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INTRASPECIFIC GENETIC DIFFERENTIATION IN CALIFORNIA SEA LIONS (*ZALOPHUS CALIFORNIANUS*) FROM SOUTHERN CALIFORNIA AND THE GULF OF CALIFORNIA

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ABSTRACT

Intraspecific patterns of mitochondrial DNA sequence variation were determined among California sea lions (*Zalophus californianus californianus*) from three colonies along the Pacific coast of southern and Baja California and one colony in the Gulf of California. We found no variation in 368 base pairs (bp) of cytochrome b sequence among 40 sea lions from these localities, but analysis of 360 base pairs of control region revealed eleven genotypes. The four genotypes found in the Gulf of California population were unique and phylogenetically distinct from those found in sea lions along the Pacific coast. The average sequence divergence between Gulf and Southern California genotypes was 4.3%, suggesting a relatively long period of isolation. However, colonies along the Pacific coast, which are less than 200 km apart, shared mtDNA genotypes, indicating that recent genetic exchange has occurred between them. Therefore, we suggest that regional female philopatry exists in California sea lions. Regional boundaries may be related to oceanic currents or patchiness in the distribution of resources. Further research is needed to better understand the underlying causes of genetic differentiation in the California sea lion.

Key words: California sea lion, *Zalophus californianus,* phylogeography, population structure, mtDNA sequence variation, hypervariable control region, cytochrome b.

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Three distinct subspecies of sea lions (*Zalophus californianus*) are recognized; one is a disjunct breeding population from the Galapagos Islands (*Z. c. wollebaeki* or the Galapagos sea lion), a second is found in Baja California and along the western coast of North America (*Z. c. californianus* or the California sea lion), and the third occurs in the coastal waters of Japan and Korea (*Z. c. japonicus* or the Japanese sea lion, Reeves *et al.* 1992). The latter subspecies is thought to be extinct and there is no evidence of intermixing of sea lions between the other two (Reeves *et al.* 1992). Adult, subadult, and some juvenile California sea lions migrate between southern rookeries and northern foraging areas in the Gulf of California and along the coasts of northern California, Oregon, Washington, and British Columbia. However, it is not known whether sea lions from the Gulf of California and the Pacific coast colonies mix when breeding or foraging (Aurioles *et al.* 1983, Reeves *et al.* 1992).

Previous studies of mitochondrial DNA polymorphisms have shown that populations within species are often connected in a phylogenetic network that reflects the geographic distance between populations or the presence of topographic boundaries (Avise *et al.* 1987). That pattern, termed phylogeographic partitioning, is common in terrestrial vertebrates and has only been described for a few marine species (Bowen *et al.* 1992, Avise 1992, Baker *et al.* 1993).

We analyzed variation in cytochrome b and control region mtDNA sequences that are known to have moderate and rapid rates of evolution, respectively, and used phylogenetic methods to determine the extent of gene flow among colonies of California sea lions in the Gulf of California and along the Pacific coast of Baja and Southern California. The rapidly evolving 360 base pair (bp) sequence that we analyzed is found in the mitochondrial control region, a non-transcribed region of about 1,200 base pairs (bp) in mammals that evolves three to five times faster than the average rate of mitochondrial DNA sequence (Aquadro and Greenberg 1983; Vigilant *et al.* 1989, 1991; Horai and Hayasaka 1990). Phylogenetic analysis of mitochondrial control region sequences has revealed distinct phylogeographic structure within several vertebrate species (Baker *et al.* 1993; Brown *et al.* 1993; Wenink *et al.* 1993, 1994). The more slowly evolving region that we analyzed is a 368 bp fragment of the cytochrome b gene, a protein coding gene that has been used extensively for systematic studies of mammals (*e.g.,* Irwin *et al.* 1991). The use of sequences that evolve at different rates provides a basis for comparison with estimates of genetic differentiation in other vertebrate species.

