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Genetic Structure of Steller Sea Lion (*Eumetopias jubatus*) Rookeries in the Sea of Okhotsk

Satoshi Koyama[1,2], Shoko Fujita[2], Tadao Hirota[3], Toshiyuki Satoh[2,*], Yoshiaki Obara[2], Hiroshi Hoshino[4], Akihiko Wada[5], Vladimir N Burkanov[6,7], and Kazuo Wada[8]

[1]*United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan*

[2]*Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai, Fuchu, Tokyo 183-8509, Japan*

[3]*Department of Biology, Faculty of Science, Yamagata University, Kojirakawa-machi 1-4-2, Yamagata 990-8560, Japan*

[4]*Creative Research Initiative "Sousei", Hokkaido University, Kita 21 Nishi 10, Sapporo, Hokkaido 001-0021, Japan*

[5]*Hokkaido-Wakkanai Fisheries Experimental Station,Wakkanai, Hokkaido 097-0001 Japan*

[6]*Kamchatka Branch of the Pacific Institute of Geography Far East Branch, Russian Academy of Sciences, 6 Partizanskaya St., Petropavlovsk-Kamchatsky 683000, Russia*

[7]*National Marine Fisheries Service, Alaska Fisheries Science Center, National Marine Mammal Laboratory, 7600 Sand Point Way NE, Seattle, WA 98115, USA*

[8]*Tounoji-Suginyama 9-18, Inuyama, Aichi, Japan*

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Satoshi Koyama, Shoko Fujita, Tadao Hirota, Toshiyuki Satoh, Yoshiaki Obara, Hiroshi Hoshino, Akihiko Wada, Vladimir N Burkanov, and Kazuo Wada (2008) Genetic structure of Steller sea lion (Eumetopias jubatus) rookeries in the Sea of Okhotsk. *Zoological Studies* **47**(6): 781-787. Genetic relationships among 4 Steller sea lion (*Eumetopias jubatus*) rookeries in the Kuril Is. (Brat Chirpoev, Lovushki, Raykoke, and Antsiferov Is.) and 1 at Iony I. were evaluated using an inter-simple sequence repeat (ISSR)-polymerase chain reaction assay. An ISSR primer yielded 15 amplification products, almost all of which were polymorphic. An analysis of molecular variance test revealed that 94.4% of the total genetic variation was attributable to differences among individuals within rookeries, and 5.6% was attributable to those among rookeries (Φ_{ST} = 0.056). Pairwise Φ_{ST} values were relatively high between rookeries of the Kuril Is. and that on Iony I., while no significant genetic differentiation was detected among rookeries of the Kuril Is. Since previous research using mitochondrial DNA markers revealed the presence of unique haplotype(s) on each island of the Kuril Is., our results suggest that males, rather than females, contribute to gene flow among the islands.
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Currently, many species are endangered worldwide, and their conservation is urgently required (Baillie et al. 2004). Information on the genetic diversity and structure of local populations is essential for the development of effective conservation policies.

The Steller sea lion (*Eumetopias jubatus*), an endangered species, is the largest animal in the family Otariidae and is widely distributed throughout the North Pacific Ocean rim, Hokkaido I., north of the Commander Is., east across the Aleutian I. chain to mainland Alaska, and south to southern California (Loughlin et al. 1987). It is reported that the global population decreased from an estimated number of 240,000 - 300,000 in the 1960s to 116,000 in 1989 (Loughlin et al. 1992).

*To whom correspondence and reprint requests should be addressed. Tel: 81-423-675629. Fax: 81-423-675628.
E-mail:tsatoh@cc.tuat.ac.jp

Various causes for this decrease have been claimed: elimination by humans, predation by killer whales and/or sharks, environmental pollution, accidental capture in fishing nets, parasites and disease, declines in prey quantity and quality, a decline in the breeding rate, etc. (Braham et al.1980, Merrick et al. 1987, Calkins and Goodwin 1988, Lowry et al. 1989, NMFS 1992, Merrick 1995, Wada 1997, Loughlin 1998, Takahashi 1999, Kruse et al 2001, Fritz and Brown 2005), although some other important factors, which have not yet been recognized, might also be involved. To uncover the gravest causes of the decline in sea lion populations, it is necessary to understand its basic biology and ecology, including its migration patterns and gene flow among local populations.

