Ann. Zool. Fennici 47: 15–27 ISSN 0003-455X (print), ISSN 1797-2450 (online)
Helsinki 10 March 2010

Low microsatellite variation in spotted seal (*Phoca largha*) shows a decrease in population size in the Liaodong Gulf colony

Jia-Bo Han[1,2,3], Fan-Yue Sun[4], Xiang-Gang Gao[3], Chong-Bo He[3], Pi-Lie Wang[3], Zhi-Qiang Ma[3] & Zhao-Hui Wang[3]

[1)] *Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China (e-mail: jbhan@sina.com)*
[2)] *Graduate School of the Chinese Academy of Sciences, Beijing 100039, China*
[3)] *Liaoning Key Laboratory of Marine Fishery Molecular Biology, Liaoning Ocean and Fisheries Science Research Institute, Dalian 116023, China*
[4)] *College of Life Science, Liaoning Normal University, Dalian 116029, China*

Received 4 Apr. 2008, revised version received 24 Oct. 2008, accepted 24 Nov. 2008

Han, J. B., Sun, F. Y., Gao, X. G., He, C. B., Wang, P. L., Ma, Z. Q. & Wang, Z. H. 2010: Low microsatellite variation in spotted seal (*Phoca largha*) shows a decrease in population size in the Liaodong Gulf colony. — *Ann. Zool. Fennici* 47: 15–27.

We report the first investigation of nuclear genetic variability in the colony of the spotted seal (*Phoca largha*) in the Liaodong Gulf, China, where the estimated population size was under 1000 individuals during the study period (2005–2008). A total of 29 microsatellite loci from five pinniped species were employed in 176 spotted seals. Only 15 were polymorphic, with a maximum of 4 alleles detected in samples, with the mean number of alleles per locus being 2.73. Estimates of expected heterozygosity H_e for the polymorphic loci ranged from 0.24 (± 0.05 SD) to 0.72 (± 0.02 SD), with a mean H_e per polymorphic locus of 0.51 (± 0.05 SD). No deviation from the Hardy-Weinberg equilibrium was detected for any of the loci. No differences were detected for either genic or genotypic frequencies of seals from the different sampling sites and years. Several statistical methods were applied to detect the population reduction and compare it with that in other seal species. The result indicated that the population of the Liaodong Gulf spotted seal has suffered a decrease in genetic variability and population reduction over the last several decades. The present study supports a notion that a decrease in population size is the main factor accounting for the low levels of variability observed. These research results provide useful data for the conservation and management of the Liaodong Gulf colony.

Introduction

Genetic variability is fundamentally important for marine mammal population to adapt to environmental changes (Lande 1988, Hamrick *et al*. 1991, Pastor *et al*. 2004, Simmons *et al*. 2006). Populations that experience severe reduction in effective size can be subject to a multitude of changes, including increased identity by descent, a loss of molecular genetic

variation, and increased importance of stochastic changes in population size or composition (England *et al*. 1996). Among the problems that these changes can cause for the species or population involved, are increased homozygosity and expression of recessive deleterious alleles. Consequent inbreeding depression may increase the probability that a population will become extinct (Saccheri *et al*. 1998). Similarly, a loss of genetic variation can limit evolutionary potential (Frankham *et al*. 1999), and reduce the ability of a population to mount a variable response to newly introduced pathogens and parasites (O'Brien & Evermann 1988). Analyzing and assessing genetic variation is important for the conservation and management of the endangered species populations that are in need of intervention or recovery.

Molecular markers are useful for population genetic studies allowing to assess influences of various factors on genetic diversity and population structure, such as historic and demographic factors (Avise *et al*. 1994), anthropogenic stressors (Whitehead *et al*. 2003, Bagley *et al*. 2001) and artificial stocking (Englbrecht *et al*. 2000). Among many types of molecular markers (Liu & Cordes 2004), microsatellite markers have been demonstrated as useful tools for population genetic studies (Whitehead *et al*. 2003) and have been used to assess genetic variation in the grey seal (*Halichoerus grypus*) (Allen *et al*. 1995), harbor seal (*Phoca vitulina*) (Coltman *et al*. 1996, 1998), harp seal (*Phoca groenlandica*) (Kretzmann *et al*. 2006) and Mediterranean monk seal (*Monachus monachus*) (Pastor *et al*. 2004), and in other various species such as the Japanese sea urchin (*Strongylocentrotus intermedius*) (Zhou *et al*. 2008), nine-spined stickleback (*Pungitius pungitius*) (Mäkinen *et al*. 2007*)* and Siberian flying squirrel (*Pteromys volans*) (Selonen *et al*. 2005).

The spotted seal (*Phoca largha*) is one of the world's endangered mammals (IUCN 1994), which is widely distributed in the North Pacific Ocean, the Yellow Sea, Sea of Japan, and Okhotsk, Bering, Chukchi, Beaufort Seas (Shaughnessy & Fay 1977). There are eight known breeding colonies of the spotted seal (Rugh *et al*. 1997): (1) Liaodong Gulf, (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 Gulf, (6) northeast from Kronotsky Cape on the eastern side of the Kamchatka Peninsula to Olyutorski Gulf, (7) the Gulf of Anadyr in the northwest Bering Sea, and (8) from Bristol Bay, Alaska, to west of the Pribilof Islands (Fig. 1).

The distribution, abundance, movements and behavior of spotted seals in the Okhotsk, Bering and Chukchi Seas have been described by Lowry *et al*. (1998). However, the geographic structure of the distribution of the spotted-seal breeding colonies, and genetic differentiation were found based on the direct sequencing of the mtDNA from populations sampled in Kasegaluk Lagoon, Alaska, and Kamchatka, Russia (O'Corry 1994), and on the coast of Hokkaido Japan (Mizuno *et al*. 2003). Also, the species has a well documented ability to travel great distances, increasing the chances for genetic exchange between populations. One male covered over 1000 km of open water, traveling from Kasegaluk Lagoon to the Chukchi Peninsula, Russia, and back in only one month (Lowry *et al*. 1994).

Seals have been hunted for centuries in the Okhotsk and Bering Seas, and Liaodong Gulf (China). Prior to 1970, there were no harvest limits set by the Russians in the Okhotsk and Bering Seas. The harvest limits in 1970 were set at 7000 seals in the Okhotsk Sea (5000 from ships and 2000 from shore) and 8000 seals in the Bering Sea (6000 from ships and 2000 from shore), or roughly equal to 5% of the presumed total population (about 300 000 by back-calculation) (Popov 1976). At the coast of Hokkaido and in Peter the Great Bay, spotted seals were reported to have a high level of human-caused mortalities such as entanglement in the fishing net, an oil spill accident, and a damage-control kill event (Mizuno *et al*. 2001, 2002, Trukhin & Mizuno 2002). In the Hokkaido Sea (Japan), the total numebr of spotted seals estimated by aerial line-transect surveys was 13 653 in March 2000 (Mizuno *et al*. 2002). At the southern coast of Primorye (Peter the Great Bay), the population size was recently estimated to be about 1000 individuals (Trukhin & Mizuno 2002).

