Mammal Study 33: 125–129 (2008)

Short communication

Mitochondrial cytochrome *b* gene sequence diversity among Steller's sea lion rookeries in the Kuril Islands and the Sea of Okhotsk

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At various stages during the Pleistocene, the Northern Hemisphere was extensively covered with ice (e.g., Cox and Moore 2005). Glaciers developed and covered Canada, parts of the United States, and northern Europe and Asia (e.g., Williams et al. 1993). This climatic change intensely affected distribution patterns of flora and fauna, forcing them into glacial refugia (e.g., Holder et al. 1999; Abbot et al. 2000; Fedorov and Stenseth 2002; Fleming and Cook 2002), including the Kuril Islands and Sea of Okhotsk in Asia (Bezverkhniy et al. 2002).

Steller's sea lion *Eumetopias jubatus* (Schreber, 1776) is a highly mobile animal with rookeries. This species is distributed along rooky coasts from Northern California, through the Gulf of Alaska and along the Aleutian Islands into Asia's Kamchatka Peninsula, Kuril Islands, and the Sea of Okhotsk (e.g., Abe et al. 2005; Loughlin et al. 1992; Wilson and Reeder 2005). The drastic climatic change of the glacial periods restricted the distribution of Steller's sea lion into glacial refugia in which populations of this species diverged genetically (Harlin-Cognato et al. 2006). Now, there are three major geographical lineages of Steller's sea lion: 'eastern stock' (California to the southeastern Gulf of Alaska), 'western stock' (Prince William Sound to Commander Islands), and derived from western stock, 'Asian stock' (Kamchatka Peninsula, Kuril Islands, and Sea of Okhotsk) (Harlin-Cognato et al. 2006; Hoffman et al. 2006). Although the worldwide phylogeography of this species is resolved (Bickham et al. 1996; Baker et al. 2005; Harlin-Cognato et al. 2006; Hoffman et al. 2006), the phylogeography of local populations is still unclear, especially the Asian populations. During the glacial periods, the intensive population reduction would have occurred in each local population, forming the glacial refugia. Since the Steller's sea lion exhibits a tendency to remain in the native locality (philopatry) and females return to within 500 km of their natal rookeries to reproduce throughout their lifetimes (Raum-Suryan and Pitcher 2002), each natal rookery should have a unique maternal lineage. In fact, significant genetic divergences among different natal rookeries are recognized in sequences of the mtDNA control region (Baker et al. 2005).

To identify the genetic variability among rookeries in the Kuril Islands and Iony Island (Fig. 1), we analyzed complete cytochrome *b* gene sequences. During glacial periods, the shoreline of the Sea of Okhotsk was different from the present form (Bezverkhniy et al. 2002). Sakhaline Island was connected with the Siberian mainland with glaciers covering the northern part of the island (Grosswald and Hughes 2002). There was sea ice from Hokkaido to the Kuril Islands (Bezverkhniy et al. 2002). These drastic environmental changes constricted and isolated the rookeries of the Steller's sea lions (Harlin-Cognato et al. 2006). Western and Asian stocks have dramatically declined over the past three decades. Of these stocks, the rookeries of the Kuril Islands appear to

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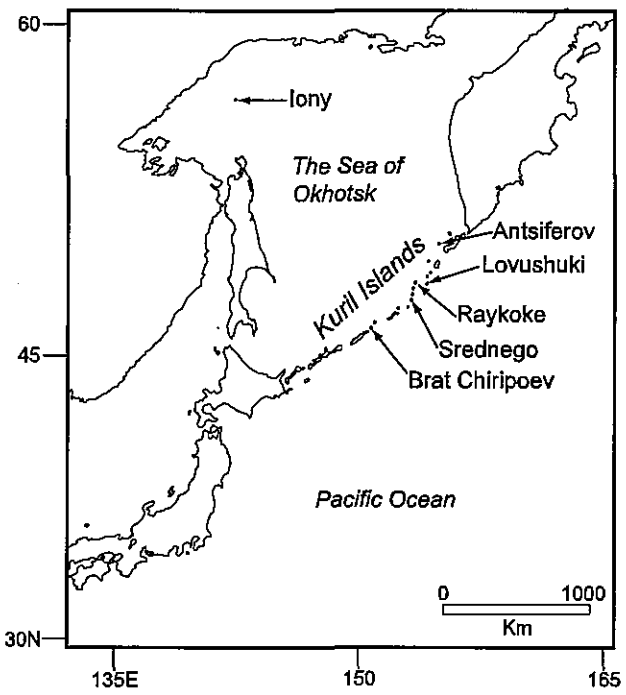

