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The distribution of nuclear genetic variation and historical demography of sea otters

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Abstract

The amount and distribution of population genetic variation is crucial information for the design of effective conservation strategies for endangered species and can also be used to provide inference about demographic processes and patterns of migration. Here, we describe variation at a large number of nuclear genes in sea otters *Enhydra lutris* ssp. We surveyed 14 variable microsatellite loci and two genes of the major histocompatibility complex (MHC) in up to 350 California sea otters *Enhydra lutris nereis*, which represents ~10% of the subspecies' population, and 46 otters from two Alaskan sites. We utilized methods for detecting past reductions in effective population size to examine the effects of near extinction from the fur trade. Summary statistic tests largely failed to find a signal of a recent population size reduction (within the past 200 years), but a Bayesian method found a signal of a strong reduction over a longer time scale (up to 500 years ago). These results indicate that the reduction in size began long enough ago that much genetic variation was lost before the 19th century fur trade. A comparison of geographic distance and pairwise relatedness for individual otters found no evidence of kin-based spatial clustering for either gender. This indicates that there is no population structure, due to extended family groups, within the California population. A survey of population genetic variation found that two of the MHC genes, DQB and DRB, had two alleles present and one of the genes, DRA, was monomorphic in otters. This contrasts with other mammals, where they are often the most variable coding genes known. Genetic variation in the sea otter is among the lowest observed for a mammal and raises concerns about the long-term viability of the species, particularly in the face of future environmental changes.

Introduction

Sea otters *Enhydra lutris* are mustelids, members of the family that also includes weasels and skunks, and are the sole representative of one of at least four extant lineages of mammals (the others being cetaceans, pinnipeds and sirenians) that have independently colonized the marine environment and adopted a marine life history. Moreover, they have done so partly through a unique thermoregulatory mechanism: dense, relatively impermeable hair, as opposed to blubber. This fur, which is the densest of any mammal, made otters the target of extensive commercial exploitation in the last century. Like other marine mammal exploitation in the north-eastern Pacific (e.g. the northern elephant seal), the fur trade pushed sea otters to the brink of extinction (Kenyon, 1969; Riedman & Estes, 1990).

The sea otter originally ranged from the central Pacific coast of Baja California, Mexico across the North Pacific rim to Asia. However, following the commercial hunting period, the species was reduced to a dozen or so remnant populations (Kenyon, 1969). Otters were believed extinct in North America south of Alaska until a small population was rediscovered in the Big Sur region of central California (CA) (roughly from Monterey to Piedras Blancas, CA, Fig. 1). From this handful of individuals, which has been described as a different subspecies (*Enhydra lutris nereis*) than sea otters in Alaska (*Enhydra lutris kenyoni*) and in Asia (*Enhydra lutris lutris*) (Wilson *et al*., 1991), the CA population has slowly recovered and now numbers between 2500 and 3000 animals (B. Hatfield, US Geological Survey, unpubl. data). Further to the north, similarly dramatic population size reductions have occurred, leading to fragmentation and the species was reduced to several remnant populations in Alaska and north-east Asia. Sea otters became extinct in the middle of their North American range in Washington, Oregon and British Columbia, although otters have since been reintroduced to these areas using animals from Alaska (Jameson *et al*., 1982). Otters were also

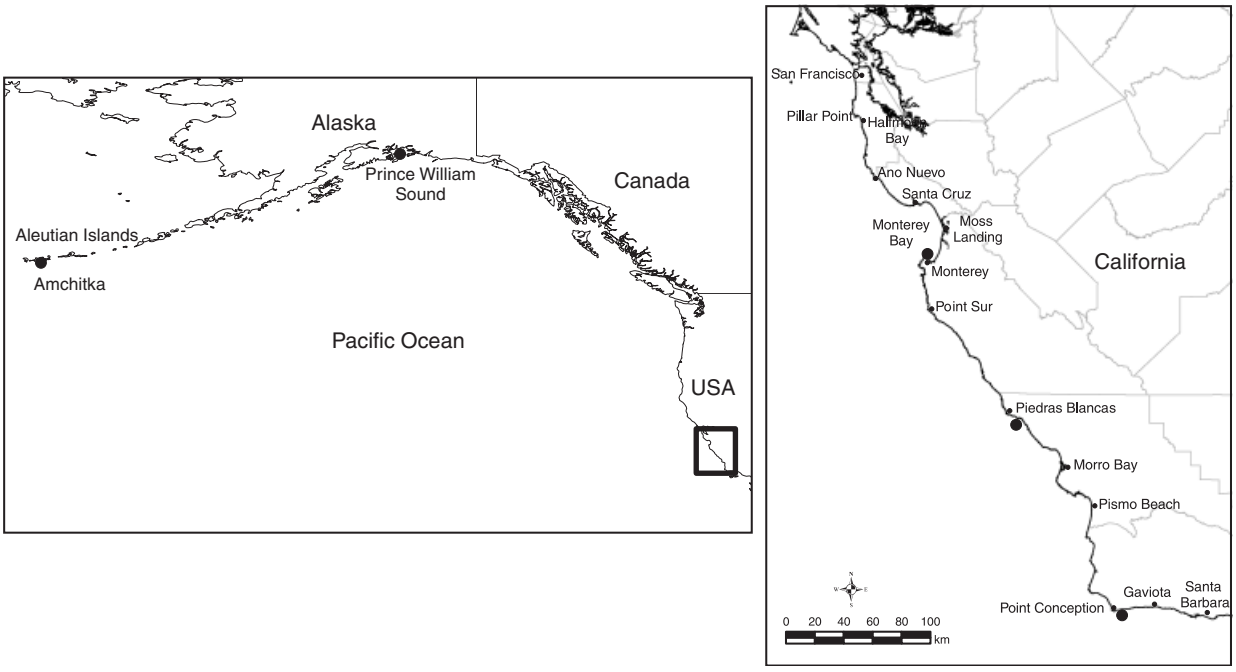

Figure 1 Sampling location of sea otters *Enhydra lutris* genotyped in this study.