Historically the species in Liaodong Gulf was apparently fairly common and its popula-

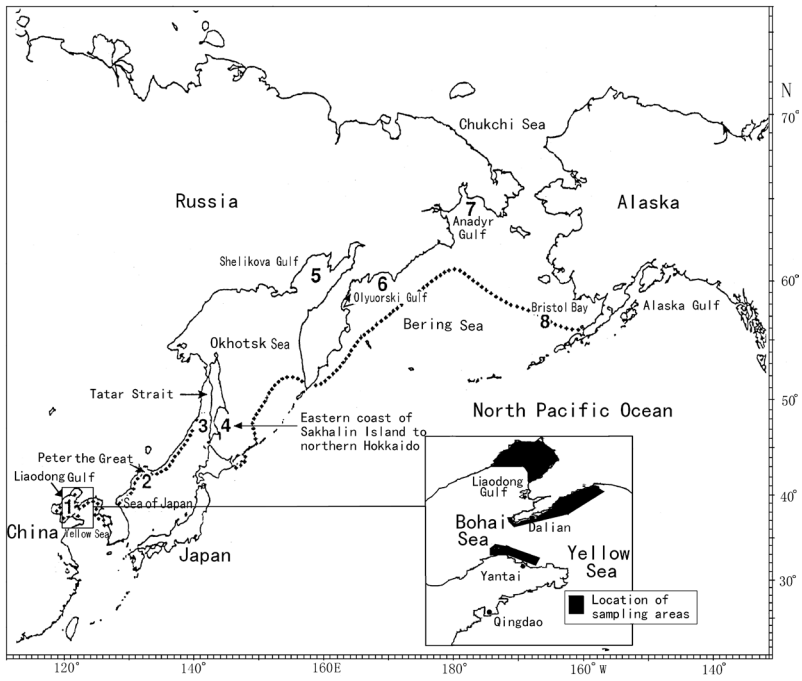

Fig. 1. Distribution of the entire range of spotted seals worldwide and sampling areas in the breeding colony of Liaodong Gulf. The eight breeding concentrations are: (1) Liaodong Gulf, (2) Peter the Great Bay near Vladivostok, (3) Tatar Strait, (4) the eastern coast of Sakhalin Island extending to northern Hokkaido, (5) Shelikova Gulf, (6) Litke Strait to the Olyuorski Guif, (7) the Guif of Anadyr, and (8) from Bristol Bay to the Pribilof Island. The dotted line shows the typical maximum extent of sea ice.

tion was estimated at 8000 individuals during 1930–1940 (Dong & Shen 1991). However, the poaching pressure on spotted seals of the Bohai Sea became high because of demands for fur, meat, oil, and male genitalia being used for traditional medicine (Wang 1986, 1993, 1998). In the 1950s and 1960s, more than 1000 seals were hunted each year and a total of about 30 000 seals were harvested from 1930 to 1990 (Dong & Shen 1991, Wang 1998). As a result of excessive harvesting and destruction of its breeding habitat, the spotted seal has declined in numbers and range, and the population declined from about 8000 individuals in the1940s to 2300 individuals in the1980s (Wang 1986, Dong & Shen 1991, Wang 1998).

The spotted seal is categorized as Critically Endangered Species in China and also in South Korea because of the habitat destruction and human harassment in the region. As a result of conservation attempts, Dalian Spotted Seal Reserve of China was established in 1992 at Liaodong Gulf in the Bohai Sea (Wang 1998, Won & Yoo 2004), where the most southern one of the eight geographic breeding colonies of the spotted seal is located (*see* Fig. 1). Seals breeding grounds are on ice floes and pups are born from January to mid-February. After breeding season, the seals remain on the ice to moult. During the non-breeding season, the spotted seals of this region are known to migrate as far south as the Yangtze River and Fujian in China (Han *et al*. 2005). Shipboard surveys suggest that spotted seals spent spring, summer and autumn feeding along the coast of Bak-ryoung Island (South Korea) and return to their breeding grounds in Liaodong Gulf in October (Won & Yoo 2004).

Our recent genetic study of the mtDNA sequence in the species (Han *et al*. 2007) showed that the genetic variation of mtDNA sequences were much lower in seals from Liaodong Gulf than that in seals from the coast of Hokkaido and the Okhotsk Sea (Mizuno *et al*. 2003), and that the spotted seal in Liaodong Gulf may be an independent colony isolated from the Hokkaido and Okhotsk Sea populations. We also found that one base pair insertion in the threonine tRNA gene (position 16296) existed in the seals from Liaodong Gulf (Han *et al*. 2007) compared with all the spotted seals from the Sea of Japan and the Okhotsk Sea (Mizuno *et al*. 2003). The information from spotted-seal satellite-linked tagging in the Liaodong Gulf and Bak-ryoung Island also indicated that the spotted seals spend the spring,

summer and autumn feeding along the coast of Bak-ryoung Island and winter breeding in the breeding colony of Liaodong Gulf (J. B. Han unpubl. data).

It seemed that the hunting is one major factor accounting for the decline in the spotted seal population in the Liaodong Gulf. However, the surviving population has further declined to fewer than 1000 individuals (Han *et al*. 2005) even though some protection measures had been introduced, and no seal hunting has been allowed in this region for the past two decades. The severity of the population reduction raises the question of whether genetic effects related to small population size, such as low ability of populations to adapt to environmental changes due to loss of genetic variability and inbreeding depression may be responsible.

In this study, we employed inter-specific pinniped microsatellite markers and the non-invasive sampling technique to describe the genetic features of the spotted seal in Liaodong Gulf. We compare this variation with that found in other pinniped species and discuss possible causes for the small population size and low levels of variation observed. Attempts are also made to find implications for the seal conservation and management in future.

Material and methods

Samples and sample collection

Althogether 176 spotted seals were collected between 2005 and 2008 from the Liaodong Gulf (China; *see* Fig. 1) colony: their sex was determined by visual examination of genitalia (Wang *et al*. 1986, Badosa *et al*. 1998). In this region, pups are born from January to February. Lanugo coat is moulted 4–5 weeks later and around the same time the pups are weaned. They remain on the pack ice for the first few weeks after weaning, which is suitable time for sampling (Wang *et al*. 1986, Wang 1990, 1993). For the DNA analysis, we collected hair samples (about 400 hairs with roots and follicles per pup) from 122 under 30-day-old individuals found on the ice pack in the coast of Liaodong Gulf. Muscle samples were obtained from 26 dead juvenile seals (under one year-old) incidentally caught in the fishing nets near the coast of Yantai Shandong, Jiaodong Peninsula. Twenty-eight muscle samples were collected from seals caught by hunters near the coast of Dalian, Liaodong Peninsula. As a result we had three groups of samples tagged as follows: Liaodong Gulf (PLG), Coast of Yantai (PCY), Coast of Dalian (PCD) (Table 1). Tissue samples were immediately preserved in 70% ethanol and, once in the laboratory, frozen at –20 °C until analysed.

DNA isolation

The DNA was extracted from the hair roots following the protocol described by Zhao and Li (2003). Briefly, the hair roots were washed in distilled water and then transferred into a 0.2-ml microcentrifuge tube containing 25 μl of rapid hair digestion buffer (2 mM $MgCl_2$, 0.02% proteinase K, 1 × PCR buffer, 50 mM KCI, 10 mM Tris-Cl, 0.01% glutin). Each tube contained 5–10 hair roots with the follicles. The samples were heated at 65 °C for 30 min, then in 95 °C for 15 min, and then coolled in 4 °C for 10 min. 2 μl supernate was used for the PCR.

