area of each polygon was measured.

Whereas multidimensional scaling is a useful method for describing data, permutation-based tests can be used to test for statistical significance without assuming independence of data points. For example, a simple Mantel procedure (Mantel 1967) assesses the significance of a regression between two distance matrices by calculating the sum of element-by-element products and then compares that statistic with a null distribution of values created from permutations of elements within one of the matrices. This approach has been extended for three or more matrices using a number of approaches (Dow & Cheverud 1985; Hubert 1985; Manly 1986; Smouse *et al*. 1986). Here, we follow the procedures of Manly (1986, 1997b) in which a standard multiple regression is conducted, but significance is assessed by comparing observed statistics for the regression against those generated by permuting the dependent variables. This particular method has been used in a number of recent studies that attempt to identify factors associated with genetic differentiation (e.g. Brown *et al*. 1991; Lugon-Moulin *et al*. 1999; Pestano & Brown 1999).

To explore the effects of biogeography, geographical distance and discontinuous habitat on genetic distance in *A. nigricaudus*, we first defined six prediction vectors reflecting distances between population pairs based on: (i) biogeography (0 = same biogeographic region, 1 = different); (ii) geographical distance (km separating the two populations); (iii) discontinuous habitat due to sandy shores (0 = population pairs separated by rocky shore or open water, 1 = population pairs separated by sandy shore); (iv) discontinuous habitat due to open water (0 = population pairs separated by rocky or sandy shore, 1 = population pairs separated by open water); (v) geographical distance * discontinuous habitat due to sandy shores; and (vi) geographical distance * discontinuous habitat due to open water. (The abbreviations BIOG, DIST, SAND, WATER, DIST * SAND and DIST * WATER are used throughout this study to refer to these six vectors.) There are three types of habitats separating the population pairs: open water, sandy shoreline and rocky shoreline. However, assigning three presence/absence binary codes, one for each habitat type (for example, for the habitat type of rock: 0 = sand or open water, 1 = rock), resulted in a design matrix that was not invertible and thus could not be solved for a least-squares solution. Therefore, the effects of the three habitat types could not be estimated independently but could be estimated only relative to each other. Thus, in order to test whether discontinuous habitat (water or sand) increases genetic distance relative to continuous habitat (rock), we created two variables, WATER and SAND, which represent the effect of open water minus the effect of rocky shore and the effect of sandy shore minus the effect of rocky shore, respectively. In both cases, a positive nonzero

correlation coefficient would indicate that the populations separated by discontinuous habitat (water or sand) are more genetically distant than populations separated by continuous habitat (rock). The interaction terms (DIST * SAND and DIST * WATER) were included because if discontinuous habitat reduces the opportunities for genetic interchange, the magnitude of the effect may be contingent upon the geographical distance. The vector of genetic distances (estimated by D_m) was used as the response variable. Thus, the full multiple regression model was:

$$Y = \beta_i x_i + \beta_{ii} x_{ii} + \beta_{iii} x_{iii} + \beta_{iv} x_{iv} + \beta_v (x_{ii} x_{iii}) + \beta_{vi} (x_{ii} x_{iv}) + e \quad (1)$$

where x_i, x_{ii}, x_{iii} and x_{iv} are the prediction vectors BIOG, DIST, SAND and WATER, respectively, β_i ... β_{vi} are the estimated partial regression coefficients, e is error and Y is genetic distance. Following Manly (1986), each vector was normalized (zero mean, unit variance). Sums of squares and associated statistics for the whole model were calculated following standard regression procedures. Significances for the whole model and partial regression coefficients were estimated by permuting the order of values in the vector of genetic distances and recalculating the regression 10 000 times using the program RT version 2.1 (Manly 1997a). This Monte Carlo procedure was used to create null distributions for all statistics reported for this model. Because each factor was expected to have a positive slope, one-sided *t*-tests were used to determine whether the observed *t*-statistic was greater than the permuted distribution of *t*-statistics. Significances of the linear regression and six partial regression coefficients were adjusted using a sequential Bonferroni correction.

