# Phylogeny and population genetics of Japanese harbour seals (*Phoca vitulina*), the southernmost population in west Pacific

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## Introduction

The harbour seal (a.k.a. common seal) *Phoca vitulina* was first placed under the phylum *Phoca* by Linnaes 1758, however they were considered as a subspecies of the spotted seal (*Phoca largha*) until Shaughnessy and Fay (1977) defined them as a separate species.

Taxonomically, the harbour seal belongs to the subfamily Phocinae (Arctic seals), tribe Phocini, subtribe Phocina, and genus *Phoca*. Genus *Phoca* comprises just two seal species: the harbour seal and the spotted seal, which split relatively recently (0.4-1.3 Ma). The primary ecological difference between them is that spotted seals give birth on ice, and new-born pups have a white coat (lanugo), while harbour seals are adapted to breeding on land, and their pups shed their lanugo *in utero*, before birth (Shaughnessy and Fay 1977; Fulton and Strobeck 2010).

Harbour seals have the widest habitat range of all pinnipeds in that they can be found in both the Atlantic and Pacific Ocean (Shaughnessy and Fay 1977; Bigg 1981; King 1983; Jefferson et al. 1993). Although the number and division of subspecies are still a subject of debate, at least four subspecies are known: two are found in the Atlantic – *Phoca vitulina concolor* in the western Atlantic and *Phoca vitulina vitulina* in the eastern Atlantic, and the other two are in the Pacific – *Phoca vitulina stejnegeri* in the western Pacific and *Phoca vitulina richardsi* in the eastern Pacific.

The Japanese harbour seal is the southernmost population of *P. v. stejnegeri*, and they are found only on the Pacific side of Hokkaido where sea ice rarely comes in winter. A total of 11 haul-out sites are known here. The southernmost haul-out site is located at Cape Erimo, in the southwestern area of the habitat range in Hokkaido. This site is 150 km away from the nearest site, which is the longest distance between two haul-out sites in this region (Kobayashi 2009). Japanese harbour seals only haul out on rocky reefs to rest, breed and moult (Kobayashi 2009; Kobayashi et al. 2014), although seals in other regions are known to haul out in a variety of habitats such as intertidal mudflats, sandbars, rocks, reefs and ice floes, as well as artefacts such as floats and log booms (Jeffries et al. 2000). Mature seals in Japan are reported to be larger and show more sexual dimorphism in body size than those of the same subspecies in the Kuril Islands (Shaughnessy and Fay 1977). In addition, Japanese harbour seals have a higher proportion of “dark phase” pelage (a black or nearly black background with light spots or rings) than “light phase” pelage (a light background colour with dark spots or blotches). The proportion of “dark phase” pelage in Japan is the highest in the Pacific (Shaughnessy and Fay 1977), indicating that Japanese harbour seals are unique in the Pacific.

Harbour seals are reported to haul out at the same sites during the breeding season in successive years (Niizuma 1986; Womble and Gende 2013), and a long-term study showed that both adult males and females tend to use their natal site or a site close to their birth place

during the breeding period, although stronger site fidelity is known in females (Härkönen and Harding 2001). In fact, harbour seals are known to show a “stepping-stone” pattern of gene flow in studies based on both maternally inherited mitochondrial DNA (mtDNA) and bi-parentally inherited nuclear microsatellite loci, where geographically closer groups show genetic similarity (Lehman et al. 1993; Lamont et al. 1996; Goodman 1998; Westlake and O’Corry-Crowe 2002).

MtDNA is maternally inherited DNA in mammals and is especially suited in phylogenetic studies as it lacks recombination, and the historical genealogical record is not mixed between different mtDNA lineages during meiosis.

Past phylogenetic studies based on the control region of mtDNA for harbour seals suggested different scenarios in how the seals extended their distribution range in the Pacific, i.e. seals dispersed from west to east (Stanley et al. 1996), east to west (Westlake and O’Corry-Crowe 2002), or entered west and east at the same time (Burg et al. 1999). These studies treated Japanese harbour seals as either a basal (ancestral) population (Stanley et al. 1996; Burg et al. 1999) or a non-basal (descendent) population (Westlake and O’Corry-Crowe 2002) in the Pacific, and they treated these seals as one lineage, although sample sizes were small ($n<14$). On the other hand, a phylogenetic study carried out using only Japanese samples and the cytochrome b region of mtDNA indicated there were two lineages of Japanese harbour seals

(Nakagawa et al. 2010), suggesting that the different perspectives in previous studies may be due to their handling of Japanese harbour seals as a single lineage. However, Nakagawa also used a small number of samples, and sample sizes differed between regions (4–23 per region).

Unlike mtDNA, nuclear microsatellite loci inherited from both males and females are often used for population genetic studies, as they are high in both polymorphism and rate of mutation, and have been adopted more frequently in studies of genealogies in recent years (Allendorf et al. 2014). In addition, comparisons of mtDNA and microsatellite markers are often used to understand sex differences in gene flow, as they have different modes of inheritance. For harbour seals, larger movement and gene flow in males are reported in the western Pacific (Burg et al. 1999; Herreman et al. 2009), which corresponds to their ecology. On the other hand, harbour seals in the North Sea, which have recently been experiencing population decline (Olsen et al. 2014, 2017), showed larger gene flow in females, while harbour seals in inland waters of Washington State in the US, which were isolated during the Last Glacial Maximum, showed the same population subdivision with both mtDNA and microsatellite loci (Huber et al. 2010, 2012).

Studies based on nuclear microsatellite markers have not yet been carried out on Japanese harbour seals, however, and comparisons with mtDNA are not possible. In addition, Japanese harbour seals experienced population fluctuation in the past few decades, which

may have influenced the population genetics of the Japanese harbour seal: The population size of harbour seals in Japan was severely depleted in the 1970s due to extensive hunting and destruction of haul-out sites (Itoo and Shukunobe 1986). After the seals were assessed as an Endangered species and became protected in 1998, their population gradually recovered, and in 2015 they were downlisted as a semi-endangered species (Japanese Ministry of the Environment 2016).

Furthermore, because harbour seals during breeding season tend to use haul-out sites where they were born, comparisons of genetic data based on the samples taken from each breeding site during the breeding season may show the genetic characteristics of breeding populations and the natal site fidelity of Japanese harbour seals, and the patterns of movement between haul-out sites during the different seasons.

Study aims in this thesis

In Chapter 1, mtDNA markers were used to focus on the divergence history of Japanese harbour seals and the relationship between seals in Japan and other locations in the world using a larger number of samples than was used in previous studies. In Chapter 2, nuclear microsatellite markers were used to evaluate the effects of recent population fluctuations on Japanese harbour seals. In Chapter 3, both mtDNA and nuclear microsatellite markers were

used to define the genetic characteristics of the breeding population of Japanese harbour seals by using only samples taken during the breeding season, and the genetic characteristics were then compared with those of the non-breeding season to investigate whether it was possible to understand seasonal movements based on genetic data.

## Chapter 1. Phylogenetic Study of Japanese Harbour Seals Using MtDNA

### Introduction

The harbour seal (*Phoca vitulina*) is an amphibious mammal that distributes across more than 16,000 km of the northern hemisphere (Fig 1-1). Although their number and division are still a subject of debate, at least four subspecies of harbour seals are known in this range of distribution (Jefferson et al. 1993). Harbour seals are widely distributed along the shore of the Pacific Ocean from Hokkaido, Japan, as the southernmost limit in the western Pacific, to California (*Phoca vitulina richardsi*), the southernmost limit in eastern Pacific (Jefferson et al. 1993). In Japan, harbour seals inhabit only the Pacific side of Hokkaido and are distributed across four administrative districts: Erimo, Akkeshi, Hamanaka, and Nemuro. Akkeshi, Hamanaka, and Nemuro are located next to each other, while Erimo is isolated and 150 km west of Akkeshi, the nearest district (Fig 1-1) (Kobayashi 2009).

Sampling locations of published sequences outside Hokkaido, Japan, used in phylogenetic analysis are indicated with stars (Accession numbers U36342–U36371 (Stanley et al. 1996)). Samples of Japanese harbour seals (*Phoca vitulina stejnegeri*) were taken from four administrative districts (Erimo, Akkeshi, Hamanaka, and Nemuro) in Hokkaido, Japan. Each district contains several haul-out sites where the seals breed.

The common ancestor of the harbour seal diverged 4.5 million years ago from *Pusa* and

*Halichoerus* lineages in the area between Greenland and the Barents Sea (Higdon et al. 2007) and entered the Pacific through the Bering Strait. When the northern pathway closed due to the formation of sea ice and continental glaciation 1.7 to 2.2 million years ago, the Atlantic and Pacific harbour seals were separated, eventually resulting in genetic differentiation between the two populations (Stanley et al. 1996).

In the Pacific, harbour seals were said to have colonised from west to east (n=9) (Stanley et al. 1996), east to west (five additional samples to the same Japanese samples as (Stanley et al. 1996)) (Westlake and O'Corry-Crowe 2002), or in both directions (same Japanese samples as (Stanley et al. 1996)) (Burg et al. 1999) which treated Japanese harbour seals as either a basal (ancestral) lineage (Stanley et al. 1996; Burg et al. 1999) or a non-basal lineage (Westlake and O'Corry-Crowe 2002) using limited number of samples. On the other hand, the phylogenetic study using only Japanese samples and cytochrome b region of mtDNA suggested there are two lineages (n=39), and populations are differentiated between Erimo and other areas (Akkeshi and Nemuro in eastern Hokkaido) (Nakagawa et al. 2010). We hypothesised the existence of the different perspectives may be due to handling Japanese harbour seals as a single lineage or not. However, comparisons of all data was not possible because the former studies used control region of mtDNA (Stanley et al. 1996; Burg et al. 1999; Westlake and O'Corry-Crowe 2002)

Therefore, the number of lineages and the phylogenetic relationship of Japanese harbour seals with neighbouring countries are still unclear.

In this study, our aim was to reach a conclusion concerning the divergence history of Japanese harbour seals and phylogenetic relationship between the seals in Japan and other countries using larger number of samples based on control region of mtDNA. We believe this will help understanding the phylogeny and the historical movement of the Pacific harbour seals as a whole in the future.

## Materials and Methods

### *Sample Area and Sample Size*

Samples were collected from four administrative districts in Hokkaido, Japan: Erimo, Akkeshi, Hamanaka, and Nemuro, each of which has several haulout sites where Japanese harbour seals breed. Three districts are located next to each other (Akkeshi, Hamanaka, and Nemuro), while Erimo is 150 km west of Akkeshi, the nearest district (Fig 1-1).

A total of 178 samples were collected from the four districts (n=50 each for Erimo and Nemuro, n=49 for Akkeshi, and n=29 for Hamanaka). Muscle samples were taken from dead seals that were incidentally caught in salmon set-nets and drowned (n=152) or found stranded (n=7), and skin samples were collected from live animals during the flipper-tagging process for academic research (n=19). 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: 039 (2009), 001 (2010) and 246 (2012) for eastern Hokkaido, and 291 (2011), 192 (2012), and 0205 (2013) for Erimo. Sampling protocols were approved by the Ethics Committee of Tokyo University of Agriculture. All samples were preserved in 70% ethanol at room temperature until DNA extraction was carried out.

***DNA extraction, PCR, and sequencing***

Genomic DNA was extracted from samples using the standard phenol-chloroform method (Green and Sambrook 2012). A total segment of the mtDNA control region was amplified using a polymerase chain reaction (PCR) with primers PvsF (5'-GTACTCATACCCATTGCCAGC-3') and PvsR (5'-GCGCGGAGGCTTGCATGTAT-3') designed for this study. PCRs were conducted in a 25 µl reaction volume containing 1.0 µl of DNA template, 2.5 µl 10X buffer, 2.0 µl dNTP (0.2 mM), 0.1 µl taq polymerase (5U/ µl), 1.25 µl (1 mM) of each primer, and 16.9 µl Mili-Q water. MtDNA amplification consisted of an initial denaturation step for 5 min at 94 °C, 30 cycles of 94 °C for 1 min, 63 °C for 1 min, 72 °C for 1 min 30 s, and a final extension at 72 °C for 5 min. PCR products were checked on agarose gel by electrophoresis and sequenced using a BigDye terminator cycle sequencing kit v3.1 (Applied Biosystems). The same forward primer and an additional reverse primer PvsFR (5'-GTAACGTAACTATGTCCCGC-3') was used for DNA sequencing, and sequences were read in both directions. Sequence editing and running CLUSTALW for alignment were implemented in MEGA version 6 (Tamura et al. 2013).

A sequence of 454 base pairs (bp) was used for analysis to examine the phylogeny of Japanese harbour seals. Only the data of Stanley et al. (Stanley et al. 1996) (GenBank accession numbers U36342–U36371) was included for the analysis since they have the longest sequence deposited in the GenBank database.

***Data analysis***

***Genetic diversity***

haplotype diversity (*H*) and nuclear diversity ($\pi$) were calculated using Arlequin version 3.5.1.2 (Excoffier et al. 1992).

***Genetic differences***

Degree of population differentiation was analyzed with Fst (Weir The and Cockerham 1984) and Φst (Kimura 1980) using Arlequin version 3.5.1.2 (Excoffier et al. 1992).

***Phylogenetic relationship***

For the phylogenetic tree, the most appropriate model of substitution was determined using the Baysian Information Criterion (BIC) in MEGA6 (Tamura et al. 2013), and the K2+G+I model was used for the maximum-likelihood (ML) tree. A tree based on the neighbour-joining (NJ) method using same substitution model (K2+G+I) was also created in MEGA6 to validate the phylogenetic tree (Tamura et al. 2013).

To visualise patterns of geographical distribution and haplotype relationships, the median-joining network (MJ Network) was generated using Network 4.6.1.3 (Bandelt et al. 1999) with

default parameters (epsilon=0, weight=10).

The results for the phylogenetic tree and network were combined to examine groupings of Japanese haplotypes. The proportions of haplotypes belonging to the different groups were then compared between the four districts to investigate trends.

***History of population expansion***

Mismatch distribution analysis, which compares the distribution of the observed numbers of pairwise differences among all haplotypes in a sample, was also conducted using Arlequin version 3.5.1.2 (Excoffier et al. 1992) to investigate past demographic fluctuations. The goodness of fit between the expected and observed values was tested using the sum of squared deviation (SSD) and Harpending's raggedness index (Hrag).

## Results

We analysed 454 bp of the mtDNA control region of 178 seals from the four districts of Erimo, Akkeshi, Hamanaka, and Nemuro in Hokkaido, Japan. Overall, 22 polymorphic sites were identified and 16 haplotypes were defined (Table 1-1). The haplotypes are deposited to GenBank (accession numbers: LC314221-LC314236). Regional trends in the haplotype frequencies for each area are shown in Table 1-2. Although nearly 70% of the seals at Erimo had haplotype JP5, this haplotype was seen in only 6% or fewer of the animals at Akkeshi and Hamanaka, and in none of the seals at Nemuro. The most common haplotypes that were found in all four areas were JP6 & JP7 of which percentages were high in Akkeshi (JP6: 18%; JP7: 45%), Hamanaka (JP6: 22%; JP7: 33%) and Nemuro (JP6: 35%; JP7: 45%), and low in Erimo (JP6: 10%; JP7: 4%). The largest number of haplotypes (NH) was found in Nemuro (n=9) while all other areas had 7 haplotypes. The number of unique haplotypes was the highest in Nemuro (n=4), followed by Erimo (n=3) and Hamanaka (n=1); there were no unique haplotypes in Akkeshi.

### Genetic *diversity*

Genetic diversity based on mtDNA differed between Erimo and other areas (Akkeshi, Hamanaka and Nemuro): in Erimo, both haplotype diversity and nuclear diversity were low ($H$= 0.509, $\pi$= 0.006), while all other areas had high haplotype diversity and low nuclear diversity ($H$= 0.749,

0.754 and 0.710; and π= 0.006, 0.006 and 0.005, respectively) (Table 1-3).

***Regional differences***

Pairwise comparisons of Fst values for mtDNA and Φst values, indicated that Erimo ($p$<0.001 after Bonferroni correction) was significantly different from the other areas (Akkeshi, Hamanaka and Nemuro) (Table 1-3). However, no significant genetic differences were found in pairwise comparisons between Akkeshi, Hamanaka, and Nemuro (Table 1-3).

***Phylogenetic relationship***

Both phylogenetic trees, using the ML and NJ methods, showed the same groupings. A single group was found in the Atlantic (Group A), while Pacific harbour seals (Group P) were divided into a minimum of two groups: the first group only contained haplotypes from Japan (Group P1), and the second group contained haplotypes solely from the eastern Pacific (Group P2) (Fig 1-2). The Japanese haplotypes other than Group P1 were located in the Group P, along with the haplotypes from Bristol Bay, and the Commander Islands.

The bootstrap values of branches of the maximum-likelihood (left) and neighbour-joining method (right). 454 bp of the control region was used to compare the phylogenetic relationships

of seals. Data outside Japan were obtained from GenBank (Accession numbers U36342–U36371 (Stanley et al. 1996)).

Groupings in the median-joining network were conducted based on the phylogenetic tree (Figure 1-3). In the haplotype network, Group P1 was connected to Atlantic, and contained only Japanese haplotypes. The haplotypes in the eastern Pacific (Group P2) and other haplotypes were then connected to Group P1. The Japanese haplotypes other than Group P1 are located in separate branches, suggesting that they diverged from multiple haplotypes: some were from Bristol Bay and others were from the same hypothetical haplotypes shared with Bristol Bay and the Commander Islands.

The node colours and sizes of circles represent the different sites, area, and sample size. The length of the node is proportional to the number of substitutions. Groupings of the nodes are based on the division of the phylogenetic tree in Figure 1-2.

The proportion of Group P1 was high at Erimo, the southernmost distribution in the range of harbour seals in the western Pacific, and decreased toward Nemuro, the easternmost sampling site in this study (Figure 1-4).

The haplogroups (Group P, A, P1 and P2) were defined in the phylogenetic tree and the median-joining network. The numbers in the bar indicate the number of samples.

***History of population expansion***

Mismatch distribution of Japanese harbour seals showed a bimodal profile, indicating secondary contact of populations after a long isolation. SSD and Hrag both supported the overall pairwise differences in the match spatial distribution model (SSD: p=0.07; Hrag: p= 0.41) (Figure 1-5) but it did significantly deviated from expectations under a sudden expansion model (SSD: p=0.02; Hrag: p=0.02).

The bar charts indicate the observed number of pairwise differences and the dashed line represents the expected distribution under a spatial expansion model (SSD: p=0.07; Hrag: p=0.41).

## Discussion

Past phylogenetic studies of harbour seals treated Japanese haplotypes as a single lineage. Some concluded that the direction of expansion occurred from west to east and that the seals in Japan represented a basal population in the Pacific (Stanley et al. 1996; Burg et al. 1999), while another study suggested that population expansion occurred in the opposite direction and that the population in Japan was not basal (Westlake and O'Corry-Crowe 2002) using control region of mtDNA. On the other hand, other study used only Japanese samples and cytochrome b region of mtDNA suggested existence of two lineages (Nakagawa et al. 2010). The number of lineages and the phylogenetic relationship of Japanese harbour seals with neighbouring countries were still unclear, because comparison of these studies was not possible.

We used relatively large number of samples (n=178) and control region of mtDNA (454bp) to clarify the divergence history of Japanese harbour seals and phylogenetic relationship between the seals in Japan and other countries.

Our results supported the result in Nakagawa et al. (2010) that the populations are differentiated between Erimo and eastern Hokkaido, and indicated there possibly are more than two lineages in Japanese harbour seals based on phylogenetic tree and haplotype network. Also, the mismatch analysis suggested secondary contact of populations after a long isolation; and increase in the population range over time and space after the restriction of original population

into a very small area.

Moreover, one of the lineage was made only by Japanese harbour seals (Group P1). The proportion of this lineage was the highest at Erimo, the southernmost distribution range of western Pacific harbour seals and gradually decreased towards the North East of Hokkaido. The Japanese haplotypes which are not in Group P1 (Figure 1-2 and 1-3) belonged to different branches, which also had haplotypes from the North Pacific suggested they have close relationship to the Northern Pacific harbour seals.

We further constructed two median joining trees using different data (Appendix 1-1; data of Figure 1-3 and (Westlake and O'Corry-Crowe 2002) (369bp) and Appendix 1-2; data of Figures 1-3 and all other data available in GenBank (370bp)) to compare with the haplotype network in result section (Figure 1-3). All new networks and Figure 1-3 showed same groupings for Japan (Group P1) and Washington (Group P2) (Fig 1-3ure, Appendix 1-1 and Appendix 1-2), and the other Japanese haplotypes showed close relationship to the seals in North Pacific.

During the Last Glacial Maximum (LGM) which was ended around 0.02 million years ago, the lowering of the sea level and the formation of the Bering land bridge connecting Eurasia and North America caused closure of Bering Strait (Peltier 1994; Hewitt 1999). At this time, the Cordilleran ice sheet covered most of North America, including the eastern Aleutian Islands but not some parts of eastern Alaska and the land bridge over the Bering Strait (Beringia) (Mann and

Peteet 1994; Peltier 1994). In addition, seasonal sea ice was extending its range from north to south in the Pacific, to as far as Erimo in Hokkaido, Japan (Ono 1984; Peltier 1994; Hewitt 2000). The animals lived over the North Pacific during this period are believed to be surviving in small, ice-free regions called refugia, and population subdivision related to refugia across the North Pacific are known in many marine and land animals (e.g. the Steller sea lion (Baker et al. 2005; Waite et al. 2011), sea otter (Cronin et al. 1996), rock ptarmigan (Holder et al. 1999), and reindeer (Flagstad and Røed 2003), as well as in subspecies of harbour seals in the eastern Pacific (Lamont et al. 1996; Huber et al. 2010)). The phylogenetic studies of chum salmon (Taylor et al. 1994; Seeb and Crane 1999; Sato et al. 2001; Beacham et al. 2009), and Pacific cod (Canino et al. 2010), which are also distributed widely over the North Pacific, suggested that animals in Hokkaido became isolated during the LGM (Taylor et al. 1994; Sato et al. 2001; Yoon et al. 2007, 2008; Beacham et al. 2009).

Fossils of harbour seals dated as 0.1 million years old were found at the Shimokita Peninsula, Aomori, which is not far from Erimo, currently the southernmost distribution range of harbour seals in the western Pacific (Figure 1-1) (Hasegawa et al. 1988; Miyazaki et al. 1994). This suggests that harbour seals already inhabited areas around Aomori long before the LGM.

Furthermore, in this study, seals in Erimo were indicated to have different history from three other regions: Seals in Erimo had low in both haplotype and nucleotide diversity (Table 1-2)

which indicate they experienced severe bottleneck and has not fully recovered (Avise 2000), while the seals in eastern Hokkaido had high in haplotype diversity but low in nucleotide diversity (Table 1-2), indicating they had sudden population expansion after bottleneck (Avise 2000). In addition, the proportion of Group PI was nearly 80% in Erimo (Figure 1-4) while the other areas had the proportion of less than 40% (Figure 1-4).

These factors suggest the history of Japanese harbour seals: the haplogroup made up only by Japanese harbour seals (Group P1) might have entered Japan before the LGM and became isolated due to the geographical boundary-sea ice, and gradually extended its range from the South West towards the North East of Hokkaido after the disappearance of the sea ice, while the seals which are not in Group P1 immigrated into Japan from the North Pacific, which are the descendent of the seals in refugia in North Pacific.

## Chapter 2. Current Population Genetics of Japanese Harbour Seals: Two Distinct Populations Found Within a Small Area

### Introduction

The harbour seal (*Phoca vitulina* Linnaeus, 1758) is a semi-aquatic mammal distributed along 16,000 km of coastline in the northern hemisphere (Shaughnessy and Fay 1977; King 1983; Jefferson et al. 1993). Although they forage in the sea, they rest, moult, and breed on haul-out sites of differing habitats including rocky reefs, intertidal sandbanks, and glacial ice floes, depending on the region (Bigg 1981; King 1983; Jefferson et al. 1993; Thompson 1993).

A long-term study showed that both adult males and females tend to use their natal site or a site close to their birth place during the breeding season, although stronger site fidelity is known in females (Härkönen and Harding 2001). In fact, harbour seals showed a "stepping-stone" pattern of gene flow in studies based on both maternally inherited mitochondrial DNA (mtDNA) and bi-parentally inherited nuclear microsatellite loci, where geographically closer groups show genetic similarity (Lehman et al. 1993; Lamont et al. 1996; Goodman 1998; Westlake and O'Corry-Crowe 2002). The population structure of harbour seals based on microsatellite loci is known to be weaker than mtDNA over the same area, which indicates higher gene flow in males (Burg et al. 1999; Herreman et al. 2009). Seals living from southeast Alaska to British Columbia, a distance of over 1,000 km, for instance, showed no significant population subdivision based on

ten microsatellite markers (Burg et al. 1999; Herreman et al. 2009), whereas several populations were found based on mtDNA control region sequence data (Burg et al. 1999; Westlake and O'Corry-Crowe 2002). In this area, more than 100,000 harbour seals are distributed almost continuously along the open area of the Pacific Coast, and various types of haul-out sites are used (Muto et al. 2017).

In Washington, harbour seals are distributed for about 200 km along the outer coast and in inland waters. Here, both mtDNA and microsatellite data indicated that the seals inhabiting the inland waters are genetically distinct from those along the Pacific coast, despite the areas being linearly only 100 km apart from each other (Lamont et al. 1996; Huber et al. 2010, 2012). The populations along the inner coast of Washington are believed to have experienced historical isolation during the Last Glacial Maximum (LGM) and as a result became a very distinct lineage (Lamont et al. 1996; Burg et al. 1999; Huber et al. 2010). In recent years, the populations of seals both in inland waters and along the outer coast of Washington have almost completely recovered after a severe decline in the first half of the 20th century (Jeffries et al. 2003).