The DNA from the muscle tissues was extracted using standard proteinase K/phenol/chloroform extraction technique (Sambrook *et al*. 2001). Briefly, The DNA was extracted from muscle samples by digestion overnight in 750 μl

Table 1. Spotted seals sampled from the Liaodong Gulf colony in China.

Group	Sampling year	Tissue sampled	Location	Number
PLG	2005–2007	Hair follicles	Liaodong Gulf	122 (53 males, 69 females)
PCY	2005	Muscle	Coast of Yantai	26 (10 males, 16 females)
PCD	2007–2008	Muscle	Coast of Dalian	28 (10 males, 18 females)
Total				176 (73 males, 103 females)

of lysis buffer (0.1 M Tris, 0.1 M EDTA, 0.25 M NaCl, 0.5% Triton, 2% sodium dodecyl sulfate, and 0.5 mg ml^{-1} proteinase K) in 50 °C, followed by phenol–chloroform–isoamyl alcohol extractions, and precipitation in 100% cold ethanol. Pellets were rinsed twice with 70% ethanol, air-dried, resuspended in Tris-EDTA and refrigerated or frozen until used.

Microsatellite analysis

Screening of microsatellite primers

A panel of 29 pairs of microsatellite primers for pinniped species (Gemmell *et al*. 1997) originally isolated from the grey seal (*Halichoerus grypus*) (Allen *et al*. 1995, Gemmell *et al*. 1997), harbor seal (*Phoca vitulina)* (Goodman 1995, Kappe *et al*. 1997, Coltman *et al*. 1996), northern elephant seal, and South American fur seal (Gemmell *et al*. 1997) were used in cross-species amplification in this study (Table 2). Primers were initially tested on samples from 12 spotted seals (four samples from three locations). PCR products were visualized under UV on agarose gels containing ethidium bromide. Loci that were amplified with specific PCR products in this limited set were used to genotype the entire samples. Each forward primer of the loci with good PCR products was labeled with TET, HEX, or FAM fluorescent dye (TaKaRa Co. Dalian China).

PCR amplification and allele separation

PCR reactions followed the protocols given in Gemmell *et al*. (1997): total volume of 25 μl, contained 2 μl supernate (hair follicles or 50–100 ng muscle DNA), 1 × buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.5 U of r*Taq* polymerase (TaKaRa Co., Dalian) and 0.5 μM of forward and reverse primers. PCR amplifications were performed in a thermal cycler Mastercycler® ep gradient S (Eppendorf Co., Germany) as follows: 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 52–58 °C (depends on the different annealing temperature) for 30 s, 72 °C for 45 s, and a final elongation of 72 °C for l0 min.

PCR products were first visualized under UV on agarose gels containing ethidium bromide and then diluted for genotyping according to amplification strength. Diluted samples (1 μl) were mixed with 10 μl of 97.6% formamide and 2.4% ROX-500 size standard (Applied Biosystems) and denatured at 94 °C for 3 min before loading in an Applied Biosystems (3130 or 3730XL) automated sequencer. Multiple loci with non-overlapping allele sizes and/or different dyes were run in the same capillary when possible. Allele size data were collected using Genescan and Genemapper software packages (Applied Biosystems). Several samples of known genotype were used to standardize slight (1–2 bp) allele size differences between instruments.

Genetic variation analysis

We assessed genetic variability as the proportion of polymorphic loci and the mean number of alleles per locus (allelic diversity). Observed heterozygosity, H_o, was compared with the unbiased estimate of heterozygosity expected under assumptions, H_e (Nei 1978). Possible departures from Hardy-Weinberg equilibrium were examined by calculating exact significance probabilities following the procedure described by Louis and Dempster (1987). For detection of genic and genotypic differences between samples in different sampling sites and years, exact tests were carried out following Raymond and Rousset (1995a). The above analyses were performed using the GENEPOP software package (ver. 3.4, probability tests using the Markov chain method) (Raymond & Rousset 1995b).

Genetic bottleneck detection

We assessed the genetic consequences of population size reduction (or bottleneck) using three methods. The first is a graphical examination of allele frequencies described by Luikart *et al*. (1998). In a population with constant size, many microsatellite alleles should be rare. In contrast, a recently bottlenecked population is expected to show fewer rare alleles, as most of them are lost quickly. A histogram of the proportion of alleles

Table 2. The 29 microsatellite loci surveyed in the Liaodong Gulf spotted seal.

Locus	Resource species	GenBank accession number	Reference	Primer sequence (5′–3′)	Polymorphic loci
SGPV2	Harbor seal	U65441	Goodman (1995)	TTGTATCAGTCACTAGCCTGGC CAAATCGAGATAACATTGCCC	2
SGPV3	Harbor seal	U65442	Goodman (1995)	ACATCAACATTCTCAGTATGGGTGG GCAGACAACACCAAGAATGAACCC	3
SGPV9	Harbor seal	G02096	Goodman (1995)	CTGATCCTTGTGAATCCCAGC TAGTGTTTGGAAATGAGTTGGC	NO
SGPV10	Harbor seal	U65443	Goodman (1998)	TTCACTTAGCATAATTCCCTC TCATGAATTGGTATTAGACAAAG	2
SGPV11	Harbor seal	U65444	Goodman (1998)	CAGAGTAAGCACCCAAGGAGCAG GTGCTGGTGAATTAGCCCATTATAAG	3
SGPV16	Harbor seal	U65445	Goodman (1995)	AGCTAGTGTTAATGATGGTGTG TCTGAGAGATTCAGAGTAACCTTC	4
SGPV17	Harbor seal	U65446	Goodman (1995)	CTGGTGTGTTAGTGAGGGTTCTGC TTAACAACTCCATTATCATTTGAGCC	2
Pvc19	Harbor seal	L40989	Coltman *et al.* (1996)	GGGTGAACAGGATTTATCC GTGCTAGATAACAATCCTAC	2
Pvc26	Harbor seal	L40988	Coltman *et al.* (1996)	TTTTCTCCATACCTACATAAT ATTGTGATCCCATTTTTGTAA	NO
Pvc29	Harbor seal	L40987	Coltman *et al.* (1996)	AATTGTGTTGTTTACATCTC AACCAGAAGAATAGAATTTGCAT	NO
Pvc30	Harbor seal	L40986	Coltman *et al.* (1996)	GCATGTGATCTTACAGCAAT CATGGGTTCTCAATAGAAGA	NO
Pvc63	Harbor seal	L40985	Coltman *et al.* (1996)	CCTGGACTTTGTTTATACCT GCATGAGTTCATCTAGGGA	2
Pvc74	Harbor seal	L40984	Coltman *et al.* (1996)	CCATCTGTGTCCTCTGATAG CTGATATTCCATGTCTGAGATA	NO
Pvc78	Harbor seal	L40983	Coltman *et al.* (1996)	GAGTATACCTCCATACTACAC AGTTGTTCTCCTGACCCAAG	3
Hgdii	Grey seal	G02095	Allen *et al.* (1995)	ACCTGCCATAGTGCTCATC GAGCCAACYAAGACAAGCC	3
Hg0	Grey seal	–	Gemmell *et al.* (1997)	AAATTGGGATTCATCAAAAC GTATGCGGTTGTTTAACGT	3
Hg1.3	Grey seal	AF055864	Gemmell *et al.* (1997)	TTTCCAAAACGGTCCAGTGG CTAGTAGATAAGAGCCACATTTCCA	NO
Hg1.4	Grey seal	AF055862	Gemmell *et al.* (1997)	TCTCCAAGACGACTGAAACCC TACCATATCTTTGTGGCTCTGT	NO
Hg2.3	Grey seal	AF055863	Gemmell *et al.* (1997)	CCAATGACAACTTACTGAGAAT TGTGAAGTGCTCTGTTTTGC	NO
Hg3.6	Grey seal	G02089	Allen *et al.* (1995)	CACATTCTTTTTATGGCTGAATA AGATGATTGGATAAAGAAGATGTG	NO
Hg3.7	Grey seal	AF055865	Allen *et al.* (1995)	CTGAATTTTCTCATTATTAGTTTTG GGTGTGTAGTAGTATCTCATCATTG	NA
Hg4.2	Grey seal	G02090	Allen *et al.* (1995)	AATCGAAATGCTGAGCCTCC TGATTTGACTTCCCTTCCCTG	NO
Hg6.1	Grey seal	G02091	Allen *et al.* (1995)	TGCACCAGAGCCTAAGCAGACTG CCACCAGCCAGTTCACCCAG	2
Hg6.3	Grey seal	G02092	Allen *et al.* (1995)	CAGGGGACCTGAGTGCTTATG GACCCAGCATCAGAACTCAAG	NO
Hg8.9	Grey seal	G02094	Allen *et al.* (1995)	TGTTAACTATCTGGCACAGAGTAAG TTTCCTATGGGTTCTACTCTC	3
Hg8.10	Grey seal	G02096	Allen *et al.* (1995)	AATTCTGAAGCAGCCCAAG GAATTCTTTTCTAGCATAGGTTG	4
M11A	Northern elephant seal	–	Gemmell *et al.* 1997	TGTTTCCCAGTTTTACCA TACATTCACAAGGCTCAA	NO
BG	Northern elephant seal	–	Gemmell *et al.* 1997	AATTAGTATGATGCTGGGCTGTC AATTGGGCATGTGATGTGATGAG	3
Aa4	South American fur seal	–	Gemmell *et al.* 1997	CTACTTCTTGGCATTTATTCAAG CATCCAACATATTTATATATAACC	NO

