An empirical genetic assessment of the severity of the northern elephant seal population bottleneck

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A bottleneck in population size of a species is often correlated with a sharp reduction in genetic variation. The northern elephant seal (*Mirounga angustirostris*) has undergone at least one extreme bottleneck, having rebounded from 20–100 individuals a century ago to over 175,000 individuals today. The relative lack of molecular-genetic variation in contemporary populations has been documented, but the extent of variation before the late 19th century remains unknown. We have determined the nucleotide sequence of a 179 base-pair segment of the mitochondrial DNA (mtDNA) control region from seals that lived before, during and after a bottleneck low in 1892. A 'primerless' PCR was used to improve the recovery of information from older samples. Only two mtDNA genotypes were present in all 150+ seals from the 1892 bottleneck on, but we discovered four genotypes in five pre-bottleneck seals. This suggests a much greater amount of mtDNA genotypic variation before this bottleneck, and that the persistence of two genotypes today is a consequence of random lineage sampling. We cannot correlate the loss of mtDNA genotypes with a lowered mean fitness of individuals in the species today. However, we show that the species historically possessed additional genotypes to those present now, and that sampling of ancient DNA could elucidate the genetic consequences of severe reductions in population size.

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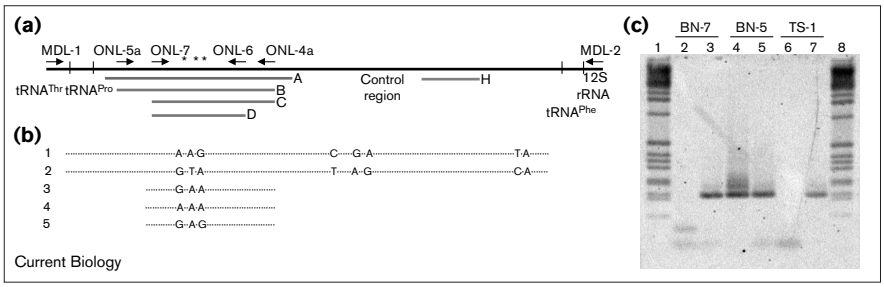
Results and discussion

To assess directly the genetic effects of the 19th century bottleneck in the northern elephant seal [1–4], we targeted for study a 300 base-pair segment of the control region of the mtDNA, which has previously been shown to be minimally polymorphic in contemporary northern elephant seals, exhibiting two distinct genotypes among 40 individuals [2]. We successfully genotyped the polymorphic portion of this segment from 11 bones and from 111 tissue samples. The bones ranged in age from ~1,000 years before present (ybp) to 30 ybp. They included five from seals that pre-dated the low point of the late 19th century bottleneck, three from seals that lived in the initial recovery phase in the 1910s, and three from seals sampled between 1960 and 1980. The tissue samples included two of the seven skin samples collected by C.H. Townsend [5] and returned to the Smithsonian Museum when the species was thought essentially extinct in 1892, and blood samples from 100 individuals living on the Channel Islands off the coast of southern California during the 1990s.

We amplified DNA from the bone samples via a sequential combination of a 'primerless PCR' [6] and a two-step nested PCR, with stringent contamination controls at each stage. Ancient DNA (aDNA) has great potential to elucidate historical population-genetic patterns [7], but is difficult to obtain from weathered specimens, particularly without contamination from modern sources [8]. A method of utilizing *Taq* DNA polymerase to reconstruct partially degraded DNA templates through a PCR-like cycling in the absence of primers was proposed by Stemmer [6], but to date not used for aDNA. We used this method by extracting DNA templates, presumably in small molar quantities and often in small fragments, from museum-catalogued northern elephant seal bones and skins. We then cycled the DNA in the presence of *Taq* polymerase, dNTPs, and $MgCl_2$-containing PCR buffer for 25 rounds of denaturation (92°C), annealing (50°C) and extension (72°C). In the absence of specific oligonucleotide primers, the polymerase will use existing fragments of DNA, even if only partially overlapping as a consequence of degradation, as templates for reconstruction of a complementary strand. For haploid loci such as mtDNA, the net result after several rounds should be an increase in the concentration of contiguous fragments of DNA, although little actual DNA amplification will result [6]. We took the outputs of this primerless PCR for bone samples and subjected them to an external and then internal PCR reactions (Figure 1) using PCR primers that allowed amplification of a 145–179 bp stretch of DNA that includes the three nucleotide positions known to be polymorphic in the mtDNA control region of extant northern elephant seals [2]. We also used this strategy to amplify the same DNA segment from two dried skin samples collected by Townsend

