Preliminary genetic status of the spotted seal *Phoca largha* in Liaodong Bay (China) based on microsatellite and mitochondrial DNA analyses

Xiang Li,[1] Athanasia C. Tzika,[2,3] Yingying Liu,[4] Karine Van Doninck,[1] Qian Zhu,[4,5] Michel C. Milinkovitch[2]

[1]Evolutionary Ecology and Genetics, URBO, Department of Biology, University of Namur (FUNDP), Namur, Belgium; [2]Laboratory of Artificial and Natural Evolution (LANE), Department of Genetics and Evolution, Sciences III, Genève, Switzerland; [3]Evolutionary Biology and Ecology, Université Libre de Bruxelles, Brussels, Belgium; [4]Ocean College, Shandong University at Weihai, Weihai, PR China; [5]Third Institute of Oceanography, State Oceanic Administration, Xiamen PR of China

Abstract

The Liaodong Bay spotted seal *(Phoca largha)* population experienced several drastic declines in the last 80 years. Recent studies are contradictory regarding the level of genetic diversity and population structure of *P. largha*, possibly because of the use of non-species-specific nuclear markers. Here, we report on i) the first isolation and characterization of 10 species-specific polymorphic microsatellite *loci* for the spotted seal, ii) sequences of a 572 bp mtDNA fragment in 25 Liaodong Bay individuals that we analyzed together with all published haplotypes from Liaodong Bay and Japan. Intermediate genetic diversity in microsatellite *loci* was found in the Liaodong Bay population and the effective population size estimates were 41.8 to 86.8 individuals. Low mtDNA genetic variability, especially nucleotide diversity, in the Liaodong Bay population was detected, but Bayesian skyline plots did not show any evidence of recent bottleneck. Both F-statistics and the haplotypic network indicate a clear differentiation between the Liaodong Bay and Japanese populations separated by a fixed mutation. Analysis of mtDNA data indicates that Liaodong Bay female seals show fidelity to their breeding site, and breeding time data suggest that this population is reproductively isolated from populations in other breeding areas. The observed low genetic diversity in mtDNA and the intermediate levels of nuclear microsatellite diversity, combined with the potential genetic isolation, suggest that the Liaodong Bay population might be at risk and that further investigation of the population genetics of spotted seals across their whole range is warranted for proper management of the species.

Introduction

Multiple populations of various seal species are threatened throughout the world because of human activities such as direct catches and accidental mortality in fishery activities, as well as modifications of habitat.[1-3] Seal stock boundaries generally are poorly understood because most species are migratory and have high dispersal abilities. The lack of objective data on seal population genetic diversity and stratification hinders achievement of sustainable management.

The spotted seal (*Phoca largha*; Pallas 1811) inhabits the ice and waters of the North Pacific Ocean and adjacent seas, and Liaodong Bay (LB) in China is the southern-most of the eight putative separate breeding areas (Figure 1).[4] Given that the abundance of spotted seals has not been extensively quantified, the species is listed as "data deficient" by IUCN (Red list; *www.iucnredlist.org*). However, it is clear that the spotted seal in China drastically declined in the last 80 years as reliable catch estimates are available for the period 1930-1990 during which 30,395 seals were caught.[5,6] Spotted seals have been hunted historically in China for their oil, fur, and genitalia. As various rough estimations performed between 1979 and 1983 indicated that the LB seal population might consist of only about 2,000 individuals, the Chinese government formally banned the hunting of spotted seals in 1982.[5,6] As a result, the size of the LB population increased rapidly and was estimated at 4,500 animals in 1990.[6] However, in the last decade, the population dramatically decreased again to an estimation of less than 1,000 individuals[7] because of several factors. First, illegal hunting of spotted seals still occurs because the demand from the Chinese traditional medicine market persists: preparations derived from seal genitalia are thought to enhance human male sexual potency. Second, the spotted seal population is threatened by massively increased human activities in LB[8]: pollution, habitat destruction, fishing activities, deliberate aggression by fishermen, gas and oil exploitation, and development of ship transportation.

Some recent studies are available on the genetic diversity and population structure of *P. largha*. First, O'Corry-Crowe and Bonin[9] obtained mtDNA sequences from 247 spotted seals and genotyped, for 18 microsatellite *loci*, 207 spotted seals (sampled from the Chukchi Sea, Bering Sea, NW Pacific Ocean, Sea of Okhotsk, Sea of Japan, and Yellow Sea), and found a significant differentiation between the southern regions (Yellow Sea and Sea of Japan) and the northern ones (Okhotsk, Bering, and Chukchi Seas). However, in that study only five individuals were collected from LB and the data is not available publically. Second, Mizuno *et al.*[10] sequenced a 571bp fragment of mtDNA control region and adja-

Correspondence: Karine Van Doninck, Evolutionary Ecology and Genetics, URBO, Department of Biology, University of Namur (FUNDP), Rue de Bruxelles 61, 5000 Namur, Belgium.
E-mail: karine.vandoninck@fundp.ac.be
Qian Zhu Third Institute of Oceanography, State Oceanic Administration, Daxue Road 178, Xiamen 361005, PR China; Ocean College, Shandong University at Weihai, Weihai 264209, PR China. E-mail: qianzhu@sdu.edu.cn

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Contributions: the work presented here was carried out in collaboration among all authors. XL, QZ, MCM conceived the study; XL, ACT, MCM designed the methods and experiments; XL carried out most of the laboratory experiments; YYL participated in the mtDNA experiments; XL, KVD, ACT, MCM analyzed the data and interpreted the results; XL drafted, and KVD, ACT, MCM revised the manuscript. All authors have approved the manuscript.