translocated from central CA to San Nicolas Island in the late 1980s, where a population of about 50 individuals persists (B. Hatfield, US Geological Survey, unpubl. data). The historical population structure of otters, as well as the frequency of migration between the CA population and those further to the north, is not known, but given the species' generally sedentary nature (Kage, 2004) and the large expanse of unoccupied habitat from northern CA through southern Washington, it is unlikely that migration between populations was high.

The entire southern sea otter population is therefore descended from the small number of individuals that survived in the Big Sur region. Populations that undergo such bottlenecks can lose much of their genetic variation. Such variation is generally believed to be beneficial in both the ability of a population to mount a variable response to novel pathogens and parasites and in its ability to adapt to a changing environment (O'Brien & Evermann, 1988).

When the changing environment consists of novel collections of pathogens and parasites, variability in the immune response may play an important role in allowing and determining the trajectory of adaptive change (O'Brien & Evermann, 1988). Genes in the major histocompatibility complex (MHC) play a central role in modulating this immune response. Genes in the MHC Class I and Class II present antigenic peptides on the surface of cells for recognition by the immune system and have been intensively studied in humans and model organisms (Trowsdale, 1995). In humans, and presumably in other species, the MHC Class I molecules primarily present endogenous antigens, such as viral particles, and the Class II molecules present exogenous parasites, such as bacteria. Alleles of both Class I and II genes are defined by variation in the antigen binding site region of the second exon and different alleles can have specificity to different antigenic peptides. It has been shown in humans that the number and variety of peptides that can be presented properly is related to the number of alleles (Zinkernagel & Doherty, 1979). Strong balancing selection for allelic diversity has led to extensive polymorphism in most outbred vertebrate populations and MHC antigen presenting loci are the most variable protein coding genes known (Apanius *et al.*, 1997). Human populations have over 200 alleles of some Class I and Class II genes and, because most MHC variation is non-synonymous, this is mainly functional variation (Robinson *et al.*, 2003). In addition, this balancing selection leads to long persistence of MHC allelic lineages with, for example, lineages predating speciation events and leading to so-called trans-species polymorphism for some MHC genes in many taxa (Klein *et al.*, 1998).

Previous population genetic studies of sea otters have yielded ambiguous results, finding levels of variation either reduced or comparable to that observed in other mammalian species. Lidicker & McCollum (1997) investigated polymorphism at 31 allozyme loci, six of which were variable. Mean heterozygosity for all 31 loci was 4.9% for CA otters and 6.8% for Alaskan otters. The percentage of polymorphic loci and mean heterozygosity were similar to that observed in other marine and terrestrial mammals. Mitochondrial DNA haplotype diversity for sea otters from California is similar to that observed in remnant otter populations from Alaska, but lower than that found in many mammals (Cronin *et al.*, 1996; Bodkin *et al.*, 1999; Larson *et al.*, 2002). A limited analysis of microsatellite

markers revealed substantial genetic differentiation between sea otters in California and Alaska, and found numbers of alleles and levels of heterozygosity that were similar to other mammalian species with large documented declines in population size (Larson *et al.*, 2002).

Here we examine the amount and distribution of genetic variation in the sea otter by surveying a large number of nuclear genes, including those that are typically among the most variable genetic loci in mammals, in a large fraction of the CA sea otter subspecies. We describe genotypic information from 14 variable microsatellite loci and sequence variation from three antigen-presenting genes of the MHC. The MHC genes that we survey are the most variable protein-coding genes known in humans and presumably in most mammals. We also evaluate patterns of genetic relatedness and geographic proximity, as well as historical demography. In addition, analysis of genetic variation from sea otter populations in Alaska provides a comparative context in which to evaluate patterns of variability observed in California.

Methods

Blood and tissue samples were taken from both live-caught and dead sea otters in the central CA area between Santa Cruz and Gaviota (Fig. 1), almost the entire range of the species in California. Samples were also obtained from live-caught sea otters in Prince William Sound (PWS; $n = 25$), and Amchitka Island (central Aleutian Islands, AMI; $n = 21$). The animals were captured using either surface tangle nets or Wilson traps operated by rebreather-equipped divers.

Sea otters in PWS were derived from a remnant colony in the south-western part of the Sound. At present, the PWS population probably contains $>10\,000$ individuals and is at or near carrying capacity (Monson *et al.*, 2000). A remnant colony also survived the fur trade at Amchitka Island. The AMI population recovered to levels at or near carrying capacity by the late 1930s or early 1940s and persisted through the 1980s, when it contained 6500 or more individuals (Kenyon, 1969; Estes, 1977). In about 1990, sea otter numbers began a period of sharp decline throughout most of south-west Alaska and the AMI population currently has 200 or fewer sea otters (Doroff *et al.*, 2003; Estes *et al.*, 2005).

