A simple method for identifying two species, *Phoca largha* and *P. vitulina*, based on mitochondrial DNA suggests their possible hybridization around Hokkaido, Japan

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Abstract — We have developed a method for identifying maternal lineage of two species, the spotted seal *Phoca largha* and the harbor seal *P. vitulina*, using restriction fragment length polymorphism of PCR products (PCR-RFLP) in mitochondrial DNA. A partial sequence of the cytochrome b gene (Cytb) was amplified and digested with *Alu*I in both species. In agarose gel electrophoresis digested PCR product from *P. largha* showed two bands, while one from *P. vitulina* showed a single band. Sequence analysis confirmed the precise detection of mitochondrial DNA lineage using PCR-RFLP methods developed in the present study. Some exceptions, where morphological character, color and pattern of fur, and mtDNA type were inconsistent, were also observed, suggesting possible hybridization of these species, as well as the possibility of misidentification by fur color and pattern.

Key words: PCR-RFLP, seals, species identification, natural hybridization

Introduction

The harbor seal, *Phoca vitulina* Linnaeus, 1758, and the spotted seal *Phoca largha* Pallas, 1811, occur in the North Pacific including the coast of Hokkaido. They are the most closely related species in the subfamily *Phocinae* (Burns 2002, Higdon et al. 2007, Dasmahapatra et al. 2009). Both species are top predators in marine ecosystems that should be carefully conserved as well as nuisances for coastal fisheries that should be controlled. To reduce the seal-fishery conflicts, species identification is essential in censuses aimed at population control and conservation. The behaviors of these two species are different with respect to breeding area, mating system, and seasonal movement (Jefferson et al. 1994, Burns 2002, Kobayashi 2009). The breeding habitats of *P. vitulina* are mainly along coastal or insular, ice-free rocky shores, except in some parts of Alaska and Greenland where they utilize icebergs. The species exhibit inter-male competition and polygyny in herds during the breeding season. Around Hokkaido, they usually utilize haul-out rockeries in areas along the coast that are rarely visited by humans throughout the year. *Phoca largha* generally use seasonal sea ice, mostly far from shore, for breeding and nursing pups, although populations of Peter the Great Bay and Liaodong Bay use haul-out

sites along the shore for whelping, nursing, and mating as well as molting (Boveng et al. 2009). They are considered to be annually monogamous. Most of the populations in the northwest Pacific Ocean migrate northward from around Hokkaido during the ice-free season and back toward the south with the ice front (Burns 2002, Kobayashi 2009). The two species have thus been thought to exhibit reproductive isolation from each other.

Several morphological characteristics, ratios of some measurements such as jugal length / condylobasal length, and non-metrical characteristics such as the shape of the skull, are said to be useful for distinguishing between these two species (Burns 2002). Nakagawa et al. (2009), however, suggested that these keys themselves were not decisive for species identification, and alternatively proposed a PCA analysis based on 21 metric cranial characteristics, which could discriminate the two species. These characteristics could not be measured in field observations or through biopsy without dissection. External morphology such fur coloration is thus so far the most reliable key in identifying these two species in field research. Each *P. vitulina* individual has a unique pattern of fine spots, often with small pale rings on the slightly darker dorsum. Their colors vary greatly, from light to dark, and most seals around Hokkaido are of the dark type. *Phoca largha*, on the other hand, is more uniform in color and pat-

terns, which is silver-gray with dark oval spots of fairly uniform size (1–2 cm) (Kobayashi 2009). The two species were distinguishable from each other in this manner around Hokkaido, but a researcher in Alaska reported that young seals without lanugo are sometimes difficult to distinguish through external morphology and that, even in adults, light-colored *P. vitulina* may be misidentified as *P. largha* (Burns 2002). Molecular markers and methods are therefore essential for decisive species identification.

The aim of the current study is to establish methods for distinguishing *P. vitulina* and *P. largha* using DNA. Given that many seals should be tested in the future, such methods need to be simpler and more cost-effective. We noticed a clear distinction between these species in their mtDNA lineage as shown in Nakagawa et al. (2010) and developed PCR-RFLP methods based on the species' sequence data. We expect that these methods will contribute to an easier way to identify species, *P. vitulina* and *P. largha*, from samples that have ambiguous morphological characteristics.