Figure 1



Detection of northern elephant seal mtDNA genotypes. **(a)** The region of mitochondrial DNA sequenced. Locations of PCR primers (MDL-1, ONL-5a, ONL-7a, ONL-6a, ONL-4a and MDL-2) used to amplify the mtDNA control region (see Supplementary material) are indicated. Segment A (300 bp) was genotyped for 40 seals by Hoelzel *et al.* [2]; three polymorphic nucleotide sites (asterisks) were detected, producing two distinct genotypes (1 and 2). In the current study, an additional 109 seals were typed for this segment. A subset of these ($n = 12$) were typed for the entire control region, for which five additional polymorphic sites but only a total of two genotypes were detected. For aDNA samples, an external PCR segment B (259 bp), and then internal PCR segments C (179 bp) and/or D (145 bp) were generated and used for genotyping archeological skins and bone. Segment H is a 300–400 bp portion of the control region that displays VNTR variation and heteroplasmy [3], and was excluded from analyses. **(b)** Representation of nucleotide sequences of the five mtDNA genotypes detected to date. The genotypes 3, 4 and 5 were detected from the archeological samples in only five pre-bottleneck seals, revealing the extreme genetic consequences of the demographic constriction(s). Dots represent (blocks of) sequences identical to the complete control region published by Hoelzel *et al.* [2]; one dot represents approximately 10 nucleotides. **(c)** Improved ability to amplify aDNA cleanly upon addition of a primerless PCR. The same DNA extracts from aDNA samples were amplified in a two-step nested PCR reaction with (lanes 3, 5 and 7), and without (lanes 2, 4 and 6) the prior reconstruction of template using a primerless PCR. For 2% agarose gel: lanes 2 and 3: BN-7; lanes 4 and 5: BN-5; lanes 6 and 7: TS-1; lanes 1 and 8: size marker (1 kb ladder). Improvement was likewise routinely observed in other aDNA templates.

[5]. The addition of the primerless PCR gave aDNA products several times when direct PCR failed (Figure 1c).

The existence of only two mtDNA genotypes among the 109 contemporary seals was confirmed by DNA sequence analysis (Figure 1b, Table 1). In the region also surveyed by Hoelzel *et al.* [2], we detected two genotypes, 1 and 2, differing in three nucleotide positions, at frequencies of 0.72 and 0.28, respectively, which are not statistically different from those reported by Hoelzel *et al.* [2] (0.73 and 0.27; $P = 0.9$, G-test of independence). The seals used in the two studies are not the same; our samples came from the main colonies of southern California, whereas those used by Hoelzel *et al.* [2] originated mainly from Año Nuevo island in central California. We detected no intercolony differences in genotype frequency ($P = 0.27$, G-test of independence). In addition, we detected five additional nucleotides within the mtDNA control region among our contemporary tissue samples that exhibited polymorphism (Figure 1b). Notably, still only two genotypes exist; each seal examined had all eight nucleotides of genotype 1 or all eight nucleotides of genotype 2. In total, the two genotypes differ by 8/1,223 bp, or 0.6% of the control region (exclusive of a 300–400 bp segment that can display heteroplasmic variable number tandem repeat (VNTR) variation [3]). Combining our samples with those of Hoelzel *et al.* [2], we conclude that additional mtDNA genotypes must be very rare to exist now in northern elephant seals but remain undetected in our studies. For example, a sample of 149 seals would have a 95% chance of detecting any third mtDNA genotype at a frequency of 2% or greater.

In sharp contrast to the two genotypes that exist in contemporary populations, we detected at least four mtDNA genotypes in the five bones that pre-date the population

Table 1

Frequencies of northern elephant seal mtDNA genotypes and diversity statistics (standard errors).

	Seal bones *ca.* 1000–1800	Seal skins 1892	Seal bones 1914–1980	Modern seals, southern California	Modern seals, central California
	This study	This study	This study	This study	Hoelzel *et al.* [2]
Genotype 1	1	0	4	78	29
Genotype 2	0	2	2	31	11
Genotype 3	2	0	0	0	0
Genotype 4	1	0	0	0	0
Genotype 5	1	0	0	0	0
Haplotype diversity, h	0.90 (0.16)	0 (0)	0.53 (0.17)	0.411 (0.038)	0.409 (0.065)
Nucleotide diversity, π	0.0065 (0014)	0 (0)	0.0086 (0.0028)	0.0067 (0.0006)	0.0066 (0.0011)