Bickham et al. (1996 1998) investigated the genetic relationship among sea lion populations by analyzing mitochondrial (mt)DNA. They suggested that sea lion populations can be divided into 2 major lineages (western and eastern), that females show a relatively high degree of philopatry, with animals returning to their natal rookeries to breed, and that the sea lions in the Kuril Is. have many endemic haplotypes. If male sea lions are also philopatric, genetic diversity would have diminished during the drastic decline in the population owing to stochastic loss of allelic diversity by genetic drift. If males are not philopatric, they will supply diversity from different populations. However, gene flow through male dispersal has not been adequately investigated in these populations.

In the present study, we analyzed the genetic relationship among 4 local populations in the Kuril Is. and 1 population on Iony I. using the inter-simple sequence repeat (ISSR) method to detect nuclear DNA polymorphisms. The ISSR technique is more reliable than random amplification of polymorphic DNA (RAPD) because of the higher annealing temperature. We compared the results to previously reported mtDNA analyses, and evaluated the contribution of males to gene flow among populations.

MATERIALS AND METHODS

Samples

During ecological research in 2001, we acquired skin tissue samples of pups from 4 rookeries in the Kuril Is. (Brat Chirpoev I., *n* = 13, Lovushki I., *n* = 17, Raykoke I., *n* = 17, and Antsiferov I., *n* = 15) and 1 rookery on Iony I. (*n* = 11) in the center of the Sea of Okhotsk (Fig. 1). Samples were collected after the sea lions had been tagged with a punch. They were stored in 100% ethanol at 4°C until DNA analysis.

DNA analysis

Each sample was minced in a 1.5 ml tube in 500 μl of 5% citric acid. After mixing the contents, the tube was centrifuged at 7000 rpm for 5 min, and the supernatant was decanted; then 600 μl of lysis buffer (50 mM Tris-HCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, and 1% sodium dodecylsulfate (SDS)) and 100 μl of proteinase solution (100 mM Tris-HCl, 12.5 mM EDTA, 150 mM NaCl, 1% SDS, and 1 μg/μl proteinase K) were added. The tube was incubated at 56°C for 30 min and at 37°C overnight. After 700 μl of phenol was added, the tube was inverted for 10 min, and centrifuged at 15,000 rpm for 10 min. The supernatant was transferred to a new tube, and was extracted with phenol/chloroform, followed by an additional extraction with chloroform. DNA was precipitated in an equal volume of isopropyl alcohol for 30 min at room temperature. The tube was centrifuged at 15,000 rpm for 15 min, the supernatant was decanted, and the precipitate was washed twice with 500 μl of 70% ethanol. The resultant DNA sample was desiccated and dissolved in 100 μl of Tris-EDTA solution (TE)

DNA was extracted using the standard proteinase K and phenolic methods (Maniatis et al., 1989). A polymerase chain reaction (PCR) was performed in a total volume of 20 μl with a single simple sequence repeat (SSR) primer (5'-$(CA)_7$GT-3'). Each reaction comprised 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 80 μM dNTP, 50 μM SSR primer, 20 ng template DNA, and 0.5 units of rTaq (TaKaRa Shuzo, Kyoto, Japan). Amplifications were carried out in an MP PCR thermal cycler (TaKaRa Shuzo) using the following protocol: an initial denaturing step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 5 min. Tubes were stored at 4°C until electrophoresis.

Data analysis

Electrophoresis was carried out on 2% (w/v) agarose TAE gels at 50 V for 1.2 h. DNA size markers (λ-Hind III digest) were loaded in the first

and last lanes. We loaded 5 μl of PCR products in the other lanes. Gels were stained using 0.5 μg/ml (w/v) ethidium bromide, and photographed by transillumination (NTM-10, Funakoshi, Tokyo, Japan) using Polaroid 667 film (Fig. 2). ISSR-PCR profiles were visually scored for the presence and absence of bands. To estimate variations due to differences among populations and among individuals within populations, an analysis of molecular variance (AMOVA) test (Excoffier et al. 1992) was performed. The significance of variance among populations was tested by a random permutation test against the null hypothesis that all individuals belonged to the same population. The AMOVA test conveys genetic differentiation among populations in terms of Φ_{ST} values that are analogous to the fixation index (F_{ST}) (Excoffier et al. 1992). We used the computer program ARLEQUIN (Schneider et al. 2000) for all AMOVA calculations.

