Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 5197–5201, May 1994
Evolution

Nuclear and mitochondrial gene genealogies and allozyme polymorphism across a major phylogeographic break in the copepod *Tigriopus californicus*

RONALD S. BURTON* AND BANG-NING LEE†

*Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093-0202; and †Department of Biology, University of Houston, TX 77204-5513

Communicated by John C. Avise, February 10, 1994 (received for review October 11, 1993)

ABSTRACT **The genetic structure of natural populations is frequently inferred from geographic distributions of alleles at multiple gene loci. Surveys of allozyme polymorphisms in the tidepool copepod *Tigriopus californicus* have revealed sharp genetic differentiation of populations, indicating that gene flow among populations is highly restricted. Analysis of population structure in this species has now been extended to include nuclear and mitochondrial gene genealogies. DNA sequences of the mtDNA-encoded cytochrome-*c* oxidase subunit I gene from 21 isofemale lines derived from seven populations reveal a phylogeographic break between populations north and south of Point Conception, California, with sequence divergence across the break exceeding 18%, the highest level of mtDNA divergence yet reported among conspecific populations. Divergence between populations based on 22 sequences of the nuclear histone H1 gene is geographically concordant with the mitochondrial sequences. In contrast with previously studied nuclear genes in other sexually reproducing metazoans, the H1 gene genealogy from *T. californicus* shows no evidence of recombination. The apparent absence of intragenic recombinants probably results from the persistent lack of gene flow among geographically separated populations, a conclusion strongly supported by allozyme data and the mitochondrial gene genealogy. Despite strong population differentiation at allozyme loci, the phylogeographic break identified by the DNA sequences was not evident in the allozyme data.**

For the past two decades, analyses of the genetic structure of marine invertebrate populations have relied primarily on surveys of electrophoretically detectable protein polymorphisms (1, 2). Despite numerous exceptions, results of these studies have led to the widely held conclusion that gene flow is more extensive among populations of species with planktonic larvae than in species lacking such dispersal stages. The recent development of molecular techniques is now allowing reexamination of this conclusion with new sets of genetic markers, such as restriction fragment length polymorphisms (RFLPs) and sequence data from nuclear and mitochondrial DNA (e.g., ref. 3). Results have been surprising. For example, based on the relative homogeneity of allozyme frequencies across populations, Buroker (4) concluded that high levels of gene flow characterize population structure in the American oyster *Crassostrea virginica*. However, subsequent mtDNA studies (5) and analyses of anonymous single-copy nuclear DNA (scnDNA) markers (6) revealed a phylogeographic break not apparent in the allozyme data. These molecular data have resulted in two conclusions which contrast markedly from that inferred from allozymes alone: (*i*) the molecular data (mtDNA and scnDNA RFLPs) suggest that there has been a historical break in the geographic distribution of the species and a current impediment to gene flow between certain geographic populations and (*ii*) similarity of allozyme frequencies across this break is more likely due to balancing selection at the allozyme loci rather than due to the homogenizing effect of gene flow (6).

Here we present DNA sequence data in the form of nuclear and mitochondrial gene genealogies sampled from populations of the copepod *Tigriopus californicus*. Previous work on the genetic structure of this species has found marked differentiation in allozyme frequencies between neighboring populations along the California coast (7, 8). Particularly remarkable is the high frequency of "private alleles" (alleles found in only one or a few populations), indicating that interpopulation gene flow in this species is highly restricted (9). The allozyme data are somewhat surprising in light of the natural history of *T. californicus* populations. The supralittoral rock pool habitat of *T. californicus* is subject to periodic drying, leading to extinction of resident *T. californicus*, which has no life stages resistant to desiccation (10). At the other extreme, wave scouring and sand transport into pools during storms can also result in local extinction (R.S.B., unpublished data). These natural history observations suggest that dispersal and recolonization following local extinction are important features of the ecology of the species. However, the high levels of allozyme differentiation suggest that local populations persist long enough to undergo extensive genetic differentiation. Hybrid breakdown of fitness-related traits in the F_2 generation of interpopulation crosses also suggest long-term genetic isolation of populations (11). To address the discrepancy between natural history in ephemeral pools and apparently long histories of population isolation, we initiated DNA sequence comparisons within and between California *T. californicus* populations for both a mitochondrial gene (cytochrome-*c* oxidase subunit I, COI) and a nuclear gene (histone H1).‡