In order to visualize the interplay of habitat type and geographical distance while controlling for biogeography, residual genetic distances were calculated as follows:

$$\text{Residuals} = Y - \beta_i x_i \quad (2)$$

where β_i is the estimated partial regression coefficient for BIOG, and Y and x_i are the normalized vectors of genetic distance and BIOG. These residual genetic distances were plotted against geographical distance for each habitat type.

Results

Descriptive statistics

A 408-bp fragment was sequenced from 105 *Axoclinus nigricaudus* individuals. The first base in our sequence corresponds to position 16619 in the sequence of Zardoya *et al*. (1995). All sequences have been deposited in GenBank, with Accession nos AF333610–AF333695. Diversity within *A. nigricaudus* mtDNA was high but was similar to diversity among other fishes for the control region (Bowen & Grant 1997; Chenoweth *et al*. 1998a; McMillan *et al*. 1999; Rocha-Olivares & Vetter 1999). Among the 105 total sequences, there were 86 haplotypes (Fig. 2). Within the 408-bp sequence, there was one indel and 72 polymorphic sites, 27 of which were singletons. There were 69 observed transitions and 11 transversions in the aligned sequences. The species-level estimate of $2N\mu$ from average pairwise differences was $\hat{\pi} = 2.71\%$ (SE = 1.38%) and from the number of segregating sites was $\hat{\theta} = 3.37\%$ (SE = 0.89%) (both expressed per site). Tajima's *D* (Tajima 1989) did not reject neutral expectations for the total data set ($D = -0.827$, $P > 0.1$), however, both Fu and Li's *D* (Fu & Li 1993), and Fu's *F* (Fu 1996), which are more sensitive to the presence of singletons in a sample, were significantly different from neutral expectations ($D = -2.843$, $P < 0.02$; $F_S = -19.904$, $P < 0.005$). These deviations indicate an excess of low frequency polymorphisms, as previously reported for mtDNA in other organisms (Excoffier 1990; Nachman *et al*. 1996) and may be consistent with the presence of mildly deleterious mutations, a recent selective sweep or nonequilibrium demographic effects (Tajima 1989). These deviations from a standard neutral model may have relatively little impact on the attainment of isolation-by-distance. Isolation-by-distance depends on a migration–drift equilibrium that may be achieved more quickly than mutation–drift equilibrium, provided that the migration rate is much higher than the mutation rate (Crow & Aoki 1984).

Genealogical estimations

All estimates of genealogical relationships showed substantial concordance with biogeography. Parsimony analyses of the 86 haplotypes identified several thousand equally parsimonious trees regardless of weighting scheme. When transversions and transitions were weighted equally, the tree lengths were 189 and the consistency indices (CI) equalled 0.42. A strict-consensus tree contained two clades, one consisting mostly of individuals from the northern Gulf and the other consisting mostly of individuals from the central Gulf. The central Gulf clade contained two smaller clades, corresponding mostly to haplotypes from western (Baja) Gulf and eastern (Sonora) Gulf populations, respectively. A haplotype network (Fig. 3A) was constructed based on a

Fig. 2 (*opposite*) Aligned haplotypes and polymorphic sites. The positions of polymorphic sites are indicated at the top of the figure. For each site, the consensus nucleotide is given; dots indicate identity to consensus and asterisks indicate single base pair deletions. A unique number is assigned to each haplotype and the collecting location and number of individuals sampled with that haplotype are reported. Haplotypes are unique to collecting locations with two exceptions; haplotype 60 is observed among one Venecia and three Kino individuals and haplotype 64 observed among one Kino and four Venecia individuals.