Materials and methods

Sampling protocols were approved by the Ethics Committee of Tokyo University of Agriculture. Samples were obtained from 5 localities around Hokkaido in 2004–2010 (Table 1, Fig. 1). Forty six samples of *P. largha*, 22 alive and 24 dead, and 24 of *P. vitulina*, 9 alive and 15 dead, were used for DNA analyses. Sample collection from live animals was carried out under the Wildlife Protection and Hunting Management Law; permission numbers obtained from the Ministry of the Environment are: 320 and 075 (2008), 128 (2009), and 422 (2010) for *P. vitulina*, and 70, 76 and 35–1 (2008), 353 and 501 (2009), and 422 (2010) for *P. largha*. Dead seals were from occasional bycatch, stranding, and extermination

Fig. 1. Sampling locations of the *Phoca largha* and *P. vitulina* examined in this study.

for population control in fishing areas. Species identification was based on external morphology, color and spot pattern; *Phoca vitulina* is in a dark color and has patterns of fine spots that vary with the individual, while *P. largha* has silver-gray fur with dark oval spots of fairly uniform size.

Skin samples collected from live seals and muscles from dead ones were preserved in 99% ethanol. Genomic DNA was extracted using standard phenol-chloroform methods from each ethanol-fixed sample. Blood samples were also used from some live seals. Genomic DNA from blood samples was extracted using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany), following the protocol supplied by the company.

To search for the PCR-RFLP marker that would make it possible to distinguish between *P. largha* and *P. vitulina*, we aligned reported sequence data of the mitochondrial Cytb gene in both species. Sequence data in *P. largha* (accession Nos. AB510423–510445, LC160126–129) and in *P. vitulina* (Nos. AB510408–510422) were retrieved from the DDBJ/GenBank and aligned. In these sequences we found that nucleotides at 16 sites were different between species but identical within each species. At one of these sites, site 573d, thymine (T) in all sequences of *P. largha* but cytosine (C) in all sequences of *P. vitulina* was found to be the candidate restriction site for PCR-RFLP with *Alu*I, which was expected to cut the sequence of AGCT in *P. largha* but to preserve AGCC in *P. vitulina*.

A pair of primers (PhocaCBf, 5′-ACC GTC ATA GCT ACA GCA TTC ATG G-3′; PhocaCBr, 5′-GTC TGA GTC GGA TAT GAT TCC GGA GG-3′) was designated for PCR amplification of the partial Cytb, 283 bp including the candidate *Alu*I site, sites 570–573d, and no other *Alu*I site in each sequence. PCR amplification was carried out in a 10 μl reaction, containing 1μl of 10x Gold buffer, 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.26 μM of each primer, 0.5 U Ampli *Taq* Gold (ThermoFisher Scientific, Waltham, MA, USA), and 2 μl of genomic DNA. The thermal-cycling profile included precycling denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 20 s, and extension at 72°C for 25 s. The PCR products were examined by electrophoresis on a 3% agarose gel. Then 5 μl of each product was digested in a 10 μl reaction with 1 μl of 10x Buffer, 0.5 μl of *Alu*I, and distilled water. Products were incubated in 37°C for 3 hours for digestion, and then checked by electrophoresis as above.