DNA was extracted from blood samples using DNeasy 96 Tissue Kits on a BioRobot 3000 (Qiagen Inc., Valencia, CA, USA). PCR was then performed on a 1:20 dilution of purified DNA. PCR reagent concentrations and thermal cycling details are available from the authors upon request. PCR products were mixed with a formamide buffer and electrophoresed on Applied Biosystems automated sequencers and individual allele calls determined using the Genotyper software package (Applied Biosystems Inc. Foster City, CA, USA). Each genotype was independently derived from the original gel file by two people and genotype scores were compared to minimize genotyping error. When discrepancies were found between genotypes from the two readers, they were resolved by mutual consensus or through genotype deletion. This involved $<2\%$ of the genotypes and most discrepancies involved only one reader recording a genotype.

Table 1 Microsatellite loci used in study

Locus name	No. of alleles	Estimated size range	Original reference
GG9	2	196–200	Davis & Strobeck (1998)
Lut435	1	116	Dallas & Piertney (1998)
Lut453	2	110–114	Dallas & Piertney (1998)
Lut715	1	118	Dallas & Piertney (1998)
Lut717	1	147	Dallas & Piertney (1998)
Lut457	3	181–185	Dallas & Piertney (1998)
Lut604	2	121–123	Dallas & Piertney (1998)
Lut615	5	247–283	Dallas & Piertney (1998)
Lut701	3	173–181	Dallas & Piertney (1998)
Lut733	1	130	Dallas & Piertney (1998)
Lut782	1	145	Dallas & Piertney (1998)
Lut832	3	193–201	Dallas & Piertney (1998)
Lut833	1	149	Dallas & Piertney (1998)
Lut902	4	154–166	Dallas *et al.* (1999)
Mer022	1	245	Fleming, Ostrander & Cook (1999)
Mer005	1	288	Fleming *et al.* (1999)
Mer030	6	224–234	Fleming *et al.* (1999)
Mvis002	1	203	Fleming *et al.* (1999)
Mvis027	2	202–204	Fleming *et al.* (1999)
Mvis072	1	272	Fleming *et al.* (1999)
Mvis075	4	166–182	Fleming *et al.* (1999)
Mvis099	1	321	Fleming *et al.* (1999)
Mvi057	3	123–127	O'Connell, Wright & Farid, (1996)
Mvi087	2	89–91	O'Connell *et al.* (1996)
TT1	4	173–179	Davis & Strobeck (1998)

Statistics calculated from California otters only.

We used a cross-species primer screening approach and initially examined 35 microsatellite primer pairs originally isolated in five other mustelids: Eurasian otter *Lutra lutra*, American mink *Mustela vison*, American ermine *Mustela erminea*, wolverine *Gulo gulo* and badger *Taxidea taxus*. These primers were screened in 24 southern sea otters. We then selected 25 of these genes (Table 1) for further genotyping in 335 otters from California and 46 from Alaska. The loci chosen were those that consistently produced one or two PCR products of approximately the predicted size. We genotyped all loci with variation present in the initial 24 CA otters (14 genes) and those for which there was not (11 genes), so as to determine whether the monomorphic loci might be variable in a larger sample or in only part of the geographic range.

Genetic variability

Observed and expected heterozygosity were estimated using the program GENETIX (Belkhir *et al.*, 2004). Allelic richness was estimated using the rarefaction method

implemented in the program HP_RARE (Kalinowski, 2005). Deviations from Hardy–Weinberg equilibrium (HWE) and pairwise linkage disequilibrium among loci were assessed with the Monte Carlo approximation of the exact test described by Guo & Thompson (1992) and implemented in GENEPOP (Raymond & Rousset, 1995). Pairwise coefficients of relatedness (r) among individuals from the CA sample were calculated using the estimator of Queller & Goodnight (1989) with the program KINSHIP. The possibility of kin- and sex-biased dispersal was examined by regressing pairwise coefficient of relatedness on geographic distance (based on a GPS-based location of sampling) separating two otters.

Evaluating historical demography

Four methods were used to assess if the genetic data from the three otter populations conformed to the expectation of a population that had experienced a recent decline in effective population size. The first is the M-ratio summary statistic method, which examines the ratio between the observed number of alleles and the allele size range for a particular microsatellite locus (Garza & Williamson, 2001). Populations with stable effective size will possess an M-ratio close to one, while recently bottlenecked populations will exhibit a reduction in the M-ratio due to the more rapid decrease in the number of alleles than the range in allele size. Significance of observed M-ratio values was assessed through simulation. This method requires specification of pre-bottleneck θ ($\theta = 4N\mu$) and the mutation parameter values for a two-phase model (TPM) of microsatellite mutation of which the stepwise mutation model (SMM) is a special case (Di Rienzo *et al.*, 1994). Four sets of parameter values were evaluated, because they have only been well estimated for humans and model organisms, and they included 80 or 90% single-step mutations, and a mean size of non-single-step mutations (Δg) of two or three repeats. For each simulation, 10 000 iterations were performed and significance was assessed as the proportion of simulated values greater than the observed M-ratio value.