The southernmost range of harbour seals in the western Pacific is at Hokkaido, Japan, where a total of 11 haul-out sites are located over the four administrative districts of Erimo, Akkeshi, Hamanaka, and Nemuro (Fig. 1). There is a single haul-out site in Erimo, which is 150 km from the closest haul-out site in Akkeshi, whereas there is a total of 10 haul-out sites interspersed

over 75 km from Akkeshi (four haul-out sites), Hamanaka (four haul-out sites) to Nemuro (two haul-out sites) (Kobayashi et al. 2014). Genetic studies based on the entire mtDNA cytochrome *b* gene (1,140 bp) (Nakagawa et al. 2010), and a portion of the control region (454 bp) (Mizuno et al. 2018) showed two lineages in Japanese harbour seals, suggesting that they are divided into two populations, one in Erimo and the other in Akkeshi, Hamanaka, and Nemuro (hereafter referred to as “eastern Hokkaido”). Furthermore, both haplotype and nucleotide diversities in Erimo were lower than in eastern Hokkaido (Nakagawa et al. 2010), and the majority of Erimo seals were thought to be the descendents of seals that first entered Hokkaido and became isolated during the LGM (Mizuno et al. 2018).

However, additional analyses using nuclear DNA, such as bi-parentally inherited microsatellite loci, are needed to better understand the current population genetics of Japanese harbour seals in Erimo and eastern Hokkaido which have different historical backgrounds.

Japanese harbour seals inhabit only the Pacific side of Hokkaido where there is an open ocean and no obvious geographical boundaries between haul-out sites. The population size decreased dramatically in the 1960s and 1970s when they were hunted for their pelage and their haul-out sites were destroyed for improving kelp production (Itoo and Shukunobe 1986). The seals were then listed as an endangered species by the Japanese Ministry of the Environment and became protected in the 1980s. The population size gradually recovered: a census carried out in 1983

found only a total of around 350 seals, but more than 1,000 seals were recorded in 2008 (Kobayashi et al. 2014). It is therefore important to evaluate the effects of recent population fluctuations using nuclear microsatellite analysis.

In this study, nuclear microsatellites were used for the first time to investigate the recent population genetic structure and the effects of a population bottleneck on Japanese harbour seals.

## Materials and methods

### *Sample collection, DNA Extraction and Amplification*

A total of 195 samples were used in this study: 49 from Erimo, 50 from Akkeshi, 46 from Hamanaka, and 50 from Nemuro (Fig. 1). Muscle samples were taken from dead seals that were fisheries bycatch (n=172) or found stranded (n=8), and skin samples were collected from live animals during flipper-tagging for academic research (n=15).

All tissue samples were stored in 70% ethanol at room temperature until DNA extraction was carried out using the standard phenol-chloroform method (Green and Sambrook 2012).

Twenty-seven published microsatellite loci – Pvc19, Pvc26, Pvc29, Pvc30, Pvc63, Pvc74, Pvc78 (Coltman et al. 1996); SGPV2, SGPV3, SGPV10, SGPV11, SGPV16, SGPV17 (Goodman 1997); Hg1.3, Hg1.4, Hg3.7, Aa4, HgO, BG (Gemmell et al. 1997); SGPV9, Hg3.6, Hg4.2, Hg6.1, Hg8.9, Hg8.10, Hgdii (Allen et al. 1995), and M11A (unpublished data by Rus Hoelzel referenced in Gemmell et al. 1997) – were first tested to determine whether they were appropriate for this study. Two steps using 32 samples (8 samples per area, half of which were males and half females) were taken to test the appropriateness: (1) all loci were checked for amplification success, and if the amplification failed, no further checking was carried out, (2) loci that were successfully amplified were genotyped again to check if the same genotypes were obtained (repeat-genotyped).

Ten microsatellite loci that were determined to be appropriate for the study – Pvc19, Pvc78, Pvc30 (Coltman et al. 1996); SGPV16, SGPV11, SGPV10 (Goodman 1997); Hg3.7, Aa4 (Gemmell et al. 1997); SGPV9 (Allen et al. 1995), and M11A (unpublished data by Rus Hoelzel referenced in Gemmell et al. 1997) – were used in the experiment. Amplification of microsatellite loci was carried out in 25 µl reaction volumes containing about 100 ng of DNA template, 2 mM of 10X *Ex Taq* Buffer ($Mg^{2+}$plus), 0.2 mM of dNTP, 0.5 U *Ex Taq* polymerase (Takara), and 1 µM of each primer (single primer pair). Each locus was amplified separately, and all PCRs included negative controls to ensure genotyping accuracy. The thermal cycler profiles for all microsatellite loci followed the original published instructions. All forward primers were fluorescently labeled on the 5' end. After amplification, a maximum of five loci, whose fluorescent label or allele size did not overlap, were co-loaded with an internal size standard (GeneScan-600 LIZ, Applied Biosystems) and run on Genetic Analyzer 3500 (Applied Biosystems). Sizing of allele fragments was determined by GeneMapper Software v4.1, and all fragment analyses included both positive and negative controls. MICRO-CHECKER (Van Oosterhout et al. 2004) was used to check for null alleles or scoring errors for each population, and deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were tested using GENEPOP (Raymond and Rousset 1995) with Markov chain parameters (1,000 dememorization steps, 100 batches, 1,000 iterations per batch), and finally, significance levels were adjusted with sequential Bonferroni correction (Rice 1989).

***Data analysis***

***Genetic diversity***

Observed heterozygosity ($H_o$) and expected heterozygosity ($H_e$) were estimated for each of the four sampling locations using Arlequin version 3.5.1.2 (Excoffier et al. 1992). Allelic richness ($A_R$) and inbreeding coefficient ($F_{IS}$) were calculated using FSTAT 2.9.3.2 (Goudet 1995).

***Population structure***

Population structure based on microsatellites was analyzed using $F_{ST}$ and $R_{ST}$ implemented in Arlequin version 3.5.1.2 (Excoffier et al. 1992). Bayesian cluster analysis using STRUCTURE 2.3.4 (Pritchard et al. 2000) was carried out to investigate the genetic structure of Japanese harbour seals using a burn-in period with the number of Markov chain Monte Carlo (MCMC) simulations set to 100,000 and 1,000,000, respectively. The admixture model with correlated allele frequencies was used, using the sampling location information model (LOCPRIOR), which allows sample locations (the four locations in this study) to be used as priors in the clustering algorithm (Hubisz et al. 2009). The value *K* was ranged from 1 to 5, and analyses were run 10 times for each *K*. The most probable number of putative populations (K) was determined based on Ln (K) and

ΔK following the method described in Evanno et al. (2005), which chooses the putative population with the largest ΔK.

***Current population status***

Potential for a recent genetic bottleneck based on heterozygosity excess was investigated in BOTTLENECK version 1.2.02. (Piry et al. 1999) for populations defined in the previous section using the two-phase mutation model (TPM) with 95% single-step mutations and 5% multiple-step mutations, and a variance among multiple steps of 12 (Piry et al. 1999). We also investigated the population bottleneck based on the allele frequency distribution using the "mode-shift" graphical descriptor (Luikart et al. 1998) that is also implemented in BOTTLENECK version 1.2.02. (Piry et al. 1999).

## Results

Ten microsatellite loci showed no null alleles or scoring errors, and none of the loci indicated significant deviation from Hardy-Weinberg equilibrium (HWE) or linkage disequilibrium. All ten loci were therefore used for the following analysis.

### *Genetic diversity*

Allele richness ($A_R$) and mean expected heterozygosity ($H_e$) were similar among all regions: $A_R$ ranged from 3.183 in Nemuro to 3.659 in Akkeshi, and $H_e$ ranged from 0.431 in Hamanaka to 0.472 in Erimo (Table 3-1). No inbreeding was observed over the four regions based on $F_{IS}$ ($p$ >0.05) (Table 3-1).

### *Population structure*

Pairwise comparisons of $F_{ST}$ values for microsatellites, and $R_{ST}$ values, indicated that Erimo ($p$ < 0.001 after Bonferroni correction) was significantly different from the other areas (Akkeshi, Hamanaka, and Nemuro) (Table 2-2).

In addition, Bayesian clustering analysis using STRUCTURE suggested that there were two subdivisions in the Japanese population based on Ln (K) and ΔK (Figure 3-2), and the two clusters

were separated into Erimo and the other areas, i.e., Akkeshi, Hamanaka, and Nemuro ("eastern Hokkaido") (Fig. 2-3).

***Current population status***

The following analyses were carried out on the two populations of Erimo and eastern Hokkaido based on the above results. No recent genetic bottlenecks were detected in either population ($p > 0.05$), and the allele frequencies were indicated to have L-shape distribution, indicating stable populations.

## Discussion

Through the use of ten microsatellite loci, our study confirmed that the current population of Japanese harbour seals was divided into two distinct populations, one in Erimo and the other in eastern Hokkaido (Akkeshi, Hamanaka, and Nemuro). This population subdivision is the same as described in a previous study based on mtDNA cytochrome b (Nakagawa et al. 2010) and the control region (Mizuno et al. 2018).

Japanese harbour seals are reported to have experienced declines in population size over the past few decades (Kobayashi et al. 2014). In the 1940s, population numbers were roughly estimated at more than 1,500, but in the 1960s and 1970s these numbers dropped to around 600–900 due to extensive hunting and destruction of haul-out sites (Itoo and Shukunobe 1986). A total of 344 seals (128 in Erimo and 216 in eastern Hokkaido) were recorded in 1983 when the population census started during the moulting season (Kobayashi et al. 2014). After the seals were listed as an endangered species by the Japanese Ministry of the Environment and became protected, the population size gradually recovered, and over 1,000 seals (524 in Erimo and 565 in eastern Hokkaido) were recorded in 2008 (Kobayashi et al. 2014). In the present study, however, no recent genetic bottlenecks were detected, the L-shape in the mode-shift model indicated stable populations, and no inbreeding or significant deviation from Hardy-Weinberg equilibrium was observed in either of the two areas.

Some species are known to have lost genetic variation after the population bottleneck. The northern elephant seal (*Mirounga angustirostris*), for example, was heavily exploited, and by 1890, fewer than 30 remained at offshore islands (Hoelzel et al. 1993, 2002). Their number has been recovering dramatically, and in 2010, the total population was estimated to be around 180,000 (Lowry et al. 2014), however, the seals lost genetic variation and a genetic bottleneck was detected (Hoelzel et al. 1993, 1999). The Hawaiian monk seal (*Monachus schauinslandi*) also experienced a genetic bottleneck after heavy exploitation, and in the 1890s, only a few animals were believed to be left at most of the islands, after which their population size recovered and was estimated at more than 1,400 in 2016, although they have low genetic variation (Kretzmann et al. 2001; Schultz et al. 2008, 2010; Carretta et al. 2019).

Similar to our study, however, spotted seals (*Phoca largha*) in Liaodong Bay, China, also experienced population decline but no genetic bottleneck was detected. Liaodong Bay spotted seals were historically hunted (>30,000 seals were killed from 1930 to 1990), and the population decreased to around 2,000 individuals between 1979 and 1983, then increased to approximately 4,500 in 1990, but after that decreased again to fewer than 1,000 seals (Li et al. 2010). The relationship between population trends and the genetic bottleneck of these three seal species suggests that the minimum number of seals during the population bottleneck is an important factor affecting the severity of the genetic bottleneck and loss in genetic variation, and the

population size of the seals in Erimo (N=128) and eastern Hokkaido (N=216) during the population bottleneck was not small enough for the analyses to detect any genetic bottleneck.

The harbour seals in Erimo and in the inland waters of Washington have similar histories, as mtDNA analysis indicated that both areas were isolated during the LGM (Lamont et al. 1996; Burg et al. 1999; Mizuno et al. 2018). In addition, microsatellite loci indicated the same subdivision that mtDNA did for both Japan and Washington. However, the degree of genetic differentiation between Erimo and eastern Hokkaido based on microsatellite DNA was larger than that of the inland waters and the outer coast of Washington: both pairwise Fst and Rst values indicated that the seals in the Strait of Georgia, in inland waters, were not significantly different from the population along the outer coast, and the individuals in each cluster defined by STRUCTURE analysis did not correspond to sampling locations (Huber et al. 2012).

Japanese harbour seals inhabit the coast, facing the open waters of the Pacific, where no obvious geographical boundaries exist, and they haul out only on rocky shores. Erimo has a single haul-out site and is 150 km from eastern Hokkaido, where 10 haul-out sites are interspersed over a distance of 75 km, and over 500 seals were recorded in each area (2008 data; Kobayashi *et al.*, 2014). On the other hand, more than 8,000 and 10,000 seals were recorded in the inland waters and along the outer coast of Washington, respectively (1999 data; Jeffries *et al.*, 2003), and these seals haul out on a variety of habitats such as intertidal mudflats, sandbars, rocks, and reefs as

well as artefacts such as floats, logbooms and ice floes (Jeffries et al. 2000), which are located almost continuously between the areas (Jeffries et al. 2000, 2003). Stronger population differentiation found in Hokkaido may be due to the much smaller number of haul-out sites and population sizes, and the fact that in Hokkaido fewer types of habitats are used for haul-out sites than in Washington.

The separation between Erimo and eastern Hokkaido populations might have been further caused by the particular position of rocky reefs or haul-out sites. At Erimo, there are numerous rocky reefs at a single haul-out site that stretch up to 1.3 km offshore from land (Fig. 1), whereas 10 haul-out sites in eastern Hokkaido, separated by a maximum of 30 km, are interspersed along 75 km of the coast (Kobayashi et al. 2014) (Fig. 1). A satellite tagging study (Kobayashi unpublished) indicated that the home range of the harbour seal in Erimo was concentrated around rocky reefs offshore, while the seals in eastern Hokkaido moved along the coast where haul-out sites are distributed (Haneda et al. 2017). Differences in the direction in which the rocky reefs stretch away from land or in which the haul-out sites are facing may be limiting the movement of Japanese harbour seals and causing the high degree of population separation between Erimo and eastern Hokkaido.

## Chapter 3. Understanding the Population Genetic Substructure of Japanese Harbour Seals in Eastern Hokkaido.

### Introduction

The harbour seal is a semi-aquatic mammal that forages and mates under water but hauls out on land to rest, give birth, rear a pup, and moult. Their lifestyle can be divided into three seasons: breeding (mating, giving birth, and rearing a pup), moulting, and other activities (mainly feeding).

Roles of hauling out can vary greatly depending on the time of year. During the breeding season, female harbour seals prioritise safety, as they give birth to a pup and raise it on land for 4-6 weeks (Boulva and McLaren 1979; Niizuma 1986; Thompson 1988). At this time, females use sheltered or isolated sites (Thompson 1989, 1993), which are often where they were born (Niizuma 1986; Härkönen and Harding 2001). Adult males are also known to be found at their natal site during the breeding season to mate, but this is less likely than females (Härkönen and Harding 2001). On the other hand, during the moulting season, seals use haul-out sites less affected by tidal cycles (Thompson 1993), as they remain most of the time on land to conserve energy, because blood flow at the skin surface increases during this time (Paterson et al. 2012). In some areas, seals stay at their breeding sites and moult there (Niizuma 1986; Thompson 1989), but switching to different haul-out sites during moulting season can also

occur depending on local environmental conditions (Thompson 1993).

Outside the breeding and moulting seasons, harbour seals are more focused on feeding than using specific haul-out sites. Switching haul-out sites can occur more easily at this time as their choice of haul-out sites is dependent on prey abundance and their movement (Thompson 1993; Peterson et al. 2012; Sharples et al. 2012). Foraging trips of adults are shorter than those of subadult seals (Thompson 1993; Thompson et al. 1994; Lowry et al. 2001; Dietz et al. 2012; Bajzak et al. 2013), and the most extensive trips are taken by first-year pups (>300km) (Thompson 1993; Lowry et al. 2001; Dietz et al. 2012), although most of them come back to their natal areas in the following breeding season (Härkönen and Harding 2001; Small et al. 2005).

In eastern Hokkaido (hereafter referred to as Eastern Hokkaido when discussing this region of haul-out sites), there are 10 haul-out sites known across three administrative districts. Four are in Akkeshi (Akkeshi A, Daikoku Is., Akkeshi B, and Akkeshi C), four are in Hamanaka (Kenbokki Is., Hamanaka A, Hamanaka B, and Hattaushi), and two are in Nemuro (Yururi Is. and Moyururi Is.) (Figure 3-1; haul-out sites are named following Koyabashi et al. 2014). The haul-out sites used during the breeding season are Akkeshi A, Daikoku Is., Kenbokki Is., Hamanaka B, Yururi Is., and Moyururi Is. (unpublished data, 2018) (Figure 3-1). The pupping season of Japanese harbour seals (*Phoca vitulina stejnegeri*) takes place in early May to the beginning of June, with

the peak pupping period occurring during the spring tide around the middle of May (Niizuma 1986). Females become oestrous towards the end of their lactation period and mate with males under water (Boulva and McLaren 1979; Niizuma 1986; Thompson 1988) from around the middle to the end of June (Niizuma 1986), and the moulting season begins soon after the end of the breeding season, from late June to August (Niizuma 1986).

Considering the ecological facts of previous studies that female harbour seals have strong natal site fidelity during the breeding season, seals at each breeding ground during the breeding season may have unique genetic characteristics. However, of the past studies that were carried out utilizing mark-recapture techniques such as Photo-ID (Niizuma 1986) and branding (Härkönen and Harding 2001), tagging studies such as satellite (Small et al. 2005; Womble and Gende 2013), VHF (Yochem et al. 1987; Cordes et al. 2011) and plastic cattle ear-tags (Thompson 1989), none of the studies considered genetic data.

MtDNA is maternally inherited DNA in mammals, not mixed between different lineages during meiosis, and is therefore suited to the study of historical movements in animals. Nuclear microsatellite loci, on the other hand, are inherited from both males and females, and are high in polymorphism and rate of mutation and are therefore suited for the study of current population genetics (Allendorf et al. 2014). Comparisons of the genetic characteristics of Japanese harbour seals across breeding areas during breeding seasons using these two

markers may thus lead to a better understanding of natal site fidelity and the seasonal movements of Japanese harbour seals.

To understand the genetic characteristics of breeding groups, it is crucial to collect samples from breeding sites during the breeding season. It is extremely difficult, however, to capture live harbour seals at haul-out sites as males can grow to around 190 cm and females can grow to 175 cm in length (Naito and Nishiwaki 1972), and they haul out on rocky shores where they are difficult to approach (Itoo and Shukunobe 1986).

The development in recent years of DNA extraction and analysis methods, however, has made it easier to obtain genetic data from animals that are difficult to capture, as these methods utilize non-invasive samples such as faeces, hairs, and feathers (Goossens et al. 1998; Murphy et al. 2002; Piggott et al. 2005; Kalz et al. 2006; Caudron et al. 2007; Yannic et al. 2011). Samples can also be obtained remotely utilizing biopsy guns and bow guns (Caudron et al. 2007; Pagano et al. 2014). The use of non-invasively collected samples, however, requires individual identification in order to avoid duplication of individuals (Waits et al. 2001), which in general is performed through the use of several microsatellite loci (Reed et al. 1997; Taberlet and Luikart 1999; Vergara et al. 2014).

In this study, the types and numbers of microsatellite loci required for individual identification of Japanese harbour seals were defined first, in order to avoid using duplicated

samples. The seals were then characterised for each breeding ground during the breeding season based on genetic data to understand natal site fidelity and the seasonal movements of the Japanese harbour seal.

## Materials and Methods

### *Types and numbers of microsatellite loci required for Individual identification of Japanese harbour seals*

To determine how many and which loci were needed to identify individual Japanese harbour seals, the probability of two randomly chosen animals having identical genotypes (probability of identity (PID(theo)) (Hanotte et al. 1991; Waits et al. 2001) and the probability of two siblings having identical genotypes (PID(sib) ) (Evett and Weir 1998) were calculated. Since the populations in Erimo and eastern Hokkaido were shown in Chapter 2 to be different, PIDs were obtained for each population. The same 10 microsatellite markers and samples (n=195) from Chapter 2 were used for calculations, as they were shown not to deviate from HW equilibrium and were therefore appropriate for analysis.

In general, PID or overall PID (multiplying PID for all loci) of <0.01 (Waits et al. 2001) is required for carrying out reliable individual identification, and normally, PID<0.01 is achieved by combining several loci that have a particularly low PID.

PID(theo) and PID(sib) for each locus were calculated with the following equations:

$$PID(theo) = \sum_i {P_i}^4 + \sum_i \sum_{j>i} (2P_iP_j)^2$$

$$PID(sib) = 0.25 + 0.5 \sum_i {P_i}^2 + 0.5(\sum_i {P_i}^2)^2 - 0.25 \sum_i {P_i}^4$$

Here, each $P_i$, $P_j$ indicates $_i$th and $_j$th alleles.

PID(obs) for each locus was also calculated by dividing the number of pairs that had identical genotypes, by the total number of pairs.

PID(obs)s were then compared with two expected PIDs, PID(theo) and PID(sib), to see which expected PID was close to PID(obs). The minimum number and types of loci required for individual identification of Erimo and Eastern Hokkaido seals were determined based on the expected PID, which showed similar trends to PID(obs). PID calculations were conducted separately for Erimo and Eastern Hokkaido using R software (ver.3.5.1) (R Core Team, 2019).

***Sample collection, DNA extraction and amplification***

***Data from Chapters 1 and 2 with the addition of samples taken in the past***

The genetic data from Chapters 1 and 2 were sorted into breeding (May to July) and non-breeding (September to November) seasons based on the month when samples were taken from the three areas of Akkeshi, Hamanaka, and Nemuro. Only animals that weighed more than 40 kg were used for the breeding season in order to avoid including yearlings (the average weight of yearlings based on unpublished data by Morohoshi (2014)). Samples taken during the moulting season (July and August) were handled as “breeding” samples, as

Japanese harbour seals were reported to be found at the same haul-out sites, or at nearby sites in the same area, during both the breeding and moulting seasons (Chishima 2008; Kimura 2014). After the data were sorted, 23 samples remained for the breeding season and 98 for the non-breeding season. Since the sample size was still small, some other samples that had been taken and stored in the past were added (breeding season: 7 in Akkeshi and 1 in Nemuro; non-breeding season: 1 in Akkeshi). The total numbers of additional samples taken previously were 31 samples for the breeding season (24 in Akkeshi, 6 in Hamanaka, and 1 in Nemuro) and 99 for the non-breeding season (22 in Akkeshi, 27 in Hamanaka, and 50 in Nemuro).

***Number of samples*** newly ***collected from breeding grounds during the breeding season***

Since the sample size for the breeding season was still too small to achieve integrity for this study, non-invasive and biopsy samples were collected from breeding grounds during the breeding season in Akkeshi, Hamanaka, and Nemuro from May to July in 2018 and 2019.

Non-invasive samples such as faeces, hairs, part of the placenta, and blood drop, plus biopsy samples such as biopsy dart and mouth swab, were collected from Akkeshi (mouth swab: n=3), Hamanaka (biopsy dart: n=1; mouth swab: n=7; faeces: n=8; placenta: n=1), and Nemuro (biopsy dart: n=7; mouth swab: n=1; faeces: n=8; hair: n=23), from May to July in 2018 and 2019. Because these samples might have been taken from the same individuals, individual

identification of the samples was carried out first, and only samples without duplications were retained for this study.

***Sample collection and storage of non-invasive and biopsy samples***

Faeces and mouth swabs were collected by rubbing the surface of faeces or wall of the oral cavity several times with cotton buds, which were then immediately placed into a 2.5 ml tube containing lysis buffer (40 mM Tris; 2 mM EDTA; 0.2 M NaCl; 10% SDS (White and Densmore 1992)). To minimise sampling multiple times from identical individuals, hairs found concentrated in one place (or a bundle of hairs, around 10~30 ) on rocks were assumed to be from the same individual and placed into small zipped plastic bags with cotton containing 99% ethanol. The placenta sample was placed into a 25 ml tube with 99% ethanol on the boat. For biopsy samples, TELINJECT BIOP-A Biopsie-caps (TELINJECT, Germany) fitted with a S100v syringe were set into a G.U.T.50 gas-operated rifle. After darts successfully hit the target animals, the darts containing skin or hair were placed into a 25 ml tube filled with 99% ethanol.

***DNA extraction of non-invasive and biopsy samples***

For muscle and flipper samples, the same procedure of DNA extraction as described in Chapter 1 was followed.

Genomic DNA from faeces was extracted using the QIAamp DNA Stool Kit (QUIAGEN). DNA from hair samples was extracted using ISOHAIR (ISOHAIR), and with the exception of hairs obtained by biopsy, a single hair was used for each extraction and fragment analysis in order to avoid mixing different individuals. DNA from mouth swabs and blood stains was extracted using QIAamp blood and tissue kit (QUIAGEN). Genomic DNA from muscle, skin, and placenta were extracted from samples using the standard phenol-chloroform method (Green and Sambrook 2012).

A total segment of the mtDNA control region was amplified using the same method as Mizuno et al. (2018), except for DNA obtained from hair when an additional 0.5 µg/µl of T4 gene protein (ISOHAIR) was added to the reaction to improve amplification.

The same 10 microsatellite loci and fragment analyses were carried out following the method described in Chapter 2, except for hair and faecal samples due to their low content of DNA and high susceptibility to genotyping errors such as allelic dropouts and false alleles (Morin et al. 2001). Amplification of microsatellite loci for hairs and faeces was carried out in 10 µl reaction volumes containing about 30 ng of DNA template, 5 µl of 2 X Multiplex PCR Buffer ($Mg^{2+}$, dNTP

plus), 0.05 µl of Multiplex PCR Enzyme Mix, and 1 µM of each primer (single primer pair), and for hair, 0.5µg/µl of T4 gene 32 protein was also added to enhance amplification success. Also, PCR and fragment analyses were carried out at least twice per locus, or repeated until the same allele profile was obtained twice at each locus.