Some samples showed inconsistency between coloration and RFLP patterns in species identification. For these samples, partial sequences of Cytb were checked to confirm the genotyping. The total Cytb gene was amplified by PCR in a 20 μl reaction, using the primer pair: forward 5′-AGG CGT CGA AGC TTG ACA TGA AAA GCC ATC GTTG-3′ and reverse 5′-CGA ATT CCA TTT TTG GTT TAC AAG AC-3′ (Árnason et al. 1995). The thermal-cycling profile included
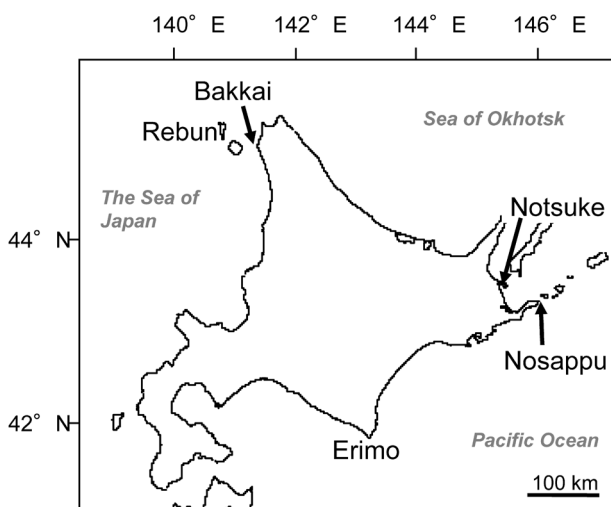
Table 1. Seal samples examined in the present study. Individuals over 130 cm in total length were adults, and others (<130 cm) were juveniles and subadults. Sample showing discrepancy between external morphology and mtDNA type is in bold letters.

sample name	locality	total length	year	type of coloration	type of mtDNA
EG0601	**Erimo**	**97.0**	**2006**	***P. largha***	***P. vitulina***
EZ0427	Erimo	109.0	2004	*P. vitulina*	*P. vitulina*
EZ0429	Erimo	128.0	2004	*P. vitulina*	*P. vitulina*
EZ05101	Erimo	105.0	2005	*P. vitulina*	*P. vitulina*
EZ05103	Erimo	120.0	2005	*P. vitulina*	*P. vitulina*
EZ05104	Erimo	108.0	2005	*P. vitulina*	*P. vitulina*
EZ0201	Erimo	no data	2002	*P. vitulina*	*P. vitulina*
EZ0417	Erimo	133.0	2004	*P. vitulina*	*P. vitulina*
EZ0422	Erimo	135.0	2004	*P. vitulina*	*P. vitulina*
EZ0423	Erimo	140.0	2004	*P. vitulina*	*P. vitulina*
EZ0602	Erimo	97.0	2006	*P. vitulina*	*P. vitulina*
BG0801	Bakkai	95.0	2008	*P. largha*	*P. largha*
BG0802	Bakkai	99.0	2008	*P. largha*	*P. largha*
BG0901	Bakkai	102.0	2009	*P. largha*	*P. largha*
BG0902	Bakkai	101.0	2009	*P. largha*	*P. largha*
BG0905	Bakkai	91.1	2009	*P. largha*	*P. largha*
BG0805	Bakkai	117.0	2008	*P. largha*	*P. largha*
BG1001	Bakkai	108.0	2010	*P. largha*	*P. largha*
BG0906	Bakkai	95.0	2009	*P. largha*	*P. largha*
NoG0803	Notsuke	134.5	2008	*P. largha*	*P. largha*
NoG0804	Notsuke	140.0	2008	*P. largha*	*P. largha*
NoG0805	Notsuke	128.1	2008	*P. largha*	*P. largha*
NoG0806	Notsuke	148.0	2008	*P. largha*	*P. largha*
NoG0809	Notsuke	126.3	2008	*P. largha*	*P. largha*
NoG0810	Notsuke	143.3	2008	*P. largha*	*P. largha*
NoG0811	Notsuke	126.7	2008	*P. largha*	*P. largha*
NoG0812	Notsuke	164.8	2008	*P. largha*	*P. largha*
NoG0813	Notsuke	119.6	2008	*P. largha*	*P. largha*
NoG0814	Notsuke	127.6	2008	*P. largha*	*P. largha*
NoZ0801	**Notsuke**	**132.5**	**2008**	***P. vitulina***	***P. largha***
NoZ0802	**Notsuke**	**132.4**	**2008**	***P. vitulina***	***P. largha***
NoZ0803	Notsuke	139.6	2008	*P. vitulina*	*P. vitulina*
NoZ0902	**Notsuke**	**132.1**	**2009**	***P. vitulina***	***P. largha***
NG0401	Nosappu	139.2	2004	*P. largha*	*P. largha*
NG0402	Nosappu	129.5	2004	*P. largha*	*P. largha*
NG0403	Nosappu	137.0	2004	*P. largha*	*P. largha*
NG0404	Nosappu	125.0	2004	*P. largha*	*P. largha*
NG0405	Nosappu	116.0	2004	*P. largha*	*P. largha*
NG0408	Nosappu	146.2	2004	*P. largha*	*P. largha*
NG0409	Nosappu	132.5	2004	*P. largha*	*P. largha*
NG0410	Nosappu	105.0	2004	*P. largha*	*P. largha*
NG0501	Nosappu	151.0	2005	*P. largha*	*P. largha*
NG0502	Nosappu	150.0	2005	*P. largha*	*P. largha*
NZ0477	Nosappu	162.2	2004	*P. vitulina*	*P. vitulina*
NZ0478	Nosappu	120.5	2004	*P. vitulina*	*P. vitulina*
NZ0479	Nosappu	162.5	2004	*P. vitulina*	*P. vitulina*
NZ0480	Nosappu	134.3	2004	*P. vitulina*	*P. vitulina*
NZ0481	Nosappu	143.5	2004	*P. vitulina*	*P. vitulina*
NZ0482	Nosappu	143.5	2004	*P. vitulina*	*P. vitulina*
NZ0483	**Nosappu**	**168.0**	**2004**	***P. vitulina***	***P. largha***
NZ0484	Nosappu	128.7	2004	*P. vitulina*	*P. vitulina*
NZ0486	Nosappu	116.6	2004	*P. vitulina*	*P. vitulina*
NZ0801	**Nosappu**	**152.6**	**2008**	***P. vitulina***	***P. largha***
ReG0802	Rebun	99.0	2008	*P. largha*	*P. largha*
ReG0803	Rebun	86.0	2008	*P. largha*	*P. largha*
ReG0804	Rebun	100.5	2008	*P. largha*	*P. largha*