Materials and Methods

Populations sampled—We collected 56 samples of skin and muscle from sea lion pup carcasses (Southern California Channel Islands) and from live juveniles from the Gulf of California (Fig. 1). DNA from 40 of those individuals was successfully amplified and used in the sequence analysis as follows: San Nicolas Island, California (n = 17); San Miguel Island, California (n = 12); Punta Banda, Baja California, Mexico (n = 3); and Bahía de los Angeles (Gulf of

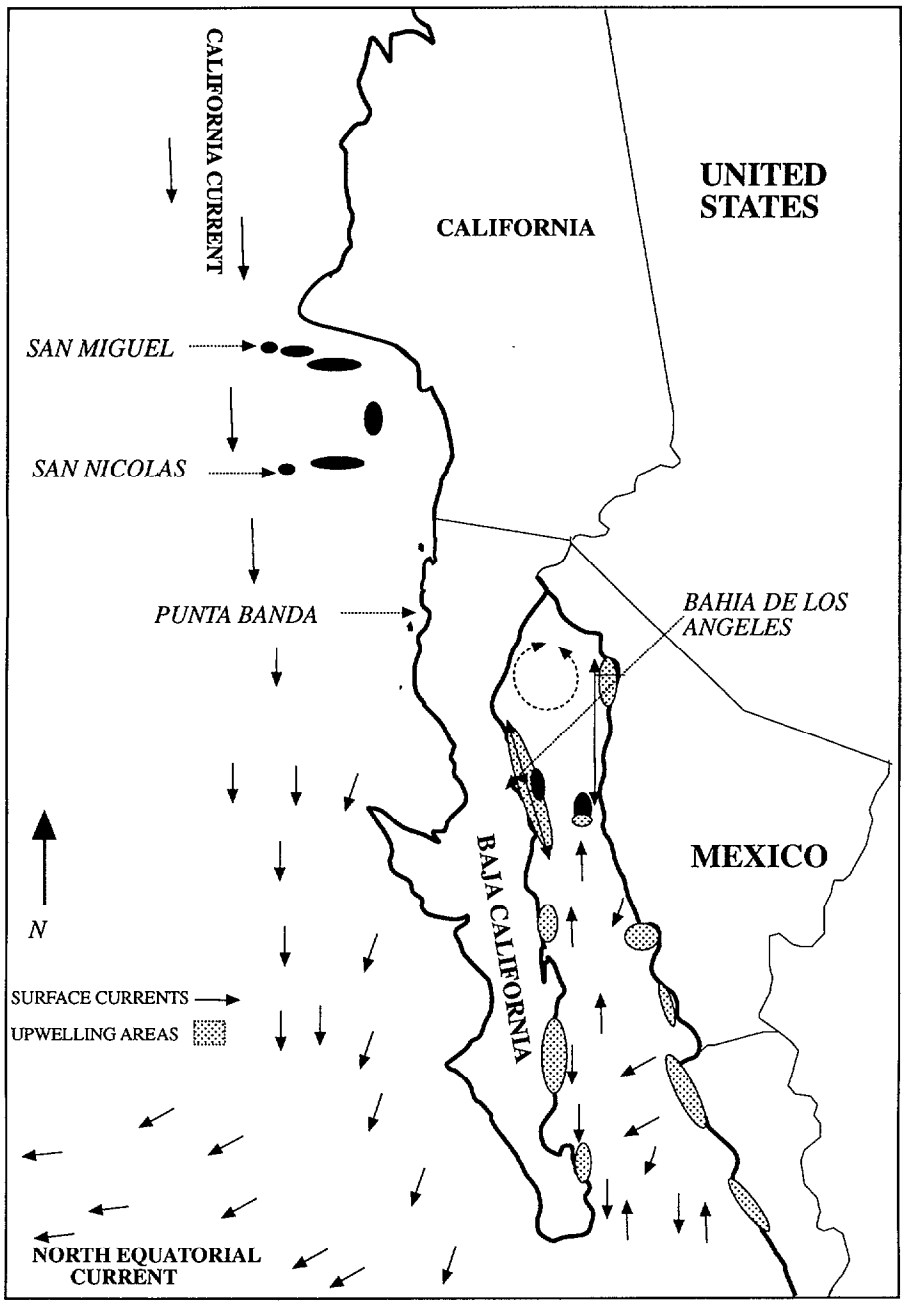

Figure 1. Surface currents and upwelling areas in the Gulf of California and the major currents of the Pacific Ocean. Sites of sample collection are labelled in italics (Adapted from Maluf 1983).