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cent tRNAs from 66 spotted seals from and around breeding area 4 (Figure 1) located along the coastal regions of Hokkaido (Japan), and found a high diversity (57 haplotypes) among individuals and no population structure. Third, Han *et al.*[11] sequenced a 717bp mtDNA control region fragment from 46 spotted seals sampled in LB, and found 17 haplotypes. These haplotypes were compared with 15 haplotypes of the 57 found in Japanese spotted seals.[10] They found a very low genetic diversity in LB with the presence of two fixed differences (one is an insertion/deletion at position 16296 of the threonine mtDNA tRNA gene, the other is a C/T transition at position 16607) between Chinese and Japanese haplotypes. However, the authors did not perform F-statistics or haplotypic network analysis and did not incorporate all available haplotypes from Japan, which may result in inaccurate conclusions. Finally, Han *et al.*[8] found a low level of genetic diversity, comparable to some documented bottlenecked mammal species, among 176 LB spotted seal individuals using 15 microsatellite *loci* isolated from other seal species. This latter result should be taken with caution as variability can be significantly underestimated when using cross-species microsatellite amplification.[12]

Here, we report on the first isolation and characterization of ten highly polymorphic species-specific microsatellite *loci* for the spotted seal *P. largha*. The obtained microsatellite data allowed us to examine the genetic status of the LB seal population and estimate its effective population size. A 572bp mtDNA fragment was sequenced from the same 25 individuals and analyzed together with all published haplotypes from LB and Japan to assess the demographic historical changes of the LB population and its genetic relationship with the Japanese one.

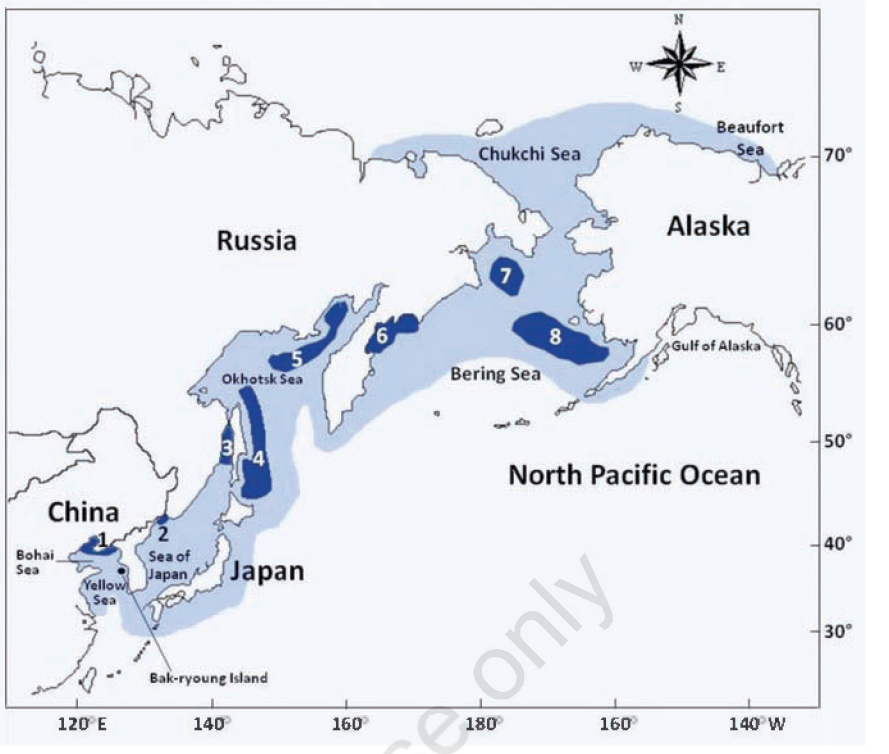

Figure 1. Spotted seal distribution (light blue) and breeding sites (numbered dark blue areas): 1) Liaodong Bay, sampling site of this study; 2) Peter the Great Bay; 3) the western coast of Sakhalin Island in the Tatar Strait; 4) the eastern coast of Sakhalin Island extending to northern Hokkaido; 5) northern Shelikova; 6) Karaginsky Bay; 7) Gulf of Anadyr; 8) east of the Bering Sea.

