Stock structure of Bryde's whales in the western North Pacific as revealed by microsatellite and mitochondrial DNA analyses

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ABSTRACT

Genetic variations at 17 microsatellite loci and 299 bp of mitochondrial DNA (mtDNA) control region were analyzed from JARPNII samples of Bryde's whales (*Balaenoptera edeni*) to describe their stock structure in the western North Pacific off Japan. Up to 50 individuals per year were collected each year from 2000 to 2007. Our main focus was to look for evidence of existence of genetically differentiated sub-stocks in the sub-area 1: one in the west of 155°E (sub-area 1W) and other in the east of 155°E (sub-area 1E). For this purpose, we conducted conventional hypothesis testing for both genetic markers and a Bayesian clustering approach for microsatellites. No genetic differences were detected among the samples collected from the different survey years within the 1W as well as 1E. We also failed to detect any evidence of genetic differentiation between the samples from the 1W and 1E, indicating these JARPNII samples came from a genetically same group of Bryde's whales. In order to examine stock differentiation in a wider geographic range in the western North Pacific, samples of Bryde's whales obtained during past commercial whaling in the central western North Pacific in 1979 and around Ogasawara Islands in 1983 and 1984 were compared to the JARPNII samples. No evidence of genetic differentiation was detected between these two and JARPNII samples. All of the results indicated that the sub-area 1 was occupied exclusively by the individuals from a single stock.

KEY WORDS: BRYDE'S WHALE, MICROSATELLITE, MTDNA, STOCK STRUCTURE, JARPNII,

INTRODUCTION

Bryde's whales (*Balaenoptera edeni*) are a unique baleen whale species because they are restricted to tropical and warm temperate waters warmer than 20°C in major oceans between approximately 40°N and 40°S year-round (Omura, 1959; Best, 1977; Kato, 2002). As typical baleen whale species, Bryde's whales undergo seasonal movement from winter breeding grounds in low latitudes to summer feeding grounds in higher latitudes. Their latitudinal migration, however, appeared to be short compared to other baleen whale species (e.g., Kishiro, 1996).

The comprehensive assessment of North Pacific Bryde's whale has begun since the 47th meeting of the International Whaling Commission (IWC) Scientific Committee (IWC, 1996, 1999, 2007). In the western North Pacific, two sub-areas (sub-area 1 and 2) divided at 180° were set within which only a single stock of Bryde's whales was highly likely distributed. Some members of the Committee, however, has argued against the single stock scenario in the sub-areas and, during *Implementation Simulation Trials* in 2007, possibility of the presence of two sub-stocks was further suggested in the sub-area 1. These two sub-stocks were thought to occupy west (1W) and east (1E) of 155°E, respectively (IWC, 2007). All of these concerns were derived from that the defined area, especially sub-area 1, was very large and wide and that supportive evidence for the scenario was limited due to lack of samples from some parts of the sub-area 1 (see Appendix 14 of Annex D in IWC, 1999).

Since 2000, JARPN second phase (JARPNII) has begun taking up to 50 Bryde's whales each year from the western North Pacific feeding ground in order primarily to study feeding ecology and fisheries interactions of whales in this area and secondary to acquire information essential for effective management of this species. The research area of JARPNII covers northwestern part of the Bryde's whales' sub-area 1 (approximately one sixth of the whole area) where no genetic samples was collected in the past.

On the basis of genetic variation at 17 microsatellite loci and 299 bp of mitochondrial DNA (mtDNA) control region sequences analyzed in individuals obtained from the western North Pacific, South Pacific, and eastern Indian Ocean, Kanda *et al.* (2007) showed that Bryde's whales were structured

within as well as between oceanic basins with evidence of limited gene flow (see also Wada & Numachi, 1991; Pastene *et al.*, 1997). Low mtDNA sequence divergence among populations and a lack of concordance between geographic and phylogenetic position of mtDNA haplotypes suggested recent separation of populations rather than frequent trans-equatorial and inter-oceanic movement. Nevertheless, the whales from the western North Pacific were shown to belong to the single stock. The western North Pacific samples in that past study included whales collected during the JARPNII surveys from 2000 to 2003.

The primary objective of this study was to better understand stock structure of Bryde's whales from the sub-area 1 of the western North Pacific using hypervariable microsatellite analysis and mtDNA control region sequencing. We first looked for any evidence of genetic differentiation between the JARPNII samples collected from the 1W and 1E. We then looked for any evidence of genetic differentiation among the samples from JARPNII and past commercial whaling conducted at outside the JARPNII area. Genetically different stocks might co-occur in the feeding ground, and incomplete knowledge about stock structure can cause unsound management, for instance, over-exploitation of certain stocks if separate stocks are erroneously treated as a single unit.

