SC/M17/ForInfo05

Updated genetic analyses based on mtDNA and microsatellite DNA suggest possible stock differentiation of Brydes whales between management sub-areas 1 and 2 in the North Pacific. Paper SC/F16/JR44 presented to the Expert Panel on JARPN II, Tokyo, Feb 20

Luis A. Pastene, Mutsuo Goto, Mioko Taguchi and Toshihide Kitakado

INTERNATIONAL
WHALING COMMISSION

SC/F16/JR/44

Updated genetic analyses based on mtDNA and microsatellite DNA suggest possible stock differentiation of Brydes whales between management sub-areas 1 and 2 in the North Pacific

Luis A. Pastene, Mutsuo Goto, Mioko Taguchi and Toshihide Kitakado

INTERNATIONAL WHALING COMMISSION

Updated genetic analyses based on mtDNA and microsatellite DNA suggest possible stock differentiation of Bryde's whales between management sub-areas 1 and 2 in the North Pacific

Luis A. Pastene[1], Mutsuo Goto[1] Mioko Taguchi[2] and Toshihide Kitakado[3]

[1] *Institute of Cetacean Research, 4-5 Toyomi-cho, Chuo-ku, Tokyo 104-0055, Japan*
[2]*Okitsunaka-cho 1347-3-302, Shimizu-ku, Shizuoka-shi, Shizuoka 424-0204, Japan*
[3] *Tokyo University of Marine Science and Technology, 1-5-7 Konan, Minato-ku, Tokyo 108-0075, Japan*

ABSTRACT

A total of 1,019 and 1,026 samples of North Pacific Bryde's whales were examined by microsatellite DNA (17 loci) and mitochondrial DNA sequencing (299bp), respectively, to examine the plausibility of four stock structure hypotheses used by the IWC SC during the 2007 RMP *Implementation*. Samples were from different sources: JARPNII (catches), Japanese dedicated sighting surveys (biopsy); IWC/POWER surveys (biopsy) and past commercial whaling (catches). No significant genetic heterogeneity was found between the Western and Eastern Sectors of sub-area 1, a result supported by high statistical power. However both genetic markers showed significant differences (for males, females and sexes combined) between sub-areas 1 and 2. Phylogenetic analysis of mtDNA haplotypes revealed no subarea-specific clades. It is proposed that a longitudinal sector around 180 degree could represent a hard boundary or a transition area where the two stocks mix. Based on these results, it is suggested that the plausibility of the stock structure hypotheses for western North Pacific Bryde's whale used in the 2007 *Implementation* whale should be re-examined. The results of this study suggest that the two-stock hypotheses (Hypotheses 2 and 3) could be more plausible than the one-stock hypothesis (Hypothesis 1) and the three-stock hypothesis (Hypothesis 4).

INTRODUCTION

The RMP *Implementation* for western North Pacific Bryde's whale was completed by the IWC SC in 2007 (IWC, 2008 pp9). One of the important sources of data and information for the discussion on stock structure came from JARPNII research.

During the *Implementation*, two sub-areas (Figure 1; IWC, 2009a pp7) and four stock structure hypotheses (Figure 2; IWC, 2007a pp8), were used. The IWC SC examined the plausibility of the four hypotheses based on genetics and non-genetics information available in 2006. That information is reproduced in Table 1 (IWC, 2007b pp95). In the context of the RMP *Implementation*, the IWC SC agreed on the plausibility of the four hypotheses as follows: Hypothesis 1: High; Hypothesis 2: High; Hypothesis 3: High; and Hypothesis 4: Medium. It should be noted that the plausibility rank was agreed in absence of any DNA data from sub-area 2.

Since the 2007 *Implementation* a substantial number of additional genetic samples have been collected from sub-area 1 through JARPNII, Japanese dedicated sighting, and IWC POWER surveys. Also samples became available from sub-area 2 through the IWC/POWER surveys. This new set of samples allowed new genetic analyses to be conducted for examining further the plausibility of the four stock structure hypotheses.

There was particular interest to test whether or not the frequencies in alleles and haplotypes in whales from the western part of sub-area 1 differ significantly from whales in the eastern part, and whether there are significant differences between sub-areas 1 and 2.

The analyses conducted in the present study considered most of the recommendations from the 2009 JARPNII review workshop (IWC, 2010) and from subsequent IWC SC Annual meetings (see Annex 5 of Tamura *et al*., 2016: SC/F16/JR1).