Polymorphic sites
Haplotype
n
Location

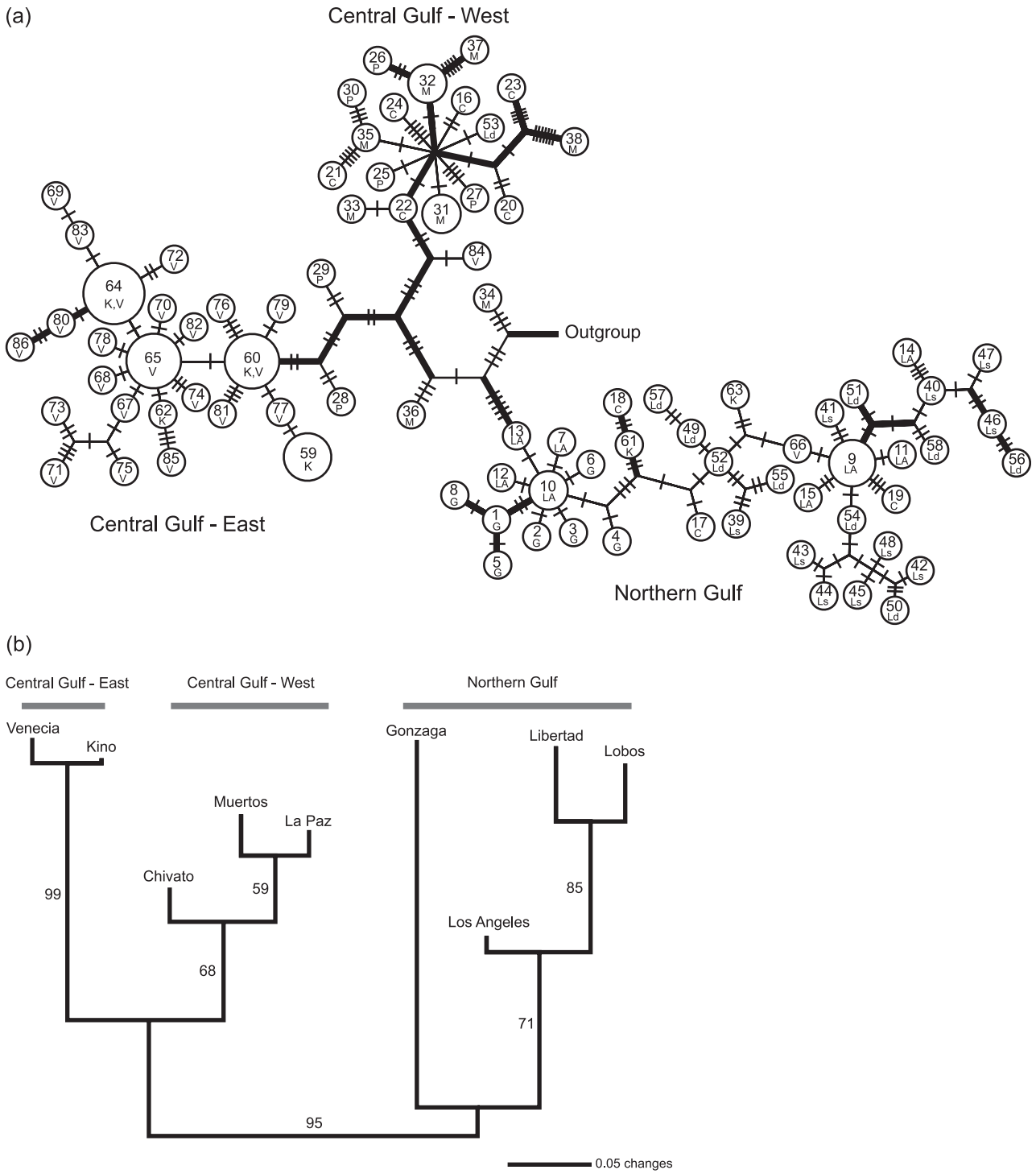

Fig. 3 (A) Haplotype network constructed from a single unweighted parsimony tree. Individual haplotypes are indicated by circles that are proportional in area to the number of individuals observed to have that haplotype. Hash marks indicate the number of mutational steps separating each haplotype. The number in each circle refers to the haplotype and the letter(s) refers to the geographical location from which it was sampled (G = Gonzaga, LA = Los Angeles, C = Chivato, P = La Paz, M = Muertos, Ls = Lobos, Ld = Libertad, K = Kino, V = Venecia). Three major clades are labelled and consist largely of individuals from the indicated geographical regions. Branches present in the strict consensus of all 1000 equally parsimonious trees are indicated in bold. (B) Neighbour-joining tree based on distances between populations estimated by K_{ST}. Support for individual branches is given by bootstrap percentages, where haplotypes were drawn randomly with replacement within each of the nine populations and K_{ST} was recalculated for each of the 1000 bootstrap replicates. Three major clades are labelled by geographical region.