California), Baja California (n = 8). Tissue samples from live, physically restrained sea lions were obtained with a hand-held biopsy dart (*cf.* Lambersten 1987, Mathews *et al.* 1988, Whitehead *et al.* 1990) and stored at −70°C.

Amplification and sequencing of mtDNA—Tissue samples were minced with a sterile dissecting blade on parafilm over a glass petri dish and were then transferred into a 1.7-ml Eppendorf tube containing 500 μl of 1× TNE pH 8.0. Genomic DNA was isolated by proteinase K digestion, followed by extraction with phenol-chloroform-isoamyl alcohol, precipitated with ethanol and resuspended in TE pH 8.0 to yield a final concentration of about 1 μg/μl (Sambrook *et al.* 1989). Two sets of universal primers were used to amplify a 398 bp region of the mitochondrial cytochrome b gene (L14724, H15149; Kocher *et al.* 1989) and a 394 bp of the control region (L15926: 5′-GAATTCCCCGGTCTTGTAAACC-3″ and H16340: 5′-CCTGAAGTAGGAACCAGATG-3′; modified from Kocher *et al.* 1989) by the polymerase chain reaction (PCR). Each PCR reaction mixture contained approximately 100 ng of genomic DNA and 1 mM dNTP mix in a reaction buffer of 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris HCl (pH 8.8), and 2.5 units of Taq DNA polymerase in a volume of 50 μl. The double-stranded amplification contained 25 pmoles of each primer and forty cycles of amplification were run in a programable Perkin-Elmer Cetus DNA thermal cycler as follows: denaturation at 94°C for 45 sec, annealing at 60°C for 30 sec, and extension at 72°C for 45 sec. The double-stranded products were separated in a 2% Nusieve (FMC Corporation, Rockland, MD) agarose gel in TAE buffer and stained with ethidium bromide. The appropriate band was excised, then purified using a Geneclean Kit from BIO 101. The sequence protocol used in this research was a modification based on the Sanger method with technical specifications according to Sambrook *et al.* (1989) and utilized a USB Sequenase kit. The sequencing reaction products were separated by electrophoresis in 6% polyacrylamide gels for 3–4 h at 55W in a Stratagene BaseAce sequencing apparatus. Sequence data are available at Genbank (accession numbers L37020–L37032).

Phylogenetic analysis and population structure—To analyze the sequence data we chose to use the unweighted maximum parsimony approach using sequences from the Steller sea lion (*Eumetopias jubatus*) as an outgroup. The branch-and-bound algorithm of PAUP (Phylogenetic Analysis Using Parsimony) version 3.1.1 for the Apple Macintosh was used to determine the most parsimonious tree (Swofford 1993). The statistical confidence of each node was judged by assessing the frequency of nodes supported in 1,000 bootstrap resamplings of our data (Felsenstein 1985). As an alternative phylogenetic approach, we used the maximum likelihood program (DNAML) of PHYLIP modified for the Apple Macintosh computer (Felsenstein 1993). This analysis attempts to identify the tree that has the highest likelihood of yielding the sequence data given a probabilistic model of sequence evolution. We used the empirically determined frequencies of nucleotides and an average transition/transversion ratio determined by pairwise comparisons of all taxa. Finally, the genetic distance between genotypes was estimated by the two-parameter model of Kimura (1981) which was then used to construct a neighbor-joining tree (Saitou and Nei 1987).

