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Abundant mitochondrial DNA variation and world-wide population structure in humpback whales

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ABSTRACT **Hunting during the last 200 years reduced many populations of mysticete whales to near extinction. To evaluate potential genetic bottlenecks in these exploited populations, we examined mitochondrial DNA control region sequences from 90 individual humpback whales (*Megaptera novaeangliae*) representing six subpopulations in three ocean basins. Comparisons of relative nucleotide and nucleotype diversity reveal an abundance of genetic variation in all but one of the oceanic subpopulations. Phylogenetic reconstruction of nucleotypes and analysis of maternal gene flow show that current genetic variation is not due to postexploitation migration between oceans but is a relic of past population variability. Calibration of the rate of control region evolution across three families of whales suggests that existing humpback whale lineages are of ancient origin. Preservation of preexploitation variation in humpback whales may be attributed to their long life-span and overlapping generations and to an effective, though perhaps not timely, international prohibition against hunting.**

Humpback whales (*Megaptera novaeangliae*) once numbered >125,000 individuals distributed into three oceanic populations: the North Pacific, the North Atlantic, and the southern oceans. Within each population, observations of migratory movement by marked individuals suggest that humpback whales form relatively discrete subpopulations that are not separated by obvious geographic barriers (1). Before protection by international agreement in 1966, the world-wide population of humpback whales had been reduced by hunting to <5000, with some regional subpopulations reduced to <200 individuals (Table 1).

To evaluate the possibility that commercial hunting reduced genetic variation in baleen whales, we examined nucleotide sequence variation in the mitochondrial (mt) DNA from 90 humpback whales collected from the three major oceanic basins. We chose humpback whales for this evaluation because their well-described subpopulation divisions and well-documented history of exploitation provide a historical framework within which to evaluate genetic data (Table 1). We chose mtDNA as a genetic marker because of its power in describing the genetic structure of maternal lineages within populations and its sensitivity to demographic changes in populations (20). To allow the use of small skin samples collected by biopsy darting, we applied the polymerase chain reaction (PCR) and direct "solid-phase" sequencing methodology (21) to the mtDNA control region or "D-loop," a noncoding region that is highly variable in most vertebrates (22).

We first verified that oceanic populations of humpback whales are independent demographic units by estimating mtDNA gene flow with a cladistic analysis of the control region sequences. We then evaluated mtDNA diversity within each oceanic population in reference to world-wide levels of variation on the assumption that loss of genetic variation would be independent within each ocean. Finally, we estimated the rate of evolution for the humpback whale control region by comparing homologous sequences in five other species of whales representing the three extant families of mysticetes. Our results show high levels of variation in humpback whales and substantial genetic differences between oceanic populations. High variation in extant populations is not due to recent divergence or frequent oceanic interchange. Instead, divergent humpback whale mtDNA lineages have persisted for millions of years.

MATERIALS AND METHODS

Samples for genetic analysis were available from 90 individual humpback whales representing three oceanic populations, six stocks, and 13 regional habitats (Table 1). Except for 2 beach-cast animals, 2 individuals entrapped in fishing nets, and 10 victims of an unusual group mortality, samples of skin tissue were collected from free-ranging whales by using a biopsy dart (23). Total cellular DNA was isolated from the epidermal layer of the skin biopsy by standard organic extraction (24).

Symmetric amplification of 100 ng of cellular DNA by the PCR followed standard protocols (25, 26). Based on initial comparison of the entire mtDNA control region of mysticete whales (27), two oligonucleotide primers were designed to amplify an internal 463-bp mtDNA segment found to include the majority of variable nucleotide positions: light-strand Dlp-10 (5′-CCACAGTACTATGTCCGTATT-3′) and heavy-strand Dlp-5 (5′-CCATCGWGATGTCTTATTTAAGRGGAA-3′). The 5′ end of the Dlp-10 primer was biotinylated to allow for attachment to paramagnetic beads coated with streptavidin (Dynal, Great Neck, NY) after symmetrical amplification (21). The solid-phase (attached) strand and the neutralized complementary strand were sequenced by standard protocols (26).