***Duplication of genotypes***

R package Allelematch (GALPERN et al. 2012) was used to check for any duplication of individuals in non-invasive and biopsy samples. Genotypes of flipper samples previously taken were also added to the analysis, as samples taken from live animals and non-invasive and/or biopsy samples might have been retaken from them. After the check for genotype duplications, samples with the same genotypes were removed from further analysis.

***Genetic characteristics of each breeding area and comparisons of genetic characteristics between breeding and non-breeding seasons***

***MtDNA***

Genetic characteristics of Eastern Hokkaido harbour seals in breeding areas during the breeding season were defined by two haplo-groups and unique haplotypes based on two

lineages (P1 and Others) found in Chapter 1.

Haplotypes obtained were divided into two groups, based on the lineage obtained in Chapter 1: Ancestral Group (P1; haplotypes B, C, D, E, L) and Others (haplotypes A, F, G, H, I, J, K, M, N, O), and unique haplotypes only seen in one area and season in each group (breeding and non-breeding season in Akkeshi, Hamanaka and Nemuro: six categories) were shown in darker colours (Figures 3-3 (a), 3-4 (a) ).

The proportion of each group and the number of unique haplotypes were compared among the three regions (Akkeshi, Hamanaka and Nemuro; Figure 3-1) during the breeding season.

Changes in the genetic characteristics between the breeding and non-breeding seasons were investigated by comparing the proportions of haplo-groups and the numbers of unique haplotypes in each area.

Tests of significance between the areas during the breeding season, and between seasons in each area, were carried out using Fisher's test with Bonferroni correction calculated by R software ver. 1.2.5033 (R Core Team 2019).

***Microsatellite***

DAPC was run using all samples in the R package Adegenet (Jombart 2008) for R software ver. 1.2.5033 (R Core Team 2019). DAPC is more suited to finding subpopulations, and can be used

for animals that have a “stepping-stone” pattern of gene flow. It finds genetically related individuals and maximises between-group variation while minimising variation within a group without making assumptions such as the type of population subdivision and model (Jombart et al. 2010). DAPC was therefore considered to be best suited for this study whose purpose is to identify the subpopulations of Japanese harbour seals in Eastern Hokkaido where breeding haul-out sites between Akkeshi, Hamanaka and Nemuro are only a maximum of about 30 km apart from each other.

To understand the genetic characteristics of eastern Hokkaido harbour seals in breeding areas during the breeding season, the number of clusters used for analysis was set to 4 (Akkeshi, Hamanaka, Nemuro, and Habomai), and evaluation of the value K was performed by looking at the trend in BIC, output by find.clusters, an R function. Habomai was added as a fourth area, as it is located near Nemuro (Figure 3-1) and migrations of seals from the Habomai Islands have been suggested based on biological data (Hokkaido 2006a; Yukiko et al. 2008). After individuals were assigned to clusters, the proportions of clusters in each area during the breeding season were compared.

Changes in the genetic characteristics between the breeding and non-breeding seasons were investigated by comparing the proportions of top clusters in each area.

Tests of significance between areas during the breeding season, and between seasons in each area, were carried out using Fisher's test with Bonferroni correction calculated by R software ver. 1.2.5033 (R Core Team 2019).

## Results

### *Types and numbers of microsatellite loci required for Individual identification of Japanese harbour seals*

Table 3-1 shows PID(obs)s, PID(theo)s and PID(sib)s calculated for each locus and overall PIDs for Erimo (n=48) and Eastern Hokkaido (n=146), listed from the lowest (at the top) to the highest (at the bottom). The relationships between the number of loci used and overall PIDs were indicated by line plot in Figure 3-2. PID(obs)s for each locus in both Erimo and Eastern Hokkaido were similar to PID(theo)s, rather than PID(sib)s, and trends of PID(obs) and PID(theo) overlapped (Figure 3-2). In Erimo, the overall PID(obs) became less than 0.01 when three loci (SGPV9, M11A, SGPV11) were used, and overall PID(theo) was less than 0.01 when four loci were used (the same three loci as PID(obs), plus Pvc78 (Table 3-1)). In Eastern Hokkaido, both PIDs also became less than 0.01 when four loci were used (M11A, Hg3.7, SGPV11, Pvc19) (Table 3-1). Out of four loci in each area, two were the same between the areas (M11A and SGPV11) and the other two loci were seen only once in each area (Table 3-1). On the other hand, in both areas, nine loci were needed (other than SGPV16 in Erimo and SGPV10 in Eastern Hokkaido) to obtain overall PID(sibs)s less than 0.01 (Figure 3-2, Table 3-1).

***Individual identification utilising non-invasive and biopsy samples***

The numbers of DNA extractions and genotyping successfully carried out from non-invasive samples collected in this study were as follows: faeces, 14 out of 16 samples; mouth swab, 11/11; biopsy (skin or hairs), 8/8; hairs, 5/23; part of placenta, 1/1; and blood drop, 1/1.

Individual identification was carried out based on the 40 successfully genotyped non-invasive (n=32) and biopsy (n=8) samples above, and flipper samples from those used in Chapters 1 and 2 (n=4). As a result, two samples from Hamanaka (a mouth swab from Hokake rock and a blood stain from Kenbokki Is.), and five samples also from Hamanaka (faeces from Kenbokki Is.) had the same genotypes (PID(theo)<0.01). Duplication of genotypes was also checked based on 10 microsatellite loci, and the same results were obtained.

After removing duplicate samples (1 from blood drop and 4 from faeces), a total of 163 samples were used for analysis, 64 for the breeding season (Akkeshi 27, Hamanaka 19, Nemuro 18) and 99 for the non-breeding season (Akkeshi 22, Hamanaka 27, Nemuro 50) (Table 3-2).

***Genetic characteristics of each breeding area***

***MtDNA***

All haplotypes found in this study matched previously known haplotypes of Japanese harbour seals deposited in GenBank by Mizuno et al. (2018) (accession numbers: LC314221-LC314236). The percentages of P1, which is thought to be an ancestral group in Japan, were 30% in Akkeshi, 33% in Hamanaka, 28% in Nemuro, and only Akkeshi had the unique haplotype of P1 (n=1) in the breeding season (Figure 3-3 (a)).

No significant differences were found in the proportion of lineages between the areas in the breeding season, and between seasons within each region ($p<0.05$).

***Microsatellite***

The K set to 4 was shown to be appropriate for this data, as the transition in BIC values between 1 and 30 clusters indicated a concave curve that showed a large decrease until K=4, and BIC started to increase from around K=10 (Appendix 3-1).

Appendix 3-2 (a) shows the population subdivision of harbour seals in Eastern Hokkaido when K was set to 4, defined by DAPC. The genetic relationships between the groups based on the

minimum spanning tree showed that all groups were close to cluster 4, and that cluster 1 was the closest to cluster 4 but diverged from clusters 2 and 3 (Appendix 3-2 (b)).

In the breeding season, the top clusters differed in all areas, which were cluster 2 in Akkeshi, cluster 3 in Hamanaka, and cluster 4 in Nemuro. The clusters that had the second largest percentage in each area were the top clusters in the area adjacent to each area that had clusters with the largest percentages (Figure 3-3 (b)), indicating a "stepping-stone" pattern of gene flow among Eastern Hokkaido harbour seals.

No significant differences were found in the proportions of clusters between the areas in the breeding season, and between seasons within each region ($p<0.05$).

***Comparisons of genetic characteristics between breeding and non-breeding seasons.***

***MtDNA***

The percentages of the "Others" category of mtDNA lineages increased from 70% in the breeding season to 82% in the non-breeding season in Akkeshi, from 72% in the breeding season to 84% in the non-breeding season in Nemuro, and in Hamanaka, the percentages were the same in the two seasons (67%). In addition, unique haplotypes belonging to the "Others" category of lineages, which were not seen in the breeding season, appeared only in the non-

breeding season in Hamanaka (n=1; 4%) and in Nemuro (n=3; 6%) (Figure 3-4 (a)). Unique haplotypes belonging to the P1 lineage were seen only during the non-breeding season in Nemuro (n=1; 2%) (Figure 3-4 (a)).

No significant differences were found in the proportions between the areas in the breeding season and between breeding and non-breeding seasons within each area ($p<0.05$).

***Microsatellite***

The top clusters in the breeding and non-breeding seasons differed in all areas. Also, for the non-breeding season, the clusters that had the highest percentage differed between Akkeshi, and Hamanaka, and Nemuro, and their percentages were much larger than those of the breeding season (Figure 3-4 (b)).

In Akkeshi, the top cluster in the non-breeding season was cluster 4, which had the largest percentage in Nemuro during the breeding season. The percentage of cluster 4 increased from 29.0% to 50.4% between the breeding and non-breeding season. In Hamanaka and Nemuro, on the other hand, the top cluster during the non-breeding season was cluster 1, which did not have large percentages in any of the areas during the breeding season, and their percentages increased from 22.2% to 42.6% in Hamanaka and from 17.9% to 31.9% in Nemuro.

No significant differences were found in proportions between regions in the breeding season, and between seasons within each area ($p<0.05$).

## Discussion

### *Individual identification of Japanese harbour seals based on microsatellite loci*

The overall heterozygosities calculated from 10 microsatellite loci were nearly the same in Erimo (He=0.472) and Eastern Hokkaido (He=0.431-0.462) (Table 2-1). However, two out of four microsatellite loci best suited for individual identification (PID(theo)<0.01) in each area differed between Erimo (SGPV9 and Pvc78) and Eastern Hokkaido (Hg3.7 and Pvc19) (Table 3-1), while the other two loci were the same, indicating microsatellite loci should be chosen depending on the region.

Previous studies based on wild populations of brown bear (*Ursus arctos*) (Waits and Leberg 2000), grey wolf (*Canis lupus*) (Forbes and Boyd 1997), and Northern hairy-nosed wombat (*Lasiorinyus krefftii*) (Taylor et al. 1994) indicated that PID(obs) lies between PID(theo) as the upper boundary and PID(sibs) as the lower boundary. These animals form a family pack structure (grey wolf), have a long parenting period (1-2 years in brown bear), or have experienced a severe population bottleneck (Northern hairy-nosed wombat). This may result in the sampling of a large proportion of closely related animals, which violates the assumption of the Hardy-Weinberg equilibrium, and the use of PID(sib) was therefore recommended (Waits and Paetkau 2005). However, this study showed that PID(obs) matches PID(theo), indicating that PID(theo) was sufficiently adequate for identifying individuals in Erimo and Eastern

Hokkaido. Since harbour seals do not form groups, they may haul out in a group irrespective of relatedness (Schaeff et al. 1999), mother and pup become separated after the nursing period of a relatively short period of time (4-6 weeks (Niizuma 1986)), and PID(obs) may match PID(theo) rather than PID(sib) in Erimo and Eastern Hokkaido harbour seals. In addition, only four loci were needed to carry out individual identification for both Erimo and Eastern Hokkaido harbour seals (PID(theo)<0.01) compared to nine loci required if there was a bias in samples (PID(sib)<0.01), indicating that individual identification could be carried out at a much lower cost than in animals that have a biology similar to harbour seals.

***Genetic characteristics of each breeding area.***

Genetic characteristics in each breeding area during the breeding season in Eastern Hokkaido were more obvious in microsatellite loci than in mtDNA. The population substructures based on mtDNA showed similar trends, while microsatellite loci showed different trends in three breeding areas during the breeding season, although there were no significant differences in the proportions within and between the regions. The different trends seen in mtDNA and microsatellite are possibly due to differences in the mutation rate, which is faster in microsatellite than in mtDNA. The differences in the mutation rate may indicate that historically seals entered Eastern Hokkaido relatively recently.

The clusters defined based on microsatellite data showed that the top clusters in all three areas were different, and that the second highest clusters in each area were the top clusters in their areas next to those with the top clusters, suggesting a “stepping-stone” gene flow. This further suggests that genetic differentiation between different breeding areas started to form due to strong natal site fidelities in the harbour seals of Eastern Hokkaido.

***Comparisons of genetic characteristics between breeding and non-breeding seasons***

In the non-breeding season, the percentages of the “Others” mtDNA lineage increased during the non-breeding season, and seals with unique haplotypes and belonged to the “Others” lineage were only seen in Hamanaka (n=1) and Nemuro (n=3). It was shown in Chapter 1 that the “Others” lineage was close to that of seals in the northern Pacific, such as at Bristol Bay in Alaska (Figures 1-2, 1-3; Appendices 1-1, 1-2). Because seals with unique haplotypes belonging to the “Others” lineage were not seen during the breeding season, they could be coming from outside Eastern Hokkaido.

All three areas in Eastern Hokkaido that showed different microsatellite clusters have the highest percentages in the breeding and non-breeding seasons. In Akkeshi, the cluster that had the highest percentage in the non-breeding season was the top cluster in Nemuro during the breeding season, indicating there are possibly movements of seals from east to west, i.e. from Nemuro to Akkeshi. Cluster 1, on the other hand, whose proportion increased suddenly during

the non-breeding season in Hamanaka and Nemuro, was not the top cluster in any of the three regions during the breeding season, and the genetic relationship of this cluster was closest to the top cluster in Nemuro in the breeding season, which suggests that seals are coming from outside Eastern Hokkaido, possibly from further north of Nemuro (Appendix 3-2).

Both mtDNA and microsatellite loci suggested that there are seasonal movements of seals from northeast of Nemuro where Habomai, a group of several small islands, is located (Figure 3-1). A relatively large breeding colony (>900) existed in Habomai, and seals from there have been suggested to be migrating to Eastern Hokkaido in autumn to forage (Hayama et al. 1986). In addition, there are set nets in Nemuro that are located next to Habomai (Wada et al. 1986), and the number of seals caught here as bycatch is much larger than the number of seals observed at haul-out sites in Nemuro (Wada et al. 1986; Kobayashi et al. 2014). Seals tagged in Habomai have also been found at Daikoku Is. (Hokkaido 2006b). The unique haplotypes seen, and the increase in clusters during the non-breeding season, may reflect the genetic characteristics of harbour seals in Habomai.

The combination of genetic data based on both mtDNA and microsatellite loci indicates that the seals in Eastern Hokkaido arrived relatively recently from the historical perspective and that not enough time had passed to form lineage differences between the areas. Microsatellite analyses, however, suggest that there are genetic characteristics in each breeding area during

the breeding season, which would indicate that Japanese harbour seals have strong natal site fidelities and further suggests a possible seasonal movement of seals between Habomai and Eastern Hokkaido. Such movement is usually shown by biological data, and sample numbers to obtain this data can often be limited. This study is therefore the first to show movements of seals based on genetic data.

## General Discussion

In this study, the divergence history, current population genetics, and genetic characteristics of Japanese harbour seals that breed in each area were investigated for the first time using genetic data.

Phylogenetic relationships based on mtDNA data indicated that the Japanese harbour seal had at least two lineages, one comprising only Japanese harbour seals and the other close to that of harbour seals in the North Pacific, such as at Bristol Bay in Alaska and the Commander Islands in Russia, and further analysis suggested that these two lineages entered Japan at different times. Harbour seals were inhabiting Japan by the Pleistocene period, as fossils dating back 0.1 million years were found at the Shimokita peninsula, south of Erimo. During the Last Glacial Maximum 0.07–0.01 million years ago, sea ice extended as far south as Erimo. Since harbour seals avoid sea ice, they were likely isolated during this time, which may have caused genetic differentiation in the lineage that contains only Japanese haplotypes. After the disappearance of the sea ice, the seals isolated in Erimo extended their range toward the north, while seals inhabiting the North Pacific entered Japan and extended their range toward the south, resulting in the existence of two different lineages in Japan.

Even though the haul-out sites in Erimo and Eastern Hokkaido are only 150 km apart and seals can easily move since there are no obvious geographical barriers between them, the

population genetic study based on microsatellite markers also indicated that seals in Erimo and Eastern Hokkaido were genetically distinct and hardly moved between the two areas. This result was also supported by satellite tracking data. Ecological characteristics uniquely seen in Japanese harbour seals may be the reason why these seals in Japan do not move between Erimo and Eastern Hokkaido. Although seals are generally known to haul out on various habitats such as intertidal mudflats, sandbars, rocks, reefs and even ice floes, as well as artefacts such as floats and log booms (Jeffries et al. 2000)(Jeffries et al. 2000)(Jeffries et al. 2000)(Jeffries et al. 2000)(Jeffries et al. 2000)(Jeffries et al. 2000)(Jeffries et al. 2000)(Jeffries et al. 2000)(Jeffries et al. 2000)(Jeffries et al. 2000)(Jeffries et al. 2000), in Japan, harbour seals haul out only on rocky reefs, and the numbers of seals and haul-out sites in Japan are much smaller than in other regions. At Erimo, there are numerous rocky reefs at a single haul-out site that stretch up to 1.3 km offshore from land, while 10 haul-out sites in Eastern Hokkaido are interspersed along 75 km of the coast. The direction in which the rocky reefs stretch away from land or in which the haul-out sites are facing may be limiting the movement of Japanese harbour seals and causing strong philopatry in Erimo and Eastern Hokkaido. Furthermore, no recent genetic bottlenecks were detected, although the seals in Erimo and Eastern Hokkaido were reported to have experienced declines in population size over the past few decades, suggesting that the population bottleneck was not large enough to affect the genetic diversity

of Japanese harbour seals.

Genetic characteristics between breeding grounds based on mtDNA suggested that the seals in Eastern Hokkaido entered the area relatively recently in the historical time scale and not enough time had passed to cause genetic differences between the breeding areas, whereas the genetic characteristics based on microsatellite loci, which mutate more easily, showed different genetic characteristics in each area, indicating that Japanese harbour seals have strong natal site fidelity. Comparisons of genetic characteristics between breeding and non-breeding seasons based on both mtDNA and microsatellite loci, on the other hand, showed that each area was dominated by seals with different genetic characteristics during the non-breeding season. Since the migration of seals between the Habomai Islands and Eastern Hokkaido has been suggested in the past, the genetic characteristics only seen in the non-breeding season may be indicating that the seals are from the Habomai Islands.

In this study, the phylo-geographical position of Japanese harbour seals was described, and the animals in Erimo were shown to have characteristics that are unique in the Pacific. Moreover, the analyses of genetic data based on the ecological data of harbour seals, which divided samples into different seasons, provided an understanding of the genetic characteristics of breeding populations, which also indicates the natal site fidelity of Japanese harbour seals and the migration of seals from different areas, outside Hokkaido. These findings

will play an important role in the future management of Japanese harbour seals.

## Summary

The harbour seal (*Phoca vitulina*) is a semi-aquatic mammal that mates and forages under water but also hauls out onto land to rest, give birth, rear their pup (breed), and moult. Harbour seals are widely distributed across the northern hemisphere, and its distribution range is the widest among the pinnipeds. Although the number and division of subspecies are still a subject of debate, at least four subspecies are known: two are found in the Atlantic – *Phoca vitulina concolor* in the western Atlantic and *Phoca vitulina vitulina* in the eastern Atlantic, and the other two are in the Pacific – *Phoca vitulina stejnegeri* in the western Pacific and *Phoca vitulina richardsi* in the eastern Pacific.

The Japanese harbour seal is the southernmost population of *P. v. stejnegeri*, and they are found only on the Pacific side of Hokkaido where sea ice rarely comes in winter. A total of 11 haul-out sites are known in this area: Erimo (1 site), Akkeshi (4 sites), Hamanaka (4 sites), and Nemuro (2 sites). Erimo is located at the southernmost habitat range in Hokkaido, 150 km away from the nearest haul-out site. The other haul-out sites are interspersed over 75 km between Akkeshi and Nemuro (Eastern Hokkaido).

Japanese harbour seals only haul out on rocky reefs to rest, breed and moult, although the seals in other regions of the world are known to haul out in a variety of habitats such as intertidal mudflats, sandbars, rocks, reefs and ice floes, as well as artefacts such as floats and

log booms. Mature seals in Japan are also reported to be larger and show more sexual dimorphism in body size than the same subspecies in the Kuril Islands. In addition, Japanese harbour seals have a higher proportion of “dark phase” pelage (a black or nearly black background with light spots or rings) than “light phase” pelage (a light background colour with dark spots or blotches). The proportion of seals with “dark phase” pelage in Japan is the highest in the Pacific.

Harbour seals are reported to haul out on the same sites during the breeding season in successive years, and a long-term study showed that both adult males and females tend to use their natal sites or sites close to their birthplace during the breeding period, although stronger site fidelity is known in females. In fact, harbour seals are known to show a “stepping-stone” pattern of gene flow in studies based on both maternally inherited mitochondrial DNA (mtDNA) and bi-parentally inherited nuclear microsatellite (MS) loci, where geographically closer groups show genetic similarity.

MtDNA is maternally inherited DNA in mammals and is especially suited to phylogenetic studies as it lacks recombination and historical genealogical records are not mixed between different lineages during meiosis. Past phylogenetic studies based on the control region of mtDNA for harbour seals suggested different scenarios in how the seals extended their distribution range in the Pacific, and the phylogenetic position of Japanese harbour seals is still

unknown. A phylogenetic study that was carried out using only Japanese samples, on the other hand, suggested that there were two lineages, but that study used a different region of mtDNA (cytochrome b) and comparison was not possible.

Unlike mtDNA, MS is inherited from both males and females, and MS is often used in population genetic studies, as they are high in both polymorphism and rate of mutation, and have thus been more suitable for genealogical studies in recent years. In addition, comparisons between mtDNA and microsatellite markers are often used to understand sex differences in gene flow, as they have different modes of inheritance. In mammals, males generally show a wider dispersion range and higher gene flow. In harbour seals, movement over longer distances and gene flow in males have also been reported in some areas (e.g. the western Pacific), however the tendency can vary depending on the historical background, and seals isolated during the Last Glacial Maximum showed the same population subdivision for both mtDNA and MS. Furthermore, Japanese harbour seals experienced population fluctuation over the past few decades. The population size of harbour seals in Japan was severely depleted in the 1970s, due to extensive hunting and destruction of haul-out sites. After the seals were assessed as an endangered species and protected in 1998, their population gradually recovered, and in 2015 they were downlisted as a semi-endangered species. This recent population decline may be affecting the genetic diversity of Japanese harbour seals. However,

no studies based on MS have yet been conducted on Japanese harbour seals, and comparisons of genetic data with mtDNA are not possible.

Since harbour seals tend to use haul-out sites where they were born, comparisons of genetic data based on samples taken from each breeding site during the breeding season may show the genetic characteristics of each breeding population. Also, the seasonal movement between haul-out sites can be visualised by comparing genetic data between breeding and non-breeding seasons. However, no studies have yet sought to find the substructure of harbour seals based on such data.

In this study, samples from major breeding haul-out sites in Japan have been taken and similar sample sizes have been used with the aim to 1) understand the divergence history of Japanese harbour seals and its relationship to other locations around the world using the control region of mtDNA, 2) investigate the effects of the recent population decline in Japanese harbour seals and understand the population genetics using MS, and finally to 3) define the genetic characteristics of Japanese harbour seals at each breeding ground during the breeding season, and use both mtDNA and MS markers to investigate seasonal migration by comparing genetic data with that of the non-breeding season.

**Chapter 1. Phylogenetic study of Japanese harbour seals using mtDNA**

Past phylogenetic studies based on the control region of mtDNA for harbour seals suggested different scenarios in how the seals extended their distribution range in the Pacific, that the seals dispersed from west to east, east to west, or entered west and east at the same time, and Japanese harbour seals were treated as either a basal (ancestral) or a non-basal (descendent) population in the Pacific. These studies treated Japanese harbour seals as one lineage, and sample sizes of Japanese harbour seals were small ($n<14$). On the other hand, a phylogenetic study that was conducted using only Japanese samples and the cytochrome b region of mtDNA suggested there were two lineages, indicating that the different perspectives in the previous studies may have been the result of their handling Japanese harbour seals as a single lineage.

The aim of this study has been to reach a conclusion concerning the divergence history of Japanese harbour seals and to identify the phylogenetic relationship between the seals in Japan and other locations in the world using a larger number of samples and the control region of mtDNA so that comparisons of genetic data with previous studies would be possible. As a result, both the phylogenetic tree and haplotype network showed that there were at least two lineages in Japanese harbour seals. In addition, the mismatch analysis suggested a secondary contact of populations after a long isolation, and an increase in the population range over time and space after the original population had been restricted to a very small area. Moreover, one of the lineages was produced only by Japanese harbour seals, and the proportion of this

lineage was the highest at Erimo, the southernmost distribution range of western Pacific harbour seals, and the lineage gradually decreased toward the northeast in Hokkaido. The other lineage included haplotypes from Japan and the North Pacific, suggesting that harbour seals in these two areas were closely related. Because fossils of harbour seals dating back 0.1 million years were found at the Shimokita peninsula, Aomori, before Last Glacial Maximum (GLM; 0.02 million years ago) and seasonal sea ice at this time was extending its southward range in the Pacific to as far as Erimo in Hokkaido, Japan, it is possible that the first seals to come to Hokkaido became isolated due to the geographical barrier produced by sea ice, and then, after the disappearance of the sea ice, gradually extended their range in Hokkaido from the southwest toward the northeast along the Pacific coast, while seals that were not in the original Japanese group extended their range to Japan from the North Pacific. The genetic distance between Erimo and Eastern Hokkaido also showed that these two populations were very distinct, suggesting that historical isolation was still having an effect on Japanese harbour seals.

**Chapter 2. Current population genetics of Japanese harbour seals**

In Chapter 1, a phylogenetic study based on mtDNA showed that there were two populations and lineages in Japanese harbour seals. Compared to mtDNA, MS has higher genetic diversity and is better suited to studying recent population genetics, however studies based on MS have

not yet been conducted on Japanese harbour seals. The population size of harbour seals in both Erimo and Eastern Hokkaido declined in the 1960s and 1970s, from 300 to 128 in Erimo and from 600 to 216 in Eastern Hokkaido, and by using MS, it is possible to evaluate the effect that population decline had on genetic diversity.