Results

Mitochondrial DNA variation and sequence divergence—We sequenced the DNA of all individuals for 368 bp of the cytochrome b gene and 360 bp of the control region. The cytochrome b sequence was conserved in California sea lions; there was no variation detected in the four colonies sampled. In contrast, the control region sequence was highly variable and we found eleven genotypes among the 40 individuals sampled (Table 1). The number of substitutions between California sea lion control region genotypes ranged from 2 to 18 (0.6% to 5.9%). The number of substitutions between Steller sea lion and California sea lion genotypes was about two to three times greater than the maximum observed within the latter species, ranging from 40 to 47 (12.9% to 15.1%). The average pairwise transition to transversion ratio between California sea lion genotypes was 2.6, and 2.3 between California and Steller sea lion genotypes (Table 1). Seven genotypes occurred in the three Pacific coast colonies of California sea lions and four were found in the Gulf of California population (Table 2). Three of the seven genotypes found in the Pacific coast were shared among San Nicolas, San Miguel, and Punta Banda colonies (PB2, PB3, SN1), and 3 were unique to the San Miguel Island colony (SM28, SM32, SM34). These three unique San Miguel genotypes were not common, being found only in single individuals. The Gulf and Pacific coast populations did not share any genotypes.

Within the four colonies the average nucleotide diversity (Nei 1987) was similar and ranged from 0.405 to 1.708 for Pacific coast colonies and 1.021 for the Gulf colony. However, the average pairwise sequence divergence was much greater between individuals from Gulf and Pacific coast populations than between individuals from Pacific coast colonies. Divergence values ranged from 1.1% to 1.4% among individuals for different Pacific coast populations and 4% and 5.3% between individuals from the Pacific and the Gulf colonies. In comparison, sequence divergence for the same segment of the control region for two species of true seals in the same subfamily, *Phoca vitulina* (harbor seal) and *Halichoerus grypus* (grey seal), is 9.3% (Árnason and Johnson 1992; Árnason, personal communication).

The division between Pacific coast and Gulf colonies was also apparent in the phylogenetic analysis of sequence data using parsimony, maximum likelihood, and neighbor-joining analyses (Fig. 2). All three phylogenetic methods support the division of the 11 genotypes into two major clades, one including 7 genotypes from the three Pacific coast colonies and the other containing the 4 genotypes found in the Gulf colony. Bootstrap resampling values significantly cluster Pacific Coast genotypes in a clade separate from Gulf genotypes. However, within these clades, the branching order of genotypes is poorly resolved and varies among the group of most parsimonious trees (Fig. 2).

Discussion

The Gulf of California population has unique control region genotypes not found in the three Pacific coast colonies that we sampled. Gulf and Pacific coast

Table 1. Kimura two-parameter divergence values (%) between selected mtDNA genotypes (above diagonal) and number of base substitutions (below diagonal). Numbers in parentheses indicate the observed number of transversions.