Table 1. Continued.

sample name	locality	total length	year	type of coloration	type of mtDNA
ReG0805	Rebun	97.5	2008	*P. largha*	*P. largha*
ReG0904	Rebun	103.3	2009	*P. largha*	*P. largha*
ReG0905	Rebun	91.1	2009	*P. largha*	*P. largha*
ReG0906	Rebun	106.6	2009	*P. largha*	*P. largha*
ReG0907	Rebun	97.6	2009	*P. largha*	*P. largha*
ReG0908	Rebun	94.3	2009	*P. largha*	*P. largha*
ReG0910	Rebun	98.4	2009	*P. largha*	*P. largha*
ReG0911	Rebun	119.2	2009	*P. largha*	*P. largha*
ReG0912	Rebun	100.1	2009	*P. largha*	*P. largha*
ReG0913	Rebun	83.5	2010	*P. largha*	*P. largha*
ReG1002	Rebun	94.0	2010	*P. largha*	*P. largha*
ReG1005	Rebun	102.3	2010	*P. largha*	*P. largha*
ReG1006	Rebun	119.0	2010	*P. largha*	*P. largha*
ReG1007	Rebun	91.8	2010	*P. largha*	*P. largha*

precycling denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 20 s, and extension at 72°C for 90 s. The PCR products were purified with magnetic beads (AMPure Agencourt; Beckman-Coulter, Brea, CA, USA) and cycle-sequenced using the forward primer and the BigDye Terminator v. 3.1 Cycle Sequencing Kit (ThermoFisher Scientific), and loaded onto an automated sequencer, ABI PRISM 3130 (ThermoFisher Scientific). The obtained sequences of the first half of Cytb were aligned and edited to 678 bp using Bioedit software (Hall 1999), and the molecular phylogeny of these sequences was tested in a neighbor-joining tree (Saitou and Nei 1987) in software MEGA6 (Tamura et al. 2013), adding the sequences derived from DDBJ. The Kimura-2-parameter model (Kimura 1980) was employed to estimate the genetic distance between sequences in the construction of the tree, and the stability of each node was tested by 1000 bootstrap replication.