In Chapter 2, 10 MS markers (Pvc19, Pvc78, Pvc30, SGPV16, SGPV11, SGPV10, Hg3.7, Aa4, SGPV9, and M11A) were used for the first time to investigate the recent population genetic structure of Japanese harbour seals. As a result, genetic diversity across both Erimo and Eastern Hokkaido were almost the same. Both Fst and Rst, and STRUCTURE analysis, showed that Japanese harbour seal populations in Erimo and Eastern Hokkaido were very distinct, which supports a previous study using mtDNA. However, Erimo and Eastern Hokkaido are only 150 km apart and have no obvious geographical barriers between them. Strong population subdivision between the two regions may thus be due to the small number of haul-out sites and population size, and that these seals in Japan use a single habitat type for their haul-out sites. At Erimo, there are numerous rocky reefs at a single haul-out site that stretch up to 1.3 km offshore from land, while 10 haul-out sites in Eastern Hokkaido are interspersed along 75 km of the coast. The direction in which the rocky reefs stretch away from land or in which the haul-out sites are facing may also be limiting the movement of Japanese harbour seals and causing strong population subdivision between Erimo and Eastern Hokkaido. No recent genetic

bottlenecks were detected, although the seals in Erimo and Eastern Hokkaido were reported to have experienced declines in population size over the past few decades, suggesting that any population bottleneck was not large enough to affect the genetic diversity of Japanese harbour seals.

**Chapter 3. Genetic characteristics of Japanese harbour seals during the breeding season across breeding areas in Eastern Hokkaido.**

Chapters 1 and 2 focused on the division of Japanese harbour seals in two populations based on both mtDNA and MS. The major breeding grounds in Eastern Hokkaido are found in Akkeshi, Hamanaka, and Nemuro, and the breeding sites are about 30 km apart. Because harbour seals are known to use the same breeding site in successive years, seals at each breeding ground during the breeding season may have unique genetic characteristics.

In this study, previously used data and collected samples were separated into breeding and non-breeding seasons. In addition, samples were collected from breeding grounds in Eastern Hokkaido during the breeding season, and the genetic characteristics of each breeding area were defined to examine the natal site fidelity of Japanese harbour seals. After that, seasonal movements between the areas were investigated by comparing the genetic characteristics between breeding and non-breeding seasons. As a result, genetic characteristics based on mtDNA showed no trends in the proportion of haplotypes belonging to the lineages of the two

regions, one in Japan and the other close to the North Pacific. On the other hand, genetic characteristics of breeding populations based on MS showed that the clusters which had the highest and the second highest proportions in all three areas (Akkeshi, Hamanaka and Nemuro) were different, but there were overlaps in the top two clusters between adjacent areas, suggesting a “stepping-stone” pattern of gene flow. Harbour seals in Eastern Hokkaido thus probably entered the area relatively recently from an historical perspective and not enough time had passed to produce differences in mtDNA genealogies between the breeding areas, and Japanese harbour seals also have strong natal site fidelity. In the non-breeding season, the largest number of unique haplotypes belonging to the lineage close to that of the North Pacific was seen in Nemuro, followed by neighbouring Hamanaka. MS also showed different trends between the areas and seasons. From the breeding to the non-breeding season, Akkeshi showed an increase in the proportion of a cluster that had the highest percentage in Nemuro in the breeding season, whereas Hamanaka and Nemuro both showed increases in the proportions of the same cluster in the non-breeding season, which was not high in any regions during the breeding season. These results, based on both mtDNA and MS, suggest that individuals could be migrating from areas outside Eastern Hokkaido. The Habomai Islands, located close to Nemuro and home to large breeding sites (>900 seals), and movements of seals from the Habomai Islands to Eastern Hokkaido have been suggested in the past. This may

explain the presence of haplotypes that are only seen during the non-breeding season in Hamanaka and Nemuro, and the cluster whose proportion increased during the non-breeding season could be the result of the genetic characteristics of harbour seals from the Habomai Islands.

**General Discussion**

In this study on Japanese harbour seals, the divergence history, current population genetics, and the genetic characteristics of seals that breed in each area were clarified for the first time.

MtDNA data indicated that the Japanese harbour seal has two lineages, one that was isolated during the LGM when seasonal sea ice extended as far south as Erimo, and another lineage that entered Japan from the north, after the disappearance of the sea ice, and the proportions of these two lineages in seals at haul-out sites in Erimo and Eastern Hokkaido are different. MS analysis also indicated that seals in Erimo and Eastern Hokkaido are genetically distinct, and that the seals rarely move between the two areas. Ecological characteristics uniquely seen in Japanese harbour seals may be the reason why there is not much gene flow between Erimo and Eastern Hokkaido, even though the two areas are only 150 km apart, have no obvious geographical barriers between them, and could easily be navigated by harbour seals. In Japan, harbour seals only haul out on rocky reefs, and the numbers of seals and haul-out sites are much smaller than other regions of the world. Also, the direction in which the rocky reefs

stretch away from land or in which the haul-out sites are facing may be limiting the movement of Japanese harbour seals and causing strong philopatry in Erimo and Eastern Hokkaido. Furthermore, no recent genetic bottlenecks were detected, although the seals in Erimo and Eastern Hokkaido were reported to have experienced declines in population size over the past few decades. Based on mtDNA, genetic characteristics between breeding grounds during the breeding season did not differ, while the same comparison based on MS showed that, although the trend was not obvious, the genetic characteristics of Japanese harbour seals between breeding populations were different, and there was a “stepping-stone” pattern of gene flow. The above suggests that the seals in Eastern Hokkaido entered the area relatively recently in historical terms and that not enough time had passed since then to produce genetic differences in mtDNA between the breeding areas, whereas the genetic characteristics based on MS, where mutation occurs more easily, indicate that Japanese harbour seals have strong natal site fidelity. Comparisons of genetic characteristics between breeding and non-breeding seasons based on both mtDNA and MS, on the other hand, showed that each area was dominated by seals with different genetic characteristics during the non-breeding season, and these are the seals possibly coming from the Habomai Islands.

This study has clarified the phylogeographical position of Japanese harbour seals, and described the seals in Erimo that have characteristics unique to the Pacific region. In addition,

analyses of genetic data based on the ecological data of harbour seals, samples of which were divided into different seasons, provided an understanding of the genetic characteristics of breeding populations, and also indicated the natal site fidelity of Japanese harbour seals and the migration of seals from different areas, outside Hokkaido. These findings will play an important role in the future management of Japanese harbour seals.

## Japanese Summary

ハーバーシール (*Phoca vitulina*) は採餌および交尾は水中で行うが，休息，出産・子育て (繁殖)，換毛を上陸場で行う半水生の哺乳類であり，北半球に広く生息する．分布域は鰭脚類の中で最大であり，亜種の数は，研究者により見解は異なるが，少なくとも太平洋および大西洋にそれぞれ 2 亜種ずつの計 4 亜種 (西太平洋：*P. v. stejengeri*，東太平洋: *P. v. richardii*，　西大西洋: *P. v. concolor*，東大西洋: *P. v. vitulina*) が知られている．北海道は西太平洋亜種 (*P. v. stejnegeri*) の生息域の最南端であり，上陸場はえりもに 1 ヵ所，厚岸に 4 ヵ所，浜中に 4 ヵ所，根室に 2 ヵ所の計 11 ヵ所が知られている．最も離れた上陸場間の距離は，えりもと厚岸の 150km で，その他の上陸場は北海道東部地域 (以下道東) の 75km 範囲に 10 ヵ所が点在する．

一般にハーバーシールは，干潟，砂州，岩，岩礁，流氷などの自然物から，ブイや丸太などの人工物など様々な場所に上陸することが知られているが，日本では岩礁のみを上陸場として利用し，そこで休息，繁殖，換毛を行う．太平洋の中でも，西太平洋亜種は，オスとメスで体長が異なり，比較的大型であることが知られているが，日本のハーバーシールの成獣はさらに体長が大きく，千島列島に生息する同亜種を上回ることが示唆されている．また，日本のハーバーシールの毛色は暗色型（黒もしくは黒っぽい背景に明るい点か輪状の模様）のほうが，明色型（明るい背景に暗い色の点や輪の模様）よりも割合が高く，暗色型の比率は太平洋で最大である．

ハーバーシールは毎年繁殖期に同じ上陸場を利用し，オスメスともに生まれた上陸場，もしくは生まれた上陸場に近い上陸場を繁殖期に利用することが示されている．また，遺伝子研究でも，母系遺伝するミトコンドリア DNA (mtDNA)と両親から遺伝するマイクロサテライト (MS) DNA 両方で，飛び石状の遺伝子流動を示し，地理的に近いグループは遺伝的により近い関係にある．

MtDNA は哺乳類で母系遺伝し，減数分裂の際に遺伝子同士の組み換えが起こらないため系統学の研究に適している．過去の mtDNA の調節領域を使用した系統学の研究では，ハーバーシールがベーリング海峡を通り太平洋に移入後，どのように分布を広げたかで異なる仮説が混在しており，太平洋における日本産ハーバーシールの分岐背景は明確にされていない．一方，日本のサンプルのみ使用した系統解析では，日本には 2 つの系統があることを示していたが，mtDNA の異なる領域を使用していたため，他地域との比較が出来ていない．

MS は，両親から遺伝し，置換速度が速く，多様性も高いため近年の集団遺伝研究に頻繁に使われてきた．また，哺乳類では一般的にオスがメスよりも広く分散し，遺伝子流動もオスで高いため，MS と mtDNA の 2 つの遺伝子マーカーを比較することで，遺伝子流動におけるオスとメスの違いを見ることができる．ハーバーシールでもオスで高い遺伝子流動を示す地域がある一方，最終氷期に集団が孤立したと考えられる集団 (東太平洋ハーバーシール) では，mtDNA と MS で同様の集団分けが示され，歴史的背景が現

代の集団遺伝に影響することが示唆されている．さらに，日本のハーバーシールは 1970 年代に個体数が減少し，1998 年に環境省の絶滅危惧種に指定され保護が始まると個体数は徐々に増加し，2015 年には準絶滅危惧種にダウンリストされている．このことから日本に生息するハーバーシールは個体数の増減の影響が遺伝的多様性に影響している可能性もある．しかしながら，現在まで日本の集団において MS を使用した研究はされておらず，mtDNA との比較ができず，集団遺伝や近年の個体数減少の影響も不明のままである．

ハーバーシールは毎年自らが出生した上陸場を繁殖上陸場として利用する傾向があり，繁殖期に，繁殖上陸場を利用する集団ごとで，それぞれ異なる遺伝的特徴を持つことが考えられる．そのため，mtDNA と MS で繁殖期の繁殖上陸場ごとの遺伝的特徴を把握することで，ハーバーシールの繁殖期の繁殖上陸場に対する依存性の高さが分かり，さらに，非繁殖期と比較することで季節移動を推定できる可能性がある．しかし，ハーバーシールの生態情報を加味し，遺伝的特徴を季節で比較した研究報告はない．

そこで本研究では，日本の主要繁殖上陸場からサンプルを収集し，地域ごとのサンプル数をそろえた上で，(1) mtDNA の調節領域を使用して，他地域に生息するハーバーシールとの系統比較を行い日本産ハーバーシールの歴史的分岐背景を明らかにし (2) MS で，近年の集団遺伝および個体数変動の影響を検証，さらに (3) mtDNA および MS によ

り，繁殖期の繁殖上陸場ごとの遺伝的特徴を把握し，非繁殖期と比較することで季節的な個体の移入を検証した.

**第 1 章　mtDNA による日本産ハーバーシールの分子系統**

これまでの mtDNA の調節領域を使用した系統学の研究では，ハーバーシールが太平洋に移入後どのように分布を広げたかについて，日本を太平洋における初期定着集団として西から東へ分布を広げた説，日本とワシントンの集団両方を初期定着集団として西と東両方に同時に移入した説，もしくは日本を後期定着集団として東から西へ分布を広げた説の 3 つの異なる説があった．これらの研究では，日本のハーバーシールを太平洋で祖先的，もしくは派生的のいずれか 1 つの系統として扱っており，サンプル数も少なかった (n<14)．一方，日本のサンプルのみで，mtDNA のシトクロム b 領域を使用した系統解析では，日本には 2 つの系統があることを示していたが．調節領域を使用した他地域との比較はできていない.

そこで第 1 章では他地域の研究と比較できるように，mtDNA の調節領域 (454bp) を使用して，日本のハーバーシール (*P. v. stejnegeri*) の分岐の歴史および，他地域に生息するハーバーシールとの系統関係を明らかにすることを目的として解析を行った. 系統樹およびハプロタイプネットワーク図ともに，日本のハーバーシールには少なくとも 2 つの系統が存在することを示し，これらの系統は長期間の孤立を経験した後，別々の時期に日本に移入してきたことがミスマッチ分析で示唆された．さらに，その 1 つの系統

は日本個体のみで構成されており (P1: 日本固有型), 日本の最南端に位置するえりもでその個体の割合が最も高く，根室に向かうにつれ徐々に減少し，もう 1 つのグループのハプロタイプはアラスカのブリストルベイやコマンドル島などの北太平洋の集団と系統的に近い関係を示した (Other: 北部由来型). ハーバーシールの化石が下北半島の最終氷期前の地層から出土したこと, また最終氷期は季節的海氷がえりも付近まで南下していたと考えられることから，日本固有型は初期に日本に定着した後，最終氷期に季節的海氷により孤立し (初期定着集団)，その後海氷が消失するともう 1 つの系統が北部から日本に移入してきたと考えられた (後期定着集団). 遺伝的分化係数でも，えりもと道東は 2 つの集団に分かれることを示し, 遺伝的多様度も 2 地域で異なる傾向を示したことから，過去の孤立が現代でも強く影響していると考えられた.

**第 2 章　日本に生息するハーバーシールの近年の集団遺伝**

第 1 章では，mtDNA から，日本のハーバーシールには 2 つの系統があり，えりもと道東は遺伝的に分化していることが示された. 一方，MS は mtDNA と比較して多様性が高く，近年の集団遺伝を示すのに適しているが日本のハーバーシールでは MS を用いた研究は行われていない. 日本のハーバーシールは 1940 年代にはえりもで 300 頭および道東で少なくとも 600 頭ほどいた個体数が 1970 年代に激減 (128 頭以下と 216 頭以下) したと言われているため，MS を使用することで，個体数減少の遺伝的多様性への影響の評価が行える可能性がある.

そこで第 2 章では，MS 10 遺伝子座 (Pvc19，Pvc78，Pvc30，SGPV16，SGPV11， SGPV10，Hg3.7，Aa4，SGPV9，M11A) を使用し，日本に生息するハーバーシールの近年の集団遺伝を把握することを目的とした．結果，遺伝的多様度は両地域で同程度だったが，遺伝的分化係数および STRUCTURE 解析による集団数の推定では，日本のハーバーシールは，mtDNA を使用した場合と同様，えりもと道東の 2 地域で遺伝的に異なることが示された．しかしながらえりもと道東は 150 ㎞しか離れておらず，これらの地域間に地理的分断を起こすような障害物はない．このような 2 地域で強い遺伝的分化を示したことは，日本におけるハーバーシールの上陸場の数および個体数は他地域と比較して少ないこと，上陸場は岩礁のみであることが要因であると考えられた．また，えりもでは，上陸場はいくつかの細かい岩礁が 1.3 ㎞にわたって連続して沖に向かって伸びているのに対し，道東では 75 ㎞範囲の沿岸に 10 の上陸場が点在していることも，日本に生息しているハーバーシールをえりもと道東で遺伝的に強く分化させたと考えられた．さらにボトルネック解析により近年の個体数減少は，遺伝的ボトルネックを起していないと判断されたことから，1970 年代のえりもおよび道東の個体数減少は，遺伝的多様性に影響するほど大きくなかった可能性が示された．

**第 3 章　繁殖期および非繁殖期における北海道道部のハーバーシールの遺伝的特徴**

第 1 章，第 2 章では，mtDNA および MS で，日本のハーバーシールはえりもと道東の 2 集団に分けられることが明らかになった．道東の主要繁殖上陸場は，厚岸，浜中，根

室それぞれで約 30km ずつ離れた場所にある．ハーバーシールは毎年繁殖期に同じ繁殖上陸場を利用することが知られているため，繁殖期に繁殖上陸場を利用する集団ごとに，それぞれで遺伝的特徴があると推察される．

そこで，第 3 章では，繁殖期に道東の主要繁殖上陸場からサンプルを集め，MS および mtDNA 両マーカーを使用して，繁殖期・繁殖上陸場ごとに遺伝的特徴を把握し，ハーバーシールの繁殖上陸場への依存性の高さを検証した．さらに，繁殖期と非繁殖期でそれらの遺伝的特徴を比較することにより，季節移動の有無も検証した．結果，繁殖期で見られた mtDNA ハプロタイプの系統 (初期定着集団および後期定着集団) の割合は地域間であまり変化はなかったが，MS をクラスター解析した結果，厚岸，浜中，根室はそれぞれ上位 2 つのクラスターの組み合わせが異なり，また，隣り合う地域同士で遺伝的特徴が類似しており，飛び石状の遺伝子流動を示した．このことから，歴史的には道東にアザラシが移入してから繁殖集団間で mtDNA の遺伝的特徴に違いが生じるような長い時間が経過していないが，mtDNA と比較して変異が起こりやすい MS で地域毎に遺伝的特徴が見られたことから，日本のハーバーシールは繁殖期に自分が出生した繁殖上陸場への依存度が高いことを示した．非繁殖期には，mtDNA で繁殖期には見られなかった，後期定着集団の特異的ハプロタイプを持つ個体が根室・浜中の順で多く見られ，さらに MS で，厚岸で，非繁殖期に根室に多かったクラスターが増加し，浜中と根室では繁殖期にはどの地域でも優勢でなかったクラスターの増加が見られたことから，他地

域からの季節移動が示された．根室のすぐ東に位置する歯舞群島には，比較的大規模の繁殖上陸場 (>900 頭) があり，過去に歯舞群島から道東への個体の移入が示唆されていることから，浜中と根室で非繁殖期にのみに見られた mtDNA の後期定着集団のハプロタイプおよび，MS で非繁殖期のみで割合が増加したクラスターは歯舞群島の遺伝的特徴を示している可能性が考えられた．

総合考察

本研究で初めて遺伝的データを元に日本のハーバーシールの分岐背景，現代の集団遺伝，繁殖期の繁殖上陸場ごとの遺伝的特徴を，多面的に理解することが出来た．

MtDNA データから，日本には最終氷期に海氷が南下してきた際に分断を受けたと考えられる初期定着集団と，海氷が消失後，北から移入してきた後期定着集団が存在し，それらの割合はえりもと道東で大きく異なっていることが示された．さらに MS を用いた解析でも，えりもと道東は遺伝的に大きく分化していることが示され，現代でも 2 地域間ではほとんど移動がないことが示された．ハーバーシールにとって，えりもと道東間の 150km は容易に移動できる距離であり，障害物がないにもかかわらず，これら地域間で遺伝子流動がほとんどないことは，日本のハーバーシール特有の生態的特徴が考えられた．日本でハーバーシールは，岩礁のみを上陸場として利用し，上陸場の数と個体数は他地域に比べて少なく，えりもと道東で上陸場の広がる方向が異なることもより定着性を強めている要因と考えられた．さらに，両地域とも 1970 年代に個体数の減少

を経験したと言われているが，MS からはその影響は見られなかった．また，mtDNA および MS を用いて繁殖期に繁殖上陸場ごとに遺伝的特徴を比較してみると，mtDNA では繁殖集団ごとに 2 つの系統の割合に違いが見られなかったが，MS では，傾向は明白でないものの，遺伝的特徴は異なる傾向を示し，近い地域間で飛び石状の遺伝子流動が見られたことから，道東にハーバーシールが移入してから，繁殖集団ごとに mtDNA に系統的な違いが出るほど歴史的には時間が経過していないこと，日本のハーバーシールは繁殖期に出生した上陸場を利用する依存度が高いことが示唆された．また，非繁殖期には，mtDNA および MS 両方で繁殖場を利用している個体とは異なる遺伝的特徴を持つ個体が各海域を利用していることが示され，それらは歯舞群島の個体である可能性を示した．

本研究では初めて日本のハーバーシールの太平洋における系統的な立位置を示し，特にえりも個体は太平洋の中でも特異的であることを示した．さらに，ハーバーシールの生態情報を加味し，季節を分けて遺伝子解析することにより，繁殖集団の遺伝的特徴の把握をし，繁殖上陸場への依存度の高さや，非繁殖期における他地域からの移入を示すことができた．このことは，ハーバーシールの個体数管理を行っていく上で重要な知見となると考えられた．

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## References


ALLEN, P. J., W. AMOS, P. P. POMEROY, AND S. D. TWISS. 1995. Microsatellite variation in grey seals (*Halichoerus grypus*) shows evidence of genetic differentiation between two British breeding colonies. Molecular ecology 4:653–662.

ALLENDORF, F. W., G. LUIKART, AND S. N. AITKEN. 2014. Conservation and the genetics of populations. Second. Wiley-Blackwell, Oxford.

ANDERSEN, L. W., C. LYDERSEN, A. K. FRIE, A. ROSING-ASVID, E. HAUKSSON, AND K. M. KOVACS. 2011. A population on the edge: genetic diversity and population structure of the world's northernmost harbour seals (*Phoca vitulina*). Biological Journal of the Linnean Society 102:420–439.

AVISE, J. C. 2000. Phylogeography — The History and Formation of Species. Harvard University Press, Cambridge, MA.

BAJZAK, C. E., W. BERNHARDT, A. MOSNIER, M. O. HAMMILL, AND I. STIRLING. 2013. Habitat use by harbour seals (*Phoca vitulina*) in a seasonally ice-covered region, the western Hudson Bay. Polar Biology 36:477–491.

BAKER, A. R. ET AL. 2005. Variation of mitochondrial control region sequences of steller sea lions: the three-stock hypothesis. Journal of Mammalogy 86:1075–1084.

BANDELT, H. J., P. FORSTER, AND A. ROHL. 1999. Median-joining networks for inferring intraspecific

phylogenies. Molecular Biology and Evolution 16:37–48.

BEACHAM, T. D., J. R. CANDY, K. D. LE, AND M. WETKLO. 2009. Population structure of chum salmon (*Oncorhynchus keta*) across the Pacific Rim, determined from microsatellite analysis. Fishery Bulletin 107:244–260.

BIGG, M. 1981. Harbour seal. Pp. 1–28 in Handbook of Marine Mammals (vol. 2) (S. H. Ridgway & R. J. Harrison, eds.). 1st edition. Academic Press, London.

BOULVA, J., AND I. A. MCLAREN. 1979. Biology of the harbor seal, *Phoca vitulina*, in eastern Canada. Department of Fisheries and Oceans Scientific Information and Publications Branch.

BURG, T. M., A. W. TRITES, AND M. J. SMITH. 1999. Mitochondrial and microsatellite DNA analyses of harbour seal population structure in the northeast Pacific Ocean. Canadian Journal of Zoology 77:930–943.

CANINO, M. F., I. B. SPIES, K. M. CUNNINGHAM, L. HAUSER, AND W. S. GRANT. 2010. Multiple ice-age refugia in Pacific cod, Gadus macrocephalus. Molecular Ecology 19:4339–4351.

CARRETTA, J. V. ET AL. 2019. U.S. Pacific Marine Mamal Stock Assessments: 2018. NOAA Technical Memorandum NMFS. NOAA-TM-NM-617:1–377.

CAUDRON, A. K., S. S. NEGRO, C. G. MULLER, L. J. BOREN, AND N. J. GEMMELL. 2007. Hair sampling and genotyping from hair follicles: a minimally-invasive alternative for genetics studies in small, mobile pinnipeds and other mammals. Marine Mammal Science 23:184–192.

CHISHIMA, J. 2008. Nemuro Hamanaka chiiki de jourikujou idou ga kakunin sareta zenigata azarasi no kotai risuto. zeniken-tsushin 21:50–59.

COLTMAN, D. W., W. D. BOWEN, AND J. M. WRIGHT. 1996. PCR primers for harbour seal (*Phoca vitulina concolour*) microsatellites amplify polymorphic loci in other pinniped species. Molecular ecology 5:161–3.

CORDES, L. S., C. D. DUCK, B. L. MACKEY, A. J. HALL, AND P. M. THOMPSON. 2011. Long-term patterns in harbour seal site-use and the consequences for managing protected areas. Animal Conservation 14:430–438.

CRONIN, M. A., J. BODKIN, B. BALLACHEY, J. ESTES, AND J. C. PATTON. 1996. Mitochondrial-DNA variation among subspecies and populations of sea otters (*Enhydra lutris*). Journal of Mammalogy 77:546–557.

DIETZ, R., J. TEILMANN, S. M. ANDERSEN, F. RIGET, AND M. T. OLSEN. 2012. Movements and site fidelity of harbour seals (*Phoca vitulina*) in Kattegat, Denmark, with implications for the epidemiology of the phocine distemper virus. ICES Journal of Marine Science 70:186–195.

EVANNO, G., S. REGNAUT, AND J. GOUDET. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular ecology 14:2611–20.

EVETT, I. W., AND B. S. WEIR. 1998. Interpreting DNA Evidence: Statistical Genetics for Forensic Scientists. Sinauer Associates Inc, Sinauer, Sunderland, MA.

EXCOFFIER, L., P. E. SMOUSE, AND J. M. QUATTRO. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491.

FLAGSTAD, O., AND K. H. RØED. 2003. Refugial origins of reindeer (Rangifer tarandus L.) inferred from mitochondrial DNA sequences. Evolution 57:658–70.

FORBES, S. H., AND D. K. BOYD. 1997. Genetic Structure and Migration in Native and Reintroduced Rocky Mountain Wolf Populations. Estructura Genetica y Migracion de Poblaciones Nativas y Reintroducidas del Lobo de las Montanas Rocallosas. Conservation Biology 11:1226–1234.