NA = no amplification.

in a dataset at different frequencies should thus reveal a deficit of rare alleles, or a "mode shift". This qualitative method can potentially identify bottlenecked populations (Luikart *et al*. 1998).

The second is the heterozygosity excess method described by Cornuet and Luikart (1996) and implemented in the Bottleneck software package (Piry *et al*. 1997). This method exploits the fact that allelic diversity is reduced faster than heterozygosity during a bottleneck, because rare alleles are lost rapidly and have little effect on heterozygosity, thus producing a transient excess in heterozygosity relative to that expected in a population of constant size with the same number of alleles (Cornuet & Luikart 1996). Several statistical tests have been proposed to evaluate such differences. We applied the Wilcoxon Signed-rank test, as it does not require a large number of polymorphic loci which are scarce in a population with low variability. Moreover, in a recent comparative analysis of statistical methods, the Wilcoxon signed-rank test performed better in identifying bottlenecked populations (Maudet *et al*. 2002). As is appropriate for microsatellites, we carried out 5000 replicates and assumed that all loci follow the two phase mutation model (TPM), in which most mutations are one-step, but a small percentage (5%–10%) are multistep (Di Rienzo *et al*. 1994).

The third method examines allele frequency distribution for gaps. In a population of constant size, most allele frequency distributions are expected to be more or less continuous, and the range in allele size, measured in repeat units, to be similar to the number of alleles. So in a population of constant size, the mean k/r ratio (k is the number of alleles and r is the range in allele size + 1, $r = S_{max} - S_{min} + 1$, where S_{max} is the size of the largest allele, and S_{min} is the size of the smallest allele in the sample) should be close to one (Garza & Williamson 2001). However, during a population size reduction, alleles are lost and, since they are not always the smallest or largest, gaps appear in the allele frequency distributions. As a result, the number of alleles decreases more rapidly than the range in allele size and the k/r ratio decreases (Garza & Williamson 2001). A simulation-based statistical test is used to evaluate the significance of the observed k/r ratio through comparison with 10 000 simulated datasets from populations at equilibrium using the program M_P_Val (Garza & Williamson 2001). When 15 variable loci are assayed and conservative assumptions about the mutation process are made, a value of $k/r \leq 0.71$ indicates that the population under study has experienced a recent reduction in size. k/r ratios were also calculated for the Mediterranean monk seal, grey and harbor seal populations for comparative purposes.

Results

Genetic variation analysis

All the initial 29 microsatellite loci (Table 2) tested in 12 samples amplifed successfully except Hg3.7. Of the 28 loci in the complete 176 samples, 15 were polymorphic (52%), while 13 were monomorphic (48%). Of the 15 polymorphic loci, six had two alleles, seven had three alleles and two had four alleles. Mean allelic diversity was 1.97 (± 0.09) when all 28 loci were considered and 2.73 (± 0.15) when only the 15 polymorphic loci were taken into account (Table 3). Two loci, SGPv17 and Pvc63, were found to be sex linked, with autosomal segregation rejected at $P = 10^{-6}$ and 10^{-10}, respectively. These loci are known to be X-linked in other pinniped species (Coltman *et al*. 1996, Gemmell *et al*. 1997, Pastor *et al*. 2004).

Observed (H_o) and expected (H_e) heterozygosities are listed in Table 3. H_o and H_e were highly correlated ($r^2 = 0.96$), so we used the latter in our analysis, as it is considered to be less biased (Nei & Roychoudhury 1974). Estimates of H_e for the polymorphic loci ranged from 0.24 (± 0.05) to 0.72 (± 0.02), with a mean H_e per polymorphic locus of 0.51 (± 0.05). Mean H_e for all 28 loci was 0.33 (± 0.13). No deviation from the Hardy-Weinberg equilibrium was detected for any of the loci (Table 3). No differences were detected for either genic ($p < 0.75$, SE = 0.002) or genotypic frequencies ($p < 0.74$, SE = 0.002) of seals from the different sampling sites and years.

In general, allele sizes in the sample were similar to those described for the same loci in other pinniped species (Coltman *et al*. 1996)

(Table 4). Moreover, some loci which were monomorphic in the source species (e.g., Pv9, Pv16, Pv17, and Pvc26) were polymorphic in the Liaodong Gulf spotted seal.

The overall allelic diversity in the Liaodong Gulf spotted seal, and also in Mediterranean monk seal, the Hawaiian monk seal and the northern elephant seal, is among the lowest recorded (Table 4). However, despite the smaller census size as in case of the Hawaiian monk seal and northern elephant seal, the Liaodong Gulf spotted seal has greater variability both in terms of the number of polymorphic loci and allelic diversity, than either of these two species.