Materials and Methods

Sampling and DNA extraction

Tissue samples (muscle and skin) were obtained from discarded or partly decayed carcasses of 20 LB spotted seals (Figure 1). Five additional samples were collected from animals that died at Penglai Aquarium, which were all caught from the wild (LB) and bred in captivity. All the 25 samples were collected at different time periods by different people and in different localities within LB during the last seven years. Genomic DNA was extracted using standard procedures.[13]

Isolation and characterization of spotted seal microsatellites

A spotted seal genomic library enriched for microsatellites was constructed as in Tzika *et al.*[14] Sequences were fed into OLIGOFAKTORY[15] to identify microsatellite repeats and their optimal flanking primers. In total, 59 *loci* were tested. Thirty of the *loci* that generated a PCR product were screened for polymorphism among eight spotted seal samples using fluorescent dUTPs (Fermentas); 10 *loci* were monomorphic, and 20 *loci* proved polymorphic.

Genotyping and microsatellite analysis

On the basis of PCR product sizes, polymorphism levels, and lacking of scoring ambiguity, 10 of the 30 microsatellite *loci* screened were selected for genotyping all 25 individuals using three multiplex-PCR reactions (Table 1). Genotyping products were separated by electrophoresis (ABI 3730 DNA Analyzer). Microsatellite allele sizes were determined with GeneMapper 3.7 (Applied Biosystems).

Presence of null alleles and allele dropout at each *locus* was tested with MICROCHECKER 2.2.3.[16] Using Arlequin 3.11,[17] we i) tested for departure from Hardy-Weinberg (HW) equilibrium (900,000 Markov chain iterations); ii) tested for independence between each possible pair of *loci* using likelihood-ratio statistics (null distribution obtained with 10,000 permutations); iii) calculated various nuclear genetic diversity statistics: number of alleles per *locus* (A) and observed (H_O) and expected (H_E) heterozygosities. Critical significance levels for multiple testing were corrected according to the Bonferroni procedure.[18]

To identify putative recent reduction of effective population size, we first used the heterozygosity excess test implemented in the software BOTTLENECK 1.2.02.[19] We used the TPM model with 20,000 replications, 95% of stepwise mutations and 5% multi-step mutations, and variance among multiple steps of 12, as recommended for microsatellites.[20] Statistical significance was assessed with a one-tailed Wilcoxon signed rank test, which recommends, to achieve high power, 15-40 individuals with 10-15 polymorphic *loci*.[19] Because our data fits those recommendations, having tested 25 individuals with 10 *loci*, we applied this test here. Second, we used the qualitative descriptor of allele frequency distribution ("mode-shift" indicator)[21] also implemented in BOTTLENECK 1.2.02.[19] However, the statistical power for this method is low for sample sizes smaller than 30 individuals.[19] Finally, we used Garza-Williamson index (M), the mean ratio of the number of alleles at a given *locus* (k) with respect to the range of allele size (r), to detect population bottlenecks

(as implemented in Arlequin 3.11).[17,22] Studies of several natural populations have shown that M is greater than 0.82 for populations that have not suffered a known reduction in size and less than 0.70 for those that went through a bottleneck.[22] Because the population size of the LB spotted seal population was roughly estimated by several incomplete investigations,[7] three genetic methods were used to estimate its effective population size (N_e). First, we used the linkage disequilibrium method associated with bias-correction[23] implemented in LDNe[24] and excluded alleles with frequency less than 0.03 because it generally provides a better balance between precision and bias for a sample size of 25.[25] Second, we used the heterozygote excess method[26] implemented in NeEstimator.[27] Third, we estimated Ne with a 95% confidence interval using the approximate Bayesian computation method[28] implemented in ONESAMP.[29]

Mitochondrial DNA sequence analyses

We amplified an mtDNA fragment containing a portion of the threonine and proline tRNA genes and part of the control region,[30] using the primers L16274 (5'-TACACTGGTCTTGTAAACC-3') and H34 (5'-CCAAATGCATGACACCACAG-3') of 25 spotted seal individuals from LB. PCR products were purified, cycle sequenced on both strands (with L16274 and H34 primers), and run on an ABI 3730 sequencer. We analyzed our LB spotted seal mtDNA sequences in combination with published sequences of 46 spotted seals from LB[11] and 66 spotted seals from the Coast of Hokkaido, Japan.[10] Haplotype (H) and nucleotide (π) diversities were computed using Arlequin 3.11.[17] Evolutionary relationships among haplotypes were examined using the median-joining network (MJN) approach[31] implemented in NETWORK 4.2.0.1 (*http://www.fluxus-engineering.com*). This approach has been proved to be one of the most reliable network procedures.[32,33] Pairwise F_{st} and Φ_{st} statistics were used to estimate genetic differentiation among populations, as implemented in Arlequin 3.11 (statistical significance evaluated using 16,000 permutations).[17] The F-statistic is based only on the difference in overall haplotype frequencies, while the Φ-statistic takes into account both haplotype frequencies and genetic distances among haplotypes.[34]

To estimate the past population dynamics of the LB population, we used the 71 mtDNA sequences to construct Bayesian skyline plots (BSPs)[35] as implemented in BEAST v1.5.4.[36] This method uses an MCMC sampling procedure to estimate the posterior distribution of the effective population size.[35] We used the HKY substitution model (determined by ModelGenerator[37]) and a strict molecular clock for the mtDNA control region sequence. Because an appropriate nucleotide substitution rate has not been estimated for this lineage, the evolutionary rate was set to 1.0 and the branch length in the plot will be in units of mutations per site. Note that the mutation rate affects only the scale of the BSPs, but not its shape. Three independent runs were performed for 1×10^8 iterations, with the first 10% discarded as burn-in, sampled every 1,000 iterations. Results of the analyses were visualized using Tracer v1.5.[38] Convergence of the chains to the stationary distribution was confirmed by visual inspection of plotted posterior estimates.