FULTON, T. L., AND C. STROBECK. 2010. Multiple fossil calibrations, nuclear loci and mitochondrial genomes provide new insight into biogeography and divergence timing for true seals (Phocidae, Pinnipedia). Journal of Biogeography 37:814–829.

GALPERN, P., M. MANSEAU, P. HETTINGA, K. SMITH, AND P. WILSON. 2012. Allelematch: an R package for identifying unique multilocus genotypes where genotyping error and missing data may be present. Molecular Ecology Resources 12:771–778.

GEMMELL, N. J., P. J. ALLEN, S. J. GOODMAN, AND J. Z. REED. 1997. Interspecific microsatellite markers for the study of pinniped populations. Molecular ecology 6:661–666.

GOODMAN, S. J. 1997. Dinucleotide repeat polymorphisms at seven anonymous microsatellite loci cloned from the European harbour seal (*Phoca vitulina vitulina*). Animal Genetics 28:310–

311.

GOODMAN, S. J. 1998. Patterns of extensive genetic differentiation and variation among European harbor seals (*Phoca vitulina vitulina*) revealed using microsatellite DNA polymorphisms. Molecular biology and evolution 15:104–18.

GOOSSENS, B., L. P. WAITS, AND P. TABERLET. 1998. Plucked hair samples as a source of DNA: reliability of dinucleotide microsatellite genotyping. Molecular ecology 7:1237–1241.

GOUDET, J. 1995. FSTAT (Version 1.2): A computer program to calculate F-S.statistics. Journal of Heredity 86:485–486.

GREEN, M. R., AND J. SAMBROOK. 2012. Molecular Cloning: A Laboratory Manual. Fourth Ed. Cold Spring Harbor Laboratory Press, New York.

HANEDA, T., K. MARI, Z. TAMURA, K. TAKADA, AND I. OGAWA. 2017. Coastal use patterns of Japanese harbor seals (*Phoca vitulina stejnegeri*) in Akkeshi Bay in Hokkaido during the spring season. Mammalian Science 57:35–43.

HANOTTE, O., T. BURKE, J. A. L. ARMOUR, AND A. J. JEFFREYS. 1991. Hypervariable minisatellite DNA sequences in the Indian peafowl Pavo cristatus. Genomics 9:587–597.

HÄRKÖNEN, T., AND K. C. HARDING. 2001. Spatial structure of harbour seal populations and the implications thereof. Canadian Journal of Zoology 79:2115–2127.

HASEGAWA, Y., Y. TOMIDA, N. KOHNO, K. ONO, H. NOKARIYA, AND T. UYENO. 1988. Quaternary

Vertebrates from Shiriya Area, Shimokita Peninsula, Northeastern Japan. Memoirs of the National Science Museum 21:36–39.

HAYAMA, S., H. UNO, AND K. WADA. 1986. A migration model based on sex and age composition of Kuril seals captured along the coast of Nemuro Peninsula, Hokkaido. Pp. 140–157 in Conservation and management of Kuril Seal (Zenigataazarashino seitaito hogo) (K. Wada, T. Itoo, A. Niizuma, S. Hayama & M. Suzuki, eds.). 1st edition. Tokai University Publisher, Tokyo.

HERREMAN, J. K., G. M. BLUNDELL, D. B. MCDONALD, AND M. BEN-DAVID. 2009. Asymmetrical male-mediated gene flow between harbor seal (*Phoca vitulina*) populations in Alaska. Canadian Journal of Zoology 87:498–507.

HEWITT, G. 2000. The genetic legacy of the Quaternary ice ages. Nature 405:907–13.

HEWITT, G. M. 1999. Post-glacial re-colonization of European biota. Biological Journal of the Linnean Society 68:87–112.

HIGDON, J. W., O. R. P. BININDA-EMONDS, R. M. D. BECK, AND S. H. FERGUSON. 2007. Phylogeny and divergence of the pinnipeds (Carnivora: Mammalia) assessed using a multigene dataset. BMC evolutionary biology 7:216.

HOELZEL, A. R. ET AL. 1993. Elephant Seal Genetic Variation and the Use of Simulation Models to Investigate Historical Population Bottlenecks. Journal of Heredity 84:443–449.

HOELZEL, A. R., B. J. LE BOEUF, J. REITER, AND C. CAMPAGNA. 1999. Alpha-male paternity in elephant

seals. Behavioral Ecology and Sociobiology 46:298–306.

HOELZEL, A. R., S. D. GOLDSWORTHY, AND R. C. FLEISCHER. 2002. Population Genetic Structure. Pp. 325–352 in Marine Mammal Biology: An Evolutionary Approach (A. R. Hoelzel, ed.). Blackwell Publishing, Oxford.

HOKKAIDO. 2006a. Report for conservation and management of seal species (in Japanese).

HOKKAIDO. 2006b. Azarashirui hogo kanri hokokusyo. Hokkaido, Japan.

HOLDER, K., R. MONTGOMERIE, AND V. L. FRIESEN. 1999. A test of the glacial refugium hypothesis using patterns of mitochondrial and nuclear DNA sequence variation in rock ptarmigan (*Lagopus mutus*). Evolution 53:1936–1950.

HUBER, H. R., B. R. DICKERSON, S. J. JEFFRIES, AND D. M. LAMBOURN. 2012. Genetic analysis of Washington State harbor seals (*Phoca vitulina richardii*) using microsatellites. Canadian Journal of Zoology 90:1361–1369.

HUBER, H. R., S. J. JEFFRIES, D. M. LAMBOURN, AND B. R. DICKERSON. 2010. Population substructure of harbor seals (*Phoca vitulina richardsi*) in Washington State using mtDNA. Canadian Journal of Zoology 88:280–288.

HUBISZ, M. J., D. FALUSH, M. STEPHENS, AND J. K. PRITCHARD. 2009. Inferring weak population structure with the assistance of sample group information. Molecular Ecology Resources 9:1322–1332.

ITOO, T., AND K. SHUKUNOBE. 1986. Number and present status of the the Kuril seal. Pp. 18–58 in Ecology and Conservaion of Kuri seal (Zenigata azarashi no seitai to hogo) (K. Wada, T. Itoo, A. Niizuma, S. Hayama & M. Suzuki, eds.). 1st edition. Tokai University Publisher, Tokyo.

JEFFERSON, T. A., S. LEATHERWOOD, AND M. A. WEBBER. 1993. FAO species identification guide. Marine mammals of the world. United Nations Environment programme food and agriculture organization of the United Nations, Rome.

JEFFRIES, S., H. HUBER, J. CALAMBOKIDIS, AND J. LAAKE. 2003. Trends and status of harbor seals in Washington state: 1978-1999. Journal of Wildlife Management 67:207–218.

JEFFRIES, S. J., P. J. GEARIN, H. R. HUBER, D. L. SAUL, AND D. A. PRUETT. 2000. Atlas of Seal and Sea Lion Haulout Sites in Washington. Washington Department of Fish and Wild life. WA.

JOMBART, T. 2008. adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24:1403–1405.

JOMBART, T., S. DEVILLARD, AND F. BALLOUX. 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC genetics 11:94.

KALZ, B., K. JEWGENOW, AND J. FICKEL. 2006. Structure of an otter (*Lutra lutra*) population in Germany – results of DNA and hormone analyses from faecal samples. Mammalian Biology - Zeitschrift für Säugetierkunde 71:321–335.

KIMURA, D. 2014. kotai shikibetu ni yoru Akkeshi Daikoku-jima no zenigata azarashi seiju-osu no

kisetsubetsu jorikujo no kaimei. Tokyo University of Agriculture.

KIMURA, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of molecular evolution 16:111–20.

KING, J. E. 1983. Seals of the world. 2nd edition. Oxford University Press, London.

KOBAYASHI, M. 2009. *Phoca vitulina* Linnaeus, 1758. Pp. 272–277 in The Wild Mammals of Japan (S. D. Ohdachi, Y. Ishibashi, M. A. Iwasa & T. Saitoh, eds.). 1st edition. SHOUKADOH Book Sellers and the Mammalogical Society of Japan, Kyoto.

KOBAYASHI, Y. ET AL. 2014. Population trends of the Kuril harbour seal *Phoca vitulina stejnegeri* from 1974 to 2010 in southeastern Hokkaido, Japan. Endangered Species Research:61–72.

KRETZMANN, M. B., N. J. GEMMELL, AND A. MEYER. 2001. Microsatellite Analysis of Population Structure in the Endangered Hawaiian Monk Seal. Conservation Biology 15:457–466.

LAMONT, M. M. ET AL. 1996. Genetic substructure of the Pacific harbor seal (*Phoca vitulina richardsi*) off Washington, Oregon, and California. Marine Mammal Science 12:402–413.

LEHMAN, N., R. K. WAYNE, AND B. S. STEWART. 1993. comparative levels of genetic variability in harbour seals and northern elephant seals as determined by genetic fingerprinting. The Zoological Society of London 66:49–60.

LI, X., A. C. TZIKA, Y. LIU, K. VAN DONINCK, Q. ZHU, AND M. C. MILINKOVITCH. 2010. Preliminary genetic

status of the spotted seal Phoca largha in Liaodong Bay (China) based on microsatellite and mitochondrial DNA analyses. Trends in Evolutionary Biology 2:6.

LOWRY, L. F., K. J. FROST, J. M. HOEP, AND R. A. DELONG. 2001. Movements of satellite-tagged subadult and adult harbor seals in Prince William Sound, Alaska. Marine Mammal Science 17:835–861.

LOWRY, M. S. ET AL. 2014. Abundance, distribution, and population growth of the northern elephant seal (*Mirounga angustirostris*) in the United States from 1991 to 2010. Aquatic Mammals 40:20–31.

LUIKART, G., F. W. ALLENDORF, J. M. CORNUET, AND W. B. SHERWIN. 1998. Distortion of allele frequency distributions provides a test for recent population bottlenecks. Journal of Heredity.

MANN, D. H., AND D. M. PETEET. 1994. Extent and Timing of the Last Glacial Maximum in Southwestern Alaska. Quaternary Research 42:136–148.

MINISTRY_OF_THE_ENVIRONMENT. 2016. Erimo area Kuril harbor seal specified rare wildlife management plan.

MIYAZAKI, S. ET AL. 1994. Summary of the fossil record of pinnipeds of Japan, and comparisons with that from the eastern North Pacific. The Island Arc 3:361–372.

MIZUNO, M., T. SASAKI, M. KOBAYASHI, T. HANEDA, AND T. MASUBUCHI. 2018. Mitochondrial DNA reveals secondary contact in Japanese harbour seals, the southernmost population in the western

Pacific. PLOS ONE 13:e0191329.

MORIN, P. A., K. E. CHAMBERS, C. BOESCH, AND L. VIGILANT. 2001. Quantitative polymerase chain reaction analysis of DNA from noninvasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes verus*). Molecular Ecology 10:1835–1844.

MURPHY, M. A., L. P. WAITS, K. C. KENDALL, S. K. WASSER, J. A. HIGBEE, AND R. BOGDEN. 2002. An evaluation of long-term preservation methods for brown bear ( *Ursus arctos* ) faecal DNA samples. Conservation Genetics 3:435–440.

MUTO, M. M. ET AL. 2017. Alaska marine mammal stock assessments, 2016. U.S. Department of Commerce, NOAA Technical Memorandum:1–366.

NAITO, Y., AND M. NISHIWAKI. 1972. The growth of two species of the harbour seal in the adjacent waters of Hokkaido. Scientific Reports of the Whales Research Institute 24:127–144.

NAKAGAWA, E., M. KOBAYASHI, M. SUZUKI, AND T. TSUBOTA. 2010. Genetic variation in the harbor seal (*Phoca vitulina*) and spotted seal (Phoca largha) around Hokkaido, Japan, based on mitochondrial cytochrome b sequences. Zoological science 27:263–268.

NIIZUMA, A. 1986. Socio-ecology and reproductive strategy of the Kuril seal. Pp. 59–102 in Conservation and management of Kuril Seal (Zenigataazarashino seitaito hogo) (K. Wada, T. Itoo, A. Niizuma, S. Hayama & M. Suzuki, eds.). 1st edition. Tokai University Publisher, Tokyo.

OLSEN, M. T. ET AL. 2017. Genetic population structure of harbour seals in the United Kingdom and

neighbouring waters. Aquatic Conservation: Marine and Freshwater Ecosystems 27:839–845.

OLSEN, M. T., L. W. ANDERSEN, R. DIETZ, J. TEILMANN, T. HÄRKÖNEN, AND H. R. SIEGISMUND. 2014. Integrating genetic data and population viability analyses for the identification of harbour seal (*Phoca vitulina*) populations and management units. Molecular ecology 23:815–31.

ONO, Y. 1984. Last Glacial Paleoclimate Reconstructed from Glacial and Periglacial Landforms in Japan. Geographical review of Japan, Series B. 57:87–100.

VAN OOSTERHOUT, C., W. F. HUTCHINSON, D. P. M. WILLS, AND P. SHIPLEY. 2004. Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4:535–538.

PAGANO, A. M., E. PEACOCK, AND M. A. MCKINNEY. 2014. Remote biopsy darting and marking of polar bears. Marine Mammal Science 30:169–183.

PATERSON, W., C. E. SPARLING, D. THOMPSON, P. P. POMEROY, J. I. CURRIE, AND D. J. MCCAFFERTY. 2012. Seals like it hot: Changes in surface temperature of harbour seals (*Phoca vitulina*) from late pregnancy to moult. Journal of Thermal Biology 37:454–461.

PELTIER, W. R. 1994. Ice age paleotopography. Science (New York, N.Y.) 265:195–201.

PETERSON, S. H., M. M. LANCE, S. J. JEFFRIES, AND A. ACEVEDO-GUTIÉRREZ. 2012. Long Distance Movements and Disjunct Spatial Use of Harbor Seals (*Phoca vitulina*) in the Inland Waters

of the Pacific Northwest. PLoS ONE 7:e39046.

PIGGOTT, M. P., S. C. BANKS, N. STONE, C. BANFFY, AND A. C. TAYLOR. 2005. Estimating population size of endangered brush-tailed rock-wallaby (*Petrogale penicillata* ) colonies using faecal DNA. Molecular Ecology 15:81–91.

PIRY, S., G. LUIKART, AND J.-M. CORNUET. 1999. Computer note. BOTTLENECK: a computer program for detecting recent reductions in the effective size using allele frequency data. Journal of Heredity 90:502–503.

PRITCHARD, J. K., M. STEPHENS, AND P. DONNELLY. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–59.

R_CORE_TEAM. 2019. R: A language and environment for statistical computing. Vienna, Australia. <https://www.r-project.org/>.

RAYMOND, M., AND F. ROUSSET. 1995. GENEPOP (Version 1.2): Population genetics software for exact tests and ecumenicism. Journal of Heredity 86:248–249.

REED, J. Z., D. J. TOLLIT, P. M. THOMPSON, AND W. AMOS. 1997. Molecular scatology: the use of molecular genetic analysis to assign species, sex and individual identity to seal faeces. Molecular ecology 6:225–234.

RICE, W. R. 1989. Analyzing tables of statistical tests. Evolution 43:223.

SATO, S., J. ANDO, H. ANDO, S. URAWA, A. URANO, AND S. ABE. 2001. Genetic Variation among Japanese

Populations of Chum Salmon Inferred from the Nucleotide Sequences of the Mitochondrial DNA Control Region. Zoological Science 18:99–106.

SCHAEFF, C. M., D. J. BONESS, AND W. D. BOWEN. 1999. Female distribution, genetic relatedness, and fostering behaviour in harbour seals,Phoca vitulina. Animal Behaviour 57:427–434.

SCHULTZ, J. K., J. D. BAKER, R. J. TOONEN, AND B. W. BOWEN. 2008. Extremely low genetic diversity in the endangered Hawaiian monk seal (*Monachus schauinslandi*). Journal of Heredity 100:25–33.

SCHULTZ, J. K., A. J. MARSHALL, AND M. PFUNDER. 2010. Genome-wide loss of diversity in the critically endangered Hawaiian monk seal. Diversity 2:863–880.

SEEB, L. W., AND P. A. CRANE. 1999. High Genetic Heterogeneity in Chum Salmon in Western Alaska, the Contact Zone between Northern and Southern Lineages. Transactions of the American Fisheries Society 128:58–87.

SHARPLES, R. J., S. E. MOSS, T. A. PATTERSON, AND P. S. HAMMOND. 2012. Spatial variation in foraging behaviour of a marine top predator (*Phoca vitulina*) determined by a large-scale satellite tagging program. PloS one 7:e37216.

SHAUGHNESSY, P. D., AND F. H. FAY. 1977. A review of taxonomy and nomenclature of North Pacific Harbor seals. Journal of Zoology 182:385–419.

SLADE, R., C. MORITZ, AND A. HEIDEMAN. 1994. Multiple nuclear-gene phylogenies: application to

pinnipeds and comparison with a mitochondrial DNA gene phylogeny. Molecular Biology and Evolution 11:341–356.

SMALL, R. J., L. F. LOWRY, J. M. HOEF, K. J. FROST, R. A. DELONG, AND M. J. REHBERG. 2005. Differential movements by harbor seal pups in contrasting Alaska environments. Marine Mammal Science 21:671–694.

STANLEY, H. F., S. CASEY, J. M. CARNAHAN, S. GOODMAN, J. HARWOOD, AND R. K. WAYNE. 1996. Worldwide patterns of mitochondrial DNA differentiation in the harbor seal (*Phoca vitulina*). Molecular biology and evolution 13:368–382.

TABERLET, P., AND G. LUIKART. 1999. Non-invasive genetic sampling and individual identification. Biological Journal of the Linnean Society 68:41–55.

TAMURA, K., G. STECHER, D. PETERSON, A. FILIPSKI, AND S. KUMAR. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular biology and evolution 30:2725–9.

TAYLOR, E. B., T. D. BEACHAM, AND M. KAERIYAMA. 1994. Population Structure and Identification of North Pacific Ocean Chum Salmon (*Oncorhynchus keta*) Revealed by an Analysis of Minisatellite DNA Variation. Canadian Journal of Fisheries and Aquatic Sciences 51:1430–1442.

THOMPSON, P. 1988. Timing of mating in the Common Seal (*Phoca vitulina*). Mammal Review 18:105–112.

THOMPSON, P. M. 1989. Seasonal changes in the distribution and composition of common seal (*Phoca vitulina*) haul-out groups. Journal of Zoology 217:281–294.

THOMPSON, P. M. 1993. Harbour seal movement patterns. Symposia of the Zoological Society of London 66:225–239.

THOMPSON, P. M., D. MILLER, R. COOPER, AND P. S. HAMMOND. 1994. Changes in the distribution and activity of female harbour seals during the breeding season: Implications for their lactation strategy and mating patterns. Journal of Animal Ecology 63:24–30.

VERGARA, M., A. RUIZ-GONZÁLEZ, J. LÓPEZ DE LUZURIAGA, AND B. J. GÓMEZ-MOLINER. 2014. Individual identification and distribution assessment of otters ( *Lutra lutra* ) through non-invasive genetic sampling: Recovery of an endangered species in the Basque Country (Northern Spain). Mammalian Biology 79:259–267.

WADA, K., S. HAYAMA, T. NAKAOKA, AND K. SHIMAZAKI. 1986. On the interactions between Kuril seals and Salmon trap net fisheries along the coastal waters of South-eastern Hokkaido. Pp. 223–244 in Conservation and management of Kuril Seal (Zenigataazarashino seitaito hogo) (K. Wada, T. Itoo, A. Niizuma, S. Hayama & M. Suzuki, eds.). 1st edition. Tokai University Publisher, Tokyo.

WAITE, J. N., L. P. WAITS, M. BOZZA, AND R. D. ANDREWS. 2011. Differentiating between Steller sea lion ( *Eumetopias jubatus* ) and northern fur seal ( *Callorhinus ursinus* ) scats through

analysis of faecal DNA. Molecular Ecology Resources 11:166–170.

WAITS, J. L., AND P. L. LEBERG. 2000. Biases associated with population estimation using molecular tagging. Animal Conservation 3:191–199.

WAITS, L. P., G. LUIKART, AND P. TABERLET. 2001. Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. Molecular Ecology 10:249–256.

WAITS, L. P., AND D. PAETKAU. 2005. Noninvasive genetic sampling tools for wildlife biologists: A review of applications and recommendations for accurate data collection. Journal of Wildlife Management 69:1419–1433.

WEIR, B., AND C. C. COCKERHAM. 1984. Estimating F-Statistics for the Analysis of Population Structure. Evolution 38:1358–1370.

WESTLAKE, R., AND G. O'CORRY-CROWE. 2002. Macrogeographic structure and patterns of genetic diversity in harbor seals (*Phoca vitulina*) from Alaska to Japan. Journal of Mammalogy 83:1111–1126.

WHITE, S. P., AND L. D. DENSMORE. 1992. Molecular Genetic Analysis of Populations: A Practical Approach. Oxford University Press, New York.

WOMBLE, J. N., AND S. M. GENDE. 2013. Post-breeding migrations of a top predator, the harbor seal (*Phoca vitulina richardii*), from a marine protected area in Alaska. PloS one 8:e55386.

YANNIC, G. ET AL. 2011. Description of microsatellite markers and genotyping performances using

feathers and buccal swabs for the Ivory gull (*Pagophila eburnea*). Molecular Ecology Resources 11:877–889.

YOCHEM, P. K., B. S. STEWART, R. L. DELONG, AND D. P. DEMASTER. 1987. Diel haul-out patterns and site fidelity of harbor seals (*Phoca vitulina richardsi* ) on San Miguel Island, California, in Autumn. MARINE MAMMAL SCIENCE 3:323–332.

YOON, M. ET AL. 2007. Congruence of Population Genetic Profiles Obtained from Mitochondrial and Microsatellite DNA Analyses in the Pacific Rim Chum Salmon Populations. North Pacific Anadromous Fish Commission Technical Repor No. 7:121–123.

YOON, M. ET AL. 2008. Mitochondrial DNA variation and genetic population structure of chum salmon Oncorhynchus keta around the Pacific Rim. Journal of Fish Biology 73:1256–1266.

YUKIKO, A., K. CHIHARU, Y. YUJEN, K. YUMI, AND S. YASUNORI. 2008. 2006 Phoca vitulina stejnegeri by Salmon Set-net Fishery in the Cape Nosappu , Japan 2006. Bulletin of the Shiretoko Museum 29:23–30.

**Figures and Tables**

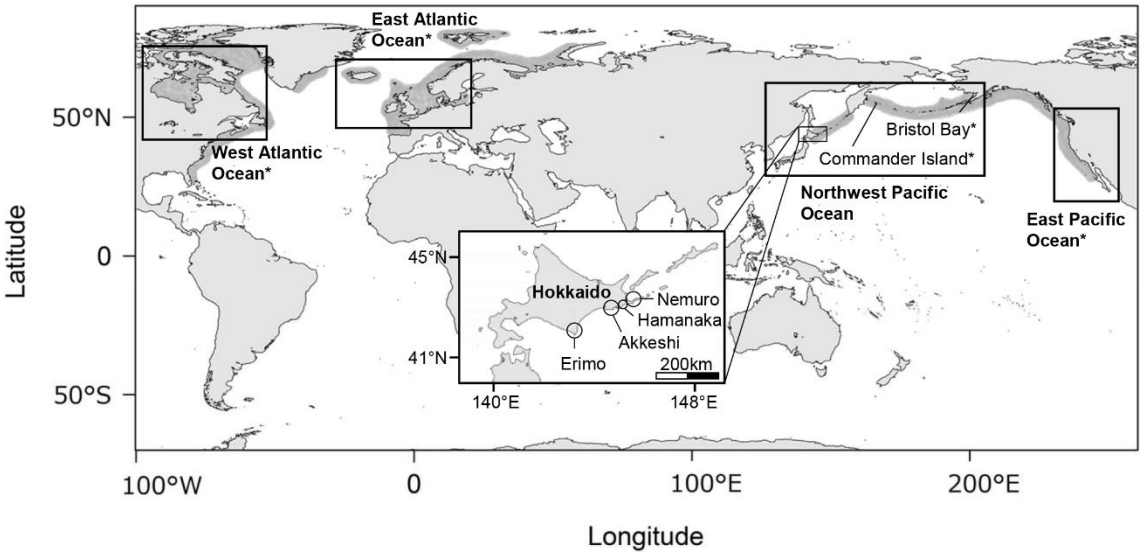



**Figure 1-1.** Distribution range of harbour seals (shaded) and sampled localities (squares). Sampling locations of published sequences outside Hokkaido, Japan, used in phylogenetic analysis are indicated with stars (Accession numbers U36342–U36371 [3]). Samples of Japanese harbour seals (Phoca vitulina stejnegeri) were taken from four administrative districts (Erimo, Akkeshi, Hamanaka, and Nemuro) in Hokkaido, Japan. Each district contains several haul-out sites where the seals breed.