Genetic bottleneck detection

The graphical representation of the allele frequencies for all 28 loci (Fig. 2) shows a deficit of

Table 3. Genetic variability measures for the polymorphic loci in the Liaodong Gulf spotted seal. * = X-linked loci; N = Number of animals successfully genotyped; A_o = observed allelic diversity; H_o = observed heterozygosity, H_e = expected heterozygosity; P = values for departure from Hardy-Weinberg equilibrium (HWE). H_{eq} is the heterozygosity expected at equilibrium obtained through coalescent simulation under the "two-phase mutation model" using the Bottleneck program for the observed number of alleles (A_o) and sample size (n). The standard deviation (SD) of H_e is used to compute the standardized difference for each locus $(H_e - H_{eq})/SD$. Heterozygosity excess is indicated by a '+' sign.

Locus	N	A_o	H_o	$H_e \pm$ SD	P	H_{eq}	$(H_e - H_{eq})$/SD	H_e excess
SGPV2	155	2	0.65	0.58 ± 0.07	0.17	0.23	0.14	+
SGPV3	157	3	0.66	0.54 ± 0.03	0.43	0.24	0.8	+
SGPV10	160	2	0.32	0.21 ± 0.05	1	0.23	–0.23	–
SGPV11	156	3	0.73	0.44 ± 0.08	0.77	0.46	1.30	+
SGPV16	162	4	0.59	0.54 ± 0.05	0.15	0.45	–0.57	–
SGPV17*	158	2	0.64	0.63 ± 0.02	0.54	0.24	1.2	+
Pvc19	155	2	0.19	0.24 ± 0.05	0.28	0.23	0.01	+
Pvc63*	153	2	0.55	0.49 ± 0.03	1	0.24	1.43	+
Pvc78	160	3	0.49	0.47 ± 0.03	1	0.24	1.40	+
Hgdii	154	3	0.65	0.59 ± 0.07	1	0.22	1.60	+
Hg0	158	3	0.39	0.35 ± 0.05	0.65	0.23	0.40	+
Hg6.1	156	2	0.54	0.48 ± 0.02	0.37	0.23	1.46	+
Hg8.9	154	3	0.71	0.23 ± 0.06	1	0.24	–0.37	–
Hg8.10	160	4	0.74	0.72 ± 0.02	0.38	0.24	1.43	+
BG	142	3	0.37	0.34 ± 0.04	1	0.39	–0.23	–
Mean		2.73	0.55	0.51 ± 0.05				

Table 4. Average allele number comparison in seven seal species for a panel of the same 24 microsatellite loci.

Same 24 loci	Liaodong Gulf seal	Mediterranean monk seal[5)]	Hawaiian monk seal[1)]	Northern elephant seal[2)]	Southern elephant seal[1)]	Grey seal[1)3)]	Harbor seal[1)4)]
No. of polymorphic loci	13	12	2	9	10	21	19
No. of loci typed	23	24	17	24	16	23	24
Overall allelic diversity	0.57	0.50	0.12	0.38	0.62	0.87	0.80
Mean A_o	2.12	1.92	1.29	1.46	3.56	4.65	4.21
Sample size	166	40–46	5	42–80	6	48	48–50
Estimated census size	800*	300[a]	1300[b]	127000[b]	664000[b]	200000[b]	> 500000[b]

Data from: [1)]Gemmell *et al.* (1997), [2)]Garza (1998), [3)]Allen *et al.* (1995), [4)]Coltman *et al.* (1996) and Forcada *et al.* (1999), [5)]Pastor *et al.* (2004). Both monk seals and northern elephant seals have suffered documented reductions in population sizes. [a] Forcada *et al.* (1999), [b] Reeves *et al.* (1992), * J. B. Han unpubl. data.

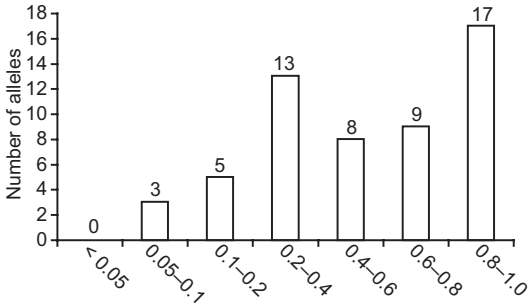

Fig. 2. Allele frequencies of 28 microsatellite loci (monomorphic included) in the Liaodong Gulf colony of spotted seals.

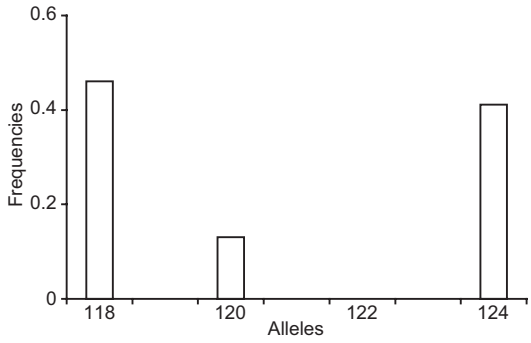

Fig. 3. Allele frequencies for a polymorphic microsatellite locus BG in the Liaodong Gulf spotted seal colony. Alleles are given in base pairs, with each allele increasingin size by two base pairs.

rare alleles (i.e., frequency less than 0.05) causing a mode-shift distortion, as is expected for recently bottlenecked populations, as opposed to an L-shaped distribution, typical of populations in equilibrium (Luikart *et al*. 1998). A statistically significant departure from mutation drift-equilibrium was detected with the heterozygosity excess method: 11 of 15 loci had an excess of heterozygosity as compared with that of a population of the same size and same number of alleles at equilibrium (Wilcoxon signed-rank test: $P = 0.00513$; one tailed for heterozygosity excess) (Table 3).

Allele size distributions were not continuous (Fig. 3). Several loci had alleles that were separated by a single repeat unit, while most had alleles that were separated by several repeats.

The values of *k*/*r* ratios calculated from 15 polymorphic microsatellite loci for the four seal species were different (Table 5). Mean *k*/*r* ratios for the current grey and harbor seal populations were close to 1 (0.88 and 0.86), as predicted for populations that have not suffered a recent bottleneck. In contrast, the mean *k*/*r* ratio for the Liaodong Gulf spotted seal was 0.68, slightly higher than that (0.65) for the Mediterranean Monk seal (Pastor *et al*. 2004). This value fell below the critical value (0.71) which indicates that the species suffered a recent reduction in population size.

Discussion

This is the first study of nuclear genetic variability in the spotted seal in the Liaodong Gulf colony. All but one of the 29 microsatellite loci (99.6%), isolated from four other pinniped species, generated specific PCR products. This high level of conservation of microsatellite loci is consistent with that found in previous studies of related species (Coltman *et al*. 1996, Gemmell *et al*. 1997, Pastor *et al*. 2004), and is most likely due to the monophyletic origin and relatively recent divergence of the major pinniped taxa (Arnason *et al*. 1995).