Results

Microsatellite variability

The cloned sequences corresponding to the 10 selected spotted-seal microsatellite polymorphic *loci* have been deposited in GenBank (accession numbers GU232531-GU232540). All 25 sampled individuals were successfully genotyped for the 10 *loci*. There was no significant evidence for null alleles, large allele dropout, or scoring errors. No *locus* showed significant deviation from HW equilibrium or linkage disequilibrium after Bonferroni

Table 1. Characterization of ten microsatellite *loci* in 25 spotted seals (*Phoca largha*) from Liaodong Bay (China).

Locus	Primer sequence (5'-3')	Repeat motif	Ta (°C)	Size range	GenBank Accession No.	A	H_O	H_E	HWE	M
PLf4	F: GTTTCTTGCCCACCATTTTCTGCTCC R: FAM-TTTTCATTACTTCTTTCTTACCC	$(GT)_{22}$	59	124-140	GU232531	6	0.72	0.71	0.56	0.67
PL65	F: GTTTCTTCATGTTCCCTGTCTCTTCCT R: PET-TGCTTCATGTCTTTTTCATTTTT	$(AC)_{21}$	59	225-247	GU232532	8	0.56	0.79	0.03	0.67
PLb10	F: GTTTCTTTTTTCTTCCTATTTTTCTACATAA R: VIC-TCAACTTCCATCTCCTACCA	$(AC)_{20}$	59	304-316	GU232533	6	0.64	0.69	0.18	0.86
PLb9	F: GTTTCTTGGGGAGGGAAATGGGGATA R: FAM-TGGGGAGGGGAGATTGTG	$(TG)^{19}$	59	147-157	GU232534	5	0.68	0.62	0.95	0.83
PLg10	F: GTTTCTTGCATTATCACACCCATTTTAC R: NED-TTCTCCACACTGCCTTAGCA	$(TG)_9(AG)_{22}$	59	191-213	GU232535	10	0.80	0.82	0.71	0.83
PLc5	F: GTTTCTTACCTCTGAAACAAATACATTGT R: PET-CCACCCCCTTCCCCTCTA	$(AAG)_7A(AAG)_{24}$	62.2	227-260	GU232536	11	0.96	0.86	0.82	0.92
PLe2	F: GTTTCTTTTTAGTCTTTTGTGTTGGTTT R: VIC-CTTCCTTCTTCTGCCCAGT	$(TG)_{19}$	59	400-422	GU232537	9	0.96	0.86	0.28	0.75
PLd7	F: GTTTCTTCAGCATTACCTACAATAGCCAAA R: NED-ATTCTTGCAACTCAATAAATCCAA	$(ATAG)_{12}$	59	222-238	GU232538	5	0.56	0.66	0.48	1.00
PL68	F: GTTTCTTTGTTTGTTGAAAATCAGGATG R: PET-CCTCTTACCCACTGCTTGT	$(TTC)_{21}(GA)_8$	58	247-270	GU232539	9	0.68	0.83	0.22	0.75
PL57	F: GTTTCTTTCCCCTTCGCCTGTTCTG R: VIC-TTTGTCTCCCTGTTTCTTTTC	$(CA)_9(GA)_{24}$	58	419-449	GU232540	10	0.80	0.77	0.99	0.63
					Mean	7.9	0.74	0.76		0.79

For each *locus* we list: the name of the *locus*; the sequences of the forward and reverse primers, the forward primer (F) includes a GTTTCTT tail (underlined) at its 5'-end to force A+ alleles and, hence, improve binning of alleles, and the reverse primer (R) was labelled fluorescently; the repeat motif column shows the repeat pattern of the cloned allele; Ta, annealing temperature; Size range, range of allele sizes (bp) observed in our study; the GenBank accession number of the genomic clone sequence on which the corresponding primers have been designed; A, number of alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, probability values of exact tests of fit to Hardy-Weinberg equilibrium; M, Garza-Williamson index; and the mean value of A, H_O, H_E, and M across the 10 *loci*. The microsatellites are separated in the three primer sets used for the multiplexing reaction.