The second method employed was the heterozygosity excess test (Cornuet & Luikart, 1996), a summary statistic method predicated on the observation that recent population bottlenecks cause rare alleles to be lost more rapidly than common alleles and that rare alleles contribute little to the observed population heterozygosity (Maruyama & Fuerst, 1985). This loss of rare alleles can lead to an excess of expected heterozygosity for a bottlenecked population when compared with an equilibrium population with the same number of alleles. This test was performed using the one-tailed Wilcoxon test for heterozygosity excess with the program BOTTLENECK (Piry, Luikart & Cornuet, 1999). Equilibrium conditions were evaluated with 1000 simulations using three models of mutation: the infinite alleles model (IAM), SMM and the TPM. Multiple parameters were evaluated for the TPM model and these included 80 and 90% single-step mutations and variances for multi-step mutations were set to 5 and 15 (assuming an exponential distribution).

The third method employed was the qualitative graphical one of Luikart *et al.* (1998). This method uses the frequency distribution of all alleles in a population genetic sample to detect the deficit of rare alleles (mode shift) expected following a rapid and severe reduction in population size.

We also applied a fourth Bayesian method (Storz & Beaumont, 2002) to evaluate the timing and extent of the population size change in sea otters. This Bayesian approach uses Markov Chain Monte Carlo (MCMC) to evaluate densities of posterior probabilities for current effective population size (N_1), pre-decline effective size (N_0), mutation rate (μ) and the time of the decline (T). Owing to the computationally intense nature of this analysis, 40 individuals (80 chromosomes) were sub-sampled from the 335 genotyped CA sea otters. Previous application of this method has shown that the posterior distributions are robust in the face of a wide array of priors (Goossens *et al.*, 2006). Nonetheless we used different priors in multiple analyses and ran independent MCMC simulations with similar priors to assess convergence of the posterior distributions (see Table 2 and 'Results'). Each MCMC was run for 2×10^9 steps and sampled every 10^5 steps (total of 20 000 observations). Posterior densities were estimated after removing the first 10% of observations using the statistical package R.

MHC

Three MHC genes – DRA, DRB and DQB that are generally variable in mammals were isolated from the southern sea otter. We targeted exon 2, which contains the peptide-binding region of these genes, using primers from other carnivores and non-stringent PCR conditions. The primers to amplify the DRA and DRB exon 2 fragments were from Garza (1998), while the primers from Hoelzel, Stephens & O'Brien (1999) were used to amplify the DQB exon 2 fragment. A single amplified fragment was observed for each gene and was then directly sequenced. PCR products were purified with the Qiagen PCR Purification kit (Qiagen Inc.) and sequenced directly with locus-specific primers on an ABI 377.

Each unique sequence was subjected to a BLAST search of the GENBANK database to verify that it corresponded to the targeted MHC gene. The sequences were then aligned with other mammalian sequences of the same gene extracted from GENBANK. Neighbor-joining trees (Saitou & Nei, 1987) were constructed with the Tamura–Nei distance measure using the MEGA3 software package (Kumar, Tamura & Nei, 2004) and node support was evaluated with 500 bootstrap replicates. Following confirmation of gene identity, a survey of variability was carried out for each of the three genes in both the California and Alaska sea otter populations.

The DRB and DQB products were found to be variable by direct sequencing. We employed a single-strand confirmation polymorphism protocol (Sunnucks *et al.*, 2000)

Table 2 Starting conditions and hyperpriors (on a log scale) for the Storz & Beaumont (2002) analysis for changes in effective population size

	Hyperpriors			
Run	log(N_0)	log(N_1)	log(μ)	log(T)
1	3 1 0 0.5	3 1 0 0.5	−3.5 0.25 0 0.5	2 1 0 0.5
2	3 2 0 0.5	3 2 0 0.5	−3.5 0.25 0 0.5	2 2 0 0.5
3	3 2 0 0.5	5 2 0 0.5	−3.5 0.25 0 0.5	2 2 0 0.5
4	3 2 0 0.5	4 2 0 0.5	−3.5 0.25 0 0.5	2 2 0 0.5
5	3 2 0 0.5	4 2 0 0.5	−3.5 0.25 0 0.5	2 2 0 0.5
6	4 2 0 0.5	3 2 0 0.5	−3.5 0.25 0 0.5	2 2 0 0.5
7	4 2 0 0.5	4 2 0 0.5	−3.5 0.25 0 0.5	2 2 0 0.5
8	3 2 0 0.5	4 2 0 0.5	−3.5 0.25 0 0.5	2 2 0 0.5
9	3 2 0 0.5	3 2 0 0.5	−3.5 0.25 0 0.5	4 2 0 0.5

Starting values are the means and variances for each independent run. Hyperpriors are the mean and variance for the mean (first two numbers) followed by the mean and variance for the variance (last two numbers).
N_0, initial population size; N_1, population size after bottleneck; μ, mutation rate; T, time since population decline. The log of starting values for each of the runs were as follows: N_0 (3 1), N_1 (3 1), μ(−3.5 1) and T(2 1), where the first number is the mean and the second the variance.

Table 3 Sample size, mean observed heterozygosity (H_o), mean expected heterozygosity (H_e), and allelic richness (A) for the three population samples of sea otters *Enhydra lutris*

Sample site	N	H_o	H_e	A
California	335	0.444	0.464	2.58
Amchitka Island	21	0.414	0.470	3.58
Prince William Sound	25	0.451	0.499	3.55

Allelic richness is calculated from the 10 variable loci in common across all population samples (see Methods/Results).

to isolate specific haplotypes. PCR products were diluted 3:5 in buffer (95% formamide, 3.2 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol), denatured at 100 °C for 3 min, placed in ice water, and loaded on 6% acrylamide gels [0.5 × TBE; 5% glycerol (v/v)] that were electrophoresed for 5–8 h at 20 W. Gels were stained with 1 × SYBR Gold (Molecular Probes, Carlsbad, CA, USA) and visualized on an FX Molecular Imager (BioRad Inc., Hercules, CA, USA). Bands were isolated directly from each gel, suspended in ddH_20 and subjected to an additional PCR amplification. PCR products were then precipitated in the presence of 20% polyethylene glycol and sequenced in each direction with BigDye (v3.1) sequencing mix (Applied Biosystems Inc.) on an ABI 377 automated sequencer.