Table 2 Within population diversities, genetic distances, and geographical distances between *Axoclinus nigricaudus* populations†

			Los		La					
	n	Gonzaga	Angeles	Chivato	La Paz	Muertos	Lobos	Libertad	Kino	Venecia
Gonzaga	10	*0.666*	121	388	731	791	156	161	261	356
Los Angeles	10	0.690*	*1.562*	267	612	672	151	134	154	241
Chivato	9	1.428**	1.093**	*2.723*	345	404	365	329	192	134
La Paz	6	1.828**	1.632*	0.000	*2.097*	64	704	670	523	433
Muertos	10	1.826**	1.782**	0.160	0.062	*1.877*	759	730	577	483
Lobos	10	1.924**	0.317	1.457**	2.043**	2.254**	*1.343*	28	183	281
Libertad	10	1.621**	0.208	1.032*	1.519**	1.743**	−0.029	1.802	154	230
Kino	10	1.810**	1.372**	1.131**	0.998*	1.271**	1.788**	1.503**	*1.365*	100
Venecia	30	2.113**	2.067**	1.778*	1.312**	1.576	2.453**	2.244**	0.115	*1.13*

†Within population nucleotide diversity, $\hat{\pi}$, is on the diagonal. Genetic distances between populations (D_m) are below the diagonal. Geographical distances (km) separating populations are above the diagonal. Significance of genetic partitioning was evaluated using the statistic Ks* with a sequential Bonferroni correction for multiple tests and is indicated by asterisks: * < 0.05, ** < 0.001. See text for further details.

randomly chosen unweighted parsimony tree. Trees based on a 6 : 1 weighting of transversions to transitions had tree lengths equal to 258 and consistency indices equal to 0.52. The consensus trees obtained with a 6 : 1 weighting contained the central western Gulf and central eastern Gulf clades, but the large northern Gulf clade collapsed into a basal paraphyletic assemblage. The neighbour-joining tree based on haplotypes contained three clades corresponding mostly to haplotypes from the northern, western-central and eastern-central areas of the Gulf. The neighbour-joining tree of populations based on K_{ST} (Fig. 3B) also contained three clades corresponding to populations from northern, western-central and eastern-central Gulf locations. Bootstrap support was high (95%) for the branch separating northern and central Gulf clades. These different genealogical analyses are concordant in suggesting separate evolutionary histories for northern and central Gulf populations and also an east–west division within the central Gulf populations.

Population subdivision

Haplotype diversity was significantly partitioned among populations and biogeographic regions in *A. nigricaudus*. In the one-factor AMOVA of all populations, much variation was explained by differences among populations ($\Phi_{ST} = 0.485$ $P < 0.001$). The two-factor AMOVA that partitioned variation among groups (biogeographic regions), among populations in groups, and within populations, revealed significant partitioning both among regions ($\Phi_{CT} = 0.307$, $P < 0.007$) and among populations ($\Phi_{ST} = 0.556$, $P < 0.001$). Haplotypes were also nonrandomly partitioned between many pairs of populations as shown by Hudson *et al.*'s (1992a) permutation test; in 28 of 36 possible populations pairs, the statistic Ks* was higher than expected by chance (Table 2). In many cases there was significant partitioning between populations, including those separated by < 200 km (Table 2). However, there was no significant genetic differentiation between populations separated by < 100 km, and some population pairs up to 483 km apart were also not significantly differentiated. For *A. nigricaudus*, species-level F_{ST} was equal to 0.536.

Biogeography, geographical distance and discontinuous habitat

A multidimensional scaling of genetic distances generated a good fit to the observed data (stress = 0.037 after 100 iterations, where a zero stress value indicates a perfect fit). The multidimensional scaling plot revealed three clusters of populations similar to those observed from genealogical analyses (Fig. 4A): northern Gulf, western-central Gulf and eastern-central Gulf. The polygon enclosing eastern Gulf populations that are separated from each other by discontinuous habitat (sand) was larger in area than the polygon enclosing western Gulf populations that are connected by almost continuous rock habitat (eastern Gulf polygon area = 1.17, western Gulf polygon area = 0.89), despite the fact that the populations separated by discontinuous habitat cover a much smaller geographical area (Fig. 1).

