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Genetic Diversity of the Spotted Seal (*Phoca largha*) in Liaodong Gulf Following Long Term Conservation Activities

Weidong Liu, Jiashen Tian, Zhen Wang, Zhongren Kong, Jiabo Han and Zhichuang Lu*

Dalian Key Laboratory of Conservation Biology for Endangered Marine mammals, Liaoning Ocean and Fisheries Science Research Institute, 50 Heishijiao Road, Shahekou District, Dalian, Liaoning, China, 116023

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Key words
Spotted seal *Phoca largha*, Liaodong Gulf, *ND4L* gene, D-loop region, Population expansion

ABSTRACT

The spotted seal (*Phcoa largha*) is the only pinniped species reproducing in the wild in China. Wild populations of this species have declined sharply in China due to anthropogenic impacts. Forty-six individuals died accidentally in Liaodong Gulf between 2018 to 2020 and were collected. The genetic diversity and population structure of this population were investigated using mitochondrial *ND4L* and D-loop sequences, and the results were compared with the same population in 2005. The haplotype diversity of Liaodong Gulf spotted seals was low compared to other spotted seal populations in other habitats. Fortunately, the genetic diversity of the current Liaodong Gulf spotted seal population was higher than 15 years ago, and the population expanded between 2005 and 2020. Our findings indicated that conservation activities for Liaodong Gulf spotted seals in recent decades have been valuable, and should persist in the future.

INTRODUCTION

The spotted seal (*Phoca largha*) is a small-bodied pinniped widespread in cold sea areas in the northern hemisphere (Jefferson *et al*., 2007). It is the only pinniped species found in China that reproduces in the wild (Rugh *et al*., 1997). The Liaodong Gulf in China is the southernmost sanctuary for spotted seals among the eight sanctuaries. Between November and May every year, spotted seals assemble in large groups in the Liaodong Gulf to mate and reproduce (Wang, 1986). Due to the destruction of their habitat by anthropogenic impacts, *P. largha* has been listed as one of the most endangered species in China (Gao *et al*., 2013). Since the 1980s, local scientific and conservation organisations have worked to protect the spotted seal population in Liaodong Bay, through the establishment of the Dalian Spotted Seal Reserve of China in 1992. Since the independent colony of spotted seals in the Liaodong Gulf, they have been isolated from the Hokkaido and Okhotsk spotted seal populations and formed an independent subspecies (Han *et al*., 2007). The surviving population declined to about 1,000 individuals by 2005, even though some protection measures had been put in place and seal hunting was prohibited in this region for the past two decades (Gao *et al*., 2015).

The genetic diversity of a population is an important indicator for assessing the environmental adaptability of individuals within the population (Markert *et al*., 2010). Our previous study indicated that the population of Liaodong Gulf spotted seals had relatively low genetic variation compared with those from the coast of Hokkaido in Japan and from the Sea of Okhotsk in Russia (Han *et al*., 2007). Following continuous efforts in recent years, the *P. largha* population in the Liaodong Gulf is currently estimated to number ~2,000 animals. From 2005 to the present, Liaodong Gulf spotted seals have undergone approximately three or four generations. Therefore, understanding the variation in genetic diversity of Liaodong Gulf spotted seals is critical for the rational conservation of this population.

Mitochondrial DNA (mtDNA) is convenient for assessing population genetics due to the characteristics of strict maternal inheritance, high uniformity in different tissues, and rapid evolution rate (Cui *et al*., 2010). The displacement loop (D-loop), a noncoding sequence, is

* Corresponding author: luzhichuang@hotmail.com
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the region with the greatest variation in mtDNA (Brown *et al*., 1986), and can be used to study the genetic diversity and population structure of spotted seals (Stanley *et al*., 1996). Another mtDNA region, the NADH dehydrogenase subunit 4L (*ND4L*) gene, has also been applied to investigate the genetic diversity of spotted seal populations (Ayako *et al*., 2003). In our previous studies, we sequenced the D-loop region and *ND4L* gene of the Liaodong Gulf spotted seal population to assess genetic diversity in 2005 (Han *et al*., 2006, 2007). In the present study, we collected several spotted seal samples from the Liaodong Gulf from 2018−2020. The genetic diversity of this population was investigated using the *ND4L* gene and D-loop region in mtDNA as molecular markers, and the results were compared with those of our previous studies. The findings illuminated variations in genetic diversity and will promote the conservation of this spotted seal population.

MATERIALS AND METHODS

Sample collection and DNA extraction

Forty-six spotted seals were obtained in the Liaodong Gulf from 2018 to 2020, all killed accidentally and found by fishermen. They were transported to the laboratory under cold conditions and abdominal muscles were collected and stored at -80°C until DNA extraction. Genomic DNA was isolated from the body wall using a TaKaRa MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Agarose gel electrophoresis (1%) was performed to confirm DNA extraction, and DNA concentration and purity were assessed by a NanoPhotometer Classic Launched instrument (IMPLEN, Germany). All DNA samples were stored at -20°C for further application.