MATERIALS AND METHODS

Over 200 *T. californicus* adults were collected from high intertidal rock pools at each of nine geographic sites along the central and southern California coast (Fig. 1) and maintained in the laboratory as breeding populations in 400-ml beakers. Within 2 weeks of collection and before any mortality was observed among the field-collected animals, allozyme frequencies were determined for eight gene loci by polyacrylamide gel electrophoresis. Protocols for electrophoretic analysis of individual adult *T. californicus* were as previously described (12), using 0.8-mm-thick vertical slab gels of 6% total acrylamide and enzyme staining by standard recipes (13,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: COI, cytochrome-*c* oxidase subunit I.
*To whom reprint requests should be addressed.
‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L31818–L31839 and L31864–L31878).

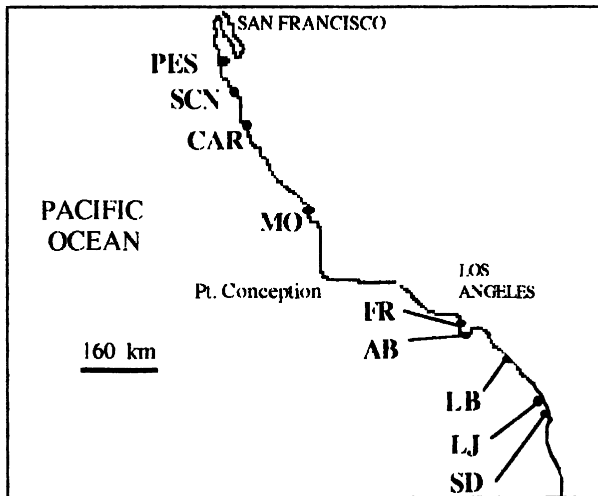

FIG. 1. Map of *T. californicus* collecting sites along the central and southern California coast. PES, Pescadero Beach; SCN, Santa Cruz; CAR, Carmel; MO, Morro Bay; FR, Flatrock Point; AB, Abalone Cove; LB, Laguna Beach; LJ, La Jolla; SD, San Diego.

14). At least 50 individuals were scored per population for each gene locus.

For DNA sequencing, we established isofemale lines from each natural population by isolating single fertilized females in Petri dishes (15 mm × 100 mm) and subsequently inbreeding the progeny. This procedure was adopted after initial attempts to amplify COI alleles from DNA extracted from single *T. californicus* adults proved inconsistent. Because mtDNA is typically maternally inherited in animals and levels of heteroplasmy are generally low, each isofemale line was expected to have only a single mtDNA haplotype. Although each line could contain as many as four alleles (two maternal and two paternal) for any nuclear gene, most lines were inbred for two or more generations before DNA sampling for H1 sequencing. Observed ambiguity in H1 sequences potentially due to multiple haplotypes segregating within isofemale lines was low, ranging from zero (13 lines) to five (2 lines) nucleotide positions per reported sequence. Since only three instances of ambiguity were observed at informative sites in the entire H1 data set (total of 22 sequences with 51 informative sites), the impact of within-line polymorphism can, for the purposes of this study, be ignored.