Results

Of the 25 microsatellite genes fully screened, 14 were variable in CA otters, with two to six alleles present (Table 1). In addition, 12 of the loci were variable in the two Alaska samples (Table 3), and 10 of these variable loci were shared between the California and Alaska samples. One of the loci (Mvis075) had a significant deviation from HWE in both the California and two Alaska populations. The deviation was a heterozygote deficiency and indicates the presence of null alleles at this locus. This locus was therefore removed from further analyses. The variable loci did not show any evidence of linkage disequilibrium in any of the samples. Observed heterozygosity was highest in the PWS sample (0.451), lowest in the AMI sample (0.414) and the CA sample was intermediate (0.444), although the differences were not significant. Allelic richness for the 10 loci variable in all populations was highest in Alaska (3.59 and 3.55 in PWS and AMI, respectively) and lowest in California (2.58).

There was no correlation between geographic distance of sampling between individuals and their pairwise coefficient of relatedness for the CA population. The regression of pairwise relatedness on distance was not significant for either all individuals analyzed together, or males and females separately (Fig. 2a–c; all $r^2 < 10^{-4}$).

The four methods used to examine sea otter historical demography gave somewhat similar results. The M-ratio analysis did not find evidence for a recent reduction in effective population size for any of the three otter populations for a wide array of mutational scenarios (Table 4). M-ratio values were high for the three otter populations, and the majority of loci possessed both relatively few alleles and had small size ranges. This is indicative of a population that has been small for an extended period of time (Garza & Williamson, 2001). The heterozygosity excess test found evidence for deviation from mutation-drift equilibrium (i.e. recent reduction in effective size) in the CA and PWS populations with the IAM model and in the CA population with some parameter values for the TPM (Table 4), but only before Bonferroni correction for multiple tests. All of the samples showed an L-shaped distribution for allele frequency distributions (no mode shift), indicative of lack of a recent loss of rare alleles.

The Bayesian approach found that the southern sea otter population has suffered a twofold order of magnitude decline in population size. Ancestral effective population sizes based on this analysis varied from 1570 to 2218 (Fig. 3a) and current effective population sizes range from 5 to 17 (Fig. 3b). The peak time of decline ranged from 119 to 549 years before present. These values represent the range from nine independent MCMC analyses, and the posterior

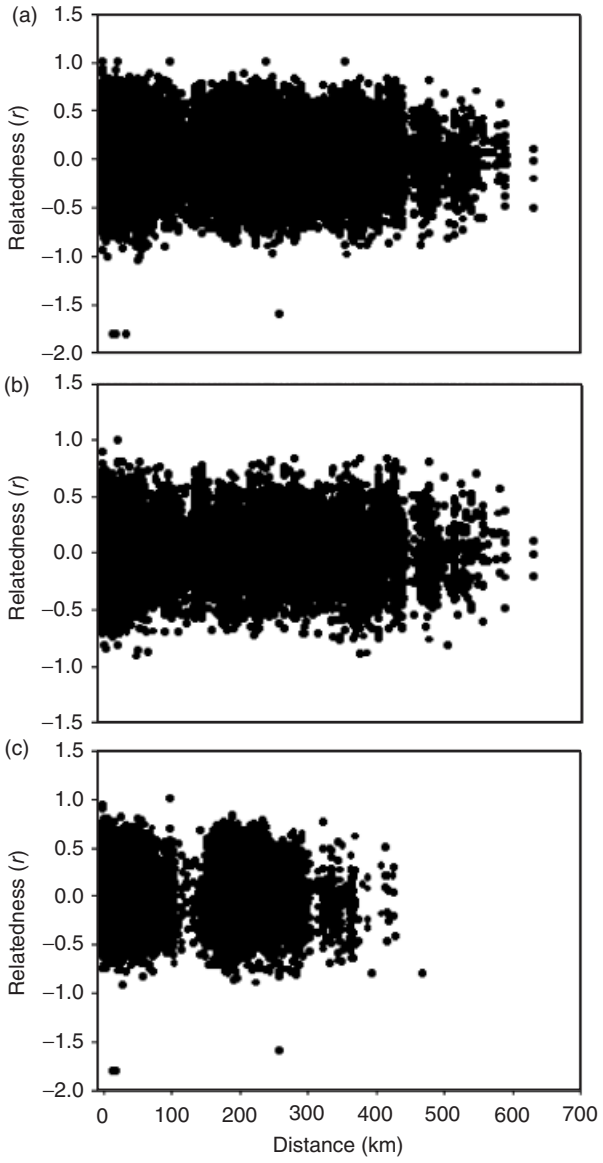

Figure 2 Plots of the relationships between pairwise coefficients of relatedness of individual otters and the geographic distance (km) between their location of sampling. (a) Relationship for all otters, (b) relationship for males, (c) relationship for females.