The level of microsatellite variation (maximum of four alleles) in the spotted seal is among the lowest as compared with that reported for marine mammals with an exception of the Mediterranean monk seal (Pastor *et al*. 2004). Similar levels have been found in only a few severely bottlenecked species, such as the northern hairy nosed wombat (*Lasiorhinus krefftii*) (Taylor *et al*. 1994) or the Kangaroo Island population of koalas (*Phascolarctos cinereus*) (Houlden *et al*. 1996). In pinnipeds, the Liaodong Gulf spotted seal also presents low variability, as do the Hawaiian monk seal (Gemmell *et al*. 1997, Kretzmann *et al*. 2001), the northern elephant seal (Garza 1998) and the Mediterranean monk seal (*Monachus monachus*) (Pastor *et al*. 2004), the three species that have gone through well documented reductions in population sizes. The amount of variability both in terms of number of polymorphic loci and allelic diversity found in the Liaodong Gulf spotted seals is slightly higher than that in the other three species. Low genetic variation may result from a small number of founders, but this is not the case here. The spot-

ted seal was apparently fairly common in Liaodong Gulf and its population was estimated in 1930–1940 at 8000 individuals (Dong & Shen 1991). Furthermore, low *k*/*r* ratios (e.g., gaps in allele frequency distribution) also suggest that estimation bias is not the cause of the low variation, as they indicate that the assayed loci have recently lost variation, not that they are inherently low in variation due to slow evolution and/or small effective sizes. Moreover, microsatellite mutation rates are generally of the same order of magnitude among mammals (Ellegren 1995, Weber & Wong 1993). The observed differences are due to properties of the particular loci sampled and not different mechanisms affecting the entire genome. In the spotted seal, the finding that variability is low at many independent nuclear loci, as well as in mitochondrial genome (Han *et al*. 2006, 2007), also contradicts the idea that the species is inherently low in variation due to low genome-wide mutation rates. Another possible explanation is small effective size in the absence of a bottleneck, which can result from strong population subdivision, high variance in reproductive success for one or both sexes, and/or low absolute numbers (Hartl & Clark 1989). Population subdivision might exist, for example, if animals always return to reproduce to the sites where they were born (Pomeroy *et al*. 2000, Mark *et al*. 1988). However, we did not detect any genetic difference between pups from the three different sampling sites of the Liaodong Gulf breeding colony. High variance in reproductive success might be due to a strongly polygynous mating system, as observed in other pinnipeds (Riedman 1990), which can greatly reduce the number of males contributing to reproduction. We, however, found a strongly monogynous mating system in the Liaodong Gulf spotted seal (J. B. Han unpubl. data). Therefore, the most likely cause for the low levels of genetic variability observed is the demographic history and the current small size — about 800 individuals in 2007 (J. B. Han unpubl. data) — of the Liaodong Gulf population.

In Hokkaido waters, spotted seals have high mobile ability (Lowry *et al*. 1998) and age-specific distribution (Mizuno *et al*. 2001). High mtDNA variation and no particular genetic structure in this population, possibly indicates lack of a population bottleneck and "random migration" among some breeding colonies in the Sea of Japan and the Okhotsk Sea (Mizuno *et al*. 2003). Our recent study, which examined the mtDNA sequence variation in the species from the Liaodong Gulf colony showed that the Liaodong Gulf spotted seal may be geographically isolated from other breeding colonies (Han *et al*. 2007). The genetic variation of mtDNA sequences we found in the seals from Liaodong Gulf was sig-

Table 5. *k*/*r* ratios in the four seal species. Data from: [1)]Pastor *et al*. (2004), [2)]Allen *et al*. (1995) and Gemmel *et al*. (1997), [3)]Coltman *et al*. (1995) and Gemmel *et al*. (1997).

Locus	Liaodong Gulf spotted seal	Mediterranean monk seal[1)]	Harbor monk seal[2)]	Grey seal[3)]
SGPV2	0.40	0.67	0.67	0.70
SGPV3	0.75	–	1	0.65
SGPV10	1	1	0.75	1
SGPV11	0.60	1	1	1
SGPV16	0.36	0.45	1	0.88
SGPV17	0.40	0.30	1	1
Pvc19	0.67	–	0.83	0.78
Pvc63	0.50	0.75	1	1
Pvc78	0.75	0.75	1	1
Hgdii	0.38	–	0.72	0.67
Hg0	0.43	–	0.68	0.57
Hg6.1	1	0.25	0.88	1
Hg8.9	1	–	0.87	0.74
Hg8.10	1	–	1	1
BG	0.75	–	0.82	0.93
Mean	0.68	0.65	0.88	0.86

nificantly lower than that in seals from the coast of Hokkaido and the Okhotsk Sea (Mizuno *et al*. 2003). Earlier we also found a population-specific marker with one base-pair insertion in the threonine tRNA gene in the Liaodong Gulf seals (Han *et al*. 2007). From this result, we at least know that there is no female seal exchange between Liaodong Gulf and adjacent Hokkaido waters (Mamiya Strai, the southern Sakhalin, Peter the Great Bay, in the Sea of Japan and Okhotsk Russia), even though the nearest breeding colony in the Peter the Great Bay (Sea of Japan) is approximately 3000 km away.

The current population-size estimate suggests that the population is only about 10% of its historical size. Much of the population size reduction occurred probably during sealing operations during 1940–1980 (Dong & Shen 1991, Wang 1986, 1998). For at least 30 years, the population has been very small and has been subject to various mortality factors such as human persecution, destruction of haul-out sites (Lu *et al*. 2002, Han *et al*. 2005, Fan *et al*. 2005).

The present study supports the notion that a decrease in population size is the main factor accounting for the low levels of variability observed in the Liaodong Gulf spotted seal. Small populations are generally considered to be susceptible to a number of genetic problems that can compromise long-term survival. Inbreeding and low levels of genetic variability were associated with low fitness in populations (Keller *et al*. 1994, Madsen *et al*. 1996). Genetic erosion was also suggested to reduce the genetic resources of populations to overcome the effects disease (O'Brien & Evermann 1988). Our finding has important conservation and management implications for spotted seals in the Bohai Sea and the Yellow Sea.

Further research is needed to clarify the role of genetic effects on the future demographic trajectory and conservation and management of this colony. This work should include an evaluation of genetic distance between the Liaodong Gulf and other spotted seal colonies. Because the spotted seal population of Liaodong Gulf is small and migrates seasonally to Bak-ryoung Island, the establishment of an international protection network and research cooperation between China and South Korea, as well as other countries, is urgently required.

Acknowledgements

The work was mainly supported by programs from International Fund for Animal Welfare and US Pacific Ocean Environmental Foundation, as well as from Project 908 of National Ocean of China (No. LN-908-01-01-06).

References

Allen, P. J., Amos, W., Pomeroy, P. P. & Twiss, S. D. 1995: Microsatellite variation in grey seals (*Halichoerus grypus*) shows evidence of genetic differentiation between two British breeding colonies. — *Molecular Ecology* 4: 653–662.

Arnason, U., Bodin, K., Gullberg, A., Ledje, C. & Mouchaty, S. 1995: A molecular view of pinniped relationships with particular emphasis on the true seals. — *J. Mol. Evol.* 40: 78–85.

Avise, J. C. 1994: *Molecular markers, natural history and evolution*. — Chapman and Hall, New York, USA.

Badosa, E., Grau, E., Aparicio, F., Layna, J. F. & Cedenilla, M. A. 1998: Individual variation and sexual dimorphism of colouration in Mediterranean monk seal pups (*Monachus monachus*). — *Mar. Mamm. Sci.* 14: 390–393.

Bagley, M. J., Anderson, S. L. & May, B. 2001: Choice of methodology for assessing genetic impacts of environmental stressors: polymorphism and reproducibility of RAPD and AFLP fingerprints. — *Ecotoxicology* 10: 239–244.