Fig. 2. PCR-RFLP patterns for partial Cytb gene in mitochondrial DNA. The rightmost is a size marker (50 bp ladder). The size of each band was calculated based on sequences derived from database.

Results and Discussion

PCR-RFLP exhibited double bands, ca. 200 and ca. 80 bp, in *P. largha* and a single band of less than 300 bp in *P. vitulina*, some of which are shown in Fig. 2. This was attributable to the nucleotide variation found in sequences from the database, where the restriction site for *Alu*I, AGCT, was at the 570–573d position in Cytb in *P. largha* but not in *P. vitulina*. According to the sequence data, the expected fragment size was 208 and 78 bp for *P. largha* and 286 bp for *P. vitulina*. This PCR-RFLP, producing bands of the exact size in an electrophoresis pattern, thus successfully distinguished *P. largha* from *P. vitulina*. When all samples were examined, in 64 individuals the mtDNA type that was detected using PCR-RFLP was consistent with the coloration. Six samples, however, exhibited inconsistency between coloration and genetic species identification (bold letters in Table 1): one indi-
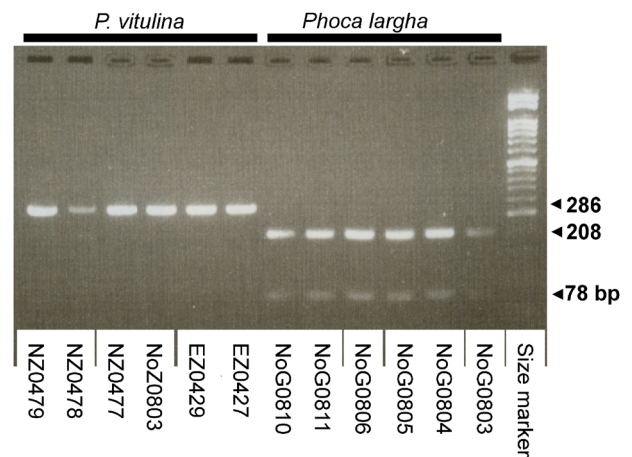
vidual, a pup, showed the coloration of *P. largha* and the mtDNA type of *P. vitulina*, while 5 adults showed the coloration of *P. vitulina* and the mtDNA of *P. largha*. These exceptions were examined by sequencing 678 bp, the first half of the Cytb gene. We identified 4 different sequences in these 6 individuals. The sequence from NoZ0902 was identical to the previously reported AB510439, AB510440, AB510441 and AB510444, and the sequences from NZ0801, NZ0483 and NoZ0801 were identical to AB519425, AB510428 and AB510438. The sequence from the sample of NoZ0802 was unique, *i.e.* different from all other sequences in the database. The sequence from EG0601 was identical to AB510411 and AB510417. The neighbor-joining (NJ) tree including sequences from the database supported the differentiation of the two species' lineage with 99% of the bootstrap value (Fig. 3). In this data set we found 38 variable sites, and 9 of the sites, including C/T at site 573d in Cytb corresponding to
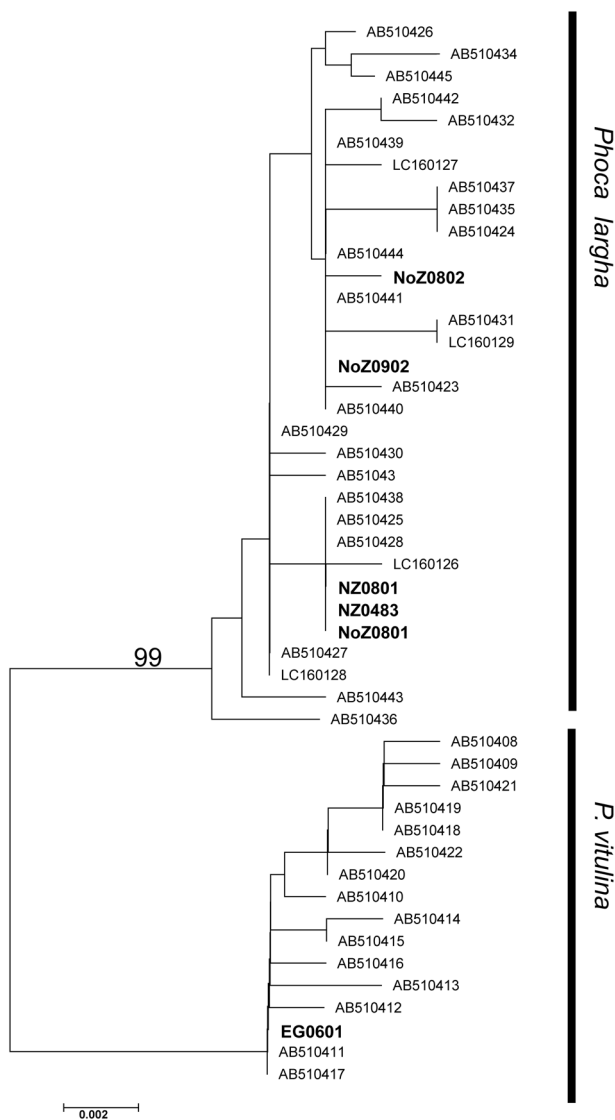
Fig. 3. Neighbor-joining tree of the first half of mitochondrial Cytb gene (678 bp) in *Phoca largha* and *P. vitulina*. OTU in the bold letters represent the name of samples sequenced in this study, while others were derived from database. The number on the main branch indicates the bootstrap value of 99%, highly supporting the separation of the two species lineages.