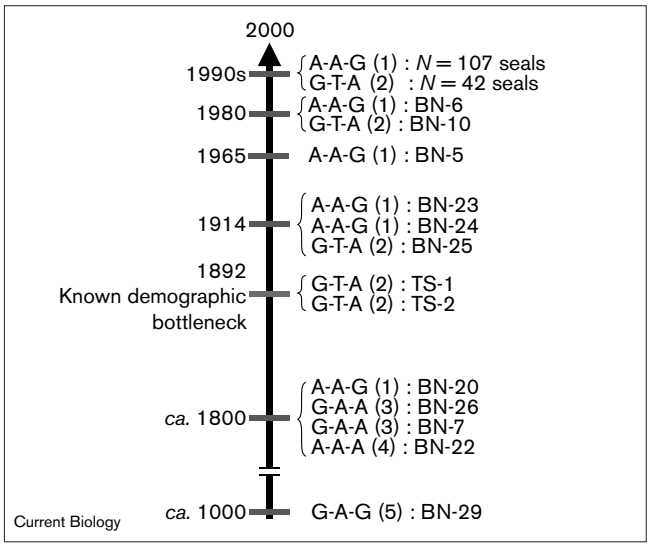
Genotypes are based on nucleotide sequences from a 179 bp segment (C) of the control region (Figure 1).

nadir in 1892 (Table 1, Figure 2). This analysis was based only on 179 bp of the region examined by Hoelzel *et al.* [2], and genotypic diversity may in fact be even greater in the entire mtDNA control region [9]. One bone (BN-20) contained the common genotype 1 in this region. Two bones (BN-7 and BN-26) contained a unique genotype (3), that differs from genotype 2 by one nucleotide. The fourth bone (BN-22) contains another unique genotype (4), that also differs from genotype 2 by one nucleotide. The fifth bone (BN-29), which is dated at least 1,000 years old, contains yet another unique genotype (5), which differs from genotype 1 by one nucleotide. All five genotypes in this segment only vary at three nucleotide sites; no other variable sites were detected. Both skin samples from the Townsend sampling possess genotype 2. For all post-bottleneck bones, including those from the early 20th century, only genotypes 1 or 2 were detected (Figure 2).

The genotypic data gathered from the northern elephant seal show that this species has experienced a severe loss of mtDNA diversity as a consequence of its late 19th century population bottleneck. We detected four mtDNA control-region genotypes in five pre-bottleneck seal bones, whereas only two such genotypes have been found in the 149 contemporary seals examined to date. The likelihood that this difference is a consequence of a biased sampling of seals on our part is extremely low. It is difficult to construct a meaningful test of whether the pre- and post-bottleneck genotype frequencies share the same underlying distribution, because only five samples exist for the former. However, the probability of drawing the post-bottleneck distribution from a simulated pool of an even distribution of genotypes 1–5 is vanishingly small for samples of more than about 10 seals. We have yet to detect the rarer modern genotype (2) in pre-bottleneck samples, leaving open the possibility that additional genotypes were present before the 19th century and will be detected with additional sampling. The observation that genotype 2 was present in 1892 supports the postulate that only two genotypes are stochastic survivors of the demographic constriction, and lessens the likelihood that the rarer genotype 2 today is a recent mutational derivative of the more common genotype 1. We guarded against the possibility that the primerless PCR was somehow generating artifactual recombinants by performing at least two independent analyses, from DNA extraction to sequence determination, on all aDNA samples. All samples generated identical sequences from replicates performed on the same bone.

The loss of genotypes in the northern elephant seal is substantial. In the pre-bottleneck sample, the haplotype (gene) diversity h, which is the heterozygosity equivalent for haploid loci [10], is 0.9 for the 179 bp segment C (Figure 1a) from the five bones, whereas in all contemporary seals it is 0.41 (Table 1), a significant decrease ($P < 0.05$, t-test). On the other hand, because the two genotypes that

Figure 2



Distribution across time of northern elephant seal mtDNA genotypes. Genotypes 1–5 in the 179 bp segment (segment C, Figure 1) are abbreviated by the nucleotide identities at the only three sites in this segment that exhibit polymorphism. Samples from the 1990s include 109 seals typed in this study, plus 40 seals typed by Hoelzel *et al.* [2]. The designation BN refers to bone samples; the designation TS refers to skin samples obtained by Townsend [5].

apparently did survive the bottleneck are relatively distinct, there is actually as much variation at the nucleotide level in contemporary seals. The nucleotide diversity, π, which estimates the average proportion of nucleotide differences between all possible pairs of sequences in the sample, is 0.0065 for the five pre-bottleneck seals, while in the post-bottleneck sample it is 0.0066 for the 149 extant seals. However, at the population level, the genetic impact of bottlenecks is more likely to be influential in terms of numbers of distinct genotypes, for these would be correlated with allelic diversity at other loci. As yet, no fitness consequences of this genetic drop are manifest in the northern elephant seal; the species numbers more than 175,000 individuals today and is still growing exponentially [11]. The species is also genetically depauperate at nuclear loci such as allozymes [1,2], minisatellite VNTR loci [4], and perhaps others as well [12–14]. Nevertheless, we still have little understanding of how neutral-marker genetic variation relates to fitness variation [15].

