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Technical Report

What do we know about whales and ecosystem in the western North Pacific Ocean? Part 1: summary of results on stock structure in baleen whale species

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ABSTRACT

Identification of biological stocks within a species is fundamental to appropriately interpret demographic parameters. Information on the temporal-spatial stock structure is crucial in establishing effective management and conservation strategies of marine living resources. This paper summarizes the recent genetic studies on stock structure of four baleen whale species in the North Pacific: right, sei, Bryde's and common minke whales. These studies used a large number of genetic samples collected mainly in surveys conducted by the Institute of Cetacean Research since 1994. Results of the studies revealed novel stock structures, and this information is contributing to stock assessments and conservation of each whale species in the North Pacific.

INTRODUCTION

Different biological stocks could have different demographic characteristics and respond in different ways to environmental stress. For this reason, the identification of stocks in a species is fundamental for the appropriate interpretation of abundance estimation and other demographic parameters. Information on the temporal-spatial stock structure is thus crucial in establishing effective management and conservation strategies. In this regard, Moritz (1994) defined management units (MUs) as historically isolated sets of populations and suggested that genetic markers be used to define management units within species: populations with a significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of the alleles.

This paper describes the outline of the recent studies which examine genetic diversities and stock structure based on the MUs defined by Moritz (1994) for the four North Pacific whale species: right (*Eubalaena japonica*), sei (*Balaenoptera borealis*), Bryde's (*B. brydei*) and common minke whales (*B. acutorostrata*).

BACKGROUND ON GENETIC STUDIES ON STOCK STRUCTURE IN NORTH PACIFIC BALEEN WHALES

North Pacific right whales

LeDuc *et al.* (2012) was the first study that focused on North Pacific right whale's stock structure and genetic diversity. The study using the mitochondrial DNA (mtDNA) control region and six microsatellite DNA (msDNA) loci, mainly showed the potential parentage and genetic diversity in 24 individual whales (23 from the eastern North Pacific and one from the western North Pacific). Their results inferred that North Pacific right whales could be isolated to some degree between the western and eastern regions based on paternity analysis. However, it was noted that the relationship of the eastern North Pacific right whales to those in the western North Pacific was largely unexamined.

Sei whales

A pioneering work on stock structure of North Pacific sei whales was carried out on the feeding grounds by Wada and Numachi (1991) using three polymorphic allozyme loci, which suggested a single stock in the area east of 160°E. The most comprehensive genetic study was performed by Kanda *et al.* (2009c) using msDNA genotypes at 17 loci and mtDNA control region sequences from a total of 790 specimens collected on the feeding grounds between 140°E and 135°W during 1972–1973 and 2002–2007. The study also showed no evidence of genetic heterogeneity in the research area. Kanda *et al.* (2013) subsequently examined genetic differences of this species both spatially and temporally. The results of the study were consistent with those found by Kanda *et al.* (2009). In addition, Kanda *et al.* (2015) examined spatial genetic differentiation using genotypes at 16 msDNA

loci by only using samples collected during the summers in 2010, 2011 and 2012 in order to eliminate temporal negative biases. The study again did not find evidence of multiple stocks of sei whales in the North Pacific.

Bryde's whales

A series of allozyme studies (Wada and Numachi, 1991; Wada, 1996) were conducted using Bryde's whale samples collected between 150°E and 160°W, on the North Pacific feeding grounds. These studies did not find evidence of genetic heterogeneity in the research area. A subsequent RFLP analyses investigated the pattern of genetic variations of Bryde's whales at intra- and inter-oceanic levels for mtDNA control region in specimens from four locations: western North Pacific, western South Pacific, eastern South Pacific and eastern Indian Oceans (Pastene *et al.*, 1997). The study showed significant genetic differences among the four regions, but did not reveal any significant genetic differences within the western North Pacific Ocean. The most recent population genetic study was performed by Kanda *et al.* (2007), using msDNA genotypes at 17 loci and mtDNA control region sequences in a sample set used by Pastene *et al.* (1997), as well as additional samples from the western North Pacific feeding grounds. The study confirmed significant genetic differentiations among oceanic regions, but not within the western North Pacific.

Common minke whales

At least two stocks of the common minke whales have been historically recognized in the western North Pacific, namely the Okhotsk Sea–West Pacific (known as the O-stock) and the Sea of Japan–Yellow Sea–East China Sea (known as the J-stock). These two stocks are differentiated morphologically, reproductively (Omura and Sakiura, 1956; Kato, 1992) and genetically (Wada and Numachi, 1991; Goto and Pastene, 1997), which suggests their reproductive isolation.