Total genomic DNA samples were obtained from groups of 15–20 adults (wet weight of an individual adult is ≈35 μg) from a single isofemale line (15). Animals were homogenized in 100 μl of 100 mM Tris/100 mM NaCl/200 mM sucrose/100 mM EDTA at pH 9.1, treated with proteinase K (100 μg/ml) for 30 min at 65°C, and then extracted once with phenol, once with phenol/chloroform (1:1), and once with chloroform/isoamyl alcohol (24:1). DNA was precipitated by addition of 0.1 volume of 3 M sodium acetate and 2 volumes of 100% ethanol and suspended in 1 mM Tris/0.1 mM EDTA at pH 8.0. Concentrations of DNA were determined by absorbance at 260 nm. Primer sequences for gene amplification and direct sequencing of the COI gene were as follows [positions in the *Drosophila yakuba* mtDNA sequence (16) are noted]: primer COIK (5′-GAGCTCCAGATATAGCATTCC-3′), 1730–1750; primer COIJ; (5′-CAATACCTGTGAGTCCTCCTA-3′), 2536–2516; primer COIL, (5′-TGAGAGATTATTC-CAAATCC-3′), 2234–2215; primer COID, (5′-AAACCAAC-TGTGAACATGTG-3′), 2357–2338. Primers K and J were designed by R. Van Syoc (California Academy of Sciences). Biotinylated COIK was paired with either COID or COIJ for gene amplification by the polymerase chain reaction (PCR) using Perkin–Elmer's GeneAmp PCR reagent kit (with 10–100 ng of template DNA) and the following thermocycle profile: 94°C, 1 min; 50°C, 1 min; 72°C, 2 min, for 35 cycles followed by 5 min at 72°C. PCR products were electrophoresed in a 2% agarose gel and extracted from the agarose with Geneclean (Bio 101). Solid-state sequencing (15) of the biotinylated strand with primers COIL and COID used Sequenase protocols (United States Biochemical) after capture of the biotinylated strand on streptavidin-coated magnetic beads (Dynal, Great Neck, NY).

A previously published sequence of a histone H1 gene from *T. californicus* (17) was used to design primers for PCR amplification and sequencing of a fragment including the 5′ end of the H1 coding region. Primers used were H1.5 (5′-ATATGTGTCGAATCGAGGGC-3′, positions 137–156 in the published sequence) and H1.3 (5′-TCTCGACCAAG-GACTTG-3′, positions 710–694). DNA samples used for H1 PCR were prepared by boiling 15 animals from a single isofemale line in 200 μl of 10% chelating resin (Sigma) for 8 min; after boiling, samples were vortexed for 10 sec and centrifuged (13,000 × *g*) for 2 min at 4°C (18). Ten microliters of the supernatant was used as template in the PCR, which used the same thermocycle profile as COI PCR. PCR products were purified with Promega's Magic PCR Preps, and direct sequencing was carried out using the *fmol* Cycle sequencing kit (Promega) and manufacturer's protocols with ^{32}P-end-labeled primers. When possible, the same isofemale lines were analyzed for both COI and H1 sequence. Unfortunately, much of the COI sequencing had been completed and a number of the isofemale lines had been lost before the H1 sequencing project was initiated. As a result, both sequences were obtained for only eight lines.

RESULTS

As has been previously observed, populations of *T. californicus* show sharp differentiation in allozyme frequencies (Table 1). This is particularly evident among the southern California populations, where fixation of alternative alleles occurs at three of the eight loci studied (APK2, GOT2, and ME). Nei's genetic distances among populations based on the allozyme data (including the monomorphic MDH and APK1 loci) were used to construct the UPGMA tree shown in Fig. 2, which was generated with the GENDIST and NEIGHBOR programs in PHYLIP (J. Felsenstein, PHYLIP version 3.4, University of Washington, Seattle, 1991). Although genetic distances among the central California populations are small, significant allelic frequency differences at one or more loci allow discrimination of all four (PES, SCN, CAR, MO) of these populations: the PGI^F allele is found only at PES, the GPT^F allele is restricted to SCN, and the GOT1^{VS} allele is significantly higher in frequency at MO than elsewhere. The geographic restriction of the PGI and GPT alleles has now been documented for >13 years (7).