Coltman, D. W., Bowen, W. D. & Wright, J. M. 1996: PCR primers for harbour seal (*Phoca vitulina concolour*) microsatellites amplify polymorphic loci in other pinniped species. — *Mol. Ecol.* 5: 161–163.

Coltman, D. W., Bowen, W. D. & Wright, J. M. 1998: Birth weight and neonatal survival of harbour seal pups are positively correlated with genetic variation measured by microsatellites. — *Proc. R. Soc. Lond. B* 265: 803–809.

Cornuet, J. M. & Luikart, G. L. 1996: Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. — *Genetics* 144: 2001–2014.

Di Rienzo, A., Peterson, A. C., Garza, J. C., Valdes, A. M., Slatkin, M. & Freimer, N. B. 1994: Mutational processes of simple-sequence repeat loci in human populations. — *Proc. Natl. Acad. Sci. USA* 91: 3166–3170.

Dong, J. H. & Shen, F. 1991: Estimates of historical population size of the harbor seal (*Phoca largha*) in Liaodong Bay. — *Marine Sciences* 15: 26–30. [In Chinese with English abstract].

Ellegren, H. 1995: Mutation rates at porcine microsatellite loci. — *Mamm. Genet.* 6: 376–377.

England, P. R., Osler, G. H. R., Briscoe, D. A. & Frankham, R. 1996: The effect of population bottlenecks on allelic diversity and heterozygosity. — *Bulletin of the Ecological Society of America* 77: 130.

Englbrecht, C. C., Freyhof, J., Nolte, A., Rassmann, K., Schliewen, U. & Tautz, D. 2000: Phylogeography of the bullhead *Cottus gobio* (Pisces: Teleostei: Cottidae)

suggests a pre-Pleistocene origin of the major central European populations. — *Mol. Ecol.* 9: 709–722.

Fan, G. K., Han, J. B., Huang, J. C. & Ma, Z. Q. 2005: Spotted seals in Miaodao Island. — *Fisheries Science* 24: 16–18. [In Chinese with English abstract].

Forcada, J., Hammond, P.S. & Aguilar, A. 1999: Population status and trends of the Mediterranean monk seal in the western Sahara during 1993–1996 and after the 1997 mass mortality. — *Mar. Ecol. Prog. Ser.* 188: 249–261.

Frankham, R., Lees, K. & Montgomery, M. 1999: Do population size bottlenecks reduce evolutionary potential? — *Animal Conservation* 2: 255–260.

Garza, J. C. 1998: *Population genetics of the northern elephant seal*. — Ph.D. thesis, University of California at Berkeley.

Garza, J. C. & Williamson, E. 2001: Detection of reduction in population size using data from microsatellite loci. — *Mol. Ecol.* 10: 305–318.

Gemmell, N. J., Allen, P. J., Goodman, S. J. & Reed, J. Z. 1997: Interspecific microsatellite markers for the study of pinniped populations. — *Mol. Ecol.* 6: 661–666.

Goodman, S. J. 1995: *Molecular population genetics of the harbour seal (Phoca vitulina) with reference to the 1988 distemper virus epizootic*. — Ph.D. thesis, Cambridge University.

Hamrick, J. L., Godt, M. J. W., Murawski, D. A. & Loveless, M. D. 1991: Correlations between species traits and allozyme diversity: implications for conservation biology. — In: Falk, D. A. & Holsinger, K. E. (eds.), *Genetics and conservation of rare plants*: 75–86. Oxford University Press, Oxford.

Han, J. B., He, C. B., Wang, Q., Ma, Z. Q. & Xu, X. H. 2006: Sequence analysis of mitochondrial ND4, tRNA-Arg, ND4L and ND3 from spotted seal (*Phoca largha*) in Liaodong Gulf. — *Fisheries Science* 25: 501–504. [In Chinese with English abstract].

Han, J. B., He, C. B., Wang, X. M., Wang, Q., Ma, Z. Q., Zhou, Z. C. & Wang, P. L. 2007: Sequence analysis of mitochondrial $tRNA^{Thr}$, $tRNA^{Pro}$ and control region from spotted seals (*Phoca largha*) in Liaodong Gulf. — *Fisheries Science* 26: 74–78. [In Chinese with English abstract].

Han, J. B., Wang, W. & Ma, Z. Q. 2005: Spotted seals in the estuary of Shuangtaizi River of Liaodong Bay. — *Marine Environmental Science* 24: 51–53. [In Chinese with English abstract].

Hartl, D. L. & Clark, A. G. 1989: *Principles of population genetics*. — Sinauer Associates, Sunderland, MA.

Houlden, B. A., England, P. R., Taylor, A. C., Greville, W. D. & Sherwin, W. B. 1996: Low genetic variability of the koala Phascolarctos cinereus in south-eastern Australia following a severe population bottleneck. — *Mol. Ecol.* 5: 269–281.

IUCN 1994: *Red list of threatened animals*. — International Union for Conservation of Nature and Natural Resources, Gland, Switzerland.

Kappe, A. L., Bijlsma, R., Osterhaus, A. D. M. E., Van Delden, W. & Van D. E. Z. L. 1997: Structure and amount of genetic variation at minisatellite loci within the subspecies complex of the harbour seal (*Phoca vitulina*). — *Heredity* 78: 457–463.

Keller, L. F., Arcese, P., Smith, J. N. M., Hochachka, W. M. & Stearns, S. C. 1994: Selection against inbred song sparrows during a natural population bottleneck. — *Nature* 372: 356–357.

Kretzmann, M., Mentzer, L., Digiovanni, J. R., Leslie, M. S. & Amato, G. 2006: Microsatellite diversity and fitness in stranded juvenile harp seals (*Phoca groenlandica*). — *Journal of Heredity* 97: 555–560.

Lande, R. 1988: Genetics and demography in biological conservation. — *Science* 241: 1455–1460.

Liu, Z. J. & Cordes, J. 2004: DNA marker technologies and their applications in aquaculture genetics. — *Aquaculture* 238: 1–37.

Louis, E. J. & Dempster, E. R. 1987: An exact test for Hardy-Weinberg and multiple alleles. — *Biometrics* 43: 805–811.

Lowry, L. F., Frost, K. J., Davis, R., Suydam, R. S. & DeMaster, D. P. 1994: *Movements and behavior of satellite tagged spotted seals (Phoca largha) in the Bering and Chukchi Seas*. — U.S. Dep. Commer., NOAA Tech. Memo. NMFS-AFSC-38.

Lowry, L. F., Frost, K. J., Davis, R., DeMaster, D. P. & Suydam, R. S. 1998: Movements and behavivor of satellite-tagged spotted seals (*Phoca largha*) in the Bering and Chukchi seas. — *Polar Biology* 19: 221–230.

Lu, S. X., Liu, Z. J. & Zong, X. B. 2002: Investigation on the resource of spotted seal (*Phoca largha*) in coast of Yantai, Shandong Province. — *Fishery economy of China* 4: 34–35. [In Chinese with English abstract].

Luikart, G. L., Allendorf, F. W., Cornuet, J. M. & Sherwin, W. B. 1998: Distortion of allele frequency distributions provides a test for recent population bottlenecks. — *J. Hered.* 89: 238–247.

Madsen, T., Stille, B. & Shine, R. 1996: Inbreeding depression in an isolated population of adders. — *Biol. Conserv.* 75: 113–118.