Kanda *et al.* (2009a; b) examined genetic variations at 16 msDNA loci in a total of 2,542 genetic samples collected in the waters around Japan and pelagic areas of the western North Pacific during 1994–2007, and succeeded in the assignment of individual to stocks using a Bayesian clustering approach, i.e. STRUCTURE analysis. The study presented the highest likelihood probability at K=2, which indicated that the samples came from two genetically distinct populations, i.e. O- and J-stocks.

Pastene *et al.* (2016a) updated these studies by increasing sample sizes, and showed a finer spatial distribution of each stock (Figure 1): J-stock whales mainly distributed in the Sea of Japan (sub-areas 6E and 10E), O-stock whales mainly distributed in the offshore North Pacific (east of sub-area 7WR). Both stocks occur along the Pacific coast of Japan (sub-areas 7CN, 7CS and 2C) and in the southern Okhotsk Sea (sub-area 11) but sub-area 2C is mainly occupied by the J-stock whales. Furthermore, the study showed the temporal distribution of the O- and J-stock whales on the Pacific side of Japan (sub-areas 2C, 7CN and 7CS) as shown in Figure 2: J-stock whales are predominant throughout the year in sub-area 2C, while the proportion of the J-stock whales increases in autumn/winter and decreases in spring/summer in sub-areas 7CS and 7CN. The reverse is true for O-stock.

A subsequent study by Pastene *et al.* (2016b) investigated the possibility of additional structure within O-stock using hypothesis testing and the discriminant analysis of principal component (DAPC) approach. A simulation exercise showed that the statistical power of

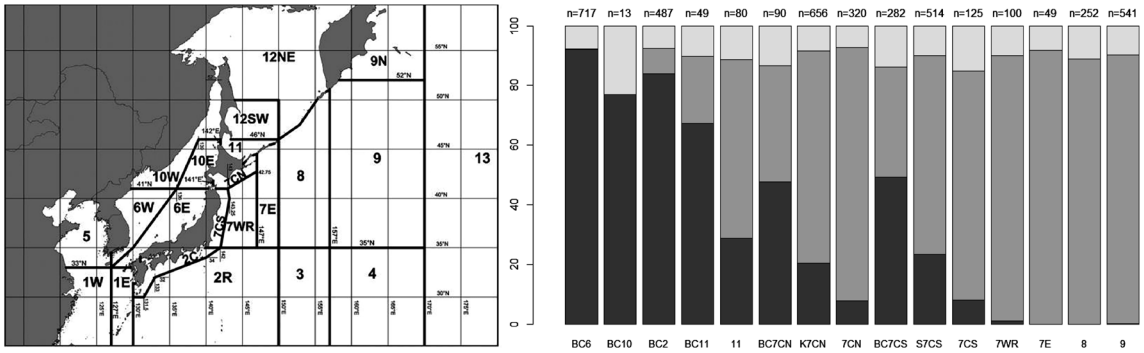

Figure 1. Management sub-areas defined by the IWC for the western North Pacific common minke whales (left), and spatial occurrence of O- and J-stocks in sub-areas (right: BC2, BC6, BC7CS, BC7CN, BC10, BC11=bycatches from the respective areas; K7CN=coastal JARPNII surveys at Kushiro; S7CS=coastal JARPNII surveys at Sanriku; 7CS, 7CN, 7WR, 7E, 8, 9 and 11=offshore JARPN/JARPNII surveys). Sample sizes are at the top of each bar. Color indicates stock assignment by STRUCTURE analysis (gray=O-stock, dark gray=J-stock, light gray=unassigned).