PCR amplification and sequencing

The *ND4L* gene and D-loop region were amplified by PCR using specific primers (Table I). PCR amplifications were carried out in an ABI2720 Thermal Cycler (Applied Biosystems, USA) in 20 μL reactions containing 10 μL of 2 × Taq Master Mix (Takara, Dalian, China), 0.5 μL of each primer, and ~50 ng template DNA. Thermal cycling parameters are listed in Table II. A negative control was included in each round of PCR to check contamination, and all negative controls failed to yield products, as expected. PCR products were analysed by electrophoresis on a 1.5% agarose gel. The main band was purified and recovered using a QIAquick Gel Extraction Kit (Qiagen, Germany). Purified PCR products were sequenced with an ABI 3730 XL automatic sequencer (Perkin Elmer, Waltham, MA, USA).

Forty-six *ND4L* and D-loop sequences were obtained in the present study. All unique sequences of both markers have been deposited in GenBank under accession numbers MZ505010-MZ505033 Information about the genetic diversity of the Liaodong Gulf spotted seal population in 2005 was obtained from our previous studies (GenBank accession numbers AB244723-AB244729, DQ153234-DQ153242, and DQ244045-DQ244052) (Han *et al*., 2006, 2007). All sequences were aligned and manually corrected using ClustalX v1.83 software (Thompson *et al*., 1997) under default settings. The number of haplotypes (h), number of polymorphic sites (S), haplotype diversity (Hd), and nucleotide diversity (Pi) were calculated using DnaSP v6.12 (Librado and Rozas, 2009). Meanwhile, Fu's *Fs* statistics (Fu, 1997) and Tajima's *D* tests (Tajima, 1989) were also performed by DnaSP v6.12 to assess the neutrality of *P. largha*. Analysis of molecular variance (AMOVA) and the fixation index (*Fst*) were employed to estimate the genetic differentiation between the Liaodong Gulf spotted seal populations in 2005 and 2020 using Arlequin v3.5.2.2 software (Excoffier *et al*., 2005). Phylogenetic trees based on the haplotypes of D-loop sequences were constructed using the maximum likelihood method by MEGA v7.0 with an appropriate substitution model sequence chosen by Modeltest v3.7 (Posada and Crandall, 1998). The robustness of the phylogenetic results was tested by bootstrap analysis with 1000 replicates.

RESULTS

Vvariations in the genetic diversity of Liaodong Gulf spotted seals

For the *ND4L* gene, one polymorphic site and two haplotypes were detected in both populations of Liaodong Gulf spotted seals in 2005 and 2020 (Table II). Two *ND4L* haplotypes detected in past and present populations were completely consistent (Fig. 1). The ND4L_1 haplotype

Table I. The primer information and thermal cycling for *ND4L* gene and D-loop region.

Target	Primer information	5′ → 3′	Thermal cycling
N4DL gene	F	CTTCCATGAGCATCGCACACAGA	94℃ 3 min; 35 cycles for 94℃ 15 s; 57℃ 20 s, 72℃ 90 s; and 72℃ 10 min.
	R	GGCTTATGCAATTGTCACCGAGT	
D-loop region	H34	CCAAATGCATGACACCACAG	94℃ 3 min; 35 cycles for 94℃ 15 s, 56℃ 45 s, 72℃ 60 s; and 72 ℃ 5 min.
	L1624	TACACTGGTCTTGTAAACC	

was present in 13 of 15 and 45 of 46 samples in 2005 and 2020 populations, respectively. In contrast, the ND4L_2 haplotype was only detected in 2 and one individual in the populations in 2005 and 2020, respectively. Moreover, lower values of Hd and Pi based on the *ND4L* gene were observed for the 2020 population (0.043 and 0.00008 for Hd and Pi, respectively) compared with 2005 (0.248 and 0.00079 for Hd and Pi, respectively; Table II).

Table II. Genetic diversity parameters for *P. largha* populations in Liaodong Gulf between 2007 and 2020 based on *ND4L* or D-loop sequences.

Population	No	h	S	Hd	Pi	Tajima's D	Fu's Fs
2005-*ND4L*	15	2	2	0.248	0.00079	-0.51271	1.188
2020-*ND4L*	46	2	2	0.043	0.00008	-1.11103	-1.567
2005-D-loop	46	10	10	0.632	0.00217	-1.37483	-4.594
2020-D-loop	46	20	37	0.866	0.00561	-2.17225*	-10.349*

*Significant at level ($p < 0.05$).