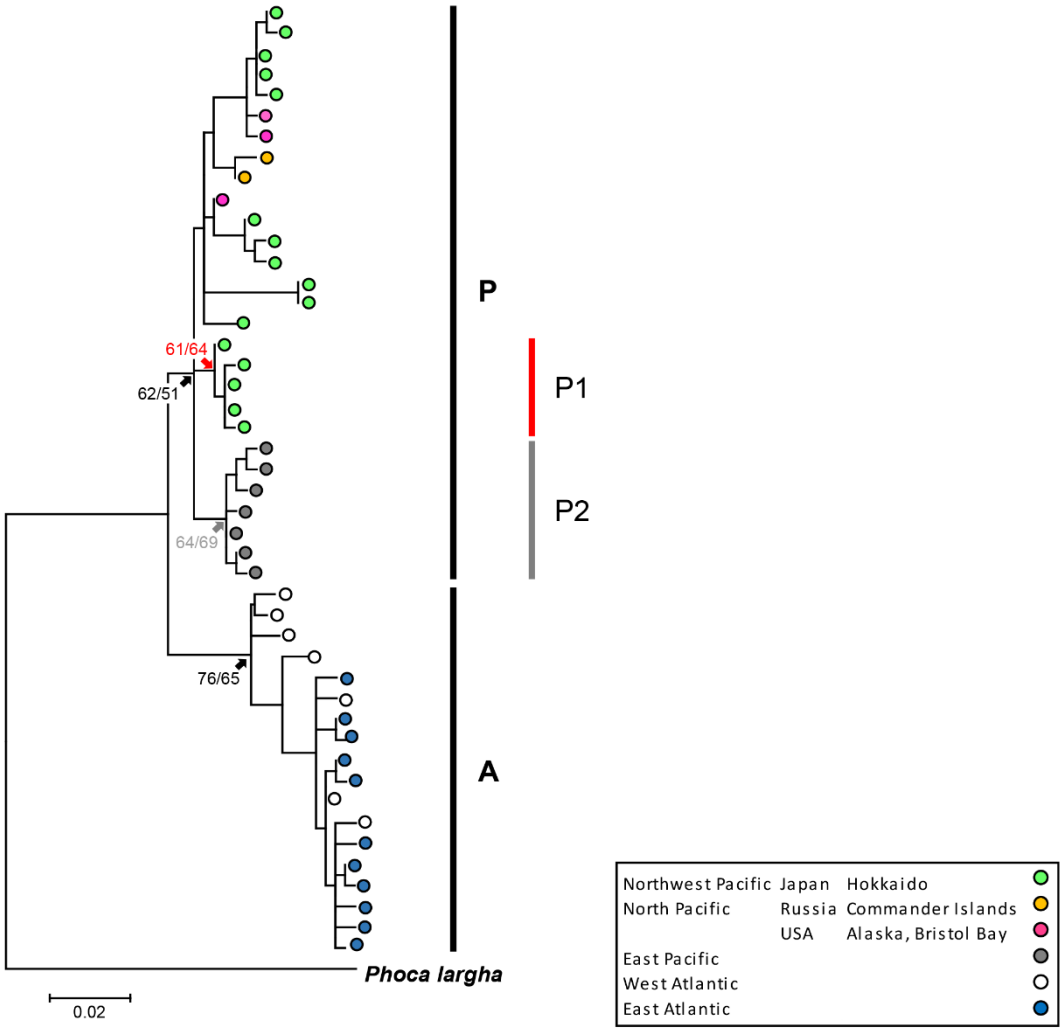



**Figure 1-2.** Phylogenetic tree of harbour seals based on the mtDNA control region. The bootstrap values of branches of the maximum-likelihood (left) and neighbour-joining method (right). 454 bp of the control region was used to compare the phylogenetic relationships of seals. Data outside Japan were obtained from GenBank (Accession numbers U36342–U36371 (Stanley et al. 1996)).

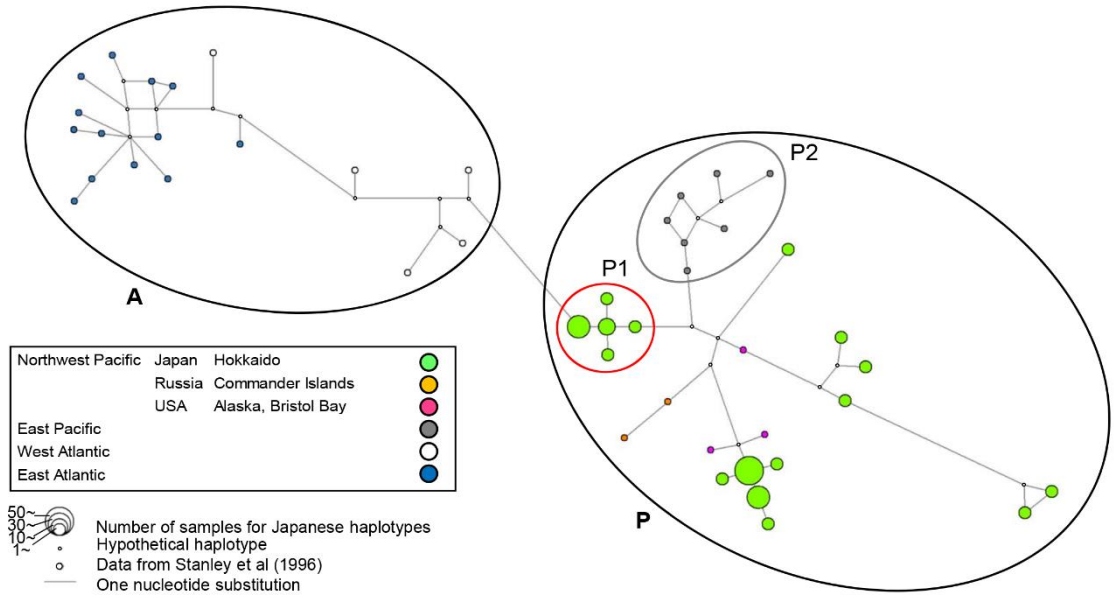



**Figure 1-3.** Median-joining network based on the mtDNA control region of harbour seals. The node colours and sizes of circles represent the different sites, area, and sample size. The length of the node is proportional to the number of substitutions. Groupings of the nodes are based on the division of the phylogenetic tree in Fig 2.

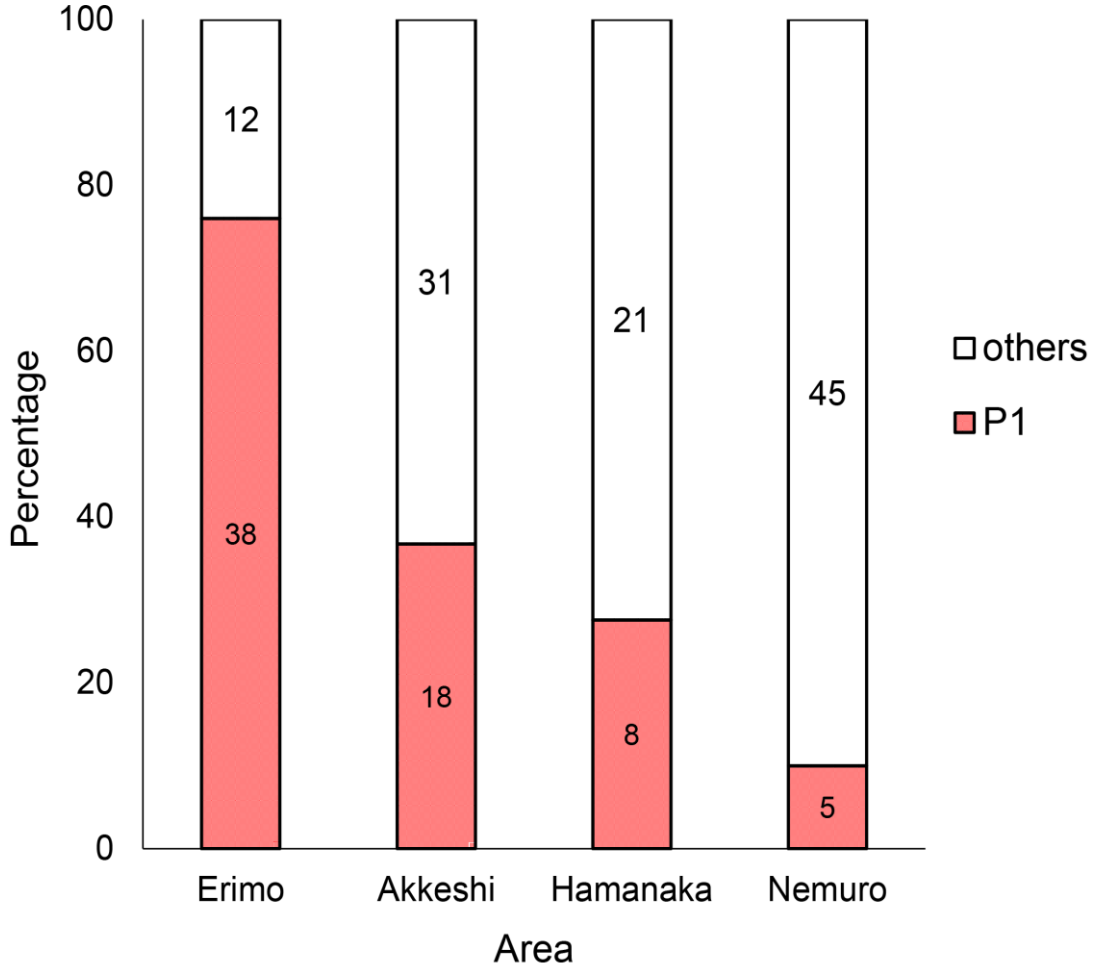



**Figure 1-4.** Proportions of haplogroups among the four districts. The haplogroups (Group P, A, P1 and P2) were defined in the phylogenetic tree and the median-joining network. The numbers in the bar indicate the number of samples.

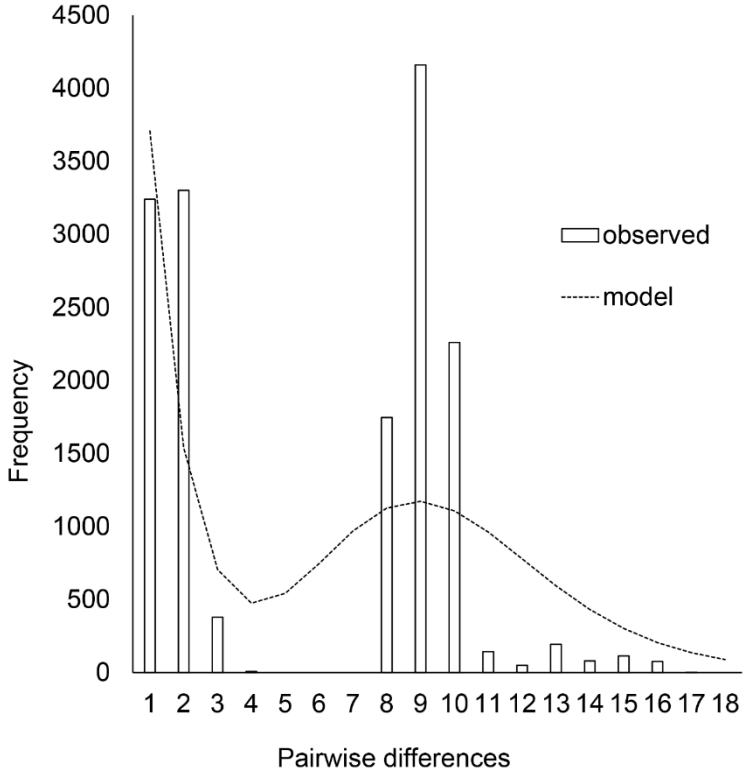



**Figure 1-5.** Mismatch distribution of mtDNA haplotypes for Japanese harbour seals. The bar charts indicate the observed number of pairwise differences and the dashed line represents the expected distribution under a spatial expansion model (SSD: p=0.07; Hrag: p=0.41).

**Table 1-1.** Polymorphic sites of the mtDNA control region detected in Japanese harbour seals.

| | Variable sites | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 16460 | 16462 | 16464 | 16471 | 16472 | 16476 | 16477 | 16479 | | 16494 | | 16552 | 16559 | 16567 | 16570 | 16579 | 16580 | 16588 | 16594 | 16607 | 16637 | 16776 | 16789 | Frequency |
| P.v.v | C | T | G | G | C | C | C | C | - | C | - | G | T | A | A | A | A | A | T | T | G | A | T | - |
| JP1 | . | . | . | A | . | . | . | - | C | . | - | A | . | . | G | G | . | C | . | . | G | C | | 2 |
| JP2 | . | . | . | . | T | . | T | . | - | . | C | . | C | . | G | G | G | . | . | . | . | . | | 1 |
| JP3 | . | . | . | . | T | . | T | . | - | . | - | . | C | . | G | G | G | . | . | . | . | . | | 24 |
| JP4 | . | . | . | . | T | . | T | . | - | . | - | . | . | . | G | G | G | . | . | . | . | . | | 4 |
| JP5 | . | . | . | . | T | . | T | . | - | . | - | . | C | . | . | G | G | . | . | . | . | . | | 39 |
| JP6 | . | . | . | . | . | T | . | . | - | T | - | . | C | G | G | G | G | . | C | A | . | . | | 37 |
| JP7 | . | . | . | . | . | T | . | T | - | . | - | A | . | . | . | . | G | . | . | . | . | . | | 56 |
| JP8 | . | . | . | . | . | T | . | . | - | T | - | . | C | G | . | G | G | . | C | A | . | . | | 4 |
| JP9 | . | . | . | A | . | . | . | T | C | . | - | A | . | . | G | G | . | C | . | . | G | C | | 1 |
| JP10 | T | . | . | . | T | . | T | . | - | . | - | . | C | . | G | G | G | . | . | . | . | . | | 3 |
| JP11 | . | . | . | . | . | T | . | . | C | T | - | . | C | G | G | G | G | . | C | A | . | . | | 2 |
| JP12 | . | C | A | A | . | T | . | . | - | . | - | . | C | G | G | G | . | . | . | . | . | . | | 1 |
| JP13 | . | C | . | A | . | T | . | . | C | . | - | . | C | . | G | G | . | . | . | . | . | . | | 1 |
| JP14 | . | C | . | A | . | T | . | . | - | . | - | A | C | G | G | G | . | . | . | . | . | . | | 1 |
| JP15 | . | . | . | . | . | T | . | . | - | T | - | . | C | G | G | . | G | . | C | A | . | . | | 1 |
| JP16 | . | C | . | . | . | T | . | . | - | T | - | . | C | G | G | . | G | . | C | A | . | . | | 1 |

**Table 1-2.** Regional differences in haplotype frequencies of Japanese harbour seals in four administrative districts of Hokkaido.

| | Erimo (%) | Akkeshi (%) | Hamanaka (%) | Nemuro (%) |
|---|---|---|---|---|
| JP1 | | | 1 (3) | 1 (2) |
| JP2 | 1 (2) | | | |
| JP3 | | 13 (27) | 7 (24) | 4 (8) |
| JP4 | 2 (4) | 2 (4) | | |
| JP5 | 35 (70) | 3 (6) | 1 (3) | |
| JP6 | 5 (10) | 9 (18) | 6 (21) | 17 (34) |
| JP7 | 2 (4) | 20 (41) | 13 (45) | 21 (42) |
| JP8 | | 1 (2) | | 3 (6) |
| JP9 | | 1 (2) | | |
| JP10 | 3 (6) | | | |
| JP11 | 2 (4) | | | |
| JP12 | | | | 1 (2) |
| JP13 | | | | 1 (2) |
| JP14 | | | | 1 (2) |
| JP15 | | | | 1 (2) |
| JP16 | | | 1 (3) | |
| NH | 7 | 7 | 5 | 9 |
| UNIQUE | 3 | 1 | 0 | 4 |
| TOT | 50 | 49 | 29 | 50 |

JP1-16 haplotypes deposited in GenBank (accession number: U36342–U36371) by Mizuno et al. (2018); NH: number of haplotypes, UNIQUE: number of unique haplotypes, TOT: total number of samples.

**Table 1-3**. Diversity indices for mtDNA in Japanese harbour seal.

| | N | *H* | | SD | π | | SD |
|---|---|---|---|---|---|---|---|
| Erimo | 50 | 0.501 | ± | 0.083 | 0.005 | ± | 0.003 |
| Akkeshi | 49 | 0.738 | ± | 0.039 | 0.006 | ± | 0.003 |
| Hamanaka | 29 | 0.719 | ± | 0.056 | 0.006 | ± | 0.003 |
| Nemuro | 59 | 0.712 | ± | 0.045 | 0.005 | ± | 0.003 |

*N*: number of samples, *H*: haplotype diversity, π: nucleotide diversity, *A*R: allelic richness, *H*o: observed heterozygosity, *H*e: expected heterozygosity, *F*IS: inbreeding coefficient, *H*ex: excess of heterozygosity.

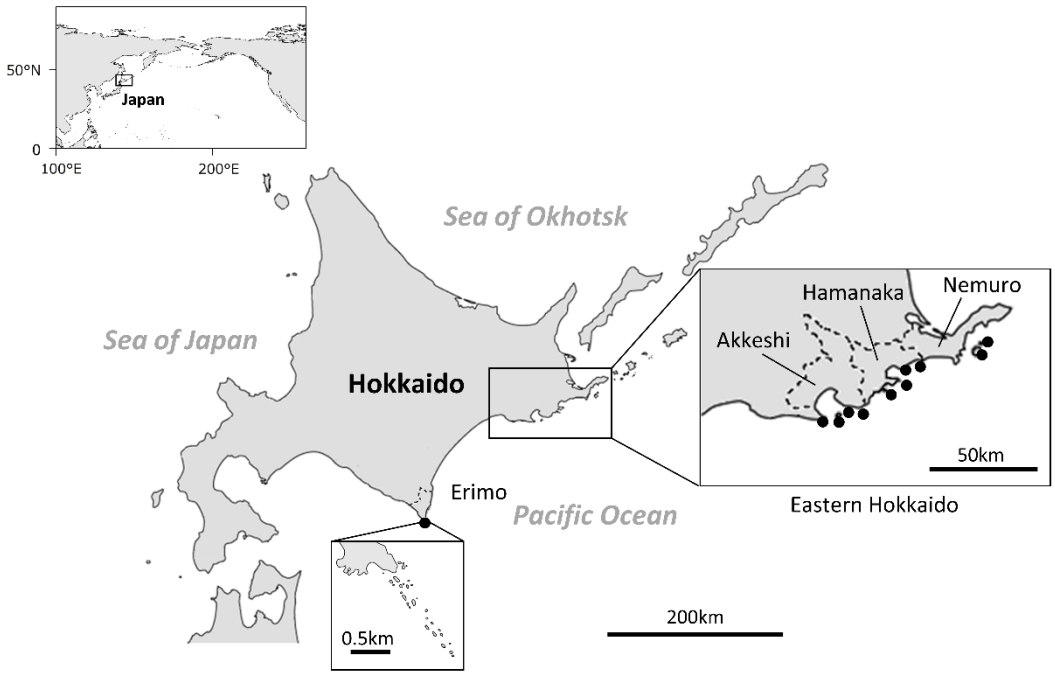



**Figure 2-1**. Locations of four administrative districts and eleven haul-out sites (black dots) of Japanese harbour seals in Hokkaido, Japan.

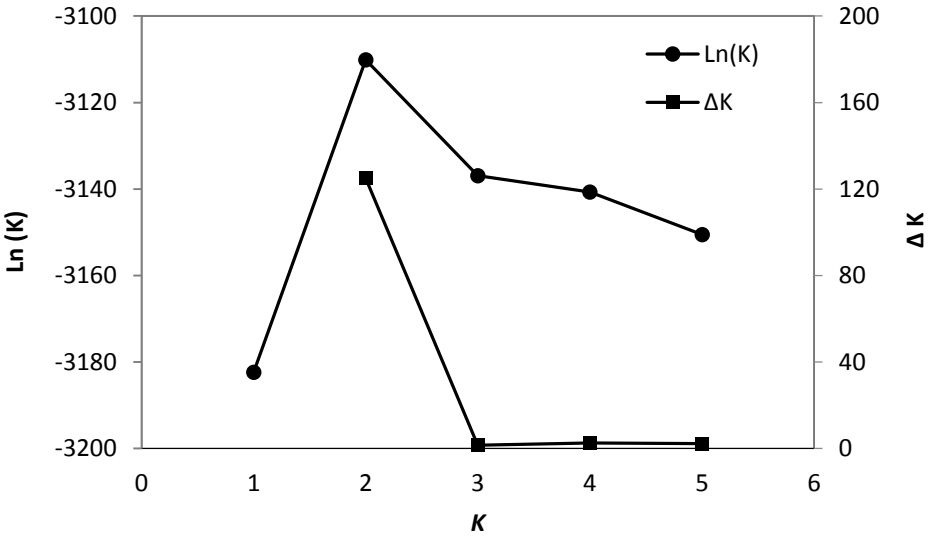



**Figure 2-2**.Putative number of populations indicated by Ln(K) and ΔK**.**

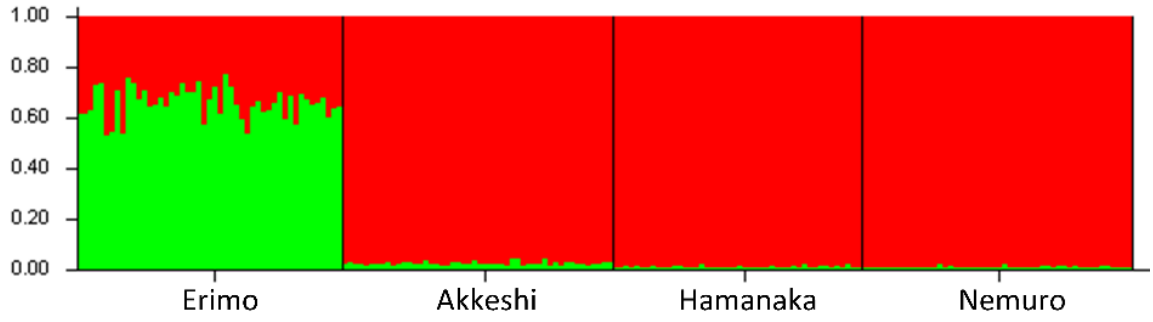



**Figure 2-3**. Genetically homogeneous groups, identified using STRUCTURE.

**Table 2-1.** Diversity indices of microsatellite loci in Japanese harbour seals of four administrative districts in Hokkaido, Japan.

| | Erimo | Akkeshi | Hamanaka | Nemuro |
|---|---|---|---|---|
| *N* | 49 | 50 | 46 | 50 |
| $A_R$ | 3.387 | 3.659 | 3.4 | 3.183 |
| $H_o$ | 0.488 | 0.462 | 0.443 | 0.434 |
| $H_e$ | 0.472 | 0.462 | 0.431 | 0.443 |
| $F_{IS}$ | -0.033 | 0.001 | -0.029 | 0.02 |

*N*: number of samples, $A_R$: allelic richness, $H_o$: observed heterozygosity, $H_e$: expected heterozygosity, $F_{IS}$: inbreeding coefficient.

**Table 2-2.** Estimates of genetic differentiation based on microsatellite loci among the four regions.

| Fst / Rst | Erimo | Akkeshi | Hamanaka | Nemuro |
|---|---|---|---|---|
| Erimo | - | 0.051*<br>0.000±0.000 | 0.037*<br>0.000±0.000 | 0.046*<br>0.000±0.000 |
| Akkeshi | 0.075*<br>0.000±0.000 | - | 0.009<br>0.027±0.002 | 0.004<br>0.123±0.003 |
| Hamanaka | 0.077*<br>0.000±0.000 | 0.009<br>0.095±0.002 | - | 0.004<br>0.166±0.004 |
| Nemuro | 0.078*<br>0.000±0.000 | 0.005<br>0.191±0.004 | -0.003<br>0.621±0.004 | - |

Pairwise Fst (above) and Rst (below).

P-values±SD are indicated under each Fst /Rst values.

Significance of P-values was determined after sequential Bonferroni correction (Rice 1989). *P<0.001

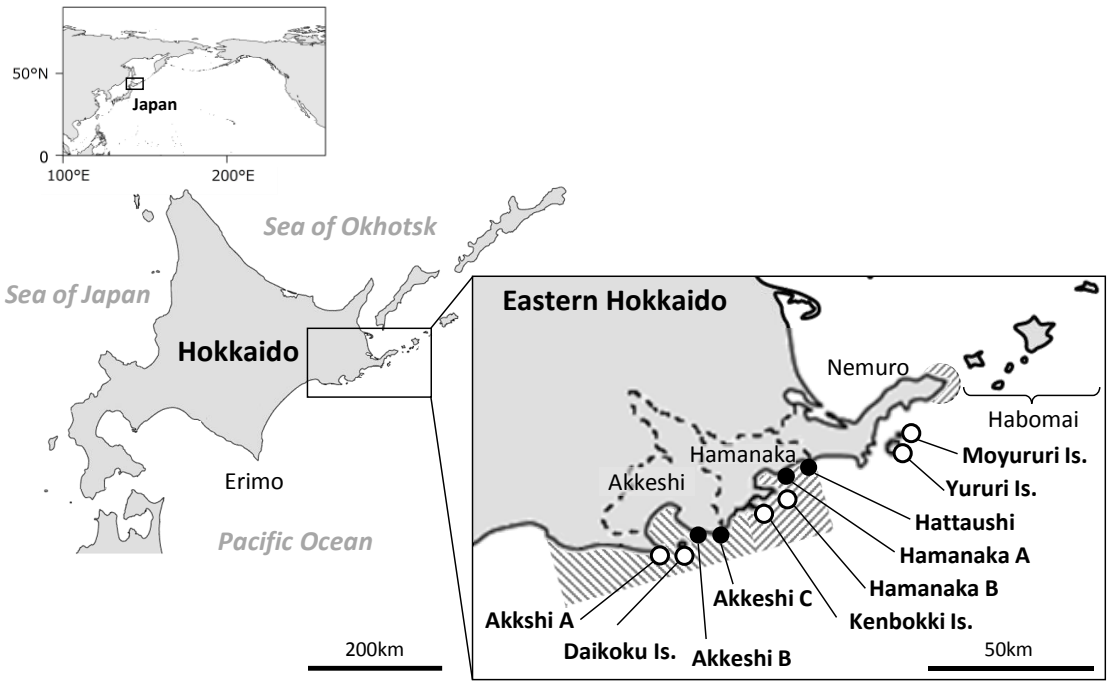
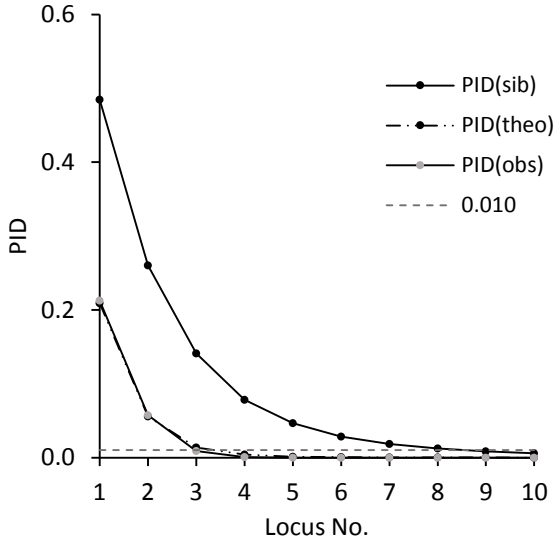
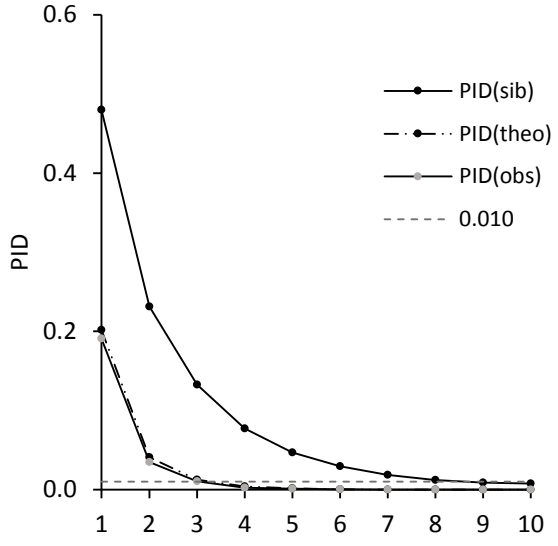



**Figure 3-1**. Locations of three administrative districts and 10 haul-out sites (circles) in Eastern Hokkaido. Shaded areas indicate approximate locations of bycatch samples used in this study. Haul-out sites are named following Kobayashi et al. (2014).