*Alu*I digestion, were different between species and identical within each species. Nucleotide substitutions at these sites contributed to the clear separation between the two lineages. The NJ tree confirmed that the mtDNA PCR-RFLP pattern corresponded precisely to the mtDNA lineage (Fig. 3). The sequences from NoZ0801, NoZ0802, NoZ0902, NZ0801 and NZ0483, which showed *P. vitulina* coloration and the RFLP pattern of *P. largha*, were included in the *P. largha* lineage, and the sequence from EG0601 was included in the *P. vitulina* lineage as the RFLP pattern suggested. The results thus indicated that the PCR-RFLP method developed in the present study is reliable for distinguishing between *P. largha* and *P. vitulina* in maternal lineage, and it is clear that some individuals showed inconsistency in species identification between mtDNA and coloration.

Minor exceptions showing this inconsistency can be attributable to the following factors: 1) recent hybridization and/or genetic introgression by past hybridization between these two species, 2) misidentification by uncertain external morphological characteristics, and 3) incomplete lineage sorting in mtDNA due to very recent separation between *P. largha* and *P. vitulina*. Among these factors, #3 is probably negated by the clear separation of the two species in the mtDNA lineage confirmed by Nakagawa et al. (2010) and our phylogenetic tree. Analyses of nuclear DNA (nDNA) are useful for comparing the possibility of #1 or #2: if an individual identified as species A in coloration has species B type in both mtDNA and nDNA, #2 is likely, or if an individual has the morphological characteristics of species A, the mtDNA of species B and the nDNA of species A or the heterozygosis of A and B, then #1 is likely. In particular, if heterozygosis of nDNA is found, it may be evidence for recent hybridization. Natural hybridization between these species may be possible in wild population. Frequent hybridization and the fertility of hybrid individuals among captive *P. vitulina* and *P. largha* described by Katsumata et al. (2003) indicated a lack of physiological or genetic barriers for hybridization between these species. In wild population, *P. largha* coming to Hokkaido have in recent years being expanding southward in increasing numbers and staying longer than before (Kobayashi, personal observation), increasing the potential for natural hybridization. Development of an effective nDNA marker to discriminate *P. largha* and *P. vitulina* seems to be the most effective approach to precisely identifying natural hybridization of these two species at the molecular level. We tried to document species discrimination and natural hybridization using microsatellite markers in nDNA but have not yet had success, because both species shared several alleles in all examined microsatellite loci (data not shown). After effective nDNA markers are established, the PCR-RFLP method developed in the present study will again be useful for the discrimination of maternal lineage in hybrids detected by the new markers.

Acknowledgements

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