RESULTS AND DISCUSSION

Variability of mtDNA Control Region Sequences. Examination of the complete mtDNA control region sequence from nine humpback whales showed that the majority of intraspecific sequence variation was located in a 283-bp region near the 5′ end (unpublished data). This variable section extends

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Abbreviations: mtDNA, mitochondrial DNA; Mya, million years ago.
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Table 1. Population information, sample locations, and nucleotide diversity for world-wide study of mtDNA in humpback whales

Ocean	Stock	Region	Population abundance			Ref(s).	Genetic sample, no. samples	Nucleotype diversity
			Unexploited	Minimum	Current			
North Atlantic			>6000	<1000	5505	2, 3	34 (15)	0.87
	Western	Gulf of Maine	NA	NA	372	3	16 (8)	0.88
		Newfoundland	NA	NA	2310	3	12 (8)	0.89
		Iceland	NA	NA	1816	4	3 (2)	NA
		Dominican Republic	NA	NA	3776	3	3 (3)	NA
North Pacific			15–20,000	<1000	≈2000	5, 6	31 (7)	0.75
	Central	Southeastern Alaska	NA	NA	547	7	5 (1)	0.00
		Hawaii	NA	NA	1407	8	7 (1)	0.00
	Eastern	California	NA	<200	253	9, 10	12 (5)	0.76
		Mexico	NA	<200	325	9, 11	7 (4)	0.86
Southern oceans			90–100,000	1700–2800	NA	12	25 (16)	0.95
	Group I–VI	Antarctic peninsula	NA	NA	NA		3 (2)	NA
	Group IV	Western Australia	12–17,000	<800	3000	13, 14	12 (10)	0.92
	Group V	Eastern Australia	10,000	200–500	≈1100	13, 15, 16	1 (1)	NA
		New Zealand	NA	NA	Low	17	1 (1)	NA
		Tonga	NA	NA	Low	18	8 (6)	0.89
World-wide			125,000	5000	NA		90 (37)	0.88

Estimated abundance is shown for the period prior to exploitation, at the lowest levels during exploitation, and as of the most recent published surveys. NA, not available. Number of samples is the total number of individual whales examined. The number of unique nucleotypes is shown in parentheses. Some nucleotypes are shared among population subdivisions. Nucleotype diversity (19) calculated for $n > 3$.

from approximately positions 16,043 to 16,307 with reference to the fin whale mtDNA genome (27). Within this region, we found 33 variable nucleotide positions defining 37 unique mtDNA sequences, referred to as nucleotypes (19), among the sample of 90 humpback whales (Fig. 1). The two most divergent humpback whale mtDNA nucleotypes differed by 15 transitions and one transversion, or 5.65% of the total sequence. The average divergence (i.e., nucleotide diversity) was 2.57% among all 90 individuals and 3.00% among the 37 unique nucleotypes. By comparison, restriction fragment length polymorphism analysis of mtDNA from northern hemisphere humpback whales showed average nucleotide diversity roughly 10-fold lower (i.e., 0.248%) for the entire mtDNA genome (28).

World-Wide Genetic Structure. We quantified the geographic differentiation of mtDNA nucleotypes by using the analysis of molecular variance model (29). This procedure calculated three genetic variance components: among regions and among regions within oceans and among oceans. The significance of the observed variance components was tested using a random permutation procedure performed on the matrix of nucleotide differences between pairs of haplotypes (program courtesy of L. Excoffier, University of Geneva, Carouge, Switzerland). The results showed that >40% of the molecular variance in the distance matrix can be accounted for by the three oceanic divisions, 2% by differences among regions within oceans, and 58% by diversity within regions. The observed partitioning of molecular variance among oceanic population was not exceeded by any of the 1000 permutations of the data matrix.

To estimate the long-term effective rate of female migration between oceanic populations, we used a cladistic measure of gene flow as inferred from the phylogeny of mtDNA nucleotypes (30). This "coalescent" approach is based on the minimum number of migration events necessary to explain the geographic distribution of nucleotypes in a phylogeny of DNA sequences. The phylogeny of humpback whale mtDNA nucleotypes was constructed by the Neighbor–Joining (31) and parsimony (32) methods and rooted to the homologous control region sequence from a North Atlantic fin whale (Fig. 2). Both methods support the division of the 37 nucleotypes into three major clades, referred to as AE, CD, and IJ based on previous restriction fragment length polymorphism analysis (28), which have diverged from each other by 4–5%.