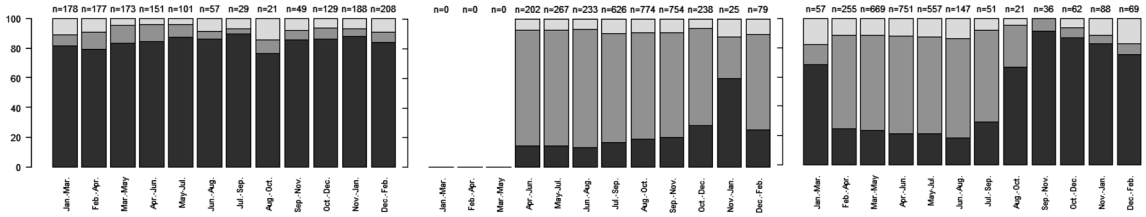

Figure 2. Monthly occurrence of J- and O-stocks in sub-areas 2C (left), 7CN (middle) and 7CS (left). Each bar is expressed as three months moving average. Sample sizes are on the top of each bar. Samples from sub-area 2C and subareas 7CN and 7CS were collected during 2001–2014 and 1994–2014, respectively. Color indicates stock assignment by STRUCTURE analysis (dark gray=J-stock, gray=O-stock, light gray=unassigned).

the hypothesis testing was high. Both analyses showed no evidence of sub-structuring within O-stock.

Tiedemann *et al.* (2017) developed a new maximum likelihood-based approach to infer Parent–Offspring (P–O) relationships using full msDNA data at 16 loci in a total of 4,554 western North Pacific common minke whales including fetuses. The study identified a total of 49 validated P–O pairs (excluding mother-fetus pairs) across sub-areas, with J-stock pairs on both sides of Japan closer to the coast, while O-stock pairs were mostly located to the east of Japan, both close to the coast as well as far offshore. The study provided no evidence for further stock structure other than O- and J-stocks.

RECENT GENETIC STUDIES ON STOCK STRUCTURE IN THE NORTH PACIFIC

Basic laboratory procedures

DNA extraction

Total genomic DNA was extracted from 0.05 g of a tissue sample using either the standard phenol-chloroform method (Sambrook *et al.*, 1989) or Gentra Puregene kits (QIAGEN) and stored in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

mtDNA

Approximately 500 base pairs of a variable part of the mtDNA control region were amplified by PCR using primers MT4 (Árnason *et al.*, 1993) and Dlp5R (5'-CCA TCG AGA TGT CTT ATT TAA GGG GAA C-3'; Pastene *et al.*, 2022). PCR amplification was carried out in 25 µl reaction mixtures containing 10–100 ng of template DNA, 0.1 µM of each primer, 0.5 units of *Ex Taq* DNA polymerase (Takara Bio Inc., Shiga, Japan), 0.2 mM of dNTPs, and 1× *Ex Taq* buffer. Each reaction was performed with an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C and 30 s at 72°C, with a final extension step at 72°C for 10 min. PCR products were purified using MicroSpin S-400HR Columns (GE Healthcare, Illinois, USA). Cycle sequencing was performed using BigDye terminator cycle sequence Kit (Applied Biosystems Inc., Massachusetts, USA), following the manufacturer's instructions. The cycle sequencing products were purified using AutoSeq G-50 spin Columns (GE Healthcare). The labeled sequencing fragments were resolved by ABI genetic analyzers (Applied Biosystems Inc.) for Sanger sequencing by capillary electrophoresis. Both strands of all samples were sequenced using the primers used for amplification. The nucleotide sequences obtained were aligned by using the program MUSCLE implemented in MEGA ver. 10.0.5 (Kumar *et al.*, 2018) or the program Sequence Navigator.

msDNA

Different nuclear msDNA loci were genotyped according to the whale species (Table 1). The polymerase chain reaction (PCR) amplifications were performed in 15 µl reaction mixtures containing 10–100 ng of DNA, 0.25 µM of each primer, 0.35 units of *Ex Taq* DNA polymerase (Takara Bio Inc.), 0.2 mM of dNTPs, and 1× *Ex Taq* buffer, with 94°C for 2 min, followed by 30 cycles at 94°C for 20 s/54–61°C for 45 s/72°C for 1 min, and a post-cycling extension at 72°C for 10 min. Negative and positive control samples, one of each, were run in parallel with all PCR amplifications. The PCR products were run on a BaseStation100 DNA fragment analyzer (Bio-Rad, California, USA) with an internal size standard (GENESCAN400HD, Applied Biosystems Inc.), or on an ABI 3500 DNA Analyzer (Applied Biosystems Inc.) with a 600 LIZ size standard (Applied Biosystems Inc.). In the former platform, alleles were visualized using Cartographer software specifically designed for the BaseStation, and the fragment sizes were determined manually in relation to the internal size standard and a positive control sample of known size that were run on each gel. In the other platform, each allele was determined by automated binning and assigned to allelic bins predefined in GeneMapper v. 4.0 (Applied Biosystems Inc.). Any misassigned scores were corrected by hand. If msDNA scores generated from both platforms were

Table 1
msDNA markers used in the recent studies on four North Pacific baleen whales.