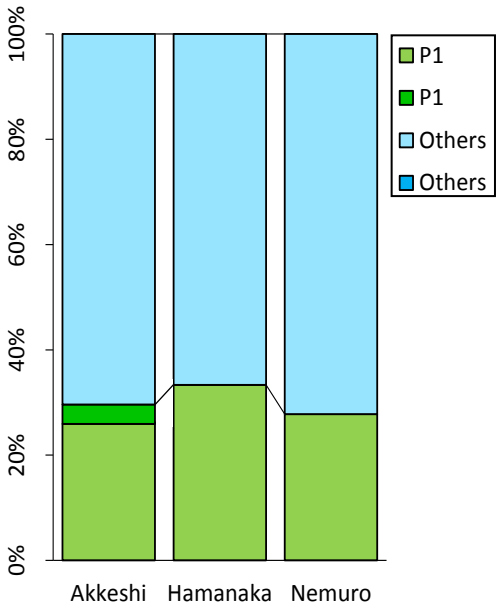



(a) Erimo

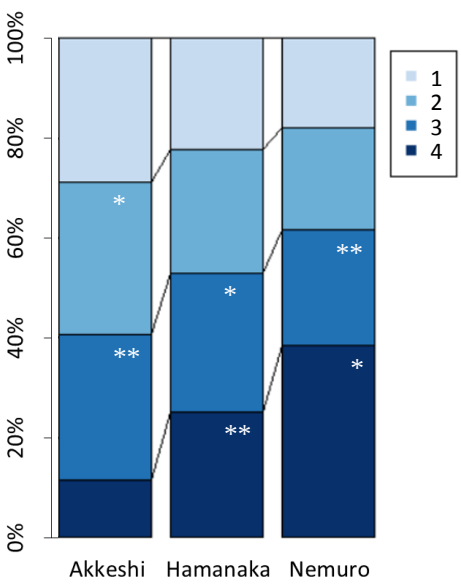



(b) Eastern Hokkaido

**Figure 3-2.** Trends and relationships among the three overall PID values: PID(obs), PID(theo) and PID(sib) across 10 loci in (a) Erimo (n=48) and (b) Eastern Hokkaido (n=146).

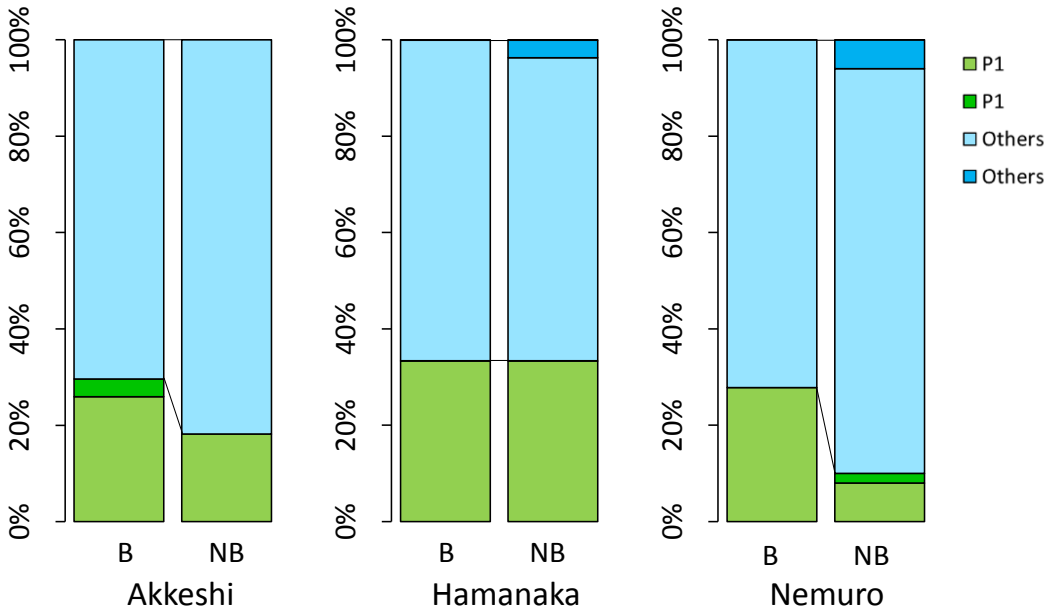



(a) Proportions of mtDNA haplo-groups in the breeding season.

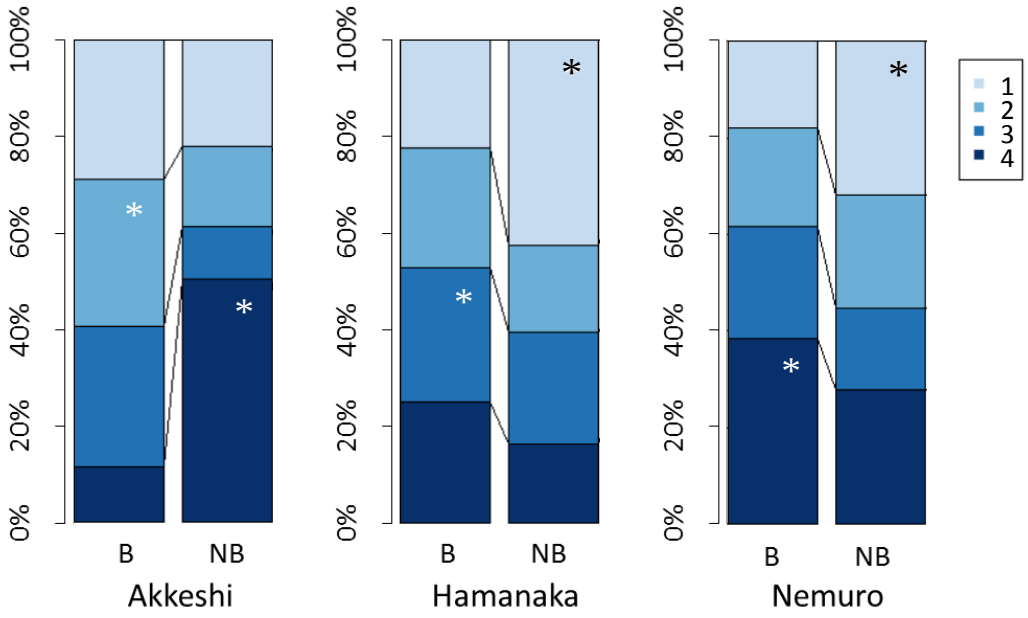



(b) Proportions of microsatellite clusters in the breeding season.

**Figure 3-3.** (a) Proportions of mtDNA haplo-groups defined in Chapter 1 (P1 and Others) during the breeding season in each area and unique haplotypes (darker colours in each lineage). (b) Proportions of microsatellite clusters during the breeding season in three regions. Asterisks indicate clusters that had the largest percentages (*) and the second largest percentages (**).




(a) Proportions of mtDNA haplo-groups in the breeding (B) and non-breeding (NB) season.




(b) Proportions of microsatellite clusters in the breeding (B) and non-breeding (NB) season.

**Figure 3-4.** (a) Proportions of mtDNA haplo-groups defined in Chapter 1 (P1 and Others) and unique haplotypes seen in each area (the darker colours in each lineage) between the breeding (B) and non-breeding (NB) season. (b) Proportions of microsatellite clusters between the breeding (B). and non-breeding season (NB) in the three regions. Asterisks indicate clusters with the largest percentage (*).

**Table 3-1.** PID values across 10 microsatellite loci arranged from the lowest PID to the highest

(a) Erimo

| | PID(obs) | | PID(theo) | | PID(sib) | |
|---|---|---|---|---|---|---|
| locus | locus | overall | locus | overall | locus | overall |
| SGPV9 | 0.213 | 0.213 | 0.209 | 0.209 | 0.485 | 0.485 |
| M11A | 0.232 | 0.049 | 0.247 | 0.052 | 0.537 | 0.260 |
| SGPV11 | 0.280 | 0.014 | 0.266 | 0.014 | 0.541 | 0.141 |
| Pvc78 | 0.259 | 0.004 | 0.291 | 0.004 | 0.555 | 0.078 |
| Hg3.7 | 0.309 | 0.001 | 0.323 | 0.001 | 0.596 | 0.047 |
| Pvc30 | 0.356 | 0.000 | 0.385 | 0.000 | 0.614 | 0.029 |
| Pvc19 | 0.384 | 0.000 | 0.396 | 0.000 | 0.648 | 0.019 |
| Aa4 | 0.450 | 0.000 | 0.444 | 0.000 | 0.664 | 0.012 |
| SGPV10 | 0.458 | 0.000 | 0.470 | 0.000 | 0.685 | 0.008 |
| SGPV16 | 0.483 | 0.000 | 0.485 | 0.000 | 0.715 | 0.006 |

(b) Eastern Hokkaido

| | PID(obs) | | PID(theo) | | PID(sib) | |
|---|---|---|---|---|---|---|
| locus | locus | overall | locus | overall | locus | overall |
| M11A | 0.191 | 0.191 | 0.202 | 0.202 | 0.480 | 0.480 |
| Hg3.7 | 0.205 | 0.039 | 0.203 | 0.041 | 0.482 | 0.231 |
| SGPV11 | 0.291 | 0.011 | 0.308 | 0.013 | 0.573 | 0.133 |
| Pvc19 | 0.308 | 0.004 | 0.315 | 0.004 | 0.582 | 0.077 |
| Aa4 | 0.359 | 0.001 | 0.370 | 0.001 | 0.609 | 0.047 |
| Pvc30 | 0.368 | 0.000 | 0.377 | 0.001 | 0.627 | 0.029 |
| SGPV9 | 0.384 | 0.000 | 0.379 | 0.000 | 0.632 | 0.019 |
| Pvc78 | 0.425 | 0.000 | 0.426 | 0.000 | 0.654 | 0.012 |
| SGPV16 | 0.488 | 0.000 | 0.500 | 0.000 | 0.724 | 0.009 |
| SGPV10 | 0.750 | 0.000 | 0.750 | 0.000 | 0.868 | 0.008 |

PID for (a) Erimo and (b) Eastern Hokkaido.

Overall PID<0.01 values are underlined.

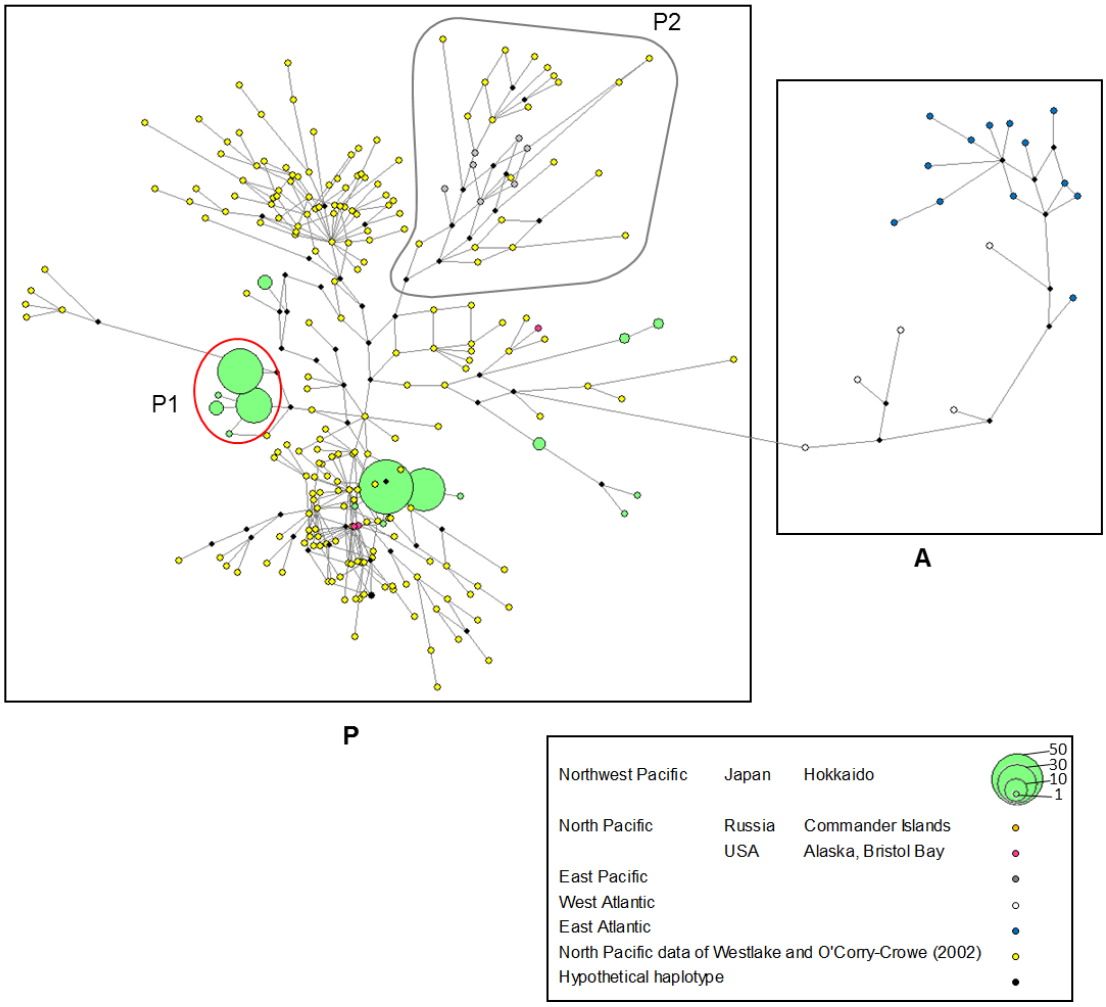
**Table 3-2**. Numbers of samples used in this study.

| | | Breeding (May-Jul) | | | Non-breeding (Sep-Nov) | | | |
|---|---|---|---|---|---|---|---|---|
| | | Akkeshi | Hamanaka | Nemuro | Akkeshi | Hamanaka | Nemuro | **Total** |
| Muscle & flipper ( )* | | **24 (4)** | **6 (0)** | **1(0)** | **22 (0)** | **27 (0)** | **50 (0)** | **130** |
| Non-invasive & biopsy | Collected | 3 | 17 | 39 | | | | 59 |
| | Used | **3** | **13** | **17** | | | | **33** |
| **Subtotal** | | **27** | **19** | **18** | **22** | **27** | **50** | **163** |
| **Total** | | | **64** | | | **99** | | |

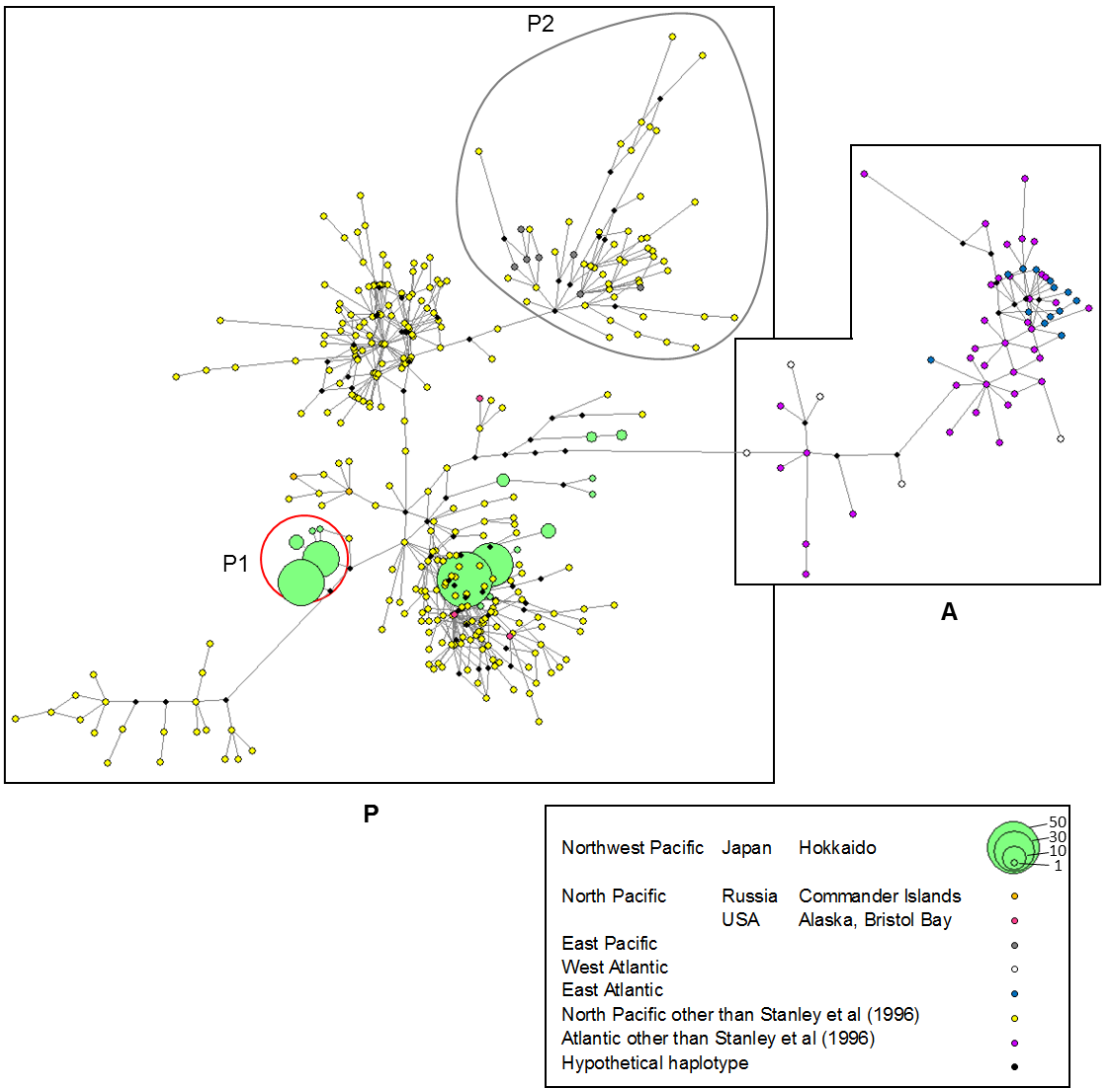
* Data from Chapters 1 and 2

( ) shows number of flipper samples.

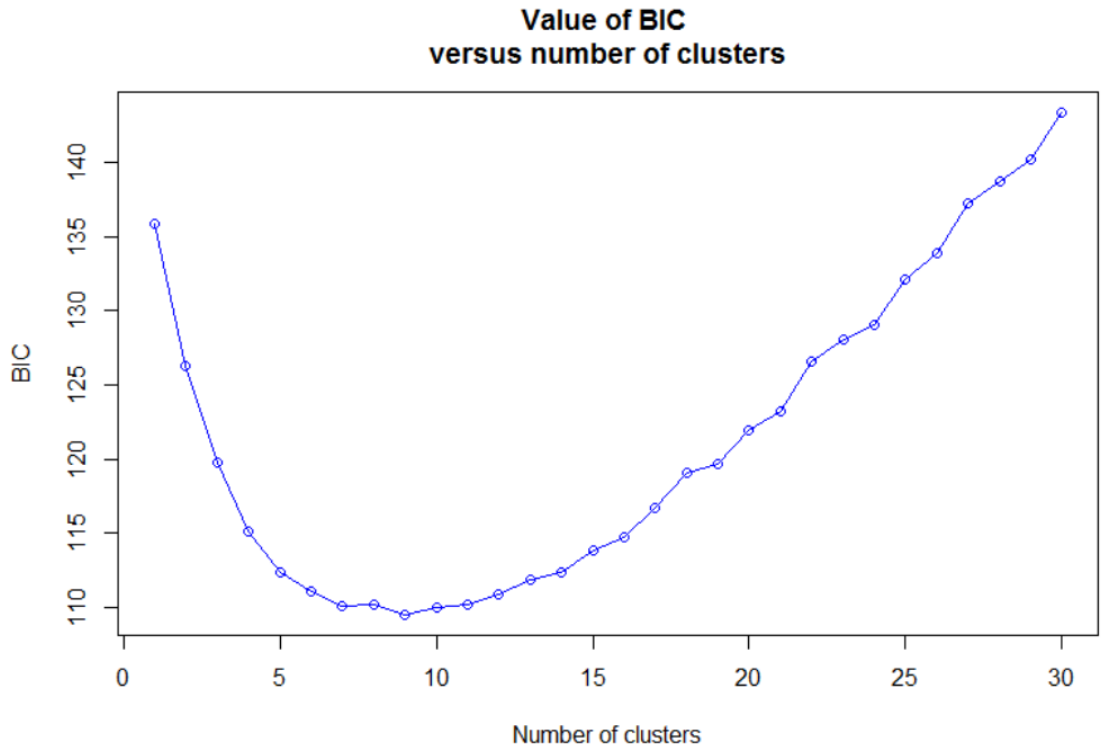
## Appendix



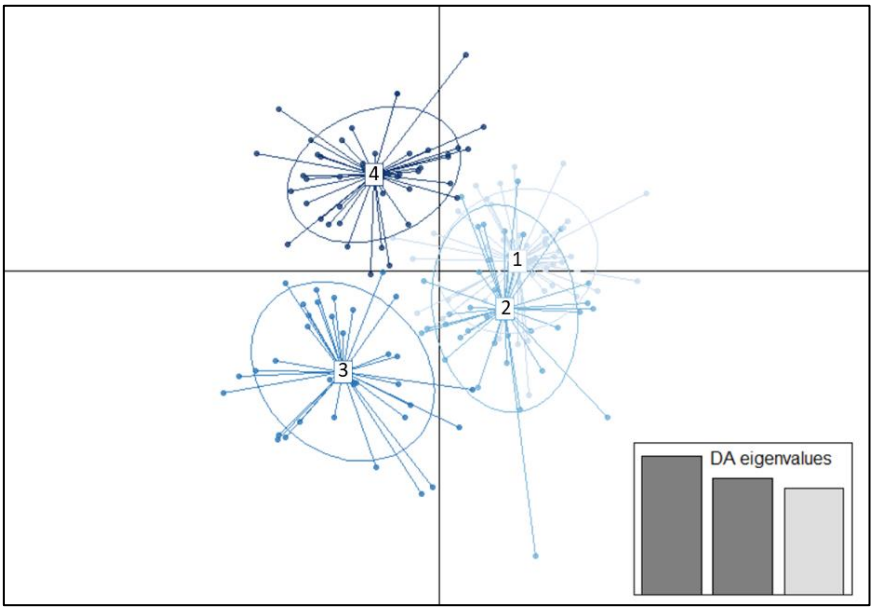

**Appendix 1-1.** Median joining tree based on the haplotypes of the Pacific harbour seals from Westlake and O'Corry-Crowe (2002), our data and Stanley *et al* (1996). Final 369bp of 255 haplotypes were used after alignment. Colouration for the haplotypes of our data and Stanley et al (1996) are same as Fig 3 for comparison. Haplotypes of Westlake and O'Corry-Crowe (2002) are shown as yellow.



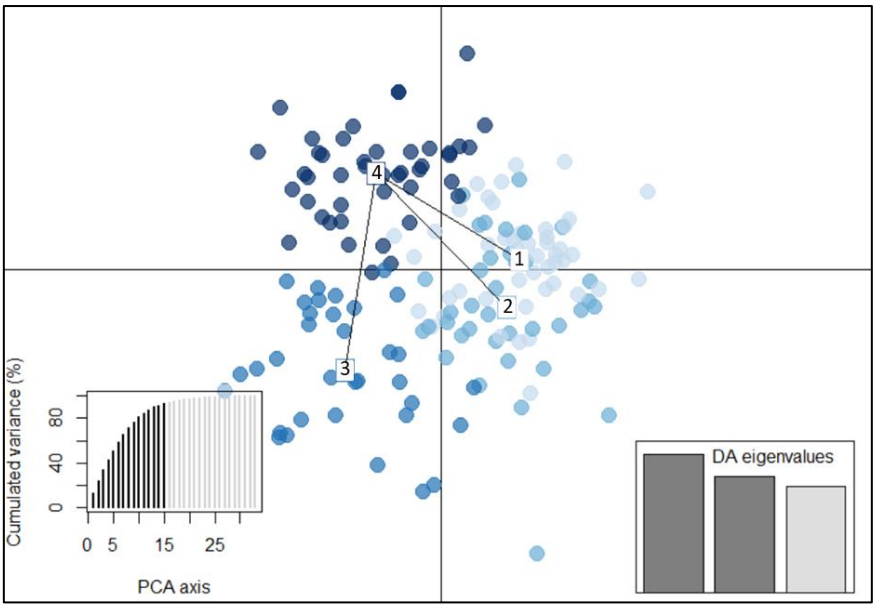

**Appendix 1-2.** Median joining tree based on all harbour seals data available in GenBank. Final sequences of 356bp 381haplotypes were used after alignment (Slade et al. 1994; Stanley et al. 1996; Burg et al. 1999; Westlake and O'Corry-Crowe 2002; Huber et al. 2010; Andersen et al. 2011). Colouration for the haplotypes of our data and Stanley et al(1996) are same as Fig 3 for comparison. Haplotypes of other studies were divided into Atlantic (purple) and Pacific (yellow).