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                    Variable Positions
                    1111111111111111111111112222
                    1124456612222333344444455666689990366
ID                  5897894501642349016704678926678993890035

HI02                GCCGGTTTCATAGTATCCAATAGTCCTACTCTAGATCTCA
CA01                ........................C............
CA04                ......................C..C............
CA05                .......T...............C............
CA08                ..TA.C.C.......C........T..G.......CT...
CA22                ...........A............C............
MX07                ...A....................C............
GM29                ..TA...C..C..........G.CT.CG.......CT.T.
GM13                ...A.CC.T..............TT.G.C.....CT...
GM14                ..TA...C..C............CT.CG.......CT.T.
GM20                ..TA...C..C..........G.CT.CG.....C.CT.T.
GM34                ..TA.C..T..............TT...C.....CT...
GM19                ...A.CC.T..............TT...C.....CT...
GM05                ...A.C..T..............TT...C.....CT...
GM41                .T.A.C..T..............TT..TC.....CTC..
NF01                ...A.C..T..............TT.G.C.....CT...
NF06                .T.A.C..T..............TT..TC.....CT...
NF08                ...A.C..T..............TT..TC.....CT...
NF09                AT.A.C..T..............TT..TC.....CT.T.
NF18                .T.A.C..T..............TT..TC.....CT.T.
IC08                ..TA......C............CT.CG.......CT.T.
DR19                .T.A.C..T............A.TT...C.....CT...
AP01                ..TAA..C.......C........TT.G.......CT...
TG02                ..TAAC.........C.........T.G...C...CT...
TG03                ..TA...C.......C....C..C..CG.......CT...
TG05                ..TA.C.C.......C........TT.G.......CT...
TG10                ...A.C..T......C........TT...C.....CT...
TG11                ..TAAC.........CT.......T..G.......CT...
TG12                ..TA...C..............T.CG.......CT...
NZ01                ..TA...C..C....C.......CT.CG.......CT...
EA15                ..TAAC.........C........T..G.......CT...
WA01                ..TA.C.C.......C........TT.G.......CTC..
WA02                ..T.....T..........G....TT.........CT...
WA07                ..T.....T..............TT.........CTC..
WA21                ...A.CC.T......C.T...G..TT...CT....CT...
WA24                ...A.C..T..............TT...C..G..CT...
WA28                ..TAAC.................T..G.......CT...
Fin                 -----....G.G.CG.T.GCC.A..T..TC....G....C
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FIG. 1. Variable sites defining 37 unique sequences within the humpback whale mtDNA control region are shown and aligned to the homologous sequence for the fin whale. Dots indicate matches with the reference sequence HI02. A short deletion in the fin whale sequence is noted as dashes. Position 29 of the humpback whale sequence corresponds to position 16,053 in the fin whale mtDNA (27).