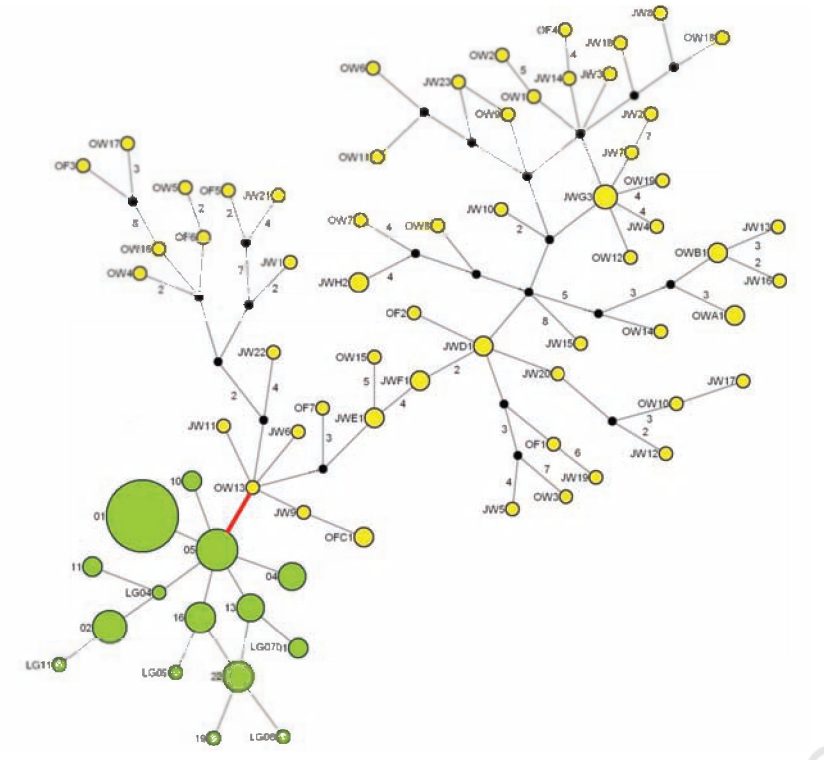

Figure 2. Median-joining network among mitochondrial *Phoca largha* haplotypes of Liaodong Bay (green) and Japan (yellow). Black spots indicate missing haplotypes. The red line shows the fixed mutation between the populations of Japan and Liaodong Bay. Circle sizes are proportional to the corresponding haplotype frequencies. The number of mutations is indicated when an edge corresponds to more than one mutation.

procedure. The number of alleles per *locus* varied from 5 to 11, with a mean number of 7.9 (Table 1). As previously demonstrated,[39,40] the *loci* with compound repeat motifs usually exhibited more alleles in our study.

The analyses of heterozygosity excess and the allele frequency distribution mode-shift did not detect any significant recent bottleneck in our population. Meanwhile, the mean Garza-Williamson index (M) was 0.79 (Table 1), slightly lower than the value for populations that have not suffered a known reduction in size (0.82) but above the critical value (0.70), so we may infer that this population did not experience any significant recent bottleneck. Expected and observed heterozygosities over all microsatellite *loci* varied from 0.62 to 0.86 and from 0.56 to 0.96, respectively. Furthermore, genetic diversity (H_E) found in our samples with species-specific primers was higher than that of another LB sample studied by Han *et al.*[8] using microsatellite *loci* isolated from other seal species (Supplementary Table 1).

The estimated mean effective size using the linkage disequilibrium method was 74.2 (95% CI=39.7-306.8, jack-knife on *loci*). The estimation based on the heterozygote excess method was 86.8 (95% CI=53.4-204.9), whereas the approximate Bayesian computation method estimate of Ne was 41.8 (95% CI=31.3-82.0). Because the three confidence intervals overlapped, there was no significant difference in the values calculated by the three different methods.

Mitochondrial sequence diversity, phylogeographical patterns, and F-statistics

The mtDNA fragment was sequenced successfully in all 25 samples. Of the 572 nucleotides scored, nine variable sites were detected, defining 10 haplotypes differing by eight transitions, no transversion, and one insertion/deletion. Nine of these 10 haplotypes overlapped with those found by Han *et al.*,[11] with the single new haplotype characterized here available in GenBank (accession number EU420021). No haplotype from the LB population corresponded to any of the 57 haplotypes found in 66 spotted seals of a Japanese population (breeding area 4 in Figure 1).[10]

The haplotype and nucleotide diversity estimate for the LB population was 0.8241±0.0371 and 0.0033±0.0022, respectively (Supplementary Table 1). Supplementary Table 1 also indicates that genetic variability in terms of haplotype and nucleotide diversity is lower in the LB population in this study and in that of Han *et al.*[11] than in the Japanese population.[10]

The median-joining network among all haplotypes (Figure 2) indicates a clear differentiation between the LB and the Japanese populations separated by a single fixed mutation (an insertion/deletion at the position 16,296 located in the tRNA-thr). In agreement with the diversity indices discussed above, the network indicates that haplotype diversity is higher in the Japanese than in the LB population.

Similarly, F-statistics indicate 1) no significant difference between our samples and those from Han *et al.*[11] (Supplementary Table 2A), but 2) significant differentiation between the populations from LB and Japan[10] (Supplementary Table 2A and B). BSPs show that the LB population maintained relatively stable population sizes over the demographic history, and no extreme size changes occurred even when considering the 95% HPD around the median BSP line (Supplementary Figure 1).