The genotypic data we present here establish at least a substantial decline in genetic diversity between the population living around 1,000 years ago and today. Thus there is no doubt that historic levels of diversity were higher, and that either a sudden reduction in the 1800s or a more long-lasting reduction over several thousand years may explain the low levels detected in extant populations. In the case of the northern elephant seal, the intriguing

possibility exists that the species has experienced multiple population crashes as a consequence of natural climatic cycles and/or persistent harvesting by native peoples along the Pacific coast [11]. In fact, repeated bottlenecks can even lead to a temporary purging of deleterious recessive alleles such that the population actually suffers minimal effects from post-bottleneck inbreeding [16,17]. Only time will tell if the genetic homogeneity of the northern elephant seal will have a negative impact on population persistence.

Materials and methods

Samples

Blood was collected from northern elephant seal populations on San Miguel Island (SMI; $n = 50$) and San Nicolas Island (SNI; $n = 50$) between 1992 and 1998, plus nine stranded seals rehabilitating at Sea World in San Diego. Seal bones came from excavations of aboriginal kitchen middens on SMI and SNI and from Isla de Guadalupe (IDG), from collections in the Los Angeles County Museum of Natural History, and from S. Schwartz, US Navy Archaeological Resources Program. All were aged by radiocarbon dating of kitchen midden strata where they were found. A description of the bones (BN-5, BN-6 and so on) and skin samples (TS-1 and TS-2) can be found in Supplementary material.

DNA extraction, amplification, and sequence analysis

DNA was obtained from bones, skin, or tissue samples by standard phenol-chloroform extraction and/or the DNeasy tissue extraction kit (Qiagen). Bones were first decalcified in 0.5 M EDTA for 5–7 days before DNA extraction. For aDNA, primerless PCR was performed by a modification of the method of Stemmer [6]. Approximately 8 µl of bone or tissue DNA extract was incubated in a 50 µl reaction volume with 1.25 U *Taq* DNA polymerase (Perkin-Elmer) in standard PCR buffer for 25 cycles of 92°C (60 sec), 50°C (60 sec), and 72°C (60 sec). Typically, 10 µl of the resulting solution was used as a template for standard PCR amplification. All seal mtDNA segments were amplified in a two-step ('nested') manner. Nucleotide sequences were obtained bidirectionally using [α-^{35}S]dATP, Sequenase 2.0 (US Biochemical) and separation on 6% polyacrylamide gels and autoradiography.

Contamination controls

All aDNA-containing samples were manipulated in dedicated aDNA facilities at SUNY Albany, which are isolated from post-amplification laboratories by at least two flights of stairs. Bone and ancient tissue handling was only performed in the mornings with personnel wearing clean, dedicated clothing and always before entry into the post-amplification laboratories. No materials, equipment or supplies were ever transferred from post-amplification laboratories to aDNA facilities. Work surfaces were routinely treated to decontamination by high pH cleaners (DNA Away) and short-wavelength UV light bathing. For initial PCR amplification, a dedicated room was used with dedicated external PCR equipment and reagents. For both the aDNA and external PCR facilities, all ordered supplies were delivered straight from the delivery dock to the dedicated facilities. Barrier pipet tips were used at all times, and all PCR reactions were set up in dedicated hoods with post-set-up UV light bathing. All bones were surface sanded extensively before internal sections were removed with disposable rotary tips of a Dremmel tool. All aDNA samples were taken from initial tissue extract to final DNA sequence at least twice to confirm that no cross-contamination or PCR artifact was present. For each stage in the genotyping of aDNA samples, multiple negative controls were used: blank tubes were left open to room air during bone manipulations, blank samples were subject to primerless and standard PCR alongside seal samples, and no-template PCR reactions were performed alongside all PCR reactions. No positive results were obtained in any of these controls. The PCR primers designed for amplification of segment C (Figure 1a) are elephant-seal specific; these primers routinely fail to generate products from closely related species, even when genomic DNA is used as a template.

Supplementary material

Supplementary material including additional methodological details is available at http://current-biology.com/supmat/supmatin.htm.

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