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Population Genetic Structure of the Spotted Seal *Phoca largha* along the Coast of Hokkaido, Based on Mitochondrial DNA Sequences

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ABSTRACT—Population genetic structure of the spotted seal, *Phoca largha*, along coastal regions of Hokkaido was investigated, using mitochondrial DNA (mtDNA) sequences. A 571-bp fragment of the mtDNA control region and adjacent threonine and proline transfer RNA genes was sequenced from 66 seals. We categorized all individuals into three groups considering sampling area and season: twenty-four seals from the Sea of Okhotsk in winter, 11 seals from the Sea of Okhotsk coast in fall, and 31 seals from the Sea of Japan coast in winter. From the 66 animals, 57 haplotypes were identified. Compared with the harbor seal sequence, all spotted seals examined shared two deletions in the control region, which distinguished between the two species. Forty-nine haplotypes were represented by a single individual, and haplotypes shared by more than two animals were generally restricted to same sampling-groups. Phylogenetic trees did not indicate clear geographic differences among the three groups. An Analysis of molecular variances (AMOVA) did not showed any significant population genetic structure in Hokkaido spotted seals (Φ_{st} = –0.003). Our results showed a high level of diversity but no genetic structure, and did not deny the possibility that seals in the Okhotsk breeding concentration mainly stayed in the fall Okhotsk and also inhabited in the winter Sea of Japan.

Key words: AMOVA, control region, Japan, marine mammal, the Sea of Okhotsk

INTRODUCTION

The spotted seal, *Phoca largha*, is widely distributed in the north and west of the North Pacific Ocean: the Huanghai (Yellow Sea), Japan, Okhotsk, Bering, Chukchi, and Beaufort Seas, and there are geographic eight breeding concentrations (Shaughnessy and Fay, 1977). Morphological differences were reported among breeding concentrations (Kosygin and Gol'tsev, 1974; Fedoseev *et al*., 1979; Fedoseev, 1984), some of which resulted them as different populations. However, it might be possible that spotted seals move around other breeding concentrations and genetic exchanges occur, because they have a high mobile ability for great distance, which was studied using satellite-linked tags (Lowry *et al*., 1998), although the genetic relationship has not yet been confirmed.

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In the coastal waters of Hokkaido (Japan) and Peter the Great Bay (Russia), spotted seals are reported to have a high level of human-caused mortality such as entanglement in the fishing net, oil spill accident, and damage control kill event (Mizuno *et al*., 2001; Trukhin and Mizuno, 2002). However, it is difficult to assess the influence on seal population continuation, since there is little understanding about the population unit and range, which is important basis in wildlife management and conservation planning. One of the reasons will be lack of genetic information in this species.

Although there have not yet been studied enough in spotted seals, mitochondrial DNA (mtDNA) control region sequences have been used as a genetic marker to identify population structure in a number of marine mammals (Dizon *et al*., 1997). Also it was reported in the harbor seal *Phoca vitulina* (Lamont *et al*., 1996; Stanley *et al*., 1996; Burg *et al*., 1999; Watanabe Y., unpublished data), which is a genetically close-related species to the spotted seal (Árnason *et al*., 1995; O'Corry-Crowe and Westlake, 1997). The only study dealing with those in the spotted seal resulted to be likewise not genetically discernable between Alaska and

Kamchatka seal (O'Corry-Crowe and Westlake, 1997). However, the whole sequence data was not shown there, and few specimens between 3 and 9 seals in each site were analyzed without enough descriptions of sampling season and method.

In this study, we describe the genetic features of spotted seals off Hokkaido waters, using the sequence of mtDNA control region, and discuss population structure and range, taking future seal conservation and management into account. In Hokkaido waters, spotted seals appear mostly in fall for following salmon, and in winter (around sea ice season) for the reproduction (Naito and Nishiwaki, 1975; Naito and Konno, 1979; Uno and Yamanaka, 1988; Mizuno *et al.*, 2001, 2002; Mizuno and Ohtaishi, 2002). We categorize these seals into several groups based on the appearance season and area, compare the genetic relationship among groups, and discuss their natal breeding concentration.