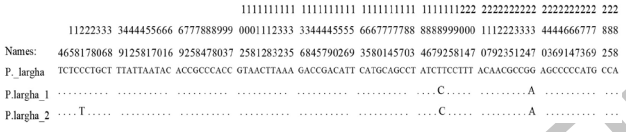

Fig. 1. The haplotypes of *ND4L* gene detected in the Liaodong Gulf spotted seal populations.

For the D-loop region, 10 polymorphic sites and 10 haplotypes were identified in the 2005 population, compared with 37 polymorphic sites and 20 haplotypes in the 2020 population (Table II). Among these haplotypes, five and 14 haplotypes were only detected in the 2005 and 2020 populations, respectively (Fig. 2). DL6 and DL3 were the most abundant haplotypes of the 2005 and 2020 populations (27 and 15 individuals, respectively; Fig. 2). Based on the D-loop sequences, the Hd and Pi of total samples were 0.632 and 0.00217 for 2005 and 0.866 and 0.00561 for 2020 populations, respectively (Table II).

Table III. Molecular variance (AMOVA) analysis for *P. largha* populations in Liaodong Gulf between 2005 and 2020 based on *ND4L* sequences.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	1	0.141	0.00420 Va	8.36
Within populations	59	2.712	0.04596 Vb	91.64
Total	60	2.852	0.05015	100

Fixation Index F_{ST}: 0.08364; p-value: 0.14761 ± 0.01174.

Variations in population genetic structure

AMOVA results indicated that 91.64% and 91.82% of total genetic variance based on the *ND4L* and D-loop sequences could be attributed to variations within populations (Tables III and IV). The fixation index *Fst* values between Liaodong Gulf spotted seal populations in 2005 and 2020 based on the *ND4L* gene and D-loop region were 0.08364 and 0.08179, respectively, and differences were significant for the D-loop region.

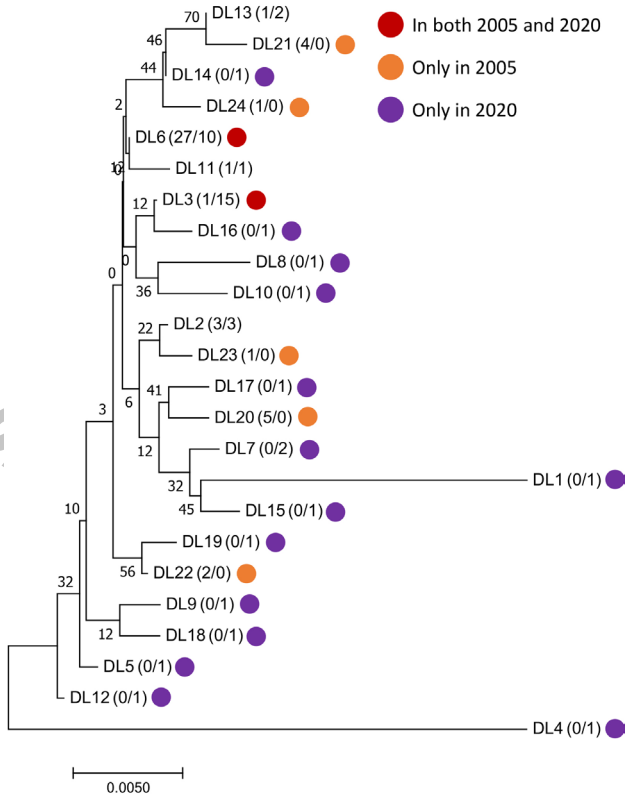

Fig. 2. Maximum likelihood tree constructed based on total 24 D-loop haplotypes of Liaodong Gulf spotted seal populations in 2005 and 2020. Numbers on the branches are bootstrap values for maximum likelihood. The numbers in the bracket mean the number of sequences belong to this haplotype obtained in 2005 and 2020, respectively. The previous number was sequence number in 2005 and the following number is that in 2020.

Tajima's *D* and Fu's *Fs* tests were performed based on the *ND4L* and D-loop sequences. For all samples, both *ND4L* and D-loop neutrality tests yielded negative Tajima's *D* values, among which the results for the 2020 population based on the D-loop region were significant (Table II). Similarly, for Fu's *Fs* tests, significantly negative values were also only obtained for the 2020 population based on the D-loop region (Table II).

Table IV. Molecular variance (AMOVA) analysis for *P. largha* populations in Liaodong Gulf between 2005 and 2020 based on D-loop sequences.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	1	5.687	0.09853 Va	8.18
Within populations	91	100.657	1.10612 Vb	91.82
Total	92	106.344	1.20465	100

Fixation Index F_{ST}: 0.08179; *p*-value: 0.00000 ± 0.00000.