Discussion

Rapid human demographic and economic development threatens the survival of marine mammals worldwide, but nowhere more than in Asia in general, and in China in particular. LB is an important breeding ground for *P. largha* and corresponds to the southern-most end of the species' range. Spotted seals spend much of their time there while bearing their pups on the ice edge along the coast, very close to human-induced perturbations. Information about the species' genetic diversity and population stratification is gradually becoming available, but species-specific nuclear markers were lacking. In this study, we isolated the first spotted seal-specific microsatellite nuclear *loci*. We also sequenced a fragment of the mitochondrial DNA in 25 samples and combined this data with previously published spotted seal homologous sequences from Japan and China.

Despite that the LB spotted seal population has drastically declined in the last 80 years, levels of genetic diversity (expected heterozygosity, H_E) found in our samples are intermediate (H_E=0.76), with values similar to those observed in other pinniped species using species-specific primers[12,41,42] and higher than in the documented bottlenecked populations of Northern elephant seals (*Mirounga angustirostris*, H_E=0.13),[1] Hawaiian monk seals (*Monachus schauinslandi*, H_E=0.03),[2] and Mediterranean monk seals (*Monachus monachus*, H_E=0.16).[3] Furthermore, no recent reduction in population size is evidenced by the molecular data. This might be because of our small sampling size causing the insufficient statistical power in the bottleneck analy-

sis "mode shift," or the methods we used to detect bottleneck are too conservative. However, the various genetic methods used in this study have shown that the effective size (N_e) estimates of the LB population varies between 41.8 and 86.8 individuals. It has been suggested that populations with Ne smaller than 50 could cause inbreeding depression and those with N_e smaller than 500 are thought to be at risk of losing genetic variation through genetic drift.[43-45] Contrasting levels of genetic diversity between our analysis and a study from the same population[8] indicate that variability can be underestimated significantly when using cross-species microsatellite amplification.

Despite that the total sample size (71) of LB in this study and that of Han *et al.*[11] is higher than that of Japan (66 samples),[10] the former population exhibits much lower mtDNA control region genetic diversity (Figure 2 and Supplementary Table 1). The observed difference in diversities in mtDNA control region (both haplotypic and nucleotidic) between the Chinese and Japanese populations is even underestimated, as many of the Japanese haplotypes probably remain to be sampled as revealed by the large number of missing haplotypes in the network (57 sampled haplotypes vs. 22 missing haplotypes; Figure 2). The nucleotide diversity estimate for the LB population (π=0.0033±0.0022) is considerably lower than reported for the control regions of other seal species; for example, grey seal (*Halichoerus grypus*),[16] harbour seal (*Phoca vitulina*),[30] ringed seal (*Phoca hispida*),[47] hooded seal (*Cystophora cristata*).[48] Furthermore, the nucleotide diversity of the LB population was even lower than that of the bottlenecked Northern elephant seal population (π=0.0065-0.0086).[49] However, BSPs investigating effective population size (N_e) over time did not detect any past bottleneck event (Supplementary Figure 1), a result similar to that obtained in the mismatch distribution analyses (data not shown). Hence, it seems that the recent reduction of the LB population size caused by human activities has not yet caused a severe bottleneck influencing the diversity indices, but rather that older and possibly recurrent events had a more profound effect on the population, as observed by mtDNA sequences. Note that the lack of a detectable signature associated with possible "recent" bottleneck does not mean that intense human activities will not have a significant impact on the levels of genetic variability and the long-term sustainability of this population.

Mitochondrial control region haplotypes of individuals from LB and Japan cluster into two distinct haplogroups separated by a fixed mutation (Figure 2). This significant differentiation between the two populations is also confirmed by the pairwise F_{ST} and Φ_{ST} statistics (Supplementary Table 2). The design of primers allowing to specifically amplify haplotypes with or without the insertion site would constitute an efficient approach to distinguish the *P. largha* populations from LB and Japan. Note that Han *et al.*[11] erroneously described another putative additional fixed transition because they included only 15 of the 57 Japanese haplotypes[10] in their analyses.

Spotted seals give birth in LB from early January to mid February, which is earlier than in the other breeding areas (February to April).[5] Hence, whereas our analyses of mtDNA data suggest that LB female seals show fidelity to their breeding site, the breeding time difference further suggests that the population in LB might be reproductively isolated from populations in other breeding areas. The observed low diversity in mtDNA and intermediate level in the microsatellite *loci* combined with genetic isolation (Figure 2) suggests that the LB population is at risk to some extent, a situation possibly comparable to the extensively characterized Peruvian populations of dusky dolphin (*Lagenorhynchus obscurus*) and Burmeister's porpoise (*Phocoena spinipinnis*).[50-52]

The set of species-specific microsatellite *loci* isolated here will be useful for further investigations on the genetic diversity and population structure of spotted seals (*P. largha*) across their whole range. Currently, few conclusions can be drawn about the possible existence of breeding isolation or regional divisions. A more elaborated study including additional samples from other breeding areas, especially spotted seals from the coast of Korea, should be conducted. Indeed, it has been suggested that about 300 spotted seals spend the spring, summer, and autumn feeding along the coast of Bak-ryoung Island off western South Korea (Figure 1) and return in October to their breeding grounds in LB, China.[53] We, unfortunately, after several attempts, never obtained any Korean sample for this study and therefore cannot investigate the relationship between these populations. Satellite-telemetric studies of spotted seals in LB could also be used in the future to clarify their migration route and investigate whether they are philopatric.