		Gulf of California				Pacific Coast						
	Steller	G1	G2	G3	G5	SN1	PB3	SM26	PB2	SM28	SM32	SM34
Steller	—	14.1	12.9	14.5	15.1	15.1	14.5	14.2	13.9	13.6	15.1	14.2
G1	44 (13)	—	1.9	1.9	2.2	4.8	4.2	4.2	4.5	3.9	5.6	4.2
G2	40 (10)	6 (3)	—	1.6	3.5	4.2	3.5	3.5	3.9	3.2	4.9	4.2
G3	45 (11)	6 (2)	5 (1)	—	3.5	5.1	4.5	4.5	4.8	4.8	5.9	4.5
G5	47 (13)	7 (2)	11 (3)	11 (4)	—	4.5	3.8	3.8	4.2	3.9	4.9	4.5
SN1	47 (14)	15 (7)	13 (4)	16 (5)	14 (5)	—	0.6	1.3	1.9	2.9	2.3	1.9
PB3	45 (13)	13 (6)	11 (3)	14 (4)	12 (4)	2 (1)	—	0.6	1.3	2.3	1.6	1.3
SM26	44 (12)	13 (5)	11 (2)	14 (3)	12 (3)	4 (2)	2 (1)	—	0.6	1.6	2	0.6
PB2	43 (11)	14 (4)	12 (1)	15 (2)	13 (2)	6 (3)	4 (2)	2 (1)	—	1.6	2.6	0.6
SM28	42 (12)	12 (6)	10 (3)	15 (4)	12 (4)	9 (3)	7 (2)	5 (1)	5 (2)	—	3.3	1.6
SM32	47 (12)	17 (6)	15 (3)	18 (4)	15 (4)	7 (2)	5 (1)	6 (1)	8 (2)	10 (1)	—	2.6
SM34	44 (13)	13 (4)	13 (3)	14 (2)	14 (4)	6 (3)	4 (2)	2 (1)	2 (2)	5 (2)	8 (2)	—

Table 2. California sea lion (*Zalophus californianus*) genotype distribution and abundance at the four sampling localities.

	Pacific Coast			Gulf of California	
Genotypes	Punta Banda	San Nicolas	San Miguel	Bahía de los Angeles	Total
PB2	1	1	1		3
PB3	1	2	3		6
SN1	1	12	4		17
SM26		2	1		3
SM28			1		1
SM32			1		1
SM34			1		1
G1				1	1
G2				3	3
G3				3	3
G5				1	1
Total	3	17	12	8	40

genotypes define two phylogenetically distinct clades that differ on average by 4.4% in DNA control region sequence suggesting a long history of isolation. In contrast, gene flow among Pacific coast colonies is substantial and, with the exception of San Miguel Island which has three individuals each with a unique genotype, no other genotypes are unique to a single Pacific coast colony (Orta 1993). Although substitution rates may be higher for the control region in humans and some other vertebrates (Vigilant 1989, 1991; Wenink *et al.* 1993), the rate for primates, rodents and cetaceans appears to be similar to that of other regions in the mammalian genome (Hoelzel *et al.* 1991). Independent divergence rate estimates for odontocete and mysticete whales suggest rates of 0.5%–1.0% per million years (Hoelzel *et al.* 1991, Baker *et al.* 1993). Therefore, the estimates of divergence time between the two California sea lion clades are about 4.4–8.8 million years ago. The Gulf of California began to separate from mainland Mexico about 4.5 million years ago, and thus the Baja Peninsula has been a geological feature of substantial dimensions since the Pliocene (Atwater 1970). However, reconstruction of sea level changes indicate that much of the spreading of the Gulf occurred more recently during late Pliocene and early Pleistocene times (less than 3 million years ago, Atwater 1970). Our results are consistent with isolation of the Gulf and Pacific populations of sea lions since the geologic origin of the Gulf. However, better fossil calibrations of the rate of control region evolution in sea lions are needed to have confidence in this conclusion.

The Gulf and Pacific coast populations do not differ in 368 bp of the cytochrome b gene. The relative rate of sequence evolution in protein coding regions such as cytochrome b is expected to be much less than that of the control region (Aquadro and Greenberg 1983, Brown 1985, Moritz *et al.* 1987). A comparison of comparable-sized control region and cytochrome b sequences in dunlins (*Calidris alpina*) found that clades separated by 20 or more control