Direct sequencing of PCR products yielded 500 bases of sequence from the coding region of the mitochondrial COI gene from 21 isofemale lines. The sequence corresponds to positions 1756–2255 in the homologous *D. yakuba* mtDNA sequence (16). Sequences were used to construct a maximum-parsimony gene tree (Fig. 3*A*) with phylogenetic analysis using parsimony (D. L. Swofford, PAUP 3.0r, Illinois Natural History Survey, Champaign, 1989). The most striking feature of the COI data is the extremely high level of divergence between the central and southern California clades. A minimum of 18% sequence divergence separates these clades. Verification of the mitochondrial nature of the sequenced PCR products was obtained by making reciprocal crosses between differentiated isofemale lines and sequencing the mtDNA of F_1 progeny. Such studies verified maternal inheritance for the PCR-generated COI fragment used in our sequence analyses (20). We also note that of 90 nucleotide

Table 1. Allelic frequencies for six polymorphic allozyme loci in eight populations of *T. californicus* along the California coast

Population	PGI			GOT2				ME				GOT1					GPT			APK2	
	F	M	S	VF	F	S	VS	VF	F	S	VS	F	S	VS	ES	SS	F	S	VS	F	S
PES	0.46	0.54				1.00				1.00			1.00					1.00			1.00
SCN		1.00				1.00				1.00		0.03	0.97				0.25	0.75			1.00
CAR		1.00				1.00				1.00			0.98	0.02				1.00			1.00
MO		1.00				1.00				1.00			0.80	0.20				1.00			1.00
FR		1.00				1.00				1.00		0.04	0.93	0.03				1.00			1.00
AB		1.00				0.99	0.01			0.98	0.02		0.07		0.93			1.00			1.00
LB	0.02	0.79	0.19	0.40	0.60			1.00				0.11	0.85			0.04		1.00		1.00	
LJ	0.06	0.94			1.00				1.00			0.19	0.79	0.02				1.00		1.00	
SD	0.22	0.78		1.00					1.00				0.88	0.12				0.94	0.06	1.00	

Allelic designations VF, F, M, S, VS, ES, and SS are in order of decreasing anodal mobility. Two additional loci, APK1 and MDH, were monomorphic. Locus designations are as follows: PGI, phosphoglucose isomerase; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; APK, arginine phosphokinase; and ME, NADP$^+$-dependent malate dehydrogenase (EC 1.1.1.40).

substitutions (of the 500 bases sequenced) distinguishing the two major clades, only three result in diagnostic amino acid substitutions (6 amino acid residues are variable of the 165 inferred from sequence data). These data clearly suggest that we were not inadvertently sequencing a pseudogene. A second remarkable feature of the COI data is the extreme divergence among the San Diego (SD) isofemale lines; the SD4 line differs from SD40 at 77 nucleotide sites (15.4%), and all these substitutions are synonymous. Although more extensive within-population sampling will require further work, the divergence at the SD site stands in marked contrast to the low levels of divergence (<1%) observed among isofemale lines from six other populations.

To assess whether or not the phylogeographic break observed in the COI data was reflected in the nuclear genome, we also sequenced ≈500 bases of a histone H1 gene, including ≈150 bases of the coding sequence and ≈350 bases of 5′ flanking region (17). PCR products from this region showed a size polymorphism, with template DNA from all 11 isofemale lines from southern California sites (SD, LJ, LB, AB, FR) producing larger PCR products than that from the four central California sites (MO, CA, SC, PES). Subsequent sequencing showed that two insertions (42–48 and 20 bases in length and ≈200 bases apart) in the 5′ flanking region were found only in the southern California samples (R.S.B., unpublished data). After elimination of these insertions/deletions from the data set, an additional 23 of 425 bases (5.4%) distinguish between central and southern California clades (Fig. 3*B*). Within the coding portion of the sequence, only one nonsynonymous substitution was observed, and it distinguishes all central from all southern sequences.