Fig. 1. Sampling localities of the Steller's sea lion (*Eumatopias jubatus*) in the Kuril Island and the Sea of Okhotsk.

be the most distinct, possessing a high number of unique haplotypes in the mtDNA control region (Bickham et al. 1998). This makes the Kuril Island stocks suitable for demonstrating the genetic variability among rookeries. We also briefly discuss the evolutionary history of Steller's sea lion in the Kuril Islands and Iony Island.

Materials and methods

Specimens

We examined a total of 104 Steller's sea lion specimens from 6 rookeries: Antsiferov Island, Lovushki Island, Raykoke Island, Srednego Island, and Brat Chiripoev Island of the Kuril Islands and Iony Island of the Sea of Okhotsk (Appendix 1 and Fig. 1). From 29 June to 6 July 2001, we collected skin samples by punching hind flippers as described in Bickham et al. (1996). Samples were preserved in 70% ethanol.

DNA extraction, amplification, and sequencing

We followed the modified phenol-chloroform method of Sasaki et al. (1995) to extract genomic DNA. This DNA was suspended in a Tris-EDTA (TE) buffer. The mtDNA fragments, including complete cytochrome *b* gene sequence, were amplified using polymerase chain reaction (PCR) with newly designed primers: SSL-Cytb F3 (5'-GTCATCATTATTCCCACATGG-3') and SSL-Cytb R3 (5'-CTTCCTTGAGTCTTAGGGAG-3'). The 50 μl reaction mixture contained 40 ng of genomic DNA, 25 μM of each primer, 0.2 mM dNTPs, 1 × PCR buffer, 50 mM KCl, 0.025 μM $MgCl_2$, and 1.5 units of *Taq* DNA polymerase (Ampli *Taq* Gold, Applied Biosystems). Amplification was carried out with a PE9700 thermocycler (Perkin-Elmer). Cycling conditions consisted of an initial denature for 10 min at 95°C followed by 30 cycles: 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The extension reaction was completed by incubation at 72°C for 10 min. Products were purified with Qiaquick PCR purification Kit (QIAGEN) and directly sequenced by an automated DNA sequencer (PRISM 3100 Genetic Analyzer, Applied Biosystems). We used four newly designed internal primers (SSL-Cytb F2: 5'-CTAACCAAGACTAATGACATG-3', SSL-Cytb In1: 5'-CCCTAACACGATTCTTCGCC-3', SSL-Cytb R2: 5'-GGGTCATGGTGTTCTCCATTT-3', and SSL-Cytb RIn3: 5'-GTCTGAGTTGGAGGAG-3') and Big Dye Terminator version 3.1 (Applied Biosystems) in sequencing.

Sequence and phylogeographic analyses

Sequence alignment was carried out using the software program CLUSTAL W (Thompson et al. 1994). To reconstruct phylogenetic relationships among haplotypes, we used a parsimonious network. Haplotype diversity and nucleotide diversity (π, the average number of nucleotide differences per site between two sequences) (Nei 1987) within populations were calculated with DnaSP 3.53 (Rozas and Rozas 1999).