Mäkinen, H. S., Välimäki, K. & Merilä, J. 2007: Cross-species amplification of microsatellite loci for nine-spined stickleback *Pungitius pungitius*. — *Ann. Zool. Fennici* 44: 218–224.

Mark, A., Hindell, G. & Little, J. 1988: Longevity, fertility and philopatry of two female southern elephant seals (*Mirounga leonina*) at macquarie island. — *Marine Mammal Science* 4: 168–171.

Maudet, C., Miller, C., Bassano, B., Breitenmosser-Würsten, C., Gauthier D., Obexer-Ruff, G., Michallet, J., Taberlet, P. & Luikart, G. L. 2002: Microsatellite DNA and recent statistical methods in wildlife conservation management: applications in Alpine ibex [*Capra ibex* (ibex)]. — *Mol. Ecol.* 11: 421–436.

Mizuno, A. W., Onuma, M., Takahashi, M. & Ohtaishi, N. 2003: Population genetic structure of the spotted seal *Phoca largha* along the coast of Hokkaido, based on mitochondrial DNA sequences. — *Zool. Sci.* 20: 783–788.

Mizuno, A. W., Suzuki, M. & Ohtaishi, N. 2001: Distribution of the spotted seal *Phoca largha* along the coast of hokkaido, Japan. — *Mammal Study* 26: 109–118.

Mizuno, A. W., Wada, A., Lshinazaka, T., Hattori, K., Watanabe, Y. & Ohtaishi, N. 2002: Distribution and abundance

of spotted seals *Phoca largha* and ribbon seals *Phoca fasciata* in the southern Sea of Okhotsk. — *Ecol. Res.* 17: 79–96.

Nei, M. 1978: Estimation of average heterozygosity and genetic distance from a small number of individuals. — *Genetics* 89: 583–590.

Nei, M. & Roychoudhury, A. K. 1974: Sampling variances of heterozygosity and genetic distance. — *Genetics* 76: 379–390.

O'Brien, S. & Evermann, J. 1988: Interactive influence of infectious disease and genetic diversity in natural populations. — *Trends Evol. Ecol.* 3: 254–259.

O'Corry-Crowe, G. 1994: *Molecular analysis of intraspecific structure of spotted seals (Phoca largha) and the phylogenetic and current relationship of spotted and harbour seals (Phoca vitulina): preliminary findings*. — Report to NMFS, Natl. Mar. Mammal Lab., Seattle, Washington.

Pastor, T., Garza, J. C., Allen, P., Amos, W. & Aguilar, A. 2004: Low genetic variability in the highly endangered Mediterranean monk Seal. — *Journal of Heredity* 95: 291–300.

Piry, S., Luikart G. L. & Cornuet, J. M. 1997: Botteneck: a computer program for detecting recent reductions in the effective size using allele frequency data. — *J. Hered.* 90: 502–503.

Pomeroy, P. P., Twiss S. D. & Redman, P. 2000: Philopatry, site fidelity and local kin associations within grey seal breeding colonies. — *Ethology* 106: 899–919.

Popov, V. N. 1976: *Status of main ice forms of seals inhabiting waters of the U.S.S.R. and adjacent to the country marine areas*. — FAO Report 51.

Raymond, M. & Rousset, F. 1995a: An exact test for population differentiation. — *Evolution* 49: 1280–1283.

Raymond, M. & Rousset, F. 1995b: GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. — *J. Hered.* 86: 248–249.

Reeves, R. R., Stewart, B. S. & Leatherwood, S. 1992: *Seals and sirenians*. — Sierra Club Books, San Francisco.

Riedman, M. L. 1990: *The pinnipeds. Seals, sea lions, and walruses*. — University of California Press. Berkeley.

Rugh, D. J., Shelden, K. E. W. & Withrow, D. E. 1997: Soptted seals, *Phoca largha*, in Alaska. — *Marine Fisheries* 59: 1–17.

Saccheri, I., Kuussaari, M. & Kankare, M. 1998: Inbreeding and extinction in a butterfly metapopulation. — *Nature* 392: 491–494.

Sambrook, J. & Russell, D. W. 2001: *Molercular cloning: a laboratory manual*, 3rd ed. — Cold Spring Harkbor Labroatory Press, New York.

Selonen, V., Painter, J. N. & Hanski, I. K. 2005: Microsatellite variation in the Siberian flying squirrel in Finland. — *Ann. Zool. Fennici* 42: 505–511.

Shaughnessy, P. D. & Fay, R. H. 1977: A review of the taxonomy and nomenclature of North Pacific harbour seals. — *J. Zool. Lond.* 182: 385–419.

Simmons, M., Mickett, K., Kucuktas, H., Li, P., Dunham, R. & Liu, Z. J. 2006: Comparison of domestic and wild channel catfish (*Ictalurus punctatus*) populations provides no evidence for genetic impact. — *Aquaculture* 252: 133–146.

Taylor, A. C., Sherwin, W. B. & Wayne, R. K. 1994: Genetic variation of microsatellite loci in a bottlenecked species: the northern hairy-nosed wombat Lasiorhinus krefftii. —*Mol. Ecol.* 3: 277–290.

Trukhin, A. M. & Mizuno, A. W. 2002: Distribution and abundance of the largha seal (*Phoca largha* Pall.) on the coast of Primorye Region (Russia): a literature review and survey report. — *Mammal Study* 27: 1–14.

Wang, P. L. 1986: Distribution, ecology and resource conservation of the spotted seal in the Huanghai and Bohai Seas. — *Acta Oceanol. Sinica* 5: 126–133. [In Chinese with English abstract].

Wang, P. L. 1993: Status and protection of Spotted sesls in the Bohai Sea. — *Fisheries Science* 12: 4–7. [In Chinese with English abstract].

Wang, S. 1998: *China Red Data Book of endangered animals: Mammalia*. — Science Press, Beijing, China. [In Chinese with English summary].

Wang, Z. M. & Wang, P. L. 1990: [The spotted seal *(Phoca largha)*]. — Ocean Publishing Company, Beijing, China. [In Chinese].

Weber, J. L. & Wong, C. 1993: Mutation of human short tandem repeats. — *Hum. Mol. Genet.* 2: 1123–1128.

Whitehead, A., Anderson, S. L., Kuivila, K. M., Roach, J. L. & May, B. 2003: Genetic variation among interconnected populations of *Catostomus occidentalis*: implications for distinguishing impacts of contaminants from biogeographical structuring. — *Mol. Ecol.* 12: 2817–2833.

Won, C. & Yoo, B. H. 2004: Abundance, seasonal haul-out patterns and conservation of spotted seals *Phoca largha* along the coast of Bak-ryoung Island, South Korea. — *Oryx* 38: 109–112.

Zhao, C. J. & Li, N. 2003: A study of a brief protocol to extract DNA from hair. — *Hereditas* (Beijing) 25: 69–70. [In Chinese with English abstract].

Zhou, Z. C., Zou, L. L., Dong, Y., He, C. B., Liu, W. D., Deng, H. & Wang, L. M. 2008: Characterization of 28 polymorphic microsatellites for Japanese sea urchin (*Strongylocentrotus intermedius*) via mining EST database of a related species (*S. purpuratus*). — *Ann. Zool. Fennici* 45: 181–184.