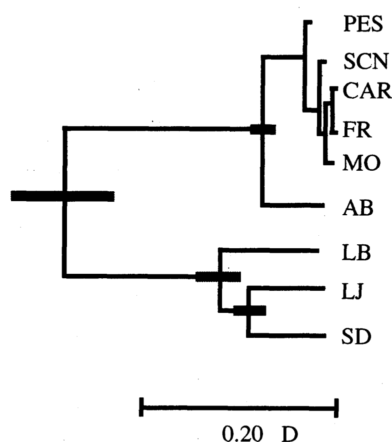

FIG. 2. Relationships among *T. californicus* populations based on allozyme frequencies presented in Table 1. The unrooted tree was constructed by applying the unweighted pair-group method with arithmetic mean (UPGMA) algorithm to the data following calculation of Nei's genetic distance from the allozyme frequencies. Scale is in units of Nei's *D*. Standard errors of major nodes were based on the jacknife method of Mueller and Ayala (19).

The two sets of sequence data are concordant with regard to the geographic position of a major phylogenetic break in *T. californicus*. In contrast, allozyme data and associated genetic distances (Fig. 2) from the same populations reveal a different (but well supported) genetic/geographic break in which the Los Angeles area populations (AB and FR) have closer affinities to the central California populations (maximum observed Nei's $D < 0.16 \pm 0.02$) than to the more southern California populations (minimum observed $D > 0.48 \pm 0.04$). Of eight allozyme loci studied (six polymorphic), there are no shared alleles between the Los Angeles (LA) populations (includes AB and FR samples) and any population to the south for three loci (GOT2, ME, and APK2).

DISCUSSION

Geographic populations of *T. californicus* are characterized by high levels of genetic differentiation (1, 7, 8). By using one or more of the six polymorphic allozyme loci, most of the study populations are easily distinguished from one another. However, because allozyme polymorphism in this species tends to be geographically restricted and frequently includes private alleles, relationships among populations are not readily apparent. One goal of the present study was to determine interpopulation relationships from DNA sequence data. In this regard, the most striking feature of the mtDNA data is the great divergence between central and southern California clades. Minimum distance between COI sequences from the two clades exceeds 18%, a value that far exceeds any previously reported intraspecific value for a protein-encoding mitochondrial gene (21, 22). Although there is no fossil record for establishing a molecular clock directly relevant to *T. californicus* populations, recent data on the COI gene in other crustacea yielded estimates of 2.2–2.6% divergence per million years (23); at such a rate of sequence evolution, a conservative date for the split between central and southern COI clades is on the order of 7 million years.

In addition to the major phylogeographic break apparent in both DNA data sets, the H1 data clearly discern structure within the set of southern populations. Los Angeles area populations (AB and FR) are well separated from those to the south (minimum sequence divergence of >2.5%), and the three SD sequences are distinct from the three LJ and LB sequences (2.0% divergence). These groupings are all robust to bootstrap resampling (relevant nodes were retained in 99–100% of 100 bootstrap replications). In contrast, no structure is apparent among the central California populations, which are characterized by low levels of within- and