Results and discussion

Complete mtDNA cytochrome *b* gene sequences were successfully determined from 104 Steller's sea lions from the Kuril Islands and the Sea of Okhotsk, resulted in seven unique haplotypes (1, 3, 4, 9, 27, 30, and 31). Of these, haplotypes 1, 3, 4, 9, and 27 are reported by Harlin-Cognato et al. (2006). Haplotypes 30 and 31 were new. Accession numbers of all sequences are in Appendix 1. Sequence alignment revealed seven variable sites. Proportions of the haplotypes in each rookery are shown in Table 1. Steller's sea lions carring haplotype 1 accounted for 59.6% of all individuals. Two other haplotypes were also common: haplotypes 4 (22.1%) and 27 (14.4%). These three haplotypes, 1, 4, and 27, were

Table 1. Number of Steller's sea lions (*Eumetopias jubatus*) in each of six rookeries in the Kuril Island and the Sea of Okhortsk carrying each haplotype

Island	Haplotype							Total
	1	3	4	9	27	30	31	
Antsiferov	9 (52.9)	1 (5.9)	5 (29.4)	–	1 (5.9)	–	1 (5.9)	17
Lovushuki	9 (62.3)	–	1 (7.1)	–	4 (28.6)	–	–	14
Raykoke	11 (61.1)	–	4 (22.2)	–	2 (11.1)	1 (5.6)	–	18
Srednego	10 (55.6)	–	3 (16.7)	1 (5.6)	4 (22.2)	–	–	18
Brat Chiripoev	14 (73.7)	–	3 (15.8)	–	2 (10.5)	–	–	19
Iony	9 (50.0)	–	7 (38.9)	–	2 (11.1)	–	–	18
Toal	62 (59.6)	1 (1.0)	23 (22.1)	1 (1.0)	15 (14.4)	1 (1.0)	1 (1.0)	104

Proportions (%) of haplotypes in each island are shown in parenthesis. Hyphens indicate haplotype was not observed.

Table 2. Intrapopulation variability of Steller's sea lions (*Eumetopias jubatus*) in each of six rookeries in the Kuril Island and the Sea of Okhortsk

Island	Nucleotide diversity	Haplotype diversity	Average number of nucleotide differences	Number of segregating sites
Antsiferov	0.00181 (0.00030)	0.662 (0.094)	2.059	6
Lovushuki	0.00176 (0.00035)	0.538 (0.115)	2.011	5
Raykoke	0.00165 (0.00029)	0.595 (0.109)	1.876	5
Srednego	0.00188 (0.00030)	0.647 (0.091)	2.144	6
Brat Chiripoev	0.00131 (0.00037)	0.444 (0.124)	2.237	6
Iony	0.00174 (0.00022)	0.621 (0.067)	1.980	5
Total	0.00166 (0.00012)	0.580 (0.042)	1.890	7

Standard deviations are in parenthesis.

found in all six rookeries. Haplotypes 3, 9, 30, and 31, however, were rare. Each minor haplotype was found in only one individual. The proportion of each was 1% (Table 1). Haplotypes 3 and 31 were found in only Antsiferov Island. Haplotypes 9 and 30 appeared in Srednego Island and Raykoke Island, respectively. Based on mtDNA control region sequences, Bickham et al. (1998) reported that Antsiferov population was highly distinct from those of Lovushuki, Raykoke, and Srednego Islands. Haplotypes 3 and 31 recognized only in Antsiferov Island may be one of evidences to support their findings. Nucleotide diversity, haplotype diversity, average number of nucleotide differences, and number of segregating sites within populations did not vary with island (Table 2). This means that each rookery population may have almost same genetic variability.

A parsimonious network of the seven haplotypes didn't show clear starburst relationships (Fig. 2). Therefore, the unique ancestral haplotype in this area was not detected. Haplotype 30, possibly endemic to Raykoke Island, was removed from haplotypes 3 or 27 by one nucleotide substitution. Haplotype 31, which was only found in the Antsiferov Island rookery, seems to have been produced from haplotype 27 by one nucleotide substitution (Fig. 2). These Asian specific haplotypes found newly in the present study would be formed in this area.