Norht Pacific right whale	Sei whale	Bryde's whale	Common minke whale
EV1	EV94	DlrFCB14	EV1
EV14	EV1	EV1	EV14
EV21	GT211	EV104	EV21
EV37	EV14	EV14	EV37
EV94	EV21	EV21	EV94
GT023	GGAA520	EV94	GATA28
GT211	GATA53	GATA28	GATA98
GT310	GT23	GATA417	GATA417
GATA028	GT575	GATA53	TAA31
DlrFCB17	GATA417	GATA98	GT23
TR3G2	GT310	GGAA520	GT195
TR2G5	EV104	GT011	GT211
TR3F2	GATA28	GT23	GT310
	GT271	GT310	GT509
	GT011	GT575	GT575
	DlrFCB17	TAA31	DlrFCB14
	GATA98		

Table 2
Genetic analyses used in the recent studies on four North Pacific baleen whales.

Analysis	North Pacific right whale		Sei whale		Bryde's whale		Common minke whale	
	mtDNA	msDNA	mtDNA	msDNA	mtDNA	msDNA	mtDNA	msDNA
Genetic diversity indices	●	●	●	●	●	●	●	●
HWE test	●	●	●	●	●	●	●	●
Hypothesis test	●		●	●	●	●	●	●
F_{ST} estimates	●		●	●	●	●	●	●
G'_{ST} estimates						●		
AMOVA			●	●	●	●		
Mantel test						●		
STRUCTURE				●				●
DAPC						●		●
sPCA								●
GENELAND								●
BAPS								●
TESS								●
Phylogenetic analyses	●				●			
PO analysis								●

contained in a single dataset, then all msDNA scores generated by the ABI 3500 DNA Analyzer were standardized to those by the BaseStation100 DNA fragment analyzer.

Basic analytical procedures

The analytical procedures used in the recent genetic studies on population structure are shown in Table 2 for each whale species.

This section briefly explains these analytical procedures.

Genetic diversity indices

mtDNA haplotype (h) and nucleotide (π) diversities (Nei, 1987) were estimated with sample standard deviations. The number of alleles (A), inbreeding coefficient (F_{IS}) and expected heterozygosity (H_E) were estimated per msDNA locus and across loci. The departure from Hardy–Weinberg equilibrium (*HWE*) was also tested for each msDNA locus and across loci.

Genetic differentiation

The genetic difference between populations was examined by several statistical tests for differences in allele/haplotype frequencies between populations, as well as by pairwise F_{ST} estimates and G'_{ST} values (Hedrick, 2005). The Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992) was used to examine the hierarchical genetic structure. A Mantel test was performed between a matrix of pairwise Edward's genetic distances and a matrix of Euclidian geographic distances to examine the pattern of isolation by distance (IBD).

Genetic clustering and stock assignment approaches

Estimation of the most likely number of clusters and/or individual-based stock assignment were performed using a Bayesian clustering analysis with an admixture model assuming correlated allele frequencies implemented in STRUCTURE program (Pritchard *et al.*, 2000).

To identify and describe clusters of genetically related individuals, a Discriminant Analysis of Principal Components (DAPC; Jombart *et al.*, 2010), which relies on data transformation using Principal Components Analysis as a prior step to discriminant analysis, was performed with or without *a priori* group assignments for msDNA genotype set. *A priori* group was determined based on geographic sampling site.

The spatially explicit clustering approaches, e.g. spatial Analysis of Principal Components (sPCA; Jombart *et al.*, 2008), GENELAND (Guillot *et al.*, 2005), TESS (François *et al.*, 2006; Chen *et al.*, 2007) and BAPS (Corander *et al.*, 2008), have been also used to investigate genetic structure and stock assignment.

Phylogeographic analysis

A statistical parsimony network (Clement *et al.*, 2000) was generated to infer the phylogenetic relationships among mtDNA haplotypes.

Parent–offspring analysis

Parent–Offspring (P–O) relationships were inferred based on a maximum-likelihood approach (Tiedemann *et al.*, 2017). mtDNA haplotypes and biological information, e.g. sex, sexual maturity and sampling position, were used to interpret the inferred P–O pairs. The geographical distribution of the pairs was discussed from the view point of stock structure.