Table 4 Results of the summary statistic methods for detection of recent reduction in effective population size for three populations of sea otters *Enhydra lutris*

	California	Amichitka Island	Prince William Sound
M ratio	0.943	0.924	0.851
TPM: 0.1, 2	NS	NS	NS
TPM: 0.1, 3	NS	NS	NS
TPM: 0.2, 2	NS	NS	NS
TPM: 0.2, 3	NS	NS	NS
Heterozygosity excess			
IAM	<0.001	0.055	0.017
SMM	0.095	0.689	0.604
TPM: 0.1, 0.05	0.040	0.633	0.425
TPM: 0.1, 0.15	0.029	0.633	0.396
TPM: 0.2, 0.05	0.016	0.604	0.339
TPM: 0.2, 0.15	0.013	0.545	0.284
Mode shift	L	L	L

For the two-phase model (TPM) parameters given are the proportion of multi-step mutations and the mean size of multi-step mutations in number of repeat units for the *M*-ratio test and the proportion of multi-step mutations and variance in multi-step mutations, for the heterozygosity excess test. For the mode shift method, L refers to the pattern expected in the absence of a recent reduction in size.
IAM, infinite alleles model; SMM, stepwise mutation model; NS, non significant.

densities from each of these runs are similar with much of their distributions overlapping.

To survey immunogenetic variation in the sea otter, sequence data from three major histocompatibility genes (DRA, DRB and DQB) was collected from a total of 24 otters from California, 21 otters from AMI, and 25 otters from PWS. The DRA locus possessed the same haplotype for all individuals in all populations. The DRB and DQB genes possessed two haplotypes in sea otters. Both DRB haplotypes were present in all three sample locations (Enlu-DRB $\times$ 01 frequency CA = 0.20, AMI = 0.18, PWS = 0.08), but the two DQB haplotypes were present only in the Alaska populations (Enlu-DQB $\times$ 01 frequency CA = 1.00, AMI = 0.95, PWS = 0.90). The MHC sequences isolated from the otter genome showed high phylogenetic similarity to the published sequences of the targeted genes from other mammalian taxa (Fig. 4) and generally grouped with sequences of other carnivores. However, the two DRB haplotypes, Enlu-DRB $\times$ 01 and Enlu-DRB $\times$ 02, were not closely related phylogenetically and differed at 25 of 215 nucleotide sites. Nineteen of the differences led to amino acid substitutions and six of the base changes were silent at the amino acid level. In contrast, the two DQB sequences possessed high phylogenetic affinity to one another with elevated bootstrap support. The DQB sequences differed by four (out of 186) bases, with three of the substitutions leading to amino acid changes and one of the changes silent.

Discussion

We examined genetic variation in the southern sea otter by surveying a large number of microsatellite loci and three well-characterized, and generally highly variable, immune system genes (DRB, DRA and DQB). Very low levels of variation were observed for both the microsatellites and MHC genes. Estimates of heterozygosity and allelic variation were similar to those observed by Larson *et al.* (2002), even though we surveyed approximately twice as many variable loci and many more individual otters.

The amount of microsatellite allelic variation observed (2.2 alleles/locus) was similar to that of other critically

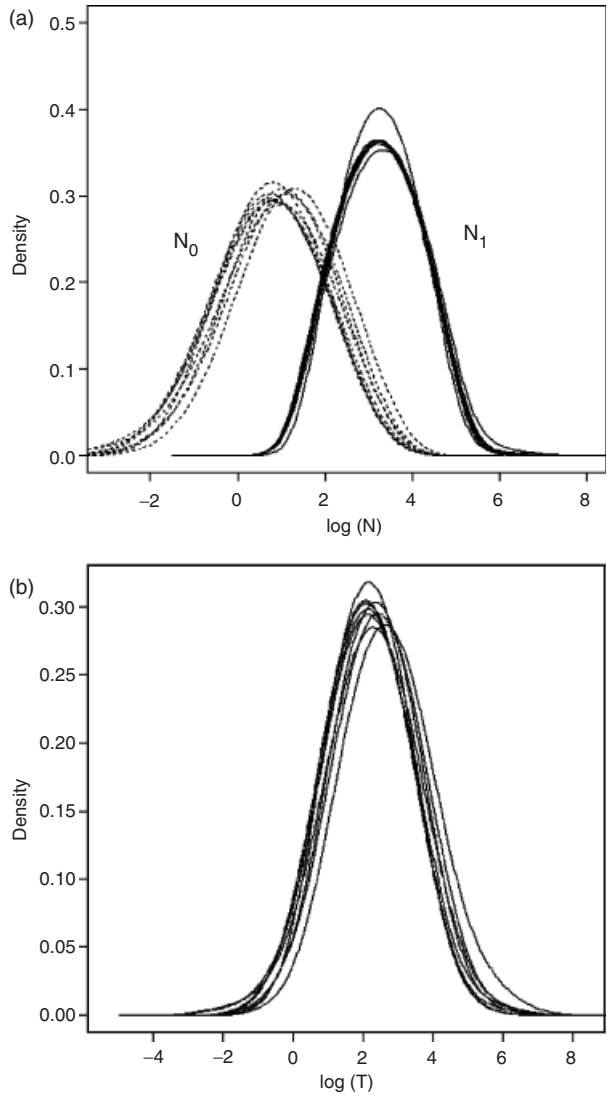

Figure 3 Posterior distributions of analysis of rate of change of population size with the method of Storz & Beaumont (2002). (a) N_0 (ancestral effective size), dashed line and N_1 (current effective size), solid line, (b) T is the time since the population size change.