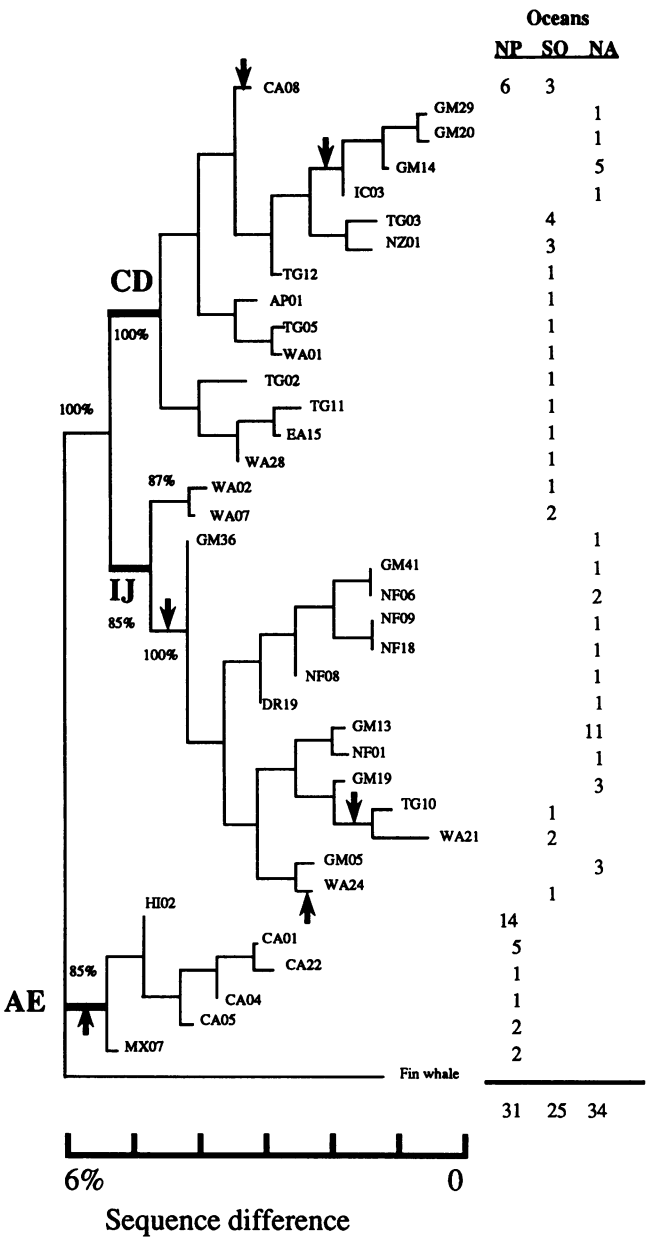

FIG. 2. Phylogenetic relationship between 37 unique mtDNA control region sequences from 90 humpback whales from the North Pacific (NP), North Atlantic (NA), and southern oceans (SO). The tree was constructed by the Neighbor–Joining method (31) and rooted with the homologous sequence of the fin whale. The branching order of the three major lineages was supported by a majority-rule consensus of Neighbor–Joining trees generated from 200 bootstrap simulations (33) of the data set using PHYLIP and by a strict consensus of 500 heuristic searches using parsimony and the randomized entry order and subtree-pruning options available in PAUP. The primary topological relationships within the control region sequence tree are concordant with a parsimony analysis of restriction fragment length polymorphism data from the entire humpback whale mtDNA genome (28). Percentages show agreement in a consensus of 500 heuristic searches using the randomized entry order and subtree-pruning options available in PAUP (34). Arrows point to the six hypothetical migration events necessary to explain the current oceanic distribution of nucleotypes.

Each oceanic population is dominated, in terms of frequency and diversity, by a different mtDNA clade, although two clades have transoceanic distributions.

The minimum number of migration events necessary to explain the current geographic distribution of nucleotypes was found to be six (Fig. 2). Of these, two have been from the southern oceans to the North Atlantic, two have been from the North Atlantic to the southern oceans, and two have been from the southern oceans to the North Pacific. The most recent historical transoceanic migration is found in the eastern North Pacific where a common nucleotype of the CD clade, referred to as CA08, is indistinguishable from nucleotypes found in the southern oceans. The transoceanic distribution of this type and observations that humpback whales migrate from the Antarctic Peninsula to the equatorial coast of Colombia (35) support the hypothesis that the Pacific coast of South America is a likely avenue for gene flow between oceans (28).

With a total sample size of 90 individuals and $s = 6$, average effective migration of females (i.e., $N_e m_e$) between the three ocean populations was estimated to be 0.5 (95% confidence limits = 0.1–1.0; computer program courtesy of M. Slatkin, University of California, Berkeley). Because the fine-scale branching of the Neighbor–Joining tree was uncertain and because a unique nucleotype could not be distinguished for all 90 individuals, this value could represent an underestimate of the true migration rate. To generate a maximum estimate of $N_e m_e$, we computed a range of minimum migration events for 330 equally parsimonious trees generated by 50 heuristic searches using the randomized entry order and subtree-pruning option available in PAUP. We found that the minimum value of s was 5 ($n = 19$), the maximum value was 7 ($n = 151$), and the modal value was 6 ($n = 160$). Using the maximum value of $s = 7$ and the minimum number of unique sequences in the three oceans, $n = 38$, we estimated $N_e m_e$ to be 1.2 (95% confidence limits = 0.3–3.1). Even the upper confidence limit of this estimate suggests that migration between oceanic populations is limited to no more than a few females per generation.

Within-Population Variation. Variation in humpback whale mtDNA control region sequences was high in both the North Atlantic and southern ocean populations relative to world-wide variation. Using the world-wide average of 3.00% nucleotide diversity as a baseline, the North Atlantic and southern ocean populations retained 82% and 76% of this total, respectively (Table 2). High levels of genetic variation were also observed within some stocks or regional habitats (Table 1). Nucleotype diversity in the Gulf of Maine and Western Australia approached that observed in some human populations (22).