References

1. Garza JC. Population genetics of the northern elephant seal. PhD dissertation, University of California, Berkeley, 1998.
2. Schultz JK, Baker JD, Toonen RJ, Bowen BW. Extremely low genetic diversity in the endangered Hawaiian Monk Seal (Monachus schauinslandi). J Hered 2009;100:25-33.
3. Pastor T, Garza JC, Allen P, et al. Low genetic variability in the highly endangered Mediterranean monk seal. J Hered 2004;95:291-300.
4. Rugh DJ, Shelden KEW, Withrow DE. Spotted seals, Phoca largha, in Alaska. Marine Fisheries Rev 1997;59:1-18.
5. Wang ZM, Wang PL. Spotted seal Phoca largha. Beijing: Ocean Press, 1990.
6. Dong JH, Shen F. Estimates of historical population size of harbour seal (Phoca largha) in Liaodong Bay. Marine Sci 1991;5:26-31.
7. Han JB, Wang W, Ma ZQ. Spotted seals in the Estuary of Shuangtaizi River of Liaodong Bay. Marine Environ Sci 2005;24:51-3.
8. Han JB, Sun FY, Gao XG, et al. Low microsatellite variation in spotted seal (Phoca largha) shows a decrease in population size in the Liaodong Gulf colony. Ann Zool Fennici 2010;47:15-27.
9. O'Corry-Crowe G, Bonin C. The biogeography and population structure of spotted seal (Phoca largha) as revealed by mitochondrial and microsatellite DNA. Draft Report to the National Marine Mammal Laboratory. Harbor Branch Oceanographic Institute, Florida Atlantic University, Fort Pierce, FL, USA 2009.
10. Mizuno AW, Onuma M, Takahashi M, Ohtaishi N. Population genetic structure of the spotted seal Phoca largha along the coast of Hokkaido, based on mitochondrial DNA sequences. Zoolog Sci 2003;20:783-8.
11. Han JB, He CB, Wang XM, et al. Sequence analysis of mitochondrial tRNAthr, tRNAPro and control region from spotted seals (Phoca largha) in Liaodong Gulf. Fisheries Sci 2007;26:74-8.
12. Garner A, Rachlow JL, Hicks JF. Patterns of genetic diversity and its loss in mammalian populations. Conserv Biol 2005;4:1215-21.
13. Sambrook J, Russell DW. Molecular cloning: a laboratory manual. 3rd edn. New York: Cold Spring Harbor Laboratory Press, 2001.
14. Tzika AC, Remy C, Gibson R, Milinkovitch MC. Molecular genetic analysis of a captive-breeding program: the vulnerable endemic Jamaican Yellow Boa. Conserv Genet 2009;10:69-77.
15. Schretter C, Milinkovitch MC. OligoFaktory: a visual tool for interactive oligonucleotide design. Bioinformatics 2006;22:115-6.
16. Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P. Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 2004;4:535-8.
17. Excoffier L, Laval G, Schneider S. Arlequin version 3: an integrated software package for population genetics data analysis. Evol