Main results

North Pacific right whales

Dataset

Pastene *et al.* (2022) summarized and analyzed all available genetic data for North Pacific right whales. After removing all duplicates (i.e. samples that were collected from the same individual) and one sample from each mother and calf pair, a single dataset was generated for the mtDNA statistical analyses, which consisted of 30 samples each in the western North Pacific (WNP: 27 new samples, one from LeDuc *et al.* [2012] and two historical baleen samples) and the eastern North Pacific (ENP: 21 samples from LeDuc *et al.* [2012], three historical baleen samples and six new samples), respectively (Figure 3); the sample size for the msDNA analysis was 19 whales for WNP.

Main results and discussion

The study found a total of 16 haplotypes among 60 individual whales. Only four haplotypes were shared between WNP and ENP right whales. The F_{ST} between WNP and ENP right whales was high (0.128) and statistically significant, and the heterogeneity test resulted in statisti-

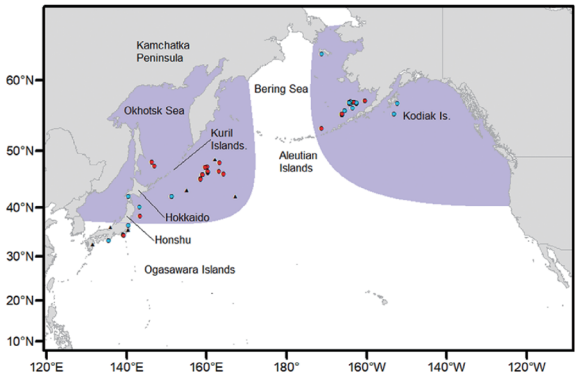

Figure 3. Approximate core distribution of North Pacific right whales in summer (July–August) based on 19th century whaling records and 20th century sighting and whaling catch data compiled by Clapham *et al.* (2004). The pattern of distribution shows high density in the western and eastern sides of the North Pacific (purple) and low density in the central area (white). The monthly plots of right whale sightings and catches by Clapham *et al.* (2004) suggested a pattern of south–north migratory movement in spring (March–May) to summer (June–August) on both sides of the North Pacific. The figure also shows the distribution of genetic samples examined in the recent study (Pastene *et al.*, 2022). Most of the samples from the eastern side are from LeDuc *et al.* (2012). Symbol color indicates sexes (red=female, blue=male, black=unidentified).

cally significant differences between whales on both sides of the North Pacific; however, there was no concordance between geography and haplotype network pattern.

The number of msDNA alleles per locus ranged from 2 to 7 (4.08 on average), and the H_E ranged from 0.285 to 0.787 (0.593 on average). The F_{IS} in each locus ranged from 0.045 to 0.350 with 0.032 on average. The study found no significant deviations from *HWE* across loci.

The mtDNA results, suggesting some degree of population structuring, were consistent with the pattern of catch and sighting data showing higher densities on either side of the North Pacific, but little in between as shown in Figure 3 (Clapham *et al.*, 2004). These findings support the hypothesis of different populations occurring in the eastern and western sides of the North Pacific. The authors also suggested the possibility of incomplete lineage sorting of ancestral polymorphisms from a lack of structure in haplotype network.

An alternative interpretation on these results was that there is a single interbreeding population in the North Pacific that is exhibiting mtDNA structuring as a result of matrilineally driven seasonal site fidelity. Although the authors could not rule out the alternative interpretation, they considered, as the more plausible, the hypothesis of two discrete breeding populations of right whales on both sides of the North Pacific since such hypothesis is supported also by the pattern of catches and sighting distribution, and different catch and recovery histories (Brownell *et al.*, 2001; Clapham *et al.*, 2004).

Sei whales

Dataset

A single dataset consisting of mtDNA control region sequences (n=1,733) and msDNA genotypes at 17 loci (n=1,729) was generated from specimens of sei whales collected from different sources (Figure 3: Appendix 7 in Tamura *et al.*, 2019). The genetic data generated from these samples were divided into three areas for the purpose of data analyses (Figure 4).

Main results and discussion

Pairwise F_{ST} estimates between areas showed no evidence of genetic heterogeneity in North Pacific sei whales for both genetic markers. These results were also supported by AMOVA and STRUCTURE analyses, which showed a lack of genetic structuring of this species in the oceanic areas of the North Pacific. These findings were also consistent with results of comparable genetic diversities among areas as well as the results from the tests of *HWE* and F_{IS} estimates. Overall, the recent refined analyses supported a single stock hypothesis as suggested previously.