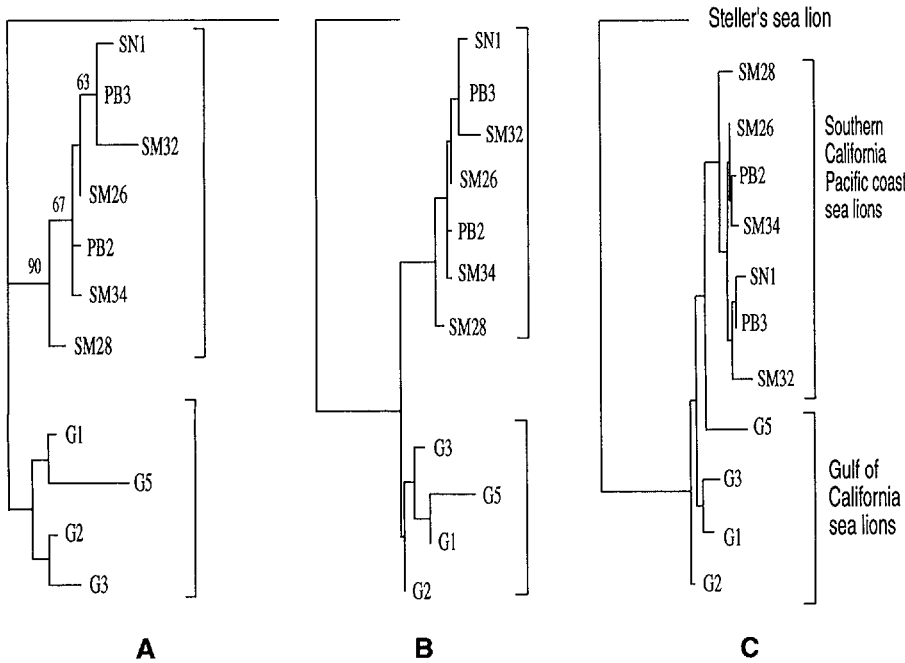

Figure 2. Three phylogenetic trees showing the relationship between genotypes found in Pacific coast and Gulf of California sea lions. (A) One of the nine branch-and-bound most parsimonious trees. Tree length = 79, overall consistency index = 0.873. Numbers at internodes refer to the percentage of bootstrap trees having the indicated groupings. (B) Phylogenetic tree generated by the maximum likelihood method, internode connecting Gulf and Pacific populations is significantly different from zero ($P < 0.01$). (C) Neighbor-joining tree based on Kimura two-parameter sequence divergence values (Table 1).

region substitutions (sequence divergence > 9%) had only 3–5 substitutions in a 302 bp region of the cytochrome b gene (Wenink *et al.* 1993). Consequently, if we assume similar relative rates of sequence evolution for California sea lions, the absence of variation in our 368 bp cytochrome b fragment might not be considered unusual, as we observed, at most, 18 control region substitutions (sequence divergence = 5.9%, Table 1).

Nonetheless, the large divergence in control region sequence between Gulf and Pacific coast genotypes suggests that females are not migrating between the two regions. Conceivably, interregion migration may have been inhibited by behavioral factors such as female philopatry that may ultimately be related to localized patterns of food abundance (Badan *et al.* 1985, Arvizu 1987). Even a single migrant per generation between populations may be sufficient to stifle genetic differentiation due to drift (Slatkin 1987). The population genetic structure of marine organisms that have a distribution around peninsular Florida also shows a sharp phylogenetic division between populations on the east and west coasts (Avise 1992). This isolation between the east and west coast populations may, in part, reflect oceanic currents that commonly circulate in opposite directions on either side of the Florida Peninsula (Avise 1992).

Similarly, studies of oceanic currents within the Gulf of California suggest that the geographic orientation of the long axis of the Gulf trough effectively excludes major Pacific ocean circulation (Fig. 1). Moreover, the midriff area, where our Gulf population was sampled, is oceanographically unique. It is characterized by forceful longitudinal tidal currents that create strong mixing leading to anomalously high bottom temperatures, salinities, and dissolved oxygen values. The depth of the Gulf trough changes dramatically in the midriff region and creates a barrier to subsurface water flow between the central and northern Gulf. Moreover, upwelling is prevalent in both summer and winter resulting in year-long fish productivity to support large numbers of sea birds and sea lions. Consequently, unlike Pacific coast populations, sea lions in the Gulf do not need to migrate to follow changing patterns in prey abundance.