Bioinform Online 2005;1:47-50.
18. Sokal RR, Rohlf FJ. Biometry: the Principles and Practice of Statistics in Biological Research. 3rd edn. New York, NY: W.H. Freeman, 1994.
19. Piry S, Luikart G, Cornuet JM. Bottleneck: a computer program for detecting recent reductions in the effective population size using allele frequency data. J Hered 1999;90:502-3.
20. Cornuet JM, Luikart G. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 1996;144:2001-14.
21. Luikart G, Allendorf FW, Cornuet JM, Sherwin WB. Distortion of allele frequency distributions provides a test for recent population bottlenecks. J Hered 1998;89:238-47.
22. Garza JC, Williamson EG. Detection of reduction in population size using data from microsatellite loci. Mol Ecol 2001;10:305-18.
23. Waples RS. A bias correction for estimates of effective population size based on linkage disequilibrium at unlinked gene loci. Conserv Genet 2006;7:167-84.
24. Waples RS, Do C. LDNE: a program for estimating effective population size from data on linkage disequilibrium. Mol Ecol Resources 2008;8:753-6.
25. Waples RS, Do C. Single linkage disequilibrium estimates of contemporary Ne using highly variable genetic markers: a largely untapped resource for applied conservation and evolution. Evol Appl 2010;3:244-62.
26. Pudovkin AI, Zaykin DV, Hedgecock D. On the potential for estimating the effective number of breeders from heterozygote excess in progeny. Genetics 1996;144:383-7.
27. Peel D, Ovenden JR, Peel SL. NeEstimator: software for estimating effective population size. Ver 1.3. Department of Primary Industries and Fisheries, Queensland Government, Queensland, 2004.
28. Beaumont MA, Zhang W, Balding DJ. Approximate Bayesian computation in population genetics. Genetics 2002;162:2025-35.
29. Tallmon DA, Koyuk A, Luikart G, Beaumont MA. ONeSAMP: a program to estimate effective population size using approximate Bayesian computation. Mol Ecol Resources 2008;8:299-301.
30. Stanley HF, Casey S, Carnahan JM, et al. Worldwide patterns of mitochondrial DNA differentiation in the harbor seal (Phoca vitulina). Mol Biol Evol 1996;13:368-82.
31. Bandelt HJ, Forster P, Röhl A. Median-Joining networks for inferring intraspecific phylogenies. Mol Biol Evol 1999;16:37-48.
32. Cassens I, Van Waerebeek K, Best PB, et al. The phylogeography of dusky dolphins (Lagenorhynchus obscurus): a critical examination of network methods and rooting procedures. Mol Ecol 2003;12:1781-92.
33. Mardulyn P, Cassens I, Milinkovitch MC. A comparison of methods for constructing evolutionary networks from intraspecific DNA sequence. In: population genetics for animal conservation. Bertorelle G, Bruford MW, Hauffe HC, Rizzoli A, and Vernesi C, eds. Cambridge: Cambridge University Press, 2009
34. Excoffier L, Smouse PE, Quattro JM. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 1992;131:479-91.
35. Drummond AJ, Rambaut A, Shapiro B, Pybus OG. Bayesian coalescent inference of past population dynamics from molecular sequences. Mol Biol Evol 2005;22:1185-92.
36. Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 2007;7:214.
37. Keane TM, Creevey CJ, Pentony MM, et al. Assessment of methods for amino acid matrix selection and their use on empirical data shows that ad hoc assumptions for choice of matrix are not justified. BMC Evol Biol 2006;6:29.
38. Rambaut A, Drummond AJ. Tracer v1.4, 2007. 39. Li YC, Fahima T, Röder MS, et al. Genetic effects on microsatellite diversity in wild emmer wheat (Triticum dicoccoides) at the Yehudiyya microsite, Israel. Heredity 2003;90:150-6.
40. Orti G, Pearse DE, Avise JC. Phylogenetic assessment of length variation at a microsatellite locus. Proc Natl Acad Sci USA 1997;94:10745-9.
41. Davis CS, Gelatt TS, Siniff D, Strobeck C. Dinucleotide microsatellite markers from the Antarctic seals and their use in other pinnipeds. Mol Ecol Notes 2002;2:203-8. [Abstract] 42. Goodman SJ. Patterns of extensive genetic differentiation and variation among European harbor seals (Phoca vitulina vitulina) revealed using microsatellite DNA polymorphisms. Mol Biol Evol 1998;15:104-18.
43. Palstra FP, Ruzzante DE. Genetic estimates of contemporary effective population size: what can they tell us about the importance of genetic stochasticity for wild population persistence? Mol Ecol 2008;17:3428-47.
44. Lynch M, Lande R. The critical effective size for a genetically secure population. Anim Conserv 1998;1:70-2. [Abstract] 45. Franklin I, Frankham R. How large must populations be to retain evolutionary potential? Anim Conserv 1998;1:69-73.
46. Graves JA, Helyar A, Biuw M, et al. Analysis of microsatellite and mitochondrial DNA in grey seals from 3 breeding areas in the Baltic Sea. Conserv Biol 2009;10:59-68.
47. Palo J. Genetic diversity and phylogeography of landlocked seals. PhD dissertation, University of Helsinki, Helsinki, Finland, 2003.
48. Coltman DW, Stenson G, Hammill MO, et al. Panmictic population structure in the hooded seal (Cystophora cristata). Mol Ecol 2007;16:1639-48.
49. Weber DS, Stewart BS, Garza JC, Lehman N. An empirical genetic assessment of the severity of the northern elephant seal population bottleneck. Curr Biol 2000;10:1287-90.
50. Cassens I, Mardulyn P, Milinkovitch MC. Evaluating intraspecific "Network" construction methods using simulated sequence data: do existing algorithms outperform the global maximum parsimony approach? Syst Biol 2005;54:363-72.
51. Cassens I, Van Waerebeek K, Best PB, et al. Evidence for male dispersal along the coasts but no migration in pelagic waters in dusky dolphins (Lagenorhynchus obscurus). Mol Ecol 2005;14:107-21.
52. Rosa S, Milinkovitch MC, Van Waerebeek K, et al. Population structure of nuclear and mitochondrial DNA variation among South American Burmeister's porpoises (Phocoena spinipinnis). Conserv Genet 2005;6:431-43.
53. Won C, Yoo BH. Abundance, seasonal haul-out patterns and conservation of spotted seals Phoca largha along the coast of Bakryoung Island, South Korea. Oryx 2004;38:109-12.