MATERIALS AND METHODS

Samples

JARPNII samples of Bryde's whales were obtained off the Japanese coast from the IWC defined sub-areas 7, 8, and 9 in the western North Pacific (Fig. 1). Up to 50 individuals per year were collected each year from 2000 to 2007. These samples were divided into sub-area 1W (West: Japanese coast and 155ºE) and 1E (East: 155ºE-180º). Table 1 shows survey year, survey period, and sample size (West, East) from 2000 to 2007. Sampling dates and locations of scientific surveys slightly differed year by year depending on the sampling plan of a given year. All samples were collected during spring and summer in the feeding ground. Details of offshore part of JARPNII survey can be found in Tamura *et al.* (2009).

In order to examine stock differentiation in a wider geographic range in the western North Pacific, samples of Bryde's whales obtained during past commercial whaling around Ogasawara Island in 1983 and 1984 (Ogasawara) and in the central western North Pacific in 1979 (pelagic) were used for comparison (Fig. 1). Individuals in the Ogasawara sample (N=102 in the microsatellite and N=103 in the mtDNA analyses) were archived specimens from past Japanese coastal commercial whaling operations. A total of 545 whales in 1983 and 528 whales in 1984 were caught according to a catch history (e.g., Table 2 of Appendix 2 in IWC, 1997). Individuals in the pelagic sample (N=98 in the microsatellite and N=113 in the mtDNA analyses) were archived specimens of past Japanese pelagic commercial whaling operations. Pelagic commercial whaling landed a total of 402 Bryde's whales in 1979 according to the catch history (IWC, 1997). More detail information on genetic characteristics of these commercial whaling samples was available in Kanda *et al.* (2007).

DNA extraction

Total DNA was extracted from 0.05 g of skin, muscle, heart, or liver tissue using the protocol of Sambrook *et al*. (1989) and then was stored in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Microsatellite analysis

Genetic variations at microsatellites were analyzed using 17 sets of primers, none of which was designed specifically from Bryde's whales: EV1, EV14, EV21, EV94, EV104 (Valsecchi & Amos, 1996), GT011 (Bérubé *et al.*, 1998), GT23, GT310, GT575 (Bérubé *et al*., 2000), GATA28, GATA53, GATA98, GATA417, GGAA520, TAA031 (Palsbøll *et al*., 1997), DlrFCB14, and DlrFCB17 (Buchanan *et al*., 1996). Primer sequences and PCR cycling profiles followed those of the original authors. PCR amplifications followed the manufacture's instructions for the use of Ex *Taq* DNA polymerase (Takara Shuzo). Amplified products were electrophoresed on a 6% polyacrylamide denaturating gel using a BaseStation 100 DNA fragment analyzer (Bio-Rad), and alleles were sized manually in relation to an internal size standard (Genescan 400HD, Applied Biosystems) and to Bryde's whale microsatellites of known size that were included on each gel.

mtDNA analysis

Sequencing analysis of the 299 bp control region of mtDNA was conducted using the primers light-strand MT4 (Árnason *et al*., 1993) and heavy-strand P2 (5'-GAAGAGGGATCCCTGCCAAGCGG-*3′* ; Hori *et al*., unpublished). PCR products were purified by MicroSpin S-400HR columns (Pharmacia Biotech).

Cycle sequencing was performed with the same primers, using BigDye terminator cycle sequence Kit (Applied Biosystems, Inc). The cycle sequencing products were purified by AutoSeq G-50 spin Columns (Pharmacia Biotech). The labeled sequencing fragments were resolved by electrophoresis through a 5% denaturing polyacrylamide matrix on an ABI 377ä or ABI3100 Automated DNA Sequencer (Applied Biosystems, Inc), following the protocols of the manufacture. For each sample both strands were sequenced.

Genetic diversity analysis

For the microsatellite analysis, the number of alleles per locus and expected heterozygosity per locus was calculated using the software FSTAT 2.9.3 (Goudet, 1995). Statistical tests for the deviations from expected Hardy-Weinberg genotypic proportions were conducted using the software GENEPOP 4.0 (Rousset, 2008). When simultaneous multiple tests were conducted, Rice (1989) correction for the multiple tests was performed.

For the mtDNA, the number of haplotypes and haplotype diversity were calculated following Nei (1987). The nucleotide diversity (Nei, 1987: equation 10.5) and its standard error for population sampling and stochastic processes were calculated from the pair-wise differences between the mtDNA sequences using the Kimura's 2- parameter adjustment (Kimura, 1980).