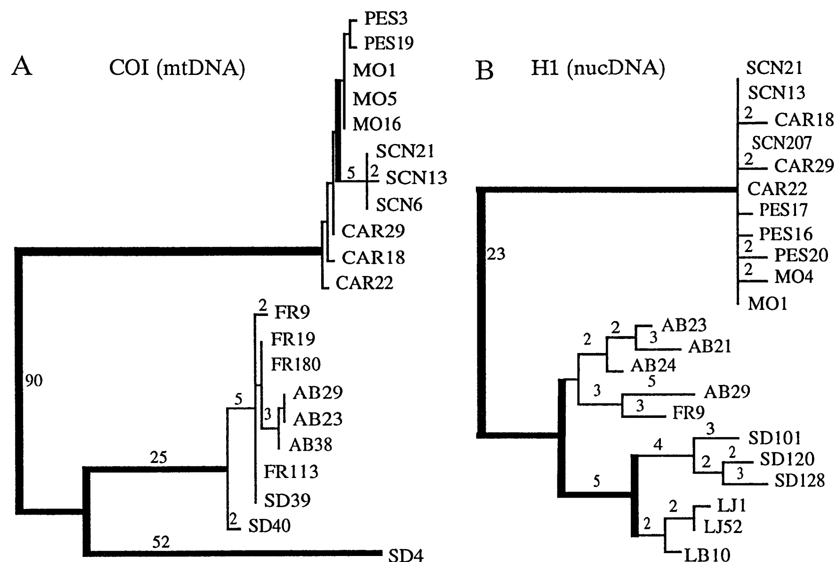

FIG. 3. Genetic relationships along *T. californicus* populations and isofemale lines. (*A*) Unrooted maximum-parsimony tree produced by the heuristic search procedure of PAUP 3.0r for 21 COI sequences. Sequences are named according to source population and numbers refer to the specific isofemale line. Branch lengths are the absolute number of nucleotide substitutions over the 500-base sequences. Tree shown is one of 5 equally parsimonious trees; all having similar topology. Bold lines indicate branches supported by bootstrap values >95%. (*B*) Unrooted maximum-parsimony tree produced by the heuristic search procedure of PAUP 3.0r for 22 H1 sequences. Sequences are named as in *A*. Branch lengths are absolute number of nucleotide substitutions over the 425 bases sequenced (after exclusion of gaps). Tree is one of 12 similar, equally parsimonious trees. Bold lines indicate branches supported by bootstrap values >95%.

between-population variation. Interestingly, the COI data do not resolve these finer relationships within the southern populations. However, the large divergence between haplotypes observed at SD warns that the present data set is probably too small to come to firm conclusions about within-region population structure.

Because interpretation of nuclear gene genealogies may be complicated by intragenic recombination, their use in analysis of population structure has been limited. Bernardi *et al.* (24) have recently reported that the nuclear lactic dehydrogenase B (LDH-B) gene genealogy of *Fundulus heteroclitus* is concordant with that of the mitochondrial cytochrome *b* gene, with a phylogenetic break along the Atlantic coast of North America reflected in both gene genealogies. However, the genealogies are based on relatively few informative nucleotide sites, and phylogeographic distinction of northern and southern alleles was strongly supported (i.e., bootstrap values >90%) only when analysis was restricted to the two extreme localities, Florida and Nova Scotia. Furthermore, >30% of alleles sampled in an intermediate New Jersey population are reportedly recombinants between two nonsynonymous substitutions at nucleotide positions <400 bases apart. Hence, the LDH-B nucleotide data from *Fundulus* had little power for inferring population structure.

In contrast, the H1 gene genealogy for *T. californicus* showed substantially more variation and geographic structuring, probably because two-thirds of the DNA fragment studied was upstream flanking region rather than coding region. In any case, no evidence for intragenic recombination has been observed to date. Given our relatively small sample of 11 central California and 11 southern California alleles, we can only conclude that if recombinant alleles do occur, they are unlikely to be common over the geographic range studied here. The most straightforward interpretation of this finding is that divergence of H1 alleles has occurred allopatrically and in the absence of gene flow, making recombination between alleles from different clades impossible. This simple explanation is consistent with the COI genealogy, which (again based on limited sampling) gives no indication of geographic mixing of central and southern California clades. Although the allozyme data do not show significant differentiation in the region between Morro Bay and Los Angeles, the frequent occurrence of alternative fixation of alleles among the southern California populations gives strong support to the hypothesis of long-term restriction of interpopulation gene flow. The phylogeographic break occurs with a region that includes Point Conception, a widely recognized zoogeographic boundary (25). The geographic concordance between the two sets of sequence data suggests that population divergence has proceeded for a sufficiently long time that concordant lineage sorting of mitochondrial and nuclear genes has taken place (26).