MATERIALS AND METHODS

Sample collection and DNA extraction: Tissue samples of muscle and skin were collected from 66 deceased spotted seals along the coast of Hokkaido between 1996 and 1998. Seals were categorized into three groups, according to sampling area, season and some circumstantial judgements (Fig. 1). Twenty-four seals were killed for damage control along the Sea of Okhotsk coast between January and February, just before breeding season (Group-code: OW), and 11 seals were incidentally caught in the fishing nets during November (Group-code: OF). We considered these two groups as a different group, because of the different sampling seasons, capture methods and age compositions (*i.e.* most of the OF seals were young, whereas the OW seals had various age structure). The remaining 31 seals were killed for damage control (n=21), found beach-cast (n=9), and incidentally caught in the fishing nets (n=1) at the Sea of Japan coast in the winter (mostly January and February). Although the capture methods for the seals in the Sea of Japan were various, we here considered them as a same group (Group-code: JW). Because they were all collected not only at the same season and area, but all young (0 or 1 yr. old), which was corresponded with the feature of haul-out seals in this area (Mizuno *et al.*, 2001). Tissue samples were stored in 70% ethanol at 5°C, and DNA was extracted using standard proteinase K/phenol/chloroform extraction (Sambrook *et al.*, 1989; Masuda and Yoshida, 1994).

Amplification and sequencing: Two PCR primers, H34 (5'-CCAAATGCATGACACCACAG-3') and L16274 (5'-TACACTGGTCTTGTAAACC-3'), were used to amplify a product containing a portion of the threonine and proline tRNA genes and part of the control region (Lamont *et al.*, 1996; Stanley *et al.*, 1996). PCR amplification was carried out, using rTaq DNA polymerase (Takara) with 3 μl of DNA extracts in 50 μl of reaction mixture and GeneAmp® PCR System 9700 (Applied Biosystems), according to manufacture's instructions. Amplification consisted of one cycle of 94°C for 5 min; 50 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s; and one final cycle of 72°C for 7 min. Sequencing was performed using an ABI PRISM(TM) 377 DNA Sequencer (Applied Biosystems).

Data analysis: Sequences were aligned using the program Clustal W 1.4 (Thompson *et al.*, 1994). Phylogenetic trees were constructed by the neighbor-joining (Saitou and Nei, 1987) and