To investigate the association between the geographical distance and genetic differentiation, the relationship of the logarithm of geographical distances between habitats and pairwise $\Phi_{ST}/(1 - \Phi_{ST})$ values among populations was analyzed by the Mantel test with exact permutations (Sokal and Rohlf 1995).

In addition, Bayesian statistical procedures have been proposed to investigate the population structure using ISSR-PCR profiles (Holsinger et al. 2002). This hierarchical approach investigates genetic data by incorporating the effect of uncertainty regarding F_{ST} and the inbreeding coefficient (F_{IS}) into the estimation of these parameters. The computer program HICKORY calculates θ^B, which represents F_{ST}, and *f* which

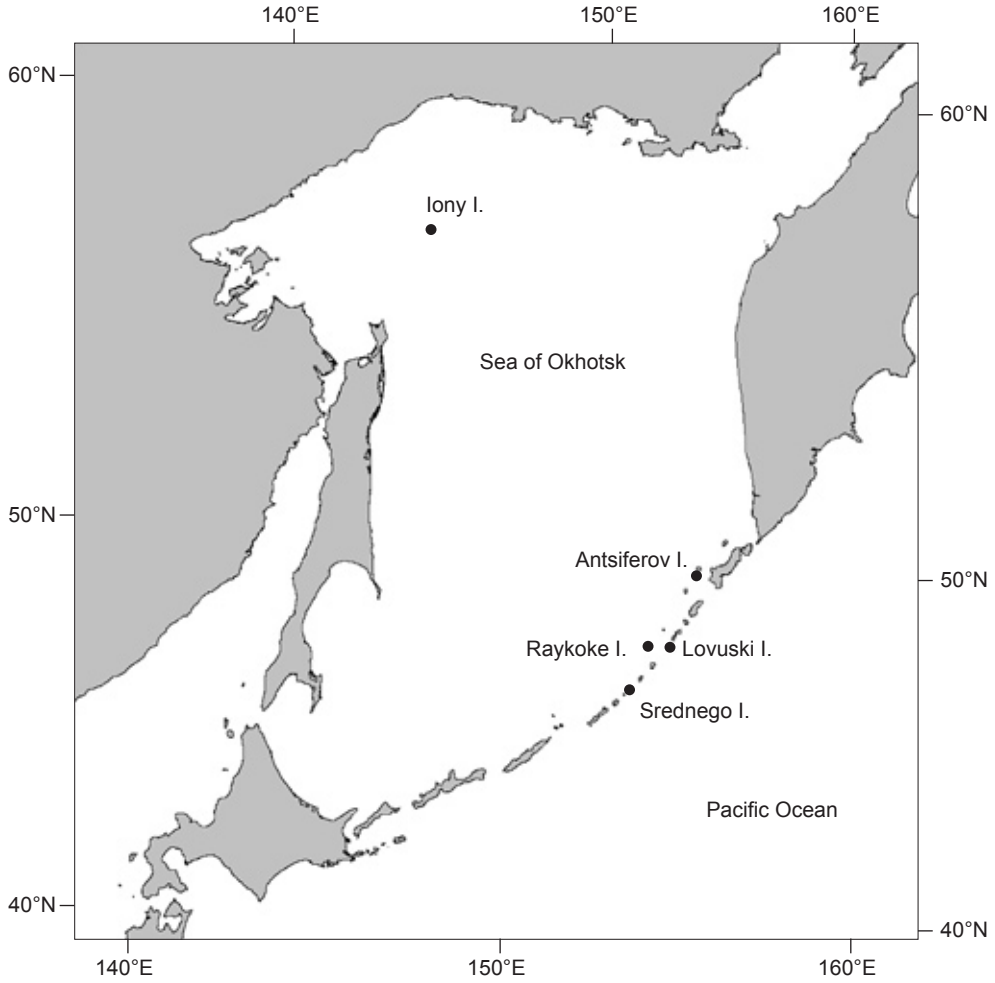

Fig.1. Sampling locations of Steller sea lions in the Sea of Okhotsk.

represents F_{IS}. The deviance information criterion (DIC) is a measure that considers both the efficiency of the model in fitting the data and the number of parameters required for this. By running different models (full, $f = 0$, $\theta^B = 0$, and *f*-free models) on the same dataset, the model with the lowest DIC was selected.

RESULTS

The ISSR-PCR indicated 15 distinguishable polymorphic bands from 73 individuals (Fig. 2). For all rookeries, 15 bands were observed, except the Iony I. population, for which only 12 bands were observed (Table 1).