The concordance between nuclear and mitochondrial gene genealogies in *T. californicus* is similar to the nuclear/mitochondrial concordance reported in the oyster system (6) discussed above. However, *T. californicus* allozyme data differ dramatically from those observed in the *Crassostrea* studies (4). While individual oyster populations were typically polymorphic at several allozyme loci and found to be relatively homogeneous across broad geographic ranges, *T. californicus* populations show low levels of within-population variation and sharp differences in allelic frequencies between populations. Both attributes of allozyme variation suggest that *T. californicus* has historically small effective population sizes leading to frequent loss of genetic variants. Although this conclusion is not unexpected, given the ephemeral nature of the habitat of *T. californicus*, it is surprising in light of the enormous number of individuals typically observed in field populations (10). What is more striking is the fact that the greatest observed divergence between populations at allozyme loci (assessed by Nei's genetic distance) does not correspond geographically with the phylogeographic break identified by both gene genealogies. Both Los Angeles populations (AB and FR) appear to show strong allozyme similarity to central California populations, whereas all DNA sequences (total of seven COI and five H1 sequences for AB and FR) are unambiguously members of southern clades. Given that the H1 gene is presumably transmitted in a manner identical to that of the allozymes, neither mode of transmission nor differences in

the effective population size between nuclear and mitochondrial genomes can account for the lack of geographic concordance between the gene genealogies and the allozyme data.

Other hypotheses to explain the discordance between allozymes and gene genealogies include the following: (*a*) the relatively lower resolving power of protein electrophoresis relative to DNA sequencing results in artifactual uniformity of allozymes (i.e., though not evident on our gels, substantial genetic differentiation actually exists at the allozyme loci among these populations); (*b*) natural selection has favored the maintenance of central California allozymes in Los Angeles populations while mtDNA and other nuclear genes have differentiated; and (*c*) differential introgression of central California allozymes (probably promoted by selection) has taken place during secondary contact of animals from central California with already differentiated Los Angeles populations. None of these hypotheses can be rejected with available data. Evaluation of hypothesis *a* requires sequence data for the allozyme loci themselves, which are not currently available. With regard to *b* and *c*, it is of interest that despite the allopatric nature of the study populations, laboratory studies have shown that all interpopulation crosses attempted among them [with a single exception (11)] have produced fertile F_2 progeny, indicating maintenance of reproductive compatibility over probably millions of years of geographic isolation (8, 11, 12). However, allozyme analyses of F_2 hybrid progeny showed sharp departures from Mendelian expectations due to genotype-specific viability differences (27), with allozymes from the more northern populations having higher relative viability than those from the southern population. Such fitness relationships would predict the introgression of northern alleles into southern populations if secondary contact between differentiated populations had occurred and are consistent with the currently observed pattern of genetic variation.

The gene genealogies presented here enhance our understanding of *T. californicus* population structure in two ways. First, the DNA sequence data clearly establish the existence of distinct central and southern California clades with an apparent geographic boundary somewhere between Los Angeles and Morro Bay. Second, the genealogies provide a framework within which we can sometimes infer population relationships. For example, despite their geographic proximity, the AB and FR populations differ dramatically in allelic frequencies at the GOT1 locus, with the $GOT1^{ES}$ allele having a frequency of 0.93 at the AB site and being absent entirely from the FR sample. The observed distribution of the $GOT1^{ES}$ allele could be the result of *in situ* population differentiation, with the allele arising at AB (or FR), achieving its high frequency at AB by selection or drift, and failing to spread to FR (or being lost from FR sometime in the past). Alternatively, the observed differentiation could be the result of the two sites having been colonized from genetically different source populations. Since both nuclear and mitochondrial gene genealogies show that FR and AB sequences are closely related, DNA sequence data strongly support the *in situ* differentiation hypothesis. This same argument applies to neighboring, genetically differentiated populations all along the coast: the gene genealogies imply that neighboring populations share phylogenetic history despite significant allozyme differentiation. Conversely, although allozymes have frequently proven to be sensitive indicators of *T. californicus* population structure, they do not appear to provide reliable insight into the phylogenetic relationships among these conspecific populations.