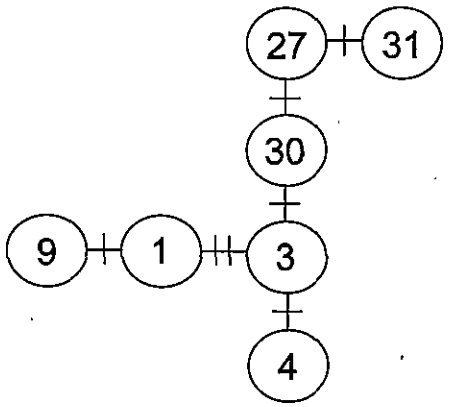

Fig. 2. Parsimonious network of seven haplotypes from 104 Steller's sea lions (*Eumatopias jubatus*) in rookeries in the Kuril Islands and the Sea of Okhotsk. Short bars on branches connecting haplotypes indicate one nucleotide substitution.

During the glacial periods, populations of Steller's sea lions could have been reduced in this area. In the last glacial period, nearly all the area of the Sea of Okhotsk

was covered by sea ice (Bezverkhniy et al. 2002). The Iony population would have disappeared. In glacial refugia in Kuril Islands, major haplotypes 1, 4, and 27 may have been kept. After glaciation, these haplotypes would have expanded in the Kuril Islands and Iony Island. Unfortunately, we could not examine sequence data from eastern and western stocks reported by Harlin-Cognato et al. (2006). They did not show exact geographical information of each haplotype. Therefore, we do not know whether the haplotype divergences found in our study were regional to the populations in the Kuril Islands and Iony Island. Also, Steller's sea lions generally conform to the metapopulation concept as described by Hanski and Simberloff (1997). They have breeding populations local to each rookery and movements among rookeries can affect local dynamics (Raum-Suryan and Pitcher 2002). In addition to this normally restricted gene flow, each breeding population must have been extremely reduced during the glacial periods. To consolidate this hypothesis, further study should examine the cytochrome *b* sequences from all populations in this area and eastern and western stocks.

Acknowledgements: We are grateful to Dr. A. Wada for collecting samples and Ms. Y. Y. Ueta for laboratory work. We thank Dr. C. L. Bridgman for her critical reading of the manuscript.

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Received 10 November 2007. Accepted 8 March 2008.

Appendix 1.

Identitiy numbers for each tissue sample from Steller's sea lions (*Eumetopias jubatus*) from six rookeries in the Kuril Islands and the Sea of Okhortsk; the numbers correspond to those of our ecological research project.

Locality	Haplotype	Identity number	Accession number
Antsiferov	1	32, 37, 40, 41, 102, 110, 199, 375, 387	DQ144995
	3	379	DQ144997
	4	25. 33. 100, 203, 204	DQ144998
	27	219	DQ145021
	31	105	AB362485
Lovushuki	1	146, 149, 161, 174, 183, 468, 470, 473, 482	DQ144995
	4	165	DQ144998
	27	170, 178, 456, 461	DQ145021
Raykoke	1	118, 119, 124, 131, 489, 495, 508, 524, 528, 530, 538	DQ144995
	4	120, 133, 136, 527	DQ144998
	27	111, 540	DQ145021
	30	14	AB362484
Srednego	1	45, 48, 62, 399, 400, 408, 411, 419, 432, 433	DQ144995
	4	57, 61, 415	DQ144998
	9	404	DQ145003
	27	407, 426, 427, 441	DQ145021
Brat Chiripoev	1	19, 21, 24, 70, 75, 77, 83, 86, 291, 296, 300, 355, 362, 365	DQ144995
	4	67, 91, 98	DQ144998
	27	89, 361	DQ145021
Iony	1	7, 244, 245, 250, 261, 267, 309, 320, 325	DQ144995
	4	247, 253, 254, 313, 329, 333, 350	DQ144998
	27	240, 259	DQ145021