Genetic divergence analysis

Conventional hypothesis testing procedure was conducted using heterogeneity test in microsatellite allele and mtDNA haplotype frequencies among the samples, respectively. Our null hypothesis to be tested is if the samples came from a genetically same group of Bryde's whales. If a statistically significant allele frequencies differences exist, then it could indicate these samples came from genetically different stocks of Bryde's whales. Probability test (or Fisher's exact test) implemented in GENEPOP was used to conduct the heterogeneity tests for the microsatellites. Sequential Bonferroni correction (Rice, 1989) was applied when multiple tests were conducted. Weir and Cockerham estimator of F_{ST} was calculated using FSTAT 2.9.3 (Goudet, 1995). The randomized chi-square test of Independence (Roff and Bentzen, 1989) was used to investigate the temporal/spatial differentiation of mtDNA variation. In each test a total of 10,000 permutations of the original data were performed. Tests were conducted for all samples combined as well for males and females separately. A p-value smaller than 0.05 was used as a criterion to reject the null hypothesis of panmixia. F_{ST} for mtDNA was calculated based on the analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992).

A Bayesian clustering approach implemented in the software STRUCTURE (Pritchard *et al.*, 2000) was also applied to determine the most likely number of genetically distinct stocks present in our samples. STRUCTURE is a model-based clustering method for inferring stock structure using multi-locus genotype data. Spatial differentiation of samples was assessed assuming that the sampled individuals belong to an unknown number of K stocks. We conducted three independent runs for each value of K between 1 and 5 with no prior information (i.e., only genetic information was considered). All of the simulations were based on a burn-in period of 10,000 iterations and runs of 100,000 iterations. The ancestry model we used for the simulation was the admixture model, which assumes individuals may have mixed ancestry. The allele frequency model used was the correlated allele frequencies model, which assumes frequencies in the different stocks are likely to be similar due to migration or shared ancestry.

RESULTS AND DISCUSSION

Genetic diversity within samples

Microsatellie. All 17 microsatellite loci were polymorphic within all the JARPNII samples (Table 2). Total number of alleles at each locus overall the samples ranged from two at DlrFCB14 to 18 at GATA28 with an average of 8.2. Expected heterozygosity ranged from 0.285 at TAA31 to 0.913 at GATA28 with an average of 0.673. None of the 17 loci showed significant deviation from expected Hardy-Weinberg genotypic proportions after correction for the simultaneous multiple tests.

The levels of allelic diversity and heterozygosity in the JARPNII samples were compatible to those species used for primer design (Buchanan *et al*., 1996; Valsecchi and Amos, 1996; Palsbøll *et al*., 1997; Bérubé *et al*., 1998, 2000) and others reported (Bérubé *et al*., 2000; Kanda *et al*., 2006). This observation was the same to that in Kanda *et al*. (2007), even the sample size had been doubled since then. Although some loci contained low number of alleles, it is unlikely that this low variability is due too the negative effect on genetic diversity at a population level. This is because the low diversity is not consistent over all of the 17 loci.

mtDNA. Sequence variations at 299bp of the mtDNA control region resulted in 38 unique haplotypes. Haplotype diversity was 0.814 and nucleotide diversity was 0.0107 (standard error = 0.00058). Genetic diversity at the mtDNA control region was similar to that of other large baleen whales in the North Pacific, such as common minke whales (Goto *et al*., 2009) and sei whales (Kanda *et al*., 2009).

Genetic divergence among samples

Bryde's whales in the JARPNII samples were divided into two on the basis of individual's sighting sites (IWC, 2007): sub-area 1W sample (1W) was those initially sighted at west of 155°E, while sub-area 1E sample (1E) was those at east of 155°E. We first tested for genetic allele frequency differences among the samples collected from different survey years within 1W as well as within 1E. No statistically significant yearly heterogeneity was detected within each after correction for multiple tests at the microsatellite as well as mtDNA sequence analysis (Table 3). We combined the year samples into one for each of the areas as 1W and 1E samples. Then, we looked for evidence of genetic differences between the 1W and 1E samples. No statistically significant heterogeneity was observed after the correction for multiple tests at the microsatellite as well as mtDNA sequence analysis (Table 3), indicating that the JARPNII samples came from a genetically same group of Bryde's whales. All of the samples were then combined into a single JARPNII sample for further analyses.