The multiple regression of six vectors (BIOG, DIST, SAND, WATER, DIST * SAND and DIST * WATER) on genetic distance was highly significant and explained 71% of the variance in D_m between populations ($R^2 = 0.705$, $F = 10.32$, $P < 0.05$). The partial regression coefficients of BIOG, DIST, WATER and DIST * SAND were significantly greater than random ($P < 0.05$; Table 3), indicating that these factors contribute to genetic distances in *A. nigricaudus*, where partial regression coefficients represent the slope of a given variable when all other variables are held constant. A scatterplot of geographical distance vs. residual genetic distance illustrates an overall pattern of increasing genetic distance with increased geographical distance (Fig. 4B).

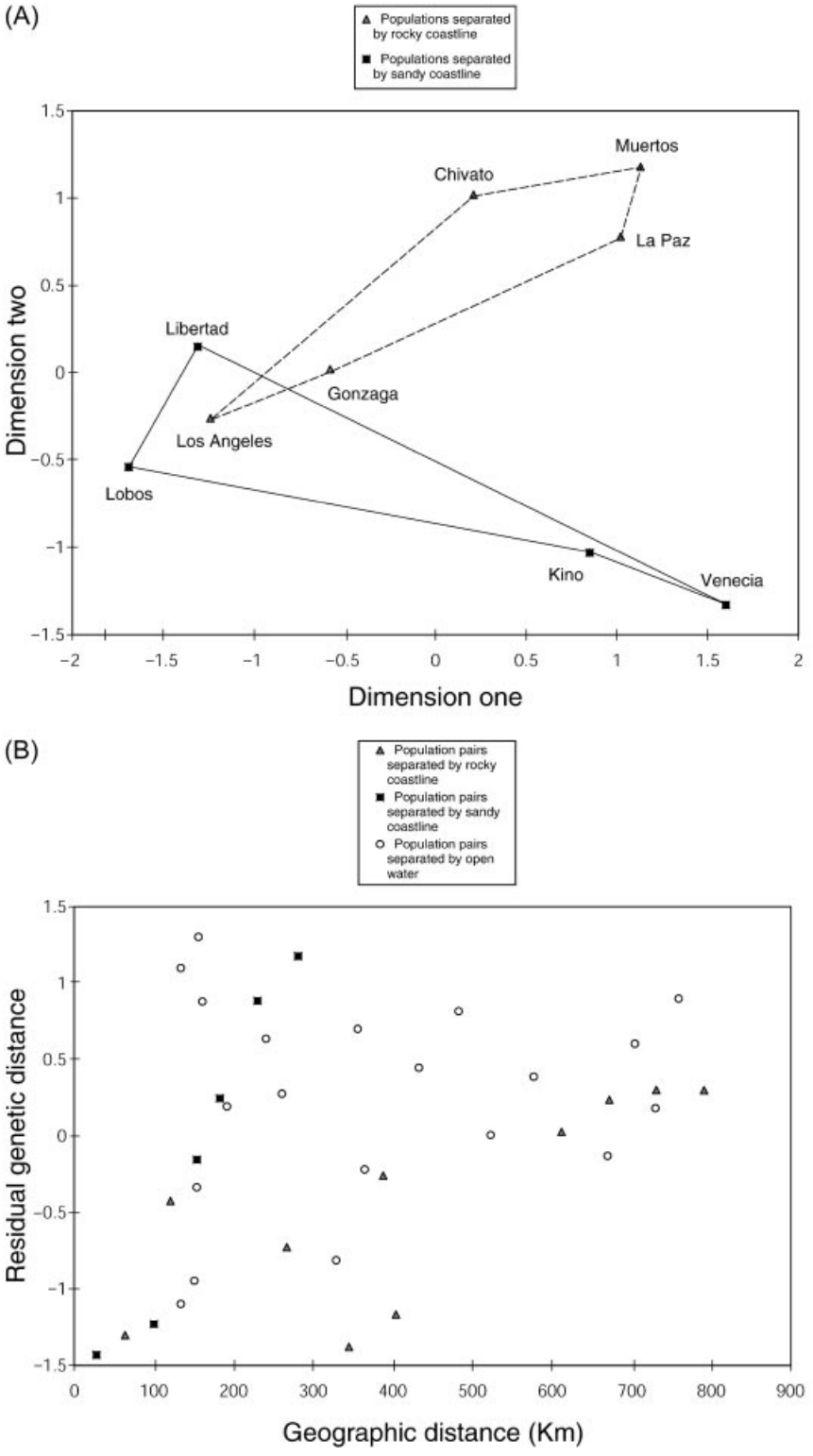

Fig. 4 (A) Multidimensional scaling plot of populations based on genetic distances (D_m) between pairs of populations. Populations from the western Gulf that are connected by rocky habitat are shown by triangles and populations from the eastern Gulf that are separated by sandy habitat are shown by squares. The area of the polygon defined by the western Gulf populations equals 0.89 and the area of the polygon defined by the eastern Gulf populations equals 1.17. Greater genetic distances among eastern Gulf populations relative to western Gulf populations are consistent with discontinuous habitat (sand) reducing genetic interchange. (B) Scatterplot of residual genetic distances (controlling for biogeography) on geographical distance for pairs of populations separated by discontinuous habitat (sand and open water) and continuous habitat (rock). A modified Mantel permutation test shows that population pairs separated by open water are more genetically distant overall than populations pairs connected by rocky coastline (Table 3: $t = 2.94$, $P < 0.05$). In addition, as geographical distance increases, populations separated by sandy coastline become more genetically distant than those population pairs connected by rocky habitat (Table 3: $t = 3.01$, $P < 0.05$). See text for further details.

Table 3 Partial regression coefficients of biogeography, geographical distance and intervening habitat for a multiple linear regression on genetic distance with probabilities determined by a modified Mantel test†

Variable‡	Partial regression coefficient (β)§	*t*	*P*¶
BIOG	0.437	3.53	0.0009*
DIST	0.472	2.39	0.0116*
SAND	−0.235	−0.93	0.8209
WATER	0.709	2.94	0.0036*
DIST * SAND	0.669	3.01	0.0019*
DIST * WATER	−0.392	−1.56	0.9331