**Appendix 3-1.** BIC values for K.




(a) DAPC scatter plot




(b) Minimum spanning tree

**Appendix 3-2.** Subpopulation of Japanese harbour seals defined by DAPC scatter plots (a) and genetically close groups shown by the minimum spanning tree based on the (squared) distance between populations inside the overall space (b).

## Sample Lists

| Larea | year | ID | month | weight | body length | sex | sampletype | Capter1 | Capter2 | Chapter3 |
|---|---|---|---|---|---|---|---|---|---|---|
| Erimo | 2004 | EZ0417 | 8 | 52 | 133 | F | muscle | ○ | ○ | |
| Erimo | 2004 | EZ0419 | 8 | 52 | 132 | F | muscle | ○ | ○ | |
| Erimo | 2004 | EZ0422 | 8 | 50 | 135 | M | muscle | ○ | ○ | |
| Erimo | 2004 | EZ0423 | 8 | 48 | 140 | M | muscle | ○ | ○ | |
| Erimo | 2004 | EZ0429 | 9 | 55 | 128 | M | muscle | ○ | ○ | |
| Erimo | 2004 | EZ0431 | 10 | 30 | 125 | F | muscle | ○ | ○ | |
| Erimo | 2005 | EZ05103 | 8 | 40 | 120 | M | muscle | ○ | ○ | |
| Erimo | 2005 | EZ05105 | 9 | 45 | 126 | M | muscle | ○ | ○ | |
| Erimo | 2011 | EZ110829-1 | 8 | 50 | 135 | F | flipper | ○ | ○ | |
| Erimo | 2011 | EZ110829-4 | 8 | 50 | 127.5 | F | flipper | ○ | ○ | |
| Erimo | 2011 | EZ110830-1 | 8 | 51 | 121.4 | F | flipper | ○ | ○ | |
| Erimo | 2011 | EZ110830-5 | 8 | 50 | 132.1 | M | flipper | ○ | ○ | |
| Erimo | 2011 | EZ1109 | 8 | 109 | 156.9 | F | muscle | ○ | ○ | |
| Erimo | 2011 | EZ110901-1 | 9 | 72 | 134.1 | M | flipper | ○ | ○ | |
| Erimo | 2011 | EZ1114 | 9 | 46 | 124.5 | F | muscle | ○ | ○ | |
| Erimo | 2011 | EZ1122 | 9 | 40 | 121.5 | M | muscle | ○ | ○ | |
| Erimo | 2011 | EZ1123 | 9 | 42 | 122.8 | F | muscle | ○ | ○ | |
| Erimo | 2011 | EZ1124nEZ110829-9 | 10 | 61 | 126.6 | M | muscle | ○ | ○ | |
| Erimo | 2011 | EZ1127 | 10 | 55 | 141.9 | M | muscle | ○ | ○ | |
| Erimo | 2012 | EZ120829-2 | 8 | 55 | 140.2 | F | flipper | ○ | ○ | |
| Erimo | 2012 | EZ120904-2 | 9 | 75 | 136.2 | M | flipper | ○ | ○ | |
| Erimo | 2012 | EZ120911-1 | 9 | 52 | 124.2 | F | flipper | ○ | ○ | |
| Erimo | 2012 | EZ121101-1 | 11 | 50 | 136.7 | F | flipper | ○ | ○ | |
| Erimo | 2012 | EZ1217 | 10 | 56 | 134.4 | F | muscle | ○ | ○ | |
| Erimo | 2012 | EZ1221 | 10 | 41 | 121.3 | M | muscle | ○ | ○ | |
| Erimo | 2012 | EZ1224 | 10 | 56 | 124.2 | M | muscle | ○ | ○ | |
| Erimo | 2012 | EZ1229 | 10 | 55 | 127.1 | F | muscle | ○ | ○ | |
| Erimo | 2013 | EZ1303 | 8 | 80 | 153 | F | muscle | ○ | ○ | |
| Erimo | 2013 | EZ1306 | 8 | 56 | 135.5 | F | muscle | ○ | ○ | |
| Erimo | 2013 | EZ130629-5 | 6 | 87 | 157 | F | flipper | ○ | ○ | |
| Erimo | 2013 | EZ1309 | 8 | 133 | 179 | M | muscle | ○ | ○ | |
| Erimo | 2013 | EZ1310 | 9 | 39.5 | 121.6 | M | muscle | ○ | ○ | |
| Erimo | 2013 | EZ131026-1 | 10 | 50 | 137 | M | flipper | ○ | ○ | |
| Erimo | 2013 | EZ131026-2 | 10 | 53 | 120 | M | flipper | ○ | ○ | |
| Erimo | 2013 | EZ1313 | 9 | 45 | 123.7 | M | muscle | ○ | ○ | |
| Erimo | 2013 | EZ1317 | 9 | 49 | 140.4 | F | muscle | ○ | ○ | |
| Erimo | 2013 | EZ1320 | 9 | 46 | 144.7 | F | muscle | ○ | ○ | |
| Erimo | 2013 | EZ1325 | 9 | 49 | 133.3 | F | muscle | ○ | ○ | |
| Erimo | 2013 | EZ1341 | 10 | 65 | 133.8 | F | muscle | ○ | ○ | |
| Erimo | 2013 | EZ1351 | 10 | 53 | 136.2 | F | muscle | ○ | ○ | |
| Erimo | 2013 | EZ1352 | 10 | 54 | 138 | M | muscle | ○ | ○ | |
| Erimo | 2013 | EZ1353 | 10 | 42 | 123.3 | M | muscle | ○ | ○ | |
| Erimo | 2013 | EZ1354 | 10 | 140 | 173.6 | F | muscle | ○ | ○ | |
| Erimo | 2014 | EZ1401 | 5 | 62 | 128.8 | M | muscle | ○ | ○ | |
| Erimo | 2014 | EZ1407 | 6 | 86 | 150 | F | muscle | ○ | ○ | |
| Erimo | 2014 | EZ1476 | 11 | 41 | 131.2 | F | muscle | ○ | ○ | |
| Erimo | 2014 | EZ1479 | 11 | 45 | 123.6 | M | muscle | ○ | ○ | |
| Erimo | 2014 | EZ1481 | 11 | 59 | 135.4 | M | muscle | ○ | ○ | |
| Akkeshi | 2007 | AZ0702 | 11 | 45.5 | 116.7 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2007 | AZ0703 | 11 | 33 | 116.5 | F | muscle | ○ | ○ | ○ |
| Akkeshi | 2007 | AZ0707 | 11 | 48 | 128.2 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2009 | AZ090405-1 | 4 | 135 | 166 | M | flipper | ○ | ○ | |
| Akkeshi | 2010 | AZ100410-1 | 4 | 85 | 162 | F | flipper | ○ | ○ | |
| Akkeshi | 2010 | AZ100416-1 | 4 | 54 | 127.5 | M | flipper | ○ | ○ | |
| Akkeshi | 2012 | AZ1201 | 4 | 46 | 118.3 | F | muscle | ○ | ○ | |
| Akkeshi | 2012 | AZ1202 | 5 | 61 | 135.5 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2012 | AZ1203-1 | 5 | 52.4 | 112.3 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2012 | AZ1203-2 | 5 | 50 | 112.3 | F | muscle | ○ | ○ | ○ |
| Akkeshi | 2012 | AZ1204 | 5 | 59 | 130.5 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2012 | AZ1205 | 5 | 57 | 124.7 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2012 | AZ120531-1 | 5 | 41 | 115.5 | F | flipper | ○ | ○ | ○ |
| Akkeshi | 2012 | AZ1206 | 5 | 65 | 123.1 | F | muscle | ○ | ○ | ○ |
| Akkeshi | 2017 | AZ120619-1 | 6 | 33 | 106.6 | - | flipper | | | ○ |
| Akkeshi | 2012 | AZ1207 | 5 | 55 | 127 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2012 | AZ1208 | 5 | 56 | 119.3 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2012 | AZ1209 | 5 | 47 | 120.1 | F | muscle | ○ | ○ | ○ |

| Larea | year | ID | month | weight | body length | sex | sampletype | Capter1 | Capter2 | Chapter3 |
|---|---|---|---|---|---|---|---|---|---|---|
| Akkeshi | 2012 | AZ1210 | 5 | 47 | 115.7 | F | muscle | ○ | ○ | ○ |
| Akkeshi | 2012 | AZ1214 | 6 | 39 | 106 | F | muscle | | ○ | |
| Akkeshi | 2012 | AZ1215 | 6 | 35 | 110.5 | M | muscle | ○ | | |
| Akkeshi | 2012 | AZ1218 | 6 | 44 | 110.7 | F | muscle | ○ | ○ | ○ |
| Akkeshi | 2012 | AZ1228 | 7 | 29 | 110 | M | muscle | ○ | | |
| Akkeshi | 2012 | AZ1229 | 7 | 32 | 107.3 | M | muscle | ○ | | |
| Akkeshi | 2012 | AZ1238 | 6 | 34.5 | 115.5 | F | muscle | ○ | ○ | |
| Akkeshi | 2013 | AZ1301-2 | 4 | 27 | 115.2 | F | muscle | ○ | ○ | |
| Akkeshi | 2013 | AZ1302 | 5 | 40 | 127 | F | muscle | ○ | ○ | |
| Akkeshi | 2013 | AZ1303 | 5 | 45 | 119.1 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2013 | AZ1304 | 5 | 54 | 115.6 | F | muscle | ○ | ○ | ○ |
| Akkeshi | 2013 | AZ1306 | 5 | 53 | 119.4 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2013 | AZ1307 | 5 | 41 | 107 | M | muscle | ○ | | ○ |
| Akkeshi | 2013 | AZ1309 | 6 | 40 | 117.4 | M | muscle | ○ | ○ | |
| Akkeshi | 2013 | AZ1310 | 6 | 35 | 111.3 | M | muscle | ○ | ○ | |
| Akkeshi | 2013 | AZ1313 | 6 | 32 | 110 | F | muscle | ○ | | |
| Akkeshi | 2013 | AZ1316 | 8 | 29 | 112.6 | F | muscle | ○ | ○ | |
| Akkeshi | 2013 | AZ1317 | 9 | 33 | 112.4 | F | muscle | ○ | | ○ |
| Akkeshi | 2013 | AZ1318 | 9 | 40 | 125.3 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2013 | AZ1319 | 9 | 31 | 118.7 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2013 | AZ1321 | 9 | 51 | 133.9 | F | muscle | ○ | ○ | ○ |
| Akkeshi | 2013 | AZ1322 | 9 | 39 | 118.9 | F | muscle | ○ | ○ | ○ |
| Akkeshi | 2013 | AZ1323 | 9 | 30.5 | 98 | F | muscle | | | ○ |
| Akkeshi | 2013 | AZ1324 | 10 | 40 | 126 | F | muscle | ○ | ○ | ○ |
| Akkeshi | 2013 | AZ1326 | 10 | 37 | 115 | F | muscle | ○ | ○ | ○ |
| Akkeshi | 2013 | AZ1327 | 10 | 49 | 128 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2013 | AZ1328 | 10 | 39 | 121 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2013 | AZ1329 | 10 | 42 | 124 | F | muscle | ○ | ○ | ○ |
| Akkeshi | 2013 | AZ1330 | 11 | 53 | 136.1 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2013 | AZ1331 | 3 | 44 | 118.8 | F | muscle | ○ | ○ | |
| Akkeshi | 2014 | AZ1401 | 4 | 42 | 116.7 | M | muscle | ○ | ○ | |
| Akkeshi | 2014 | AZ1402 | 5 | 43 | 114.3 | F | muscle | ○ | ○ | ○ |
| Akkeshi | 2014 | AZ1410 | 6 | 34 | 107.2 | F | muscle | | ○ | |
| Akkeshi | 2014 | AZ1415 | 9 | 39 | 109 | F | muscle | ○ | ○ | ○ |
| Akkeshi | 2014 | AZ1420 | 10 | 40 | 115.2 | M | muscle | | ○ | ○ |
| Akkeshi | 2014 | AZ1424 | 10 | 37 | 119.1 | M | muscle | | ○ | ○ |
| Akkeshi | 2014 | AZ1427 | 10 | 45 | 121.6 | M | muscle | ○ | ○ | ○ |
| Akkeshi | 2014 | AZ1428 | 10 | 36 | 117 | M | muscle | | ○ | ○ |
| Akkeshi | 2014 | AZ1429 | 10 | 39 | 114.3 | M | muscle | | ○ | ○ |
| Akkeshi | 2015 | AZ1501 | 5 | 45 | 113 | F | muscle | | | ○ |
| Akkeshi | 2015 | AZ1514 | 9 | 49 | 128.7 | F | muscle | | ○ | ○ |
| Akkeshi | 2017 | AZ170527-1 | 5 | 13 | 76.7 | - | flipper | | | ○ |
| Akkeshi | 2017 | AZ170527-2 | 5 | 28 | 97.2 | - | flipper | | | ○ |
| Akkeshi | 2017 | AZ1717 | 6 | 41 | <155 | M | muscle | | | ○ |
| Akkeshi | 2019 | AZ1902(mum) | 5 | 158 | 167 | F | muscle | | | ○ |
| Akkeshi | 2019 | AZ1902(pup) | 5 | 19 | 98 | M | muscle | | | ○ |
| Akkeshi | 2018 | D180527-69 | 5 | - | - | - | mucus | | | ○ |
| Akkeshi | 2018 | D180527-70 | 5 | - | - | - | mucus | | | ○ |
| Akkeshi | 2018 | D180527-75 | 5 | - | - | - | mucus | | | ○ |
| Hamanaka | 2019 | 1906210 | 6 | - | - | - | faece | | | ○ |
| Hamanaka | 2019 | 1906214 | 6 | - | - | - | faece | | | ○ |
| Hamanaka | 2019 | 1906217 | 6 | - | - | - | faece | | | ○ |
| Hamanaka | 2019 | 1906218 | 6 | - | - | - | faece | | | ○ |
| Hamanaka | 2019 | 190621-8 | 6 | - | - | - | epiderm | | | ○ |
| Hamanaka | 2012 | AZ1219 | 6 | 33 | 109.8 | M | muscle | ○ | ○ | |
| Hamanaka | 2012 | AZ1223 | 6 | 44 | 108.4 | M | muscle | ○ | ○ | ○ |
| Hamanaka | 2012 | AZ1224 | 6 | 39 | 116.9 | F | muscle | ○ | ○ | |
| Hamanaka | 2012 | AZ1225 | 7 | 32 | 106.1 | M | muscle | ○ | ○ | |
| Hamanaka | 2012 | AZ1226 | 7 | 35 | 107 | M | muscle | ○ | ○ | |
| Hamanaka | 2012 | AZ1227 | 7 | 32 | 108.8 | F | muscle | ○ | ○ | |
| Hamanaka | 2012 | AZ1230 | 8 | 39 | 126.8 | M | muscle | ○ | ○ | |
| Hamanaka | 2012 | AZ1231 | 9 | 40 | 126.1 | M | muscle | ○ | ○ | ○ |
| Hamanaka | 2012 | AZ1232 | 9 | 53 | 120.4 | M | muscle | ○ | ○ | ○ |
| Hamanaka | 2012 | AZ1234 | 10 | 35 | 112.5 | F | muscle | ○ | ○ | ○ |
| Hamanaka | 2012 | AZ1235 | 10 | 39 | 117.2 | M | muscle | ○ | ○ | ○ |
| Hamanaka | 2012 | AZ1236 | 10 | 58 | 128.2 | F | muscle | ○ | ○ | ○ |

| Larea | year | ID | month | weight | body length | sex | sampletype | Capter1 | Capter2 | Chapter3 |
|---|---|---|---|---|---|---|---|---|---|---|
| Hamanaka | 2013 | AZ1311 | 6 | 40 | 111 | M | muscle | ○ | ○ | |
| Hamanaka | 2013 | AZ1314 | 7 | 33 | 105 | F | muscle | ○ | ○ | |
| Hamanaka | 2013 | AZ1320 | 9 | 33 | 115.1 | M | muscle | ○ | ○ | ○ |
| Hamanaka | 2013 | AZ1325 | 10 | 56 | 137.4 | F | muscle | ○ | ○ | ○ |
| Hamanaka | 2014 | AZ1403 | 5 | 74 | 138.2 | F | muscle | ○ | ○ | ○ |
| Hamanaka | 2014 | AZ1404 | 6 | 45 | 117 | M | muscle | ○ | ○ | ○ |
| Hamanaka | 2014 | AZ1406 | 6 | 39 | 115.9 | F | muscle | ○ | ○ | |
| Hamanaka | 2014 | AZ1409 | 6 | 29 | 95.4 | F | muscle | ○ | ○ | |
| Hamanaka | 2014 | AZ1411 | 6 | 33 | 107.3 | M | muscle | ○ | ○ | |
| Hamanaka | 2014 | AZ1414 | 7 | 37 | 118.1 | M | muscle | ○ | ○ | |
| Hamanaka | 2014 | AZ1417 | 9 | 31 | 104.3 | M | muscle | ○ | ○ | ○ |
| Hamanaka | 2014 | AZ1418 | 9 | 71 | 135 | M | muscle | | ○ | ○ |
| Hamanaka | 2014 | AZ1419 | 9 | 33 | 112.8 | F | muscle | ○ | ○ | ○ |
| Hamanaka | 2014 | AZ1421 | 10 | 27 | 95.8 | M | muscle | ○ | ○ | ○ |
| Hamanaka | 2014 | AZ1422 | 10 | 36 | 110.9 | M | muscle | ○ | ○ | ○ |
| Hamanaka | 2014 | AZ1423 | 10 | 40.5 | 114.7 | M | muscle | ○ | ○ | ○ |
| Hamanaka | 2014 | AZ1425 | 10 | 60 | 130.5 | F | muscle | ○ | ○ | ○ |
| Hamanaka | 2014 | AZ1426 | 10 | 38 | 117 | M | muscle | ○ | ○ | ○ |
| Hamanaka | 2015 | AZ1504 | 6 | 46 | 123.3 | M | muscle | | ○ | ○ |
| Hamanaka | 2015 | AZ1505 | 6 | 57 | 124 | M | muscle | | ○ | ○ |
| Hamanaka | 2015 | AZ1515 | 9 | 57 | 132.5 | F | muscle | | ○ | ○ |
| Hamanaka | 2015 | AZ1516 | 9 | 37 | 113.1 | M | muscle | | ○ | ○ |
| Hamanaka | 2015 | AZ1517 | 9 | 38 | 104 | F | muscle | | ○ | ○ |
| Hamanaka | 2015 | AZ1518 | 9 | 34 | 107.7 | M | muscle | | ○ | ○ |
| Hamanaka | 2015 | AZ1519 | 9 | 100 | 155.9 | M | muscle | | ○ | ○ |
| Hamanaka | 2015 | AZ1520 | 9 | 50 | 117.1 | F | muscle | | ○ | ○ |
| Hamanaka | 2015 | AZ1521 | 9 | 72 | 139.4 | F | muscle | | ○ | ○ |
| Hamanaka | 2015 | AZ1522 | 9 | 46 | 118.7 | F | muscle | | ○ | ○ |
| Hamanaka | 2015 | AZ1523 | 10 | 56 | 138 | M | muscle | | ○ | ○ |
| Hamanaka | 2015 | AZ1524 | 10 | 32 | 119.2 | F | muscle | | ○ | ○ |
| Hamanaka | 2015 | AZ1525 | 10 | 64 | 142.4 | M | muscle | | ○ | ○ |
| Hamanaka | 2016 | AZ1606 | 10 | 35 | 112.1 | F | muscle | | ○ | ○ |
| Hamanaka | 2017 | AZ1704 | 6 | 34 | 116.1 | F | muscle | | ○ | |
| Hamanaka | 2017 | AZ1719 | 6 | 53 | 128.5 | M | muscle | | ○ | ○ |
| Hamanaka | 2018 | H180515-1 | 5 | - | - | - | mucus | | | ○ |
| Hamanaka | 2018 | H180515-2 | 5 | - | - | - | mucus | | | ○ |
| Hamanaka | 2018 | H180515-3 | 5 | - | - | - | mucus | | | ○ |
| Hamanaka | 2018 | H180515-4 | 5 | - | - | - | mucus | | | ○ |
| Hamanaka | 2018 | H180515-5 | 5 | - | - | - | mucus | | | ○ |
| Hamanaka | 2018 | H180516-6 | 5 | - | - | - | placenta | | | ○ |
| Hamanaka | 2019 | JPN81 | 6 | - | - | - | mucus | | | ○ |
| Hamanaka | 2019 | JPN82 | 6 | - | - | - | mucus | | | ○ |
| Nosappu | 2002 | NZ0252 | 11 | 100 | 161 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2003 | NZ0356 | 10 | - | 158.5 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2003 | NZ0364-1 | 11 | 100 | 180 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2004 | NZ0445 | 9 | 84 | 160.7 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2004 | NZ0448 | 9 | 105 | 158 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2004 | NZ0454 | 10 | 91 | 162.4 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2004 | NZ0465 | 10 | 148 | 175 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2004 | NZ0466 | 10 | 134 | 175.2 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2004 | NZ0468 | 10 | 128 | 170.5 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2004 | NZ0473 | 11 | 87 | 167 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2004 | NZ0476 | 11 | 122 | 173.5 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2004 | NZ0477 | 11 | 95 | 162.2 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2004 | NZ0479 | 11 | 128 | 162.5 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0501 | 9 | 50 | 135.5 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0503 | 11 | 97 | 170 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0507 | 9 | 87 | 181.5 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0508 | 9 | 55 | 162.5 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0509 | 9 | 77 | 169.5 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0511 | 9 | 89 | 188 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0516 | 9 | 56 | 163 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0517 | 9 | 104 | 185.5 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0519 | 9 | 71 | 163 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0526 | 9 | 88.3 | 169 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0530 | 9 | 79 | 171 | M | muscle | ○ | ○ | ○ |

| Larea | year | ID | month | weight | body length | sex | sampletype | Capter1 | Capter2 | Chapter3 |
|---|---|---|---|---|---|---|---|---|---|---|
| Nosappu | 2005 | NZ0531 | 9 | 71 | 168.4 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0534 | 9 | 52 | 142 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0536 | 9 | 73 | 171.8 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0537 | 9 | 83 | 177 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0538 | 9 | 63 | 165.4 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2015 | NZ0539 | 5 | 55 | 135.5 | M | muscle | | | ○ |
| Nosappu | 2005 | NZ0541 | 9 | 62 | 163 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0542 | 9 | 68 | 167.5 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0544 | 9 | 51 | 164 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0548 | 9 | 59 | 141.8 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0550 | 9 | 54 | 146.5 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0553 | 9 | 71 | 171.8 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0556 | 9 | 102 | 182.8 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0557 | 9 | 74 | 171.3 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0558 | 9 | 72 | 137.3 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0564 | 10 | 117 | 187.5 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0566 | 10 | 80 | 180 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0567 | 10 | 60 | 145.5 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0569 | 10 | 59 | 160.9 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0571 | 10 | 51 | 160.3 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0573 | 10 | 61 | 165.3 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0574 | 10 | 51 | 136.9 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0577 | 10 | 85 | 170.1 | M | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0580 | 10 | 132 | 201.4 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0581 | 10 | 59 | 160.5 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0583 | 11 | 99 | 180.5 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2005 | NZ0584 | 11 | 126 | 188 | F | muscle | ○ | ○ | ○ |
| Nosappu | 2019 | 190619-10 | 6 | - | - | - | epiderm | | | ○ |
| Nosappu | 2019 | 190619-8 | 6 | - | - | - | epiderm | | | ○ |
| Nosappu | 2019 | 190622-15-1 | 6 | - | - | - | epiderm | | | ○ |
| Nosappu | 2019 | 190622-2 | 6 | - | - | - | faece | | | ○ |
| Nosappu | 2019 | 190622-3 | 6 | - | - | - | faece | | | ○ |
| Nosappu | 2019 | 190622-4 | 6 | - | - | - | faece | | | ○ |
| Nosappu | 2019 | 190622-5 | 6 | - | - | - | faece | | | ○ |
| Nosappu | 2019 | 190702-2 | 7 | - | - | - | faece | | | ○ |
| Nosappu | 2019 | 190702-6 | 7 | - | - | - | epiderm | | | ○ |
| Nosappu | 2018 | M180523-1 | 5 | - | - | - | faece | | | ○ |
| Nosappu | 2018 | M180607 | 6 | - | - | - | epiderm | | | ○ |
| Nosappu | 2019 | NZ190527 | 5 | - | - | - | mucus | | | ○ |
| Nosappu | 2019 | NZ190622-13 | 6 | - | - | - | hair | | | ○ |
| Nosappu | 2019 | NZ190622-8 | 6 | - | - | - | hair | | | ○ |
| Nosappu | 2019 | NZ190702-1 | 7 | - | - | - | hair | | | ○ |
| Nosappu | 2019 | NZ190702-10 | 7 | - | - | - | hair | | | ○ |
| Nosappu | 2019 | NZ190702-8 | 7 | - | - | - | hair | | | ○ |