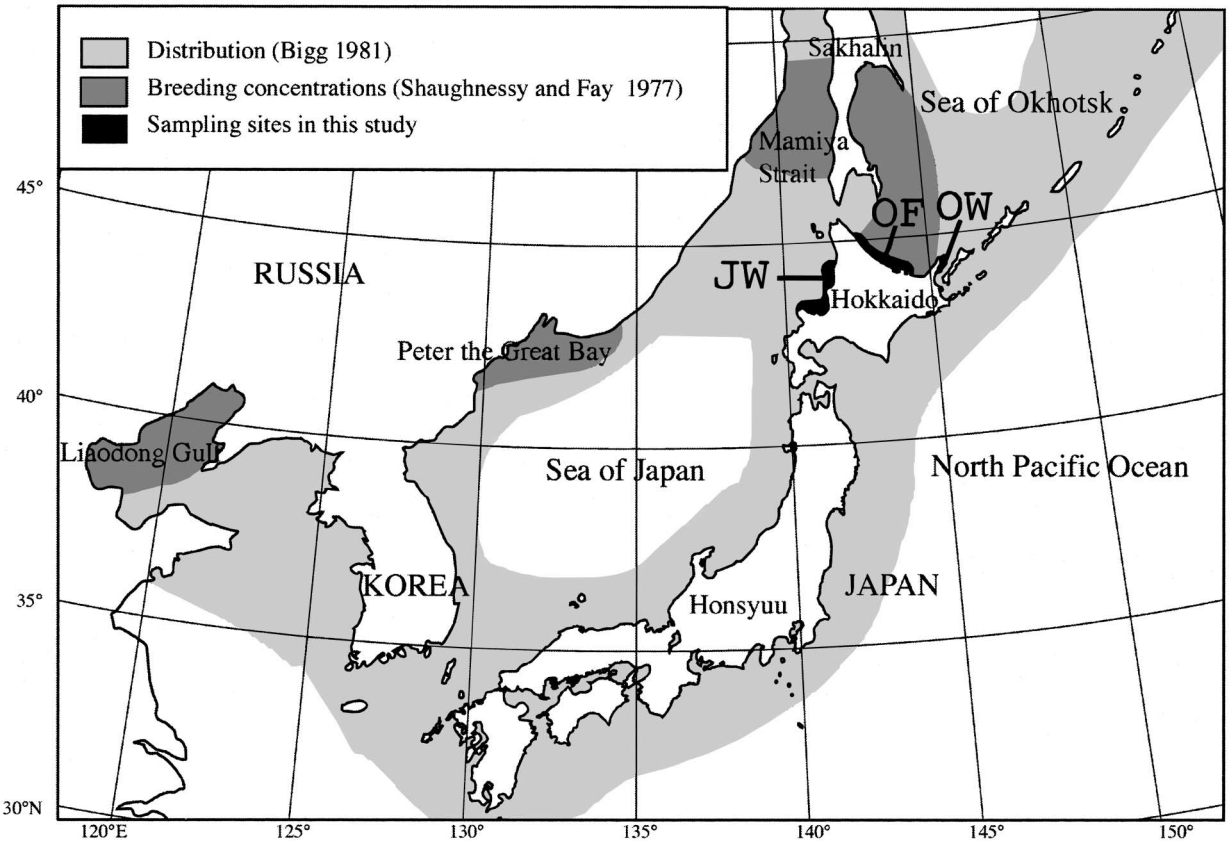

Fig. 1. The geographic distribution of the spotted seal around Japan and the sampling sites for this study. OW = The Sea of Okhotsk coast in winter, OF = The Sea of Okhotsk coast in fall, JW = the Sea of Japan coast in winter.

UPGMA (Sokal and Michener, 1958) using the program MEGA version 2.1 (Kumar *et al*., 2001). Number of nucleotide substitutions per site was estimated for multiple substitutions using the Kimura's two parameter methods (Kimura, 1980). The reliability of the topology of trees obtained was assessed by 1,000 bootstrap replications (Felsenstein, 1985). Because of its close relationship to the spotted seal (Árnason *et al*., 1995; O'Corry-Crowe and Westlake, 1997), sequence from the harbor seal *Phoca vitulina vitulina* (Árnason and Johnsson, 1992) was used as an outgroup for the analysis. The extent of population subdivision among groups was examined using analysis of molecular variance (AMOVA; Excoffier *et al*., 1992) implemented in ARLEQUIN (Schneider *et al*., 2000). The analysis was carried out for three comparisons; between seasonal different groups in the Sea of Okhotsk (OF-OW), between different area groups in winter season (JW-OW), and among the all groups (OF-OW-JW). The standard variance and haplotypic correlation measures (Φ-statistics) can be used to infer the degree of population subdivision. The significance of the resultant Φ-statistics and variance components were tested with 1,023 permutations.

RESULTS

A total of 571 base pairs of 5' end of the mtDNA control region, adjacent proline and a portion of the threonine tRNA genes were sequenced for all 66 spotted seals. This fragment was equivalent to the positions 16 273 – 16 826 and 1 – 31 of the published harbor seal sequence (Árnason and Johnsson, 1992). Sixty-six variable sites were observed that define 57 haplotypes (Fig. 2). Sixty-one variable sites were transition substitutions, 2 were transversions, and 3 were length changes (positions 16 484 – 16 485, 16 489, and 16 498 – 16 499) (Fig. 2). Within the 571 base pairs examined, the observed number and the percentage of pairwise differences among haplotypes ranged from 2 to 16, and from 0.2% to 4.0% (average=1.7%, and SD=0.7%), respectively. Comparing with the harbor seal sequences, all 66 spotted seals had three deletions; one base pair long in the threonine tRNA gene (position 16 296), and 3 and 11 base pairs long in the mtDNA control region adjacent to the proline tRNA gene (positions 16 378 – 16 380, 16 384 – 16 394) (Fig. 2).

Most of the 57 haplotypes were unique to single animals. Only 8 were shared between two or three individuals (Fig. 2; Haplotypes A ~ H). Six of 8 shared haplotypes were found in the seals belonging to the same group; 2 (A, B), 1 (C), and 3 (D, E, F) haplotypes (Fig. 2) were found in seals at the Okhotsk coast in winter (OW), in fall (OF), and at the