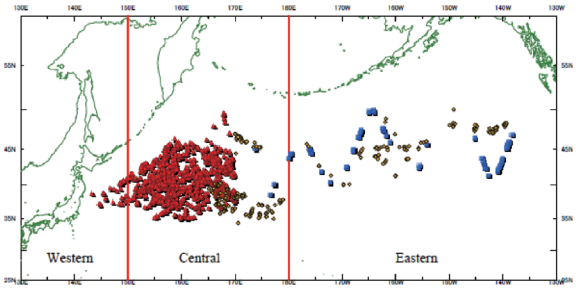

Figure 4. Sampling position of sei whales used in the recent genetic analyses (Appendix 7 in Tamura *et al.*, 2019). Color indicates sample source; yellow: historical commercial whaling during 1972–1973, red: JARPNII during 2002–2016, and blue: POWER during 2010–2013. Solid red line shows subareas. The genetic data was stratified according to the three areas, i.e. western, central and eastern, for data analyses.

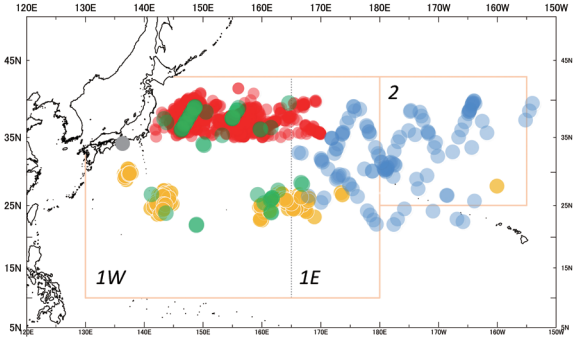

Figure 5. Sampling position of Bryde's whales used in the genetic analysis in Taguchi *et al.* (in press). Color indicates sample source; yellow: historical commercial whaling operated in 1979 and 1983–1984, red: JARPNII during 2000–2016, blue: POWER during 2013–2016, green: dedicated sighting survey in 2012 and 2014, and gray: bycatch in 2010. Areas enclosed by solid and dashed lines are sample stratification according to management areas for the western North Pacific Bryde's whales defined by the IWC scientific committee (IWC, 2008; 2018). Multiple genetic samples that were collected outside of these areas were assigned to the longitudinally closest area, i.e. area 2.

Bryde's whales

Dataset

In the most recent study (Taguchi *et al.*, in press), a total of 1,182 msDNA genotype sets at 17 loci and 1,195 mtDNA control region sequences were analyzed (Figure 5). These data were divided into the three management areas for the purpose of data analyses (Figure 5).

Main results and discussion

According to Taguchi *et al.* (in press), genetic diversities were similar among areas and a haplotype network did not show any geographic structure. However, an AMOVA found evidence of genetic structure in this species. Pairwise F_{ST} and G'_{ST} estimates, as well as heterogeneity tests, attributed this genetic structure to weak, but significant differentiation between areas 1W and 2. Furthermore, a Mantel test and a high-resolution analysis of genetic diversity statistics showed a weak spatial cline of genetic differentiation.

Given that this study focused on whales in their feeding grounds, these findings could be reconciled by two possible scenarios: (1) the occurrence of a single population with kin-association being responsible for feeding ground preference, and (2) the occurrence of two populations, with feeding ground preference for either area 1W or area 2; both populations mix geographically through area 1E. The results from the DAPC were not inconsistent with these scenarios. Given an estimated dispersal rate of less than 2% between areas 1W and 2, which indicates some demographic independency among whales in these two areas, both scenarios should be considered based on the precautionary principle in stock assessments.

Common minke whales

Recent genetic works have focused on refining the two-stock hypothesis, O- and J-stocks, as well as investigating whether additional structure exists within each stock.

Dataset

Recent studies have been based on mtDNA control region sequences (n=4,706) and msDNA genotype sets at 16 loci (n=4,707). The genetic samples were collected during the Japanese Whale Research Programs under Special Permit in the western North Pacific, Phases I and II (JARPN/JARPNII) conducted systematically in the western North Pacific during spring–summer from 1994 to 2016, as well as from bycatches which occurred along the Japanese coast from 2001 to 2016.