†Multiple regression: $R^2 = 0.705$, $F = 10.32$, *$P = 0.0001$. ‡BIOG, biogeography: DIST, geographical distance: SAND, sandy shore: WATER, open waters. See Materials and Methods for a full explanation. §Equivalent to β values in full regression model. See Materials and Methods. ¶Probability was assessed by following a Monte Carlo procedure where observed *t* and *F*-values were compared against a null distribution of *t* and *F*-values based on 10 000 randomizations of response values (genetic distances). Asterisks indicate values that remain significant ($P < 0.05$) following a sequential Bonferroni correction.

Although population pairs from different biogeographic regions are generally more distant than pairs from the same region, the significant positive coefficients for both BIOG and DIST demonstrate that each factor independently contributes to genetic distance. Because WATER represents the difference between population pairs separated by open water (discontinuous habitat) and rocky shore (continuous habitat), the positive partial regression coefficient for WATER indicates that population pairs separated by open water are on average more genetically distant than those connected by continuous habitat (Fig. 4B). The positive coefficient for DIST * SAND demonstrates that, as geographical distance increases, the difference in genetic distances between populations separated by sand (discontinuous habitat) and rocky shore increases (Fig. 4B).

Discussion

In *Axoclinus nigricaudus*, we find that there is substantial partitioning of genetic variation over relatively short geographical distances (< 200 km, in some cases), and that the patterns of genetic differentiation are best explained by the combined effects of biogeography, geographical distance and discontinuous habitat.

Biogeography, geographical distance and habitat discontinuities

Phylogenetic relationships among mtDNA haplotypes revealed a deep division between northern and central Gulf populations. Unweighted parsimony and neighbour-joining analyses of haplotypes, as well as a neighbour-joining tree of populations, produced trees that contained two large clades corresponding mostly to northern and central Gulf haplotypes and populations. Among all parsimony trees, northern and central Gulf clades were separated by nine mutational steps (Fig. 3A), suggesting separate evolutionary histories for these two groups. The two-factor AMOVA, based on haplotype distances, also reflected the strong partitioning of haplotypes between biogeographic regions ($\Phi_{CT} = 0.307$, $P < 0.007$). Overall, the concordance between *A. nigricaudus* genealogy and previously identified biogeographic regions is striking.