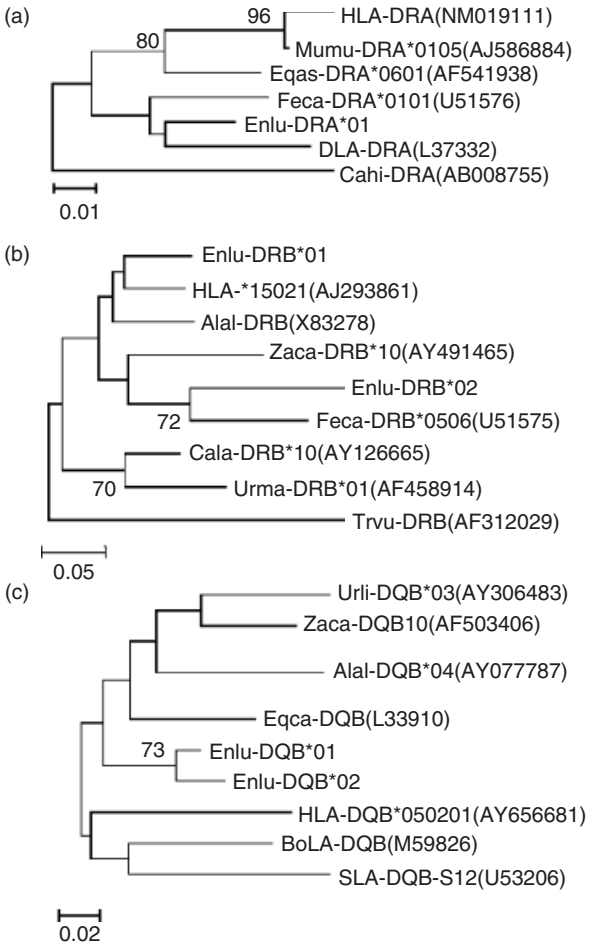

Figure 4 Neighbor-joining trees of the alleles from three MHC class II genes isolated from sea otters and sequences of the same genes from other representative mammalian species. (a) DRA, (b) DRB, (c) DQB. Bootstrap support above 70% is shown. GENBANK accession numbers follow sequences in parentheses. Alal, *Alces alces*; BoLA, *Bos taurus*; Cahi, *Capra hircus*; Cala, *Canis latrans*; DLA, *Canis familiaris*; Eqas, *Equus asinus*; Eqca, *Equus caballus*; Enlu, *Enhydra lutris*; Feca, *Felis catus*; HLA, *Homo sapiens*; Mumu, *Macaca mulatta*; SLA, *Sus scrofa*; Trvu, *Trichosurus vulpecula*; Urma, *Ursus maritimus*; Urli, *Urocyon littoralis*; Zaca, *Zalophus californianus*.

endangered mammals, such as the Mediterranean monk seal (Pastor *et al*., 2004; 1.9 alleles/locus) and northern hairy-nosed wombat (Taylor *et al*., 1994; 1.8 alleles/locus), and other species known to have undergone recent, severe reductions in population size, such as the northern elephant seal (Garza, 1998; 1.5 alleles/locus) and the Scandinavian wolverine (Walker *et al*., 2001; 3.0 alleles/locus). However, caution must be taken when comparing levels of genetic variation at microsatellite loci in different species, particularly when the number of loci is limited and the strategy for ascertaining the microsatellite loci is unknown. This is because a large number of representative microsatellite loci must be surveyed before estimates of allelic variation begin to asymptote to the parametric value. In addition, some published data report on only the most variable microsatellite loci, whereas others report results from all variable loci. This second scenario is the strategy employed here, as well as for at least the northern elephant seal (Garza, 1998) and Mediterranean monk seal (Pastor *et al*., 2004) studies. Values from these studies should then be roughly comparable and reinforces the dramatic effect that population decline has had on both neutral and functional genetic variation in the southern sea otter.

Comparison of allelic richness and number of observed alleles in the three otter populations indicates that the CA population has the lowest level of variation of the three. A slightly lower mean number of alleles was observed in the

CA otters (data not shown), in spite of a sample size >10 times larger than in the two Alaska populations. Allelic richness, which measures allelic variability while taking into account different sample sizes, was much lower in California (2.58) than in Alaska (3.58 and 3.55). This is somewhat in contrast with the results of Larson *et al.* (2002) who found nearly identical measures of allelic diversity in California and Alaska otter populations. Possible explanations for this discrepancy are that a much larger number of, and different, microsatellite loci are used in the current study and/or that Larson *et al.* (2002) only presents number of alleles, in spite of differences in sample size.

Results from the methods that detect recent changes in effective population size were contrasting but informative. The summary statistic methods generally did not find strong evidence for recent reductions in effective size for the three otter populations, although the heterozygosity excess method did find marginally significant results in CA sea otters with some parameter values before correction for multiple tests. The small range in allele size for most of the loci is responsible for the lack of significant results with the *M*-ratio test. Such a result, in combination with the low allelic variability, indicates that the long-term effective population size is low and suggests that the population bottleneck that occurred due to the fur trade affected a population that already had a very small effective population size. Otherwise, at least some of the loci would be expected to have larger allele size ranges with significant gaps. It is highly unlikely that a severe episode of genetic drift would remove only alleles that were on the extremes of the size range and leave no gaps in the allele size distribution. In addition, rare alleles are generally lost most rapidly during bottlenecks, yet the CA sea otter population does not have a deficit of rare alleles that 'distorts' the frequency distribution (Luikart *et al.*, 1998), indicating that many of the rare alleles have arisen by mutation since and during the episodes of severe genetic drift. The lack of a general signature of a recent population bottleneck found here is similar to what has been found in the European otter *L. lutra* (Pertoldi *et al.*, 2001).