The commercial whaling samples of Bryde's whales obtained around Ogasawara Island in 1983 and 1984 (Ogasawara) and in the central western North Pacific in 1979 (pelagic) were used to examine stock differentiation in a wider geographic range in the subarea 1. The Ogasawara sample was collected from west of 155°E and the pelagic sample from east of 155°E, and these samples corresponded to the SWNP and CWNP samples in Kanda *et al*. (2007), respectively. No evidence of genetic differentiation was observed among the JARPNII, Ogasawara, and pelagic samples after the corrections for multiple loci. These results indicated that all the three samples were obtained from a genetically same group of Bryde's whales. F_{ST} values calculated from microsatellite allele and mtDNA haplotype frequencies, respectively, between pairs of these three samples were not statistically different from zero (Table 4). Bayesian clustering analyses conducted without information on geographic sampling origins presented the lowest likelihood probability at K = 5 and the highest likelihood probability at K = 1 (Table 5). This result also provided support for a single stock.

Both conventional hypothesis testing and Bayesian clustering method failed to detect evidence of existence of multiple stocks in the sub-area 1 in this study. Kanda *et al*. (2007) has already reached the same conclusion that only a genetically single stock occupies the JARPNII survey area using the samples collected from 2000 to 2003. Because sampling locations of the 2000 to 2003 samples were restricted to west of 160°E, however, the conclusion of the single stock was tentative. In the current study, Bryde's whales in the samples of another four years from 2004 to 2007 included those collected further east up to 170°E (eastern boundary of sub-area 9). We, therefore, think that these individuals from JARPNII and commercial whaling samples fairly well covered the sub-area 1, allowing us to detect whether or not multiple sub-stocks existed in the sub-area 1. All of the results indicate that the sub-area 1 is occupied exclusively by the individuals from a single stock.

As described in Kanda et al. (2007), our results were consistent with previous genetic studies of Bryde's whale (Wada and Numachi, 1991; Pastene *et al*., 1997) as well as field studies (Nemoto, 1959; Ohsumi, 1978; Kishiro, 1996). Wada (1996) analyzed *GOT-1* allozyme allele frequencies in 2521 individuals collected from 1974 to 1984, divided along the 10-degree squares based on their catch positions between 20°N and 40°N and between 140°E and 160°W, and failed to show existence of multiple stocks in the western North Pacific. Past mark-recapture data demonstrated that tagged whales regularly moved within the sub-area 1 as well as from near the equator to coastal and pelagic areas off the coast of Japan.

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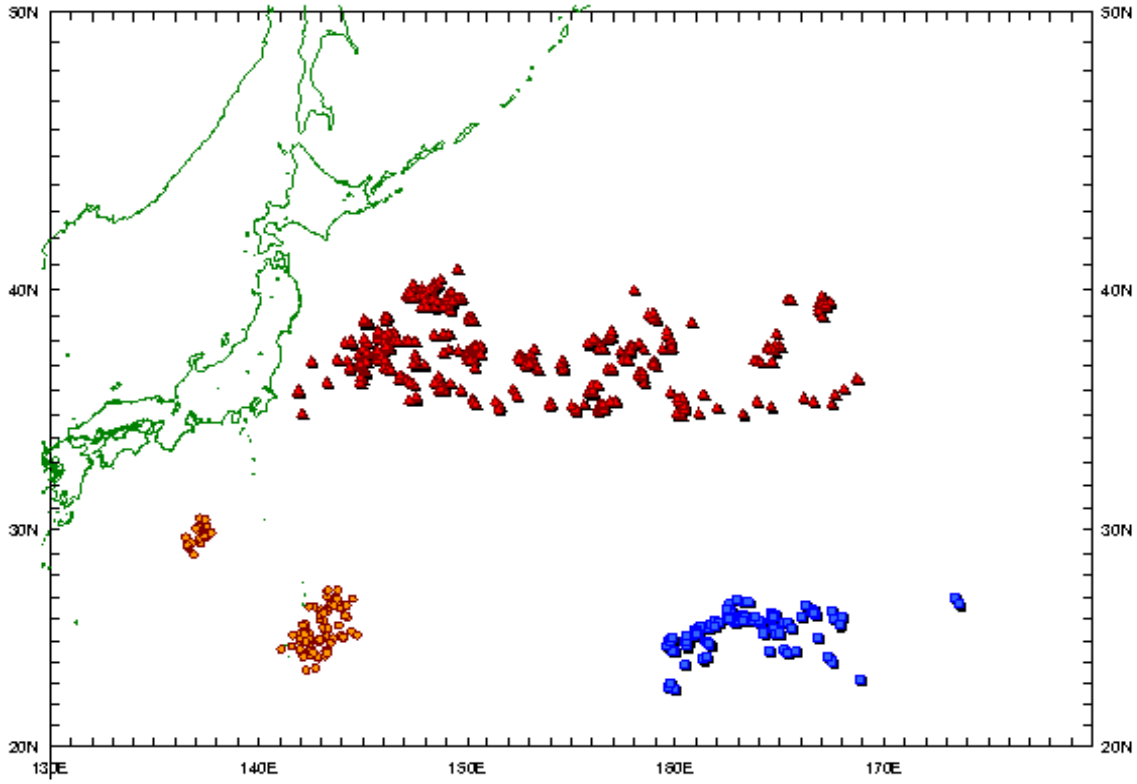

Fig. 1. Sampling locations of Bryde's whales used in this study.
Triangle = JARPNII, diamond = Ogasawara, square = pelagic.