Main results and discussion

P–O inferences and their geographical distribution

Goto *et al.* (2019) updated the analysis by Tiedemann *et al.* (2017), and identified a total of 40 and 13 P–O pairs for O- and J-stocks, respectively. The O-stock pairs were found between coastal and offshore waters, while J-stock pairs were distributed within and between the Sea of Japan and the Pacific side of Japan (Figure 6). These results provided no evidence for further stock structure other than O- and J-stocks.

Spatially explicit clustering analyses

The results of sPCA in conjunction with those of DPAC suggested the occurrence of the O- and J-stocks but provided no evidence for additional structure in each stock (Taguchi *et al.*, 2019a), which was also supported by the mtDNA analyses. These findings indicated a low possibility that multiple stocks exist (other than the J- and O-stocks) in the study area with overlapping geographic ranges.

A study by de Jong and Hoelzel (2019) using three other spatially explicit clustering tools including GENELAND, TESS and BAPS showed different patterns of clustering, and the authors stated that the most informative approach was GENELAND using the mixture model with correlated allele frequency model which supported K=4. However, an additional work subsequently undertaken by Taguchi *et al.* (2019b) examined the correspondence of the four clusters by GENELAND with the available genetic and non-genetic information, and concluded that the

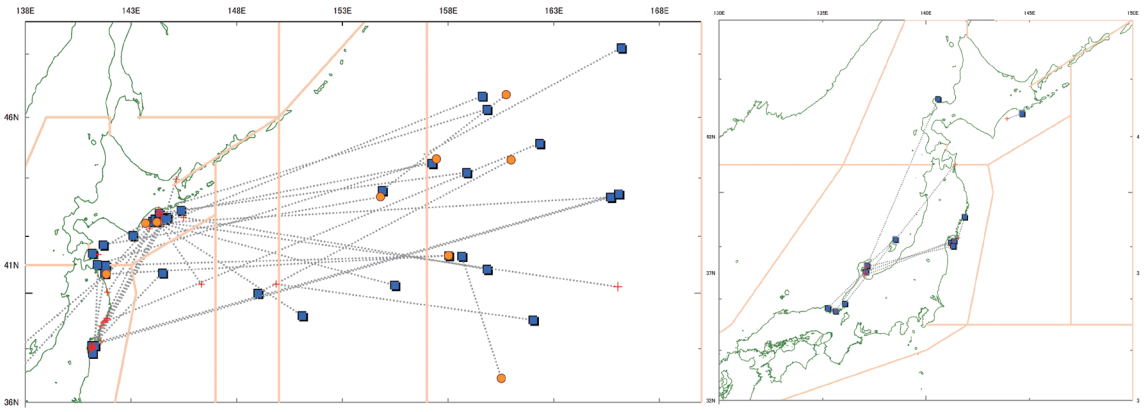

Figure 6. Distribution of parent–offspring pairs of O- (left) and J-stocks (right) in western North Pacific common minke whale (blue square: parent, orange circle: mature offspring, red cross: immature or unknown offspring).

most plausible scenario was for two populations (O- and J-stocks) with complex spatial and temporal mixing along the Pacific coast of Japan. de Jong and Hoelzel (2019) further noted that some of the analyses conducted were consistent with a scenario of coastal areas containing genetically admixed individuals, and recommended further analyses under the GENELAND as well under the TESS and BAPS.

CONCLUDING REMARKS

Over the last two decades, many genetic studies focused on stock identification and structure in western North Pacific baleen whales have been undertaken. The driving force behind these analyses was to obtain information to assist in determining effective conservation and management. Of necessity, these studies were based on genetic samples collected on feeding grounds and migratory corridors. In this context, they are based on the concept of MUs described by Moritz (1994). The recent studies summarized in this paper used several increasingly sophisticated clustering approaches for the purpose of identifying stocks, which showed statistical results consistent with this criterion for defining stocks and contributed to their stock assessment. It is also important to make effort to investigate the stock structure of baleen whales distributed in unsurveyed areas including breeding grounds, and the genetic relationship of them with whales distributed around Japan.

FUTURE WORKS

Genetic samples for other baleen whale species in the western North Pacific (i.e. humpback whales, fin whales and blue whales) are also available at the ICR. The genetic data generated from these samples will be analyzed in the near future to investigate their stock structure.

The development of a new genetic marker, which is less error-prone than msDNA, i.e. SNP (Single Nucleotide Polymorphism) is on-going at the ICR. This marker will be used to perform the genetic analyses for all whale species of interest. Such analyses will aid in a better understanding of stock structure of baleen whales in the western North Pacific.

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