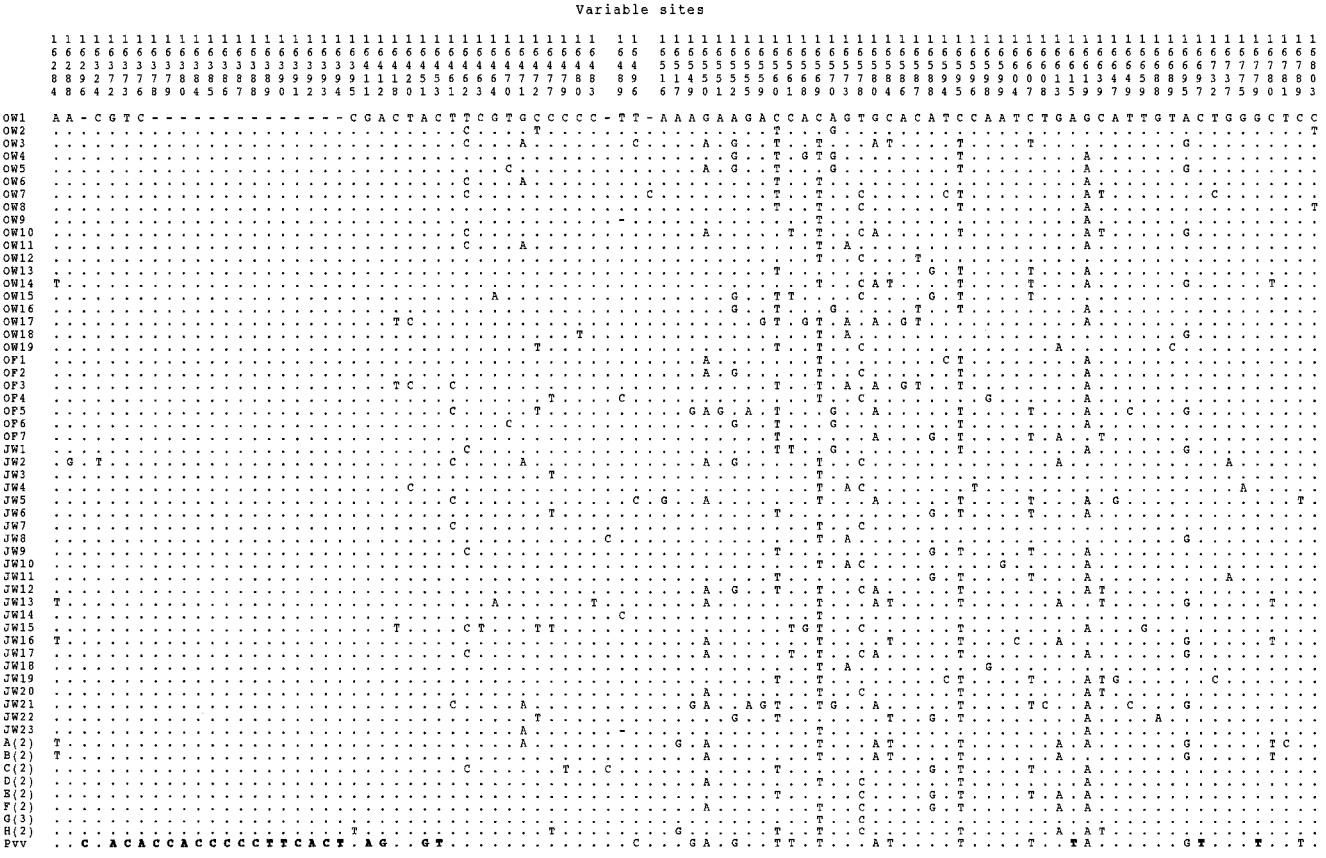

Fig. 2. Aligned mitochondrial control region and adjacent proline and threonine transfer RNA gene sequences from 66 spotted seals, showing variable sites and deletion events (–). Dots (•) represent the same base with those of the haplotype OW1. The 8 shared haplotypes (letters A–H) are also given. Each of the 49 unique haplotypes found in a single spotted seal is identified by a letter code (corresponding to the sampling sites and seasons, in details see text) followed by a number. Variable sites within the 571-bp fragment spanning positions 16 273 – 16 826 and 1 – 31 of the published mitochondrial genome (Pvv) are numbered according to the published harbour seal *Phoca vitulina vitulina* sequence (Árnason and Johnsson 1992).

Sea of Japan coast in winter (JW), respectively. The other two shared haplotypes were found in seals from both the Sea of Okhotsk and the Sea of Japan; haplotypes G and H (Fig. 2) consisted of OF-JW, and OW-JW, respectively.

In the neighbor-joining and UPGMA trees, there was no obvious phylogenetic structure in each sex, and then we analyzed all individuals together, which also represented no obvious structure.

The AMOVA analyses did not reveal any significant population genetic structure with low Φ_{st} value for all comparisons; OF-OW, JW-OW , and OF-OW-JW (p=0.545, 0.236, and 0.603, Φ_{st} = –0.003, 0.006 and –0.003, respectively) (Table 1). More than 99 percentages of the variance was accounted for within populations in all comparisons.