Table 1. Survey year and period, sample size (west, east)* of Bryde's whales collected from JARPNII surveys from 2000 to 2007.

Year	Survey period	Sample size (west, east)*
2000	August - September	42 (42, 0)
2001	May - July	43 (43, 0)
2002	July - August	50 (36, 14)
2003	May - July	50 (37, 13)
2004	June - September	50 (0, 50)
2005	June - July	50 (46, 4)
2006	June - August	50 (12, 38)
2007	June - August	50 (20, 30)

* west=west of 155°E, east=east of 155°E

Table 2. The number of alleles (A), expected heterozygosity (He), and test result for expected Hardy-Weinberg genotypic proportions (HW) at 17 microsatellite loci analyzed in the JARPNII samples of Bryde's whales. n.s. = not significant.

	A	He	HW
DlrFB14	2	0.490	n.s.
DlrFB17	15	0.885	n.s.
EV1	11	0.735	n.s.
EV104	7	0.755	n.s.
EV14	8	0.769	n.s.
EV21	7	0.645	n.s.
EV94	8	0.549	n.s.
GATA28	18	0.913	n.s.
GATA53	7	0.617	n.s.
GATA98	7	0.796	n.s.
GATA417	8	0.755	n.s.
GGAA520	7	0.765	n.s.
GT23	8	0.741	n.s.
GT011	3	0.495	n.s.
GT310	5	0.608	n.s.
GT575	11	0.646	n.s.
TAA31	7	0.285	n.s.
average	8.2	0.673	
mtDNA			
Haplotype number		38	
Haplotype diversity		0.814	
Nucleotide diversity		0.0107	
(standard error)		(0.00058)	

Table 3. Test results (p-values) for genetic differentiation at the 17 microsatellite loci and mtDNA sequences among the samples of Bryde's whales.

Locus	1W	1E	1Wx1E	Sub-area 1*
Microsatellite				
DlrFB14	0.014	0.310	0.411	0.894
DlrFB17	0.811	0.490	0.043	0.922
EV1	0.987	0.152	0.893	0.321
EV14	0.430	0.316	0.686	0.526
EV21	0.616	0.780	0.143	0.629
EV94	0.183	0.100	0.309	0.004
EV104	0.284	0.190	0.529	0.848
GATA28	0.522	0.333	0.138	0.328
GATA53	0.218	0.078	0.639	0.771
GATA98	0.147	0.688	0.241	0.080
GATA417	0.079	0.026	0.904	0.651
GGAA520	0.589	0.594	0.723	0.158
GT23	0.334	0.128	0.537	0.113
GT011	0.502	0.713	0.793	0.043
GT310	0.513	0.523	0.778	0.749
GT575	0.723	0.814	0.765	0.302
TAA31	0.620	0.204	0.461	0.216
mtDNA	0.332	0.160	0.225	0.055

* among JARPNII, Pelagic, and Ogasawara (see text for sample details)

Table 4. Level of genetic differentiation (F_{ST}) calculated from mtDNA haplotype frequencies (above diagonal) and microsatellite allele frequencies (below diagonal) among the samples of Bryde's whales from the sub-area 1.

	JARPNII	Pelagic-E	Ogasawara-W
JARPNII	-----	-0.0020	0.0035
Pelagic-E*	-0.0002	-----	-0.0021
Ogasawara-W**	0.0004	-0.0009	-----

* Pelagic-E: commercial whaling in the central western North Pacific in 1979 (sub-area 1 east)
** Ogasawara-W: commercial whaling around Ogasawara island in 1983 and 1984 (sub-area 1 west)

Table 5. Estimated posterior probability of number of stocks (K) for the pooled samples of Bryde's whales collected from JARPNII and past commercial whaling computed using STRUCTURE.

K	Log P (x/k)	s.d.	Probability
1	-28477.4	64.7	~1.0
2	-28791.8	849.9	~0.0
3	-29177.9	1694.6	~0.0
4	-30503.8	4420.5	~0.0
5	-32488.0	8446.0	~0.0