The AMOVA test revealed that genetic variation was predominantly due to differences among individuals within populations (variance component of 94.4%; Table 2) and partially due to differences among populations (variance component of 5.6%). Although Φ_{ST} was relatively small, the random permutation test revealed significant genetic differentiation among populations ($p < 0.01$).

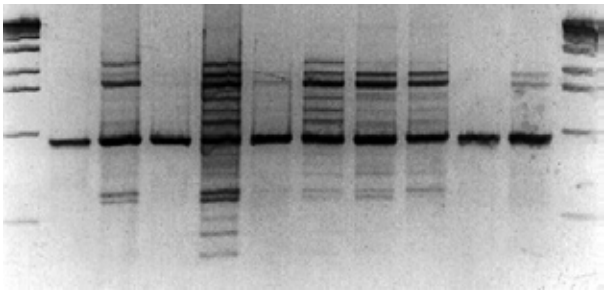

Fig. 2. Amplified fragment profile.

Pairwise Φ_{ST} values between populations ranged from - 0.03 to 0.17 (Table 3). The random permutation test revealed that Φ_{ST} was significant only between the Iony and Raykoke populations ($p < 0.05$). Pairwise $\Phi_{ST}/(1 - \Phi_{ST})$ values were marginally correlated with the logarithm of geographical distance between populations (one-tailed Mantel test, $p = 0.09$, Fig. 3). If we removed the data between the Raykoke and Lovuski populations (leftmost point in figure3), a distinct correlation was detected (one-tailed Mantel test, $p = 0.001$).

We calculated θ^B and *f* using 4 models (Table 4); the full model was only slightly preferred since the DIC with this model was lower than those from the alternative models, i.e., $\theta^B = 0$ or $f = 0$ (Spiegelhalter et al. 2002). No evidence of inbreeding within the populations was indicated since the DIC was almost identical with the full model and the $f = 0$ model. The models also indicated genetic differentiation among populations

Table 1. Frequency of each band

	n	Frequency of band														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Antsiferov I.	15	0.2	0.6	0.5	0.7	0.3	0.2	0.8	1	0.1	0.3	0.8	0.6	0.1	0.5	0.5
Lovuski I.	17	0.5	0.7	0.7	0.8	0.4	0.2	0.5	1	0.4	0.2	0.9	0.7	0.1	0.5	0.5
Raykoke I.	17	0.4	0.7	0.8	0.8	0.1	0.1	0.2	1	0.2	0.5	0.8	0.8	0.5	0.5	0.9
Brat Chirpoev I.	13	0.5	0.8	0.6	0.7	0.3	0.3	0.6	1	0.2	0.4	0.8	0.8	0.2	0.6	0.6
Iony I.	11	0.3	0.5	0.3	0.3	0	0.1	0.5	1	0	0	0.7	0.5	0.6	0.5	0.5

Table 2. Analysis of molecular variance

Source of variation	*d.f.*	Sum ofsquares	Variance ofcomponents	%	Φ_{ST}	*P**
Among populations	4	1.6	0.01	5.6	0.056	< 0.01
Among individuals within populations	68	14.0	0.21	94.4		
Total	72	15.6	0.22			

*After 10,000 random permutations

since the DIC of the full model was lower than that of the $\theta^B = 0$ model (i.e., $\theta^B > 0$).

DISCUSSION

In the current study, we investigated the genetic structure of local populations of the Steller sea lion. Pairwise Φ_{ST} values were relatively large between the populations on Iony I. and the Kuril Is., although none was significant, except that of the Raykoke population. In contrast, low genetic distances were observed among populations of the Kuril Is. Interestingly, the genetic distance between the Raykoke and Lovuski populations was relatively high, despite being the nearest rookeries. Bickham et al. (1998) also using an mtDNA analysis revealed that the genetic identity between the Raykoke and Srednego populations was higher than that between Raykoke and Lovuski, although Srednego I. is distant from Raykoke and Lovuski Is. (Fig. 3). Intraspecific differentiation among rookeries in the Kuril Is. and Iony I. might have been caused by the presence of a separate glacial refugium (Bickham et al 1998). Bickham et al. (1996) suggested that the mtDNA of the Steller sea lion had diverged within the past 3.84×10^5 yr. The ISSR analysis, however, was unable to reveal a date when these populations became genetically