Genetic evidence consistent with a population bottleneck was found only in the Central North Pacific (i.e., Hawaii and southeastern Alaska) where mtDNA nucleotype diversity was zero (Table 2). This low genetic variation may reflect the results of commercial exploitation during two periods of intense harvesting in this century (5). It is not clear, however, why the Central North Pacific population should have been more susceptible to hunting than other surveyed subpopulations. A second explanation is suggested by Herman's (36) review of the historic ecology of humpback whales in Hawaii. Based on the absence of native Hawaiian legends and words

Table 2. Average percent differences (i.e., nucleotide diversity, π) of all sequenced nucleotypes and unique mtDNA nucleotypes (shown in parentheses) within and between populations of humpback whales

	% difference		
	North Pacific	North Atlantic	Southern oceans
North Pacific	1.353 (1.543)	1.406 (1.990)	1.029 (1.529)
North Atlantic	3.100 (3.808)	2.035 (2.455)	0.745 (0.567)
Southern oceans	2.848 (3.472)	2.905 (2.966)	2.285 (2.344)

Sample sizes are shown in Table 1. Data within and between populations of whales are shown on the diagonal (underlined) and below, respectively. Genetic distances adjusted for within-region variation are above the diagonal.

describing baleen whales and the absence of humpback whale sightings or catches in the logbooks of 19th century whalers, Herman (36) proposed that humpback whales began migrating to the main Hawaiian islands only during this century. Such a recent colonization event could have isolated a single maternal lineage that existed previously elsewhere in the North Pacific.

Tempo of Control Region Evolution. To estimate the age of the three major humpback whale mtDNA lineages, we calibrated the divergence rate of the 283-bp segment of the control region by sequencing the homologous region from five other whales, the fin *Balaenoptera physalus*, the blue *Balaenoptera musculus*, the sei *Balaenoptera borealis*, the gray *Eschrichtius robustus*, and the bowhead *Balaena mysticetus*. Published control sequence from the killer whale *Orcinus orca* (37) was used as an outgroup. Phylogenetic reconstructions based on maximum likelihood (38), Neighbor–Joining (31), and parsimony (32) analyses are consistent with the major features of the fossil record (Fig. 3*A*). The family Balaenidae are apparent in the fossil record by the early Miocene, 20–25 million years ago (Mya), whereas fossil Balaenopteridae are not distinct as a family until the mid-late Miocene, 10–15 Mya (40). The humpback genus *Megaptera*

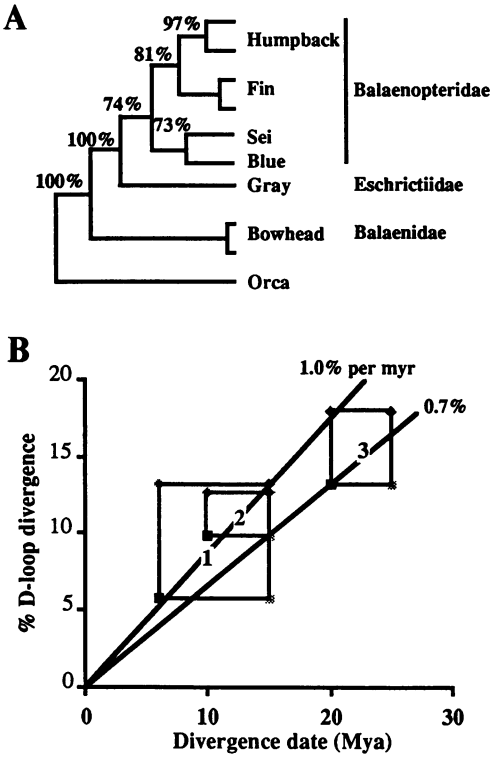

FIG. 3. (*A*) Phylogenetic relationship of variable mtDNA control region sequences among mysticete whales, constructed using maximum likelihood (38) and parsimony (32) analyses. The tree is based on a 254-bp sequence of the control region beginning at position 29 in relation to the humpback whale sequence (Fig. 1). Sequences from more than one individual were used for the humpback (n = 3), fin (n = 2), and bowhead (n = 2) whales. The relative ranges of intraspecific variation are indicated by the depth of the branches in the cladogram. Transversions were weighted 10:1 over transitions in the data matrix. Percentages show agreement in a consensus of 500 bootstrap simulations of the data set using the branch and bound search option available in PAUP (34). (*B*) Divergence rate calibration for mysticete control regions. Sequence differences for control region data were calculated using equation 5 of Li *et al.* (39). Divergence dates (Mya) are minimum and maximum times from the fossil record for the genera or families compared, as described in text. The diagonal lines are the steepest and shallowest lines that pass through all data boxes and represent our best estimate of divergence rates.