DISCUSSION

In the present study, two and 20 haplotypes of *ND4L* genes and D-loop regions were respectively identified in the Liaodong Gulf spotted seal population in 2020. In the present study, the genetic diversity indices based on the *ND4L* gene of spotted seal population in 2020 showed slight lower than those in 2005. However, we speculated that the lower genetic diversity based on the *ND4L* gene could be a mask due to the increase of detected individuals in 2020 (46 vs 17). Meanwhile, the *ND4L* genes were completely consistent between the Liaodong Gulf spotted seal populations in 2005 and 2020. Generally, the evolution rate of mitochondrial DNA is very fast, and the mitochondrial DNA in the same population will also be highly diverse (Broughton and Gold, 2000). However, the *ND4L* gene is the most conserved region in mitochondria, which is highly consistent within the same species and is used for taxonomic identification (Yu *et al*., 1999). Thus, we believed that the genetic diversity of Liaodong Gulf spotted seal population based on the *ND4L* gene remained stable between 2005 and 2020.

In contrast to *ND4L* gene, the D-loop region, which exhibits the greatest variation among mtDNA regions, is often used to assess differences in genetic diversity among different populations of a given species (Zhang *et al*., 2021). In 2003, a study investigated the genetic diversity of spotted seal population in the seas of Japan and Okhotsk based on the D-loop region, and 57 haplotypes were found in 66 individuals (Ayako *et al*., 2003). This implied that the current genetic diversity in the Liaodong Gulf spotted seal population remains at a relatively low level (20 of 46 vs. 57 of 66). There are some potential reasons that may explain the lower genetic diversity of Liaodong Gulf spotted seals. Particularly, the Liaodong Gulf is a narrow habitat located at the margin of the spotted seal living area, resulting in reproductive isolation from other populations. In addition, severe environmental pollution and intensive fishing activities in the Liaodong Gulf have resulted in too few successfully breeding adults in the existing population.

Although the genetic diversity of D-loop region in the Liaodong Gulf spotted seal population was relatively low, the obvious increase of it in 2020 compared to 2005 was observed in the present study. For the obtained haplotypes of D-loop region, the unique haplotypes emerged in 2020 almost detected in only one individual. In contrast, the disappeared haplotypes in 2020 were detected in several individuals in 2005. This could be the reason for the higher genetic diversity indices based on the D-loop region in the Liaodong Gulf spotted seal population in 2020 compared to 2005. The *Fst* value is an effective index for assessing differentiation among populations (Wright, 1972). The significant *Fst* value for D-loop sequences between the populations investigated in the present study indicated appreciable genetic differentiation between the Liaodong Gulf spotted seal populations in 2005 and 2020. In addition, neutrality tests, such as Tajima's *D* and Fu's *Fs* tests, were used to examine recent population expansion when the null hypothesis was rejected (Zhang *et al*., 2017). Negative and significant neutrality test values indicated that sequences contained more nucleotide changes than would be predicted based on the neutral evolution model, which may suggest a population expansion event in recent history (Zhang *et al*., 2017). Herein, both Tajima's *D* and Fu's *Fs* values based on D-loop sequences in the 2020 population were significantly negative. In addition, the actual individual number of the Liaodong Gulf spotted seal population was increased from less than 1000 in 2005 to more than 2000 in 2020. These results further indicated that a population expansion event could have occurred recently.

CONCLUSION

It is well-known that the spotted seal population in the Liaodong Gulf has rapidly declined in the past few decades. Systematic conservation activities have been conducted, and the population of Liaodong Gulf spotted seals has increased, genetic diversity is lower than in other spotted seal populations. Fortunately, the genetic diversity of the Liaodong Gulf spotted seal population based on the D-loop region in now higher than it was in 2005. Our findings confirmed that conservation activities for Liaodong Gulf spotted seals are valuable and should be continued in the future.

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Ethic approval

Samples collection for spotted seals collected in Liaodong Bay, China, were authorized under the Ministry of Agriculture and Rural Affairs of the People's Republic of China, permit number: 1376. This study was conducted under a permit issued by Liaoning Fisheries Administration Bureau, Liaoning Province, China (approval number: LSYXFZ20111105).

Data availability statement

All sequences have been deposited in GenBank at https://www.ncbi.nlm.nih.gov/nuccore/ (accession numbers MZ505010-MZ505033). Information about the genetic diversity of the Liaodong Gulf spotted seal population in 2005 was obtained from our previous studies (GenBank, https://www.ncbi.nlm.nih.gov/nuccore/, accession numbers AB244723-AB244729, DQ153234-DQ153242, and DQ244045-DQ244052).

Statement of conflict of interest

The authors have declared no conflict of interest.

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