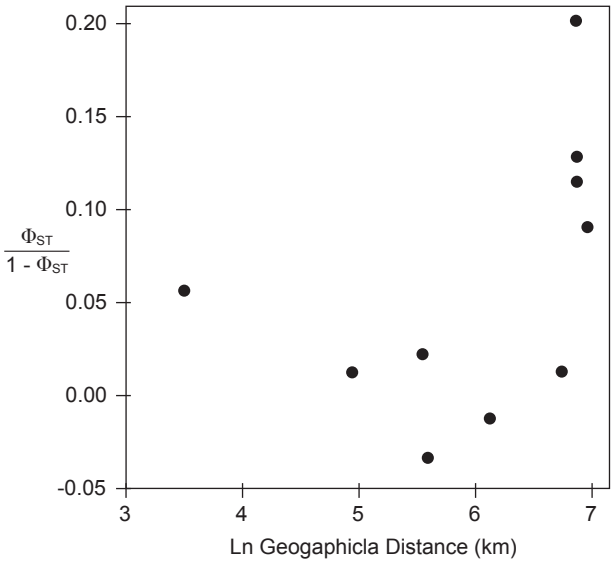

Fig. 3. Relationship between the pairwise $\Phi_{ST}/(1 - \Phi_{ST})$ values and the logarithm (ln) of geographical distances among populations.

Table 3. Pairwise population Φ_{ST} values

	Lovuski I.	Raykoke I.	Brat Chirpoev I.	Iony I.
Antsiferov I.	0.01	0.11	-0.01	0.01
Lovuski I.		0.03	-0.03	0.12
Raykoke I.			0.02	0.17*
Brat Chirpoev I.				0.09

*$p < 0.05$, after 10,000 random permutations. p values were corrected using the sequential Bonferroni method (Rice 1989).

Table 4. Estimation of F_{ST} by Bayesian statistical procedures. Means and 95% confidence ranges are shown for θ^B analogous to F_{ST}. f is analogous to F_{IS}. The deviance information criterion (DIC) is a model selection criterion. Smaller DIC values indicate a better model

	θ^B				
Mode	Mean	2.50%	97.50%	f	DIC
Full	0.0524	0.017	0.1017	0.7479	307
$f = 0$	0.036	0.0123	0.0697	-	308
$\theta^B = 0$	-	-	-	0.7488	328
f free	0.053	0.0196	0.0997	0.521	314

distinct. Further examination using sequence analysis is necessary to estimate the period when segregation occurred among these populations.

Bickham et al. (1998) investigated the genetic structure of Steller sea lion populations, using maternally inherited mtDNA. They found high genetic differentiation among local populations. On the other hand, the analysis using ISSR markers in the present study revealed no significant genetic differentiation among populations of the Kuril Is. The difference between our results and those of Bickham et al. (1998) is due to the nature of the markers used. ISSR markers deal with genetic structure caused by both sexes, whereas mtDNA analysis shows the structure responsible for the females' genetic contribution. Consequently, the observed genetic structure is explained by male dispersal and female philopatry.

Since several factors, including inbreeding avoidance, facilitate sex-biased dispersal (Hirota 2004 2005), further studies are required to determine the factors that are crucial to the evolution of male-biased dispersal observed in Steller sea lions. Nevertheless, this dispersal pattern would reduce the probability of inbreeding, which increases the risk of population extinction (Saccheri et al. 1998). This is consistent with the result that DIC values with the full model and the $f = 0$ model were almost the same, which indicates no evidence of inbreeding.

Although the AMOVA test detected significant genetic differentiation among populations, the high genetic diversity that was observed is mainly attributable to individual variations within populations. It follows that abundant genetic diversity remains in these populations despite the significant population decline in the past 3 decades. Although Lidicker et al. (1981) showed a low level of genetic variability in an allozyme of the Steller sea lion in a limited portion of the eastern stock, Bickham et al. (1996 1998b) confirmed the occurrence of a high level of haplotypic diversity in the mtDNA of Steller sea lion populations. In the current study, the high genetic diversity observed using ISSR markers could support Bickham et al.'s (1996 1998b) conclusion that the recovery of Steller sea lions remains possible if the population decline can be arrested or reversed.

We have provided evidence for male dispersal and the structure of local populations of Steller sea lions. We hope that the information presented in this study contributes to an appropriate conservation plan for this endangered animal.

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