We thank D. Brown and D. Wells for H1 primers and help with the histone sequencing and M. Haygood and R. Van Syoc for COI primers and help with the COI sequencing. The thoughtful comments of J. C. Avise, D. Brazeau, H. Ganz, M. Hellberg, B. Palenik, W. Swanson, and two anonymous reviewers greatly improved the manuscript. This work was supported by National Science Foundation Grants BSR-8741308, DCB-8811227, and IBN-92-96185.

1. Burton, R. S. (1983) *Mar. Biol. Lett.* **4,** 193–206.
2. Hedgecock, D. (1986) *Bull. Mar. Sci.* **39,** 550–564.
3. Palumbi, S. R. (1992) *Trends Ecol. Evol.* **7,** 114–118.
4. Buroker, N. E. (1983) *Mar. Biol.* **7,** 99–112.
5. Reeb, C. A. & Avise, J. C. (1990) *Genetics* **124,** 397–406.
6. Karl, S. A. & Avise, J. C. (1992) *Science* **256,** 100–102.
7. Burton, R. S. & Feldman, M. W. (1981) *Evolution* **35,** 1192–1205.
8. Burton, R. S. (1986) *Bull. Mar. Sci.* **39,** 526–535.
9. Slatkin, M. (1985) *Evolution* **39,** 53–65.
10. Vittor, B. A. (1970) Thesis (Univ. of Oregon, Eugene).
11. Burton, R. S. (1990) *Evolution* **44,** 1814–1822.
12. Dill, M. M. & Burton, R. S. (1984) *Biochem. Genet.* **22,** 339–347.
13. Harris, H. & Hopkinson, D. A. (1976) *Handbook of Enzyme Electrophoresis in Human Genetics* (North-Holland, Amsterdam).
14. Bulnheim, H. P. & Scholl, A. (1981) *Mar. Biol.* **64,** 105–115.
15. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds. (1992) *Short Protocols in Molecular Biology* (Wiley, New York), 2nd Ed.
16. Clary, D. O. & Wolstenholme, D. R. (1985) *J. Mol. Evol.* **22,** 252–271.
17. Brown, D., Cook, A., Wagner, M. & Wells, D. (1992) *DNA Sequence* **2,** 387–396.
18. Walsh, P. S., Metzger, D. A. & Higuchi, R. (1991) *BioTechniques* **10,** 506–513.
19. Mueller, L. D. & Ayala, F. J. (1982) *Genet. Res.* **40,** 127–137.
20. Lee, B.-N. (1993) Ph.D. dissertation (Univ. of Houston, Texas).
21. Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., Reeb, C. A. & Saunders, N. C. (1987) *Annu. Rev. Ecol. Syst.* **18,** 489–522.
22. Harrison, R. G. (1989) *Trends Ecol. Evol.* **4,** 6–11.
23. Knowlton, N., Weigt, L. A., Solorzano, L. A., Mills, D. K. & Bermingham, E. (1993) *Science* **260,** 1629–1632.
24. Bernardi, G., Sordino, P. & Powers, D. A. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9271–9274.
25. Valentine, J. W. (1966) *Limnol. Oceanogr.* **11,** 198–211.
26. Neigel, J. E. & Avise, J. C. (1986) in *Evolutionary Processes and Theory*, eds. Karlin, S. & Nevo, E. (Academic, Orlando, FL), pp. 515–534.
27. Burton, R. S. (1987) *Evolution* **41,** 504–513.