Delineations of biogeographic regions in the Gulf of California have been largely based on changes in fish community composition and do not necessarily point to an underlying cause of the north–central division. Ecological differences, particularly cold winter temperatures, probably prevent range expansion of many tropically derived species into the northern Gulf (Thomson & Lehner 1976). Although temperature may contribute to changes in community composition, it seems unlikely that selection due to environmental factors (such as temperature) would cause a deep divergence among *A. nigricaudus* mtDNA haplotypes. The observed partitioning of *A. nigricaudus* mtDNA between biogeographic regions is also puzzling because there are no obvious geological events or oceanographic circulation patterns (Badan-Dangon *et al*. 1985; Bray 1988; Paden *et al*. 1991) that point to a northern–central Gulf division. In addition, there are no known sister taxa on opposite sides of this division, and northern–central Gulf partitioning has not been observed in *Malacoctenus hubbsi* (C. Riginos unpublished) or *Girella nigrican*s (Terry *et al*. 2000), the only other species surveyed for genetic variation across both regions. Nonetheless, the genetic partitioning of *A. nigricaudus* populations between northern and central Gulf regions suggests that genetic interchange across this boundary may be more restricted than previously recognized. If genetic surveys of additional taxa reveal other examples of northern–central Gulf divergence, this pattern would point to an historical factor affecting many species.

Although 31% of genetic variation in *A. nigricaudus* was partitioned between biogeographic regions, discontinuous habitat and geographical distance also contributed to

divergence among populations. Overall, genetic distance increased with geographical distance (Fig. 4B), consistent with isolation-by-distance. Although BIOG and DIST were correlated ($R^2 = 0.181$), the significant partial regression coefficient for DIST ($t = 2.39$, $P < 0.05$) showed that there was an effect of geographical distance beyond that explained by biogeography. Studies of coconut crabs (Lavery *et al*. 1995, 1996) also demonstrate additive effects from geographical distance and biogeographic regions, where genetic distances between Pacific populations and a single Indian Ocean population are greater than genetic distances among Pacific populations.

Discontinuous habitat also accounted for substantial genetic divergence. The partial regression coefficient of WATER was significantly greater than zero ($t = 2.94$, $P < 0.05$), indicating that population pairs separated by open water were more genetically distant, on average, than population pairs connected by rocky shoreline. However, there was much scatter among the open water data points, with large genetic distances found both over small and large geographical distances (Fig. 4B). This scatter was largely due to differing patterns of genetic connectivity across open water within each biogeographic region. Distinct central-west Gulf and central-eastern Gulf groups were present in all genealogical estimations and in a multidimensional scaling plot, and population distances were high between these regions ($D_m \geq 0.998$, Table 2). This west–east division suggests that the open waters of the central Gulf are a strong barrier to genetic interchange. In contrast, among northern Gulf fish, several haplotypes from Los Angeles (west) were most closely related to eastern haplotypes from Lobos and Libertad (Figs 2 and 3A), resulting in low D_m values (Table 2: $D_m \leq 0.317$). It is likely that stepping-stone populations on the islands Isla de la Guarda and Isla Tiburon facilitate limited genetic interchange between Los Angeles and north-eastern populations (Fig. 1). Overall, genetic distances were higher across open water than across rocky shoreline, and this result is consistent with other studies that have demonstrated a reduction in gene flow across open water in coastal marine organisms (e.g. Bell *et al*. 1982; Stepien & Rosenblatt 1991; Doherty *et al*. 1995; Johnson & Black 1995).