Table 1. Analysis of Molecular Variance within and among three spotted seal groups (OW, OF, and JW).

Variance component	Variance	%Total	Φ_{st} statistics	P
OF and OW				
Among groups	–0.014	–0.00	–0.003	0.545
Within groups	4.766	100.00		
JW and OW				
Among groups	0.028	0.59	0.006	0.236
Within groups	4.683	99.41		
OF, OW, and JW				
Among groups	–0.013	–0.28	–0.003	0.603
Within groups	4.644	100.28		

DISCUSSION

This study provided sequence data of 571 base pairs of the mtDNA control region, proline tRNA and a portion of the threonine tRNA genes in the spotted seal, which have not ever been reported. There was not obvious phylogenetic structure, nor subdivision, which was reflected in the very low estimates of Φ-statistics, among three groups along the Hokkaido coast. Thus, we were not able to deny the possibility that seals in the Okhotsk breeding concentration (OW) mainly stayed in the fall Okhotsk (OF) and also inhabited in the winter Sea of Japan (JW). On the other hand, six of 8 shared haplotypes were found in the seals belonging to the same group. Although each shared haplotypes consisted of a few seals (2–3), it might provide the possibility that the spotted seal form the genetically close related small group.

Comparing with the harbor seal sequence (Árnason and Johnsson, 1992), two deletions in the control region were found in the all 66 spotted seals in this study. These deletions were corresponded with those reported in the Alaska and Kamchatka spotted seals (O'Corry-Crowe and Westlake, 1997). Therefore, the result in the present study supported the previous statement (O'Corry-Crowe and Westlake, 1997) that the deletions proved to be a useful species-specific marker between the spotted seal and the harbor seal.

Since the intra-specific genetic study of the spotted seal have not been enough reported, we here discuss those referring to the close related species, the harbor seal. First, we compared our results to those of the sympatric harbor seal (the Kuril Seal *Phoca vitulina stejnegeri*) in Hokkaido waters. We found 57 haplotypes in the mtDNA of 66 spotted seals, whereas in the Hokkaido Kuril seal, only 11 haplotypes with 16 variable sites were found in the almost same mtDNA regions from 110 animals, where the pairwise differences ranged from 1 to 9 (Watanabe Y., unpublished data). Watanabe (unpublished data) discussed that little numbers of haplotypes and strong population structure depending on the geographical area in Kuril seals caused from the population bottleneck and the ecological features. The Kuril seal haul out and breed on certain rocky reef, with strong fidelity to the haul-out site (Wada *et al*., 1986). Because of these ecological features, the Kuril seal might have been easily affected from human activities in Hokkaido. Their abundance was drastically declined by hunting, habitat destruction, and unknown causes between 1940s and 1970s (Itoo and Shukunobe, 1986), although it has been increasing in recent years (Nakaoka, 1997). On the other hand, spotted seals breed on the pack ice (Shaughnessy and Fay, 1977), and have a high mobile ability (Lowry *et al*., 1998) without year-round fidelity to the haul-out site (Aoki, 1996). In Hokkaido, this feature of the spotted seal might contribute to reduce damages from habitat disturbance and to keep their various mtDNA haplotypes, in contrast to the Kuril seal. Spotted seal genetic diversity is, however, comparable to that found among the eastern Pacific harbor seal that have not experienced a significant reduction in numbers, where Lamont *et al*. (1996) found 30 variable sites and 47 haplotypes within the 320 bp of the control region of 86 seals, with pairwise divergence levels (ranged from 1 to 16) similar to those we found among spotted seals.

Considering spotted seal's high mobile ability (Lowry *et al*., 1998) and age specific distribution (Mizuno *et al*., 2001), the large amount of mtDNA variation and no genetic structure in this study suggest that their population range will not be limited to Hokkaido waters. These genetic features also might indicate possibly due to lack of a severe population bottleneck at some time in the past, or/and due to their "random migration" among some breeding concentrations.

Further genetic intra-specific study will clarify the relationship among the breeding concentrations adjacent Hokkaido waters; the Mamiya Strait, the southern Sakhalin, Peter the Great Bay, and the Liaodong Gulf. Combination with the behavioral approach such as satellite-linked tagging (in the Bering and Chukchi Seas: Frost *et al*., 1993; Lowry *et al*., 1998), we will have non-biased information of spotted seal population structure and the range, which contribute to define the management unit for wildlife conservation. It will work for risk assessment against the human activities such as incidental catches in fishing net and damage control kills

on seal survival.

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