Behavioral factors may also be very important in causing long term isolation among marine organisms. For example, the green turtle (*Chelonia mydas*) was studied throughout its Atlantic range for mitochondrial and nuclear variation (Bowen *et al.* 1992, Karl *et al.* 1992). The pattern of mitochondrial variation suggested extreme female philopatry as nearly all genotypes assorted to different areas where females lay eggs. Apparently, female turtles return to the beach where they were born to lay their eggs some 30 or more years after birth. Similarly, philopatry might explain the long term separation of Gulf and Pacific coast populations without the necessity for more proximate physical or biological explanations. However, the original impetus for the philopatric pattern may have had a physical explanation that is no longer apparent.

Males may migrate between populations more freely than females. In fact, in green turtles, nuclear markers were not strongly partitioned among localities, suggesting males were not philopatric (Karl *et al.* 1992). Thus, sea lions need to be surveyed for nuclear markers in order to determine if males are similarly restricted to breeding in Gulf or Pacific coast regions.

Gulf and Pacific coast populations of California sea lions have similar levels of mitochondrial DNA diversity despite different demographic histories. Populations in southern California and along the Pacific coast of Baja California declined during the 19th and early 20th centuries due to hunting, but there is no evidence that the Gulf population was so reduced (Townsend 1918, Stewart *et al.* 1993). Sea lion numbers have increased rapidly since the cessation of commercial exploitation in the mid-1900s, particularly in Southern California (*e.g.,* Stewart *et al.* 1993), and they were estimated to number over 174,000 in United States and Mexican waters in the early 1980s (Aurioles *et al.* 1983, Le Boeuf *et al.* 1983). The northern elephant seal (*Mirounga angustirostris*) experienced a more extreme population bottleneck in the same area and at the same time, also due to hunting, and was reduced to one breeding colony (Stewart *et al.* 1994). The extant population that numbers now over 120,000 (Stewart *et al.* 1994) has little genetic variability (Hoelzel *et al.* 1993, Lehman *et al.* 1993). Analysis of 300 bp of control region sequence in elephant seals revealed only 2 genotypes in 67 northern elephant seals. This contrasts with the southern elephant seal (*M. leonina*), which was never reduced to such low numbers and showed 26 genotypes among 48 individuals from two populations (Hoelzel *et al.* 1993). We found eleven genotypes among 40 California sea lions which

suggests that the reduction in abundance of this species was not as severe as that of the northern elephant seal. But the amount of sequence divergence between the two southern elephant seal populations separated by 2,000 km was similar to that observed between Gulf and Pacific California sea lions separated by only several hundred kilometers. The maximum population divergence was 4.7% between southern elephant seals at South Georgia and Peninsula Valdez, Argentina, compared to 4.4% between California sea lion populations in the Gulf and Pacific coasts. The value of sequence divergence between Gulf and Pacific coast sea lion populations is about half that of grey seals and harbor seals that are classified in different genera (Árnason and Johnson 1992; Árnason, personal communication).

In conclusion, our results suggest that a significant phylogenetic division exists between the population of California sea lions in the Gulf of California and those along the Pacific coast of North America. The degree of sequence divergence is similar to that found between widely separated colonies of southern elephant seals. These results indicate that the two regional populations of California sea lions may have been isolated for sufficient time for behavioral, physiological, and ecological divergence to have occurred (*cf.* Wayne *et al.* 1989). Consequently, the two populations are evolutionarily independent of one another and should be treated as separate genetic units for conservation management. Reintroduction or translocation plans should avoid mixing individuals from different regions as the presumably unique history of adaptations in each region may be obscured and may lead to limited outbreeding depression (*e.g.,* Avise and Ball 1990, Dizon *et al.* 1992). Further sampling and analyses of nuclear and mitochondrial DNA are needed to better define the geographic boundaries of the two regional populations of California sea lions and to determine the relationships of these populations to the Galapagos Island race of this species.

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