is known from the late Miocene, ≈6 Mya (41). Although the sole member of the family Eschrichtiidae (i.e., the gray whale) is often described as "primitive" (42), its fossil record can be traced back only into the late Pleistocene (40). Our phylogeny agrees that the Balaenidae, as represented by the bowhead, is an early divergence of the common mysticete lineage and suggests a close relationship between the Eschrichtiidae and the Balaenopteridae (43).

The divergence dates from the fossil record were compared to the pair-wise sequence divergences between the three families of baleen whales to estimate a range of divergence rates for the control region (Fig. 3*b*). We calculated the average pair-wise sequence divergence at three taxonomic levels: (*i*) between a trio of the most divergent humpback whales and the genus *Balaenoptera*, represented by the blue, sei, and fin whales (sequence difference = 5.8–13.2%); (*ii*) between these four species of the family Balaenopteridae and the family Eschrichtiidae, i.e., the gray whale (sequence difference = 9.8–12.7%); and (*iii*) between the combined Balaenopteridae and Eschrichtiidae species and the family Balaenidae, represented by the bowhead (sequence difference = 13.2–17.9%). As a conservative approach, we took the upper and lower ranges of the divergence values from each of the three comparisons and divided them by the upper and lower ranges of divergence times estimated from the fossil record. The divergence rate estimate consistent with all three comparisons is 0.7–1.0% per Myr, comparable to that estimated for odontocetes (37). The slowest and fastest rates consistent with any comparison are 0.4% and 1.8%, respectively. Even the fastest estimated rate for the mysticete whales is nearly 10-fold slower than the rate of divergence of the human mtDNA control region that has been used for population comparisons (22).

The rate of humpback whale control region divergence can be used to infer a time scale for the divergence of humpback whale nucleotypes. Based on our estimated rates of 0.7–1.0% per Myr, we calculate that the last common maternal ancestor of the world-wide population of humpback whales was during the late Miocene, ≈5 Mya. Using the maximum rate consistent with any of our data, the age of these lineages is 2–3 Myr. In contrast, the age of the last common maternal ancestor of the world-wide human population is estimated to be 166,000–249,000 years ago (22, 44).

Conservation Genetics of Exploited Humpback Whale Populations. How have most populations of humpback whales survived near-extinction without major loss of mtDNA variation? One possibility is that insufficient time has passed since exploitation for the effects of drift to be measured. Humpback whales have a minimum age of sexual maturity of 4–6 years (13), suggesting an average generation time on the order of 5–10 years. In the three decades since most populations were at their lowest level, there have been only 3–6 humpback generations and each of these were largely overlapping. Given the >30-year potential life span of humpback whales (13), some individuals that survived commercial hunting during the early 1960s are probably still alive today.

The long generation time and age structure of humpback whale populations may have allowed them to pass through a brief bottleneck without major loss of genetic variation (45). Although humpback whales were hunted commercially as early as 1611 (46), major harvests have been recent and relatively short-lived. Since protection from hunting was extended to all oceans in 1966, many populations have increased measurably in abundance (Table 1), although all are still considered to be endangered (47). As a result, most stocks of humpback whales have been spared the prolonged low-level exploitation that may be responsible for the absence of recovery in the North Atlantic right whale *Eubalaena glacialis* (48).

Humpback whales have survived the rapacious past of the commercial whaling industry without a major loss of genetic diversity in most populations. This is due in part to life-history parameters that have mitigated the effects of genetic drift in postexploitation populations and in part to effective, though perhaps not timely, international protection. With the possible important exception of the central North Pacific stock, we find little reason to suspect that loss of genetic variation through inbreeding will limit recovery in populations of humpback whales. Full population recovery is more likely to depend on the strength of international agreements prohibiting further hunting, the regional protection of coastal marine habitats critical for feeding and reproduction, and the global protection of the ocean ecosystem.

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