Multidimensional scaling (Fig. 4A) showed that population pairs connected by sandy shoreline were more genetically distant than population pairs connected by rocky coast, even though rocky coast populations span a greater geographical distance than sandy shore populations (791 and 281 km, respectively, Fig. 1). In the multiple regression model, in contrast, the partial regression of SAND was not significant ($P > 0.05$, Table 3). The interaction DIST * SAND, however, was significant ($t = 3.01$, $P < 0.05$). This interaction is best understood by examining Fig. 4(B): the partial residuals of genetic distance (with the effect of biogeography removed) for population pairs separated by both sand and rock habitats showed a generally linear relationship of increasing genetic distance with increased geographical distance. However, the partial regression slope for population pairs separated by sand was greater than the slope for populations separated by rock; this difference in slope is expressed by the statistical significance of the DIST * SAND interaction term. Over increased distance, sandy shoreline reduces genetic interchange among *A. nigricaudus* populations. Similar differences in isolation-by-distance slopes are observed between estruarine and shoreline populations of an atherinid fish (Johnson *et al*. 1994) and a littorinid snail (Johnson & Black 1998b). This intraspecific pattern is also concordant with the observation that many hypothesized sister groups of eastern tropical Pacific blennioids are separated by large stretches of sand (Walker 1960; Rosenblatt 1967) and suggests that, over large distances, sandy coastal areas are partial barriers to gene flow for hard-substrate fishes. The nonsignificant slope of DIST * WATER indicated that the effect due to open water is not contingent upon the geographical distance crossed. Overall, geographical distance, discontinuous habitat and a biogeographic boundary explain 71% of the variation in levels of genetic divergence among *A. nigricaudus* populations.

Extreme genetic partitioning in A. nigricaudus

In *A. nigricaudus*, a very high level of genetic partitioning was often found over relatively small distances (< 200 km; Table 2). Differences among populations explained 48.5% of the variation (one-factor AMOVA, $P < 0.001$), and Hudson *et al*.'s (1992a) permutation test provided strong statistical support for differences between many pairs of populations (Table 2). In fact, the degree of genetic partitioning found in *A. nigricaudus* is higher than that observed for nearly all marine fishes. In *A. nigricaudus* $F_{ST} = 0.536$, where F_{ST} (Hudson *et al*. 1992b) for mtDNA estimates the parameter $1/(2Nm + 1)$, N is the female effective population size and m is the female migration rate. Our estimate of 0.536 corresponds to a value of 0.224 for autosomal loci, assuming a sex ratio of one and equal migration of males and females. This estimate of partitioning is generally higher than reported values for reef fishes based on allozymes (F_{ST} ranges from 0.002 to 0.032, Waples 1987; average $G_{ST} = 0.062$, Ward *et al*. 1994; F_{ST} ranges from 0.0023 to 0.1124, Shulman 1998) or mtDNA (e.g. Φ_{ST} ranges from −0.012 to 0.172, Shulman & Bermingham 1995). Both *Acanthochromis polyacanthus* ($F_{ST} = 0.7919$, Doherty *et al*. 1995) and *Embiotocidae jacksoni* ($F_{ST} = 0.444$, Waples 1987) populations have greater population structure than *A. nigricaudus*, but both also lack a planktonic larval stage. *A. nigricaudus* has a planktonic larval stage, but its duration is unknown. Among fishes that do have a larval stage, the single example of an F_{ST} greater than that observed for

A. nigricaudus is Abrolhos Islands *Craterocephalus capreoli* (F_{ST} = 0.437 over 35 km), although surprisingly F_{ST} was considerably less when *C. capreoli* were sampled over a greater geographical range (F_{ST} = 0.073 over 850 km, Johnson *et al*. 1994).

Two aspects of *A. nigricaudus* life history may contribute to the high degree of genetic partitioning. First, *A. nigricaudus* larvae probably remain close to shore after hatching (Brogan 1994), which might reduce migration. Second, the species range of *A. nigricaudus* is geographically complex, spanning two parallel shorelines, a number of islands and two ecologically distinct regions (Fig. 1). Here, we decomposed some of that geographical complexity into three factors (biogeography, geographical distance and discontinuous habitat) and found that each factor contributed to population divergence. These results suggest that where multiple factors can limit dispersal, a planktonic larval stage does not preclude substantial genetic partitioning over short geographical distances.

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This research is part of a larger project that looks at how extrinsic and intrinsic factors shape genetic variation in Gulf of California blennioid fishes. This work represents part of Cynthia Riginos's PhD dissertation. Michael W. Nachman's research interests include molecular population genetics and the genetics of speciation.