In contrast, the Bayesian method (Storz & Beaumont, 2002) found evidence for a twofold order of magnitude decrease in effective population size for CA sea otters. Such a decrease would be, for example, from thousands of breeding individuals to tens of individuals. This magnitude of population size decrease agrees with the historical declines reported for southern sea otters (Kenyon, 1969; Riedman & Estes, 1990). The Bayesian analysis also suggests that the time since the decline was *c.* 120–550 years ago (the range of posterior distribution means). The known peak of human sea otter exploitation in California corresponds to the later part of this range, but the bulk of the estimates are earlier than the known period of decline (Kenyon, 1969). This result, coupled with the *M* ratio, heterozygosity excess and qualitative allele frequency analyses suggest that the decline in effective population size for CA sea otters began before the documented reduction due to the fur trade. This may have been due to a sustained small breeding population or a severe reduction in size that predates the fur trade. There is evidence that native people exploited otter populations (Simenstad, Estes & Kenyon, 1978), and otter bones are found in more than 100 archaeological sites in California (T. L. Jones, Cal Poly, pers. comm.) and it is possible that such exploitation contributed to a reduction in effective size before the fur trade in California, as it did with other marine mammals (Jones *et al.*, 2004). Populations which have been small for a long time have generally purged many of the deleterious recessive alleles and mitigated many of the other effects of inbreeding (Charlesworth & Charlesworth, 1987), and this may mean that southern sea otters are less susceptible to the genetic effects of small population size than other species that have suffered recently reduced effective population size.

We found no evidence for geographically limited and/or sex-biased dispersal in the CA sea otters, as there was a lack of any relationship between pairwise genetic relatedness and geographic distance. Adult male sea otters have high dispersal rates and disperse greater distances than females (Jameson, 1989; Kage, 2004), but this was not reflected in our results. To the extent that this result represents a general lack of subdivision or geographic panmixia (i.e. that the probability of a particular otter mating with any other otter is not correlated with where it is born) for the population, then the inbreeding inherent in subdivision will be avoided and effective population size will be maximized for CA sea otters.

We found a very low level of variation at MHC genes in sea otter populations. This is not surprising given the substantial population declines sea otters have experienced, but emphasizes the dramatic reduction in functionally important genetic variation that has been experienced in sea otters, relative to outbred mammalian species. It is possible that more allelic variation is present in sea otters, as we have only surveyed the most variable second exon in a number of the best-characterized MHC genes. This, in fact, does appear to be the case, as more allelic variation is observed at expressed MHC genes in otters (Bowen *et al.*, 2006), but the additional variation present is very likely minimal and would not substantially change the result of a depauperate suite of antigen-presenting genes in sea otter populations.

It is unclear at this time what the locus and allelic relationships are for the alleles isolated from the variable MHC loci (DQB and DRB). In many mammalian species, the MHC class II genes are present in multiple copies (Kumánovics, Takada & Fischer-Lindahl, 2003), and there is also variation in number of copies per chromosome within populations. A high amount of divergence was observed for the two DRB alleles, possibly indicating the presence of at least two DRB loci in sea otters. It may also indicate the preservation of two alleles from divergent lineages of the same locus, as has been observed for many taxa where the long persistence of MHC allelic lineages leads to trans-species polymorphism (Klein *et al.*, 1998). We did observe a close phylogenetic affinity of sea otter MHC sequences to those of other mammalian taxa and an increase in the

number of non-synonymous (amino acid changing) substitutions between alleles of the DRB and DQB genes, indicating that these are functional gene copies and not pseudogenes.

In spite of the extremely low amount of genomic variation uncovered in the southern sea otter, the 14 variable microsatellite loci have sufficient power for use as genetic tags (Palsboll, 1999). Two pairs of sea otters samples from this study were found to have matching genotypes at all 14 loci. These loci are sufficiently powerful to successfully identify individuals [P(ID) = 0.0005] with the method of Paetkau & Strobeck (1994), so these matches are unlikely to be due to chance alone. In addition, the collection location and gender of these pairs of samples is consistent with their being from recaptures of the same animal.

The low level of genetic variation discovered in southern sea otters is unusual for a mammal, and raises concern about the ability of the population/subspecies to avoid extinction in the face of environmental change and novel pathogens. There is a high incidence of pathogen-mediated mortality in CA sea otters, due to protozoal parasites (*Toxoplasma gondii* and *Sarcocystis neurona*) and a number of other novel disease agents (Lindsay, Thomas & Dubey, 2000; Miller *et al*., 2002; Kreuder *et al*., 2003; Stavely *et al*., 2003; Kreuder *et al*., 2005). It is unclear if there is a specific, underlying genetic mechanism that contributes to this potential increase in mortality, but reduced genetic variation and a recent increase in prevalence of pathogens, due to anthropogenic forces (e.g. pollution), may play a role (Jessup *et al*., 2004).

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