MATERIALS AND METHODS

Samples

JARPNII samples (n=679) of Bryde's whales obtained from 2002 to 2014 were collected from the western North Pacific (Table 2 and Figure 3). Although sampling dates and locations of the surveys slightly differed year by year depending on the sampling plan of a given year, samples were taken in the northwestern North Pacific in the range 35°05'N-49°35'N, and 143°19'E-169°58'E. Skin biopsy samples of Bryde's whales were obtained during the IWC/POWER and Japanese dedicated sighting surveys. POWER survey covered the area south of 40°N from 165°E to 155°W (POWER 2013-14). A total of 82 POWER biopsy samples were used (Table 2 and Figure 3). Japanese dedicated sighting surveys conducted in 2012 and 2014 covered the areas south of 40°N between 140°E and 170°E. A total of 58 biopsy samples from this source was used. Finally, a total of 200 samples from past commercial whaling (1979 pelagic; 1983/84 coastal) were used (Table 2 and Figure 3).

DNA extraction

The IWC guidelines for DNA data quality (IWC, 2009b) were followed as much as possible (see Kanda *et al*., 2014).

Total DNA from each of the whales was extracted from 0.05g of skin tissue in the JARPNII samples, skin biopsy in the POWER samples and muscle tissue in the commercial samples, using the protocol of Sambrook *et al*. (1989). Extracted DNA was stored in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Microsatellite analysis

Genetic variation at microsatellite DNA was analyzed using 17 loci, none of which was designed specifically from Bryde's whales: EV1, EV14, EV21, EV94, EV104 (Valsecchi and Amos, 1996), GT011 (Bérubé *et al*., 1998), GT23, GT211, GT271, GT310, GT575 (Bérubé *et al*., 2000), GATA28, GATA53, GATA98, GATA417, GGAA520 (Palsbøll *et al*., 1997), and DlrFCB17 (Buchanan *et al*., 1996). Primer sequences and PCR cycling profiles generally followed those of the original authors.

PCR amplifications were performed in 15μl reaction mixtures containing 10-100ng of DNA, 5 pmole of each primer, 0.625 units of Ex Taq DNA polymerase (Takara Shuzo), and 2mM of each dNTP, and 10x reaction buffer containing 20mM $MgCl_2$ (Takara Shuzo). Amplified products with internal size standard (GENESCAN400HD, Applied Biosystems Japan) were run on a 6% polyacrylamide denaturating gel (Long Ranger) using BaseStation100 DNA fragment analyzer (Bio-Rad). Although alleles were visualized using Cartographer software specifically designed for the BaseStation, allelic sizes were determined manually in relation to the internal size standard and Bryde's whale's DNA of known size that were rerun on each gel.

Data analysis

Level of polymorphisms

The number of alleles per locus, expected heterozygosity per locus and inbreeding coefficient was calculated using FSTAT 2.9.3 (Goudet, 1995). Statistical tests for the deviations from expected Hardy-Weinberg genotypic proportions were conducted using GENEPOP 4.0 (Rousset, 2008).

Homogeneity test

Conventional hypothesis testing procedure was conducted using heterogeneity test in microsatellite allele frequencies among samples. The null hypothesis to be tested was whether or not the samples came from a genetically same group of Bryde's whales. If statistically significant allele frequency differences exist, it could indicate these samples came from genetically different stocks of Bryde's whales. Probability test (or Fisher's exact test) implemented in GENEPOP 4.0 (Rousset, 2008) was used to conduct the heterogeneity tests. Statistical significance was determined using the chi-square value obtained from

summing the negative logarithm of *p*-values over the 17 microsatellite loci (Sokal and Rohlf, 1995). The False Discovery Rate (FDR) approach (Benjamini and Yekutieli, 2001) was used for adjustment of *p*-value in case of multiple comparisons. F_{ST} value was calculated using FSTAT 2.9.3 (Goudet, 1995).

There were three cases of re-sampling, two involving the same year and one involving more than one year. In these cases only one individual was used in the statistical analyses.

Assessment of the statistical power

In order to assess the statistical power for the homogeneity test (e.g., Waples and Gaggiotti, 2006), genotypic data were generated using the computer software EASYPOP (Balloux, 2001), and heterogeneity tests were conducted with these generated data. Two stocks (1W and 1E) were assumed which consists of diploid individuals with constant sizes and equal sex ratio with random mating. Ratios of effective population size to census population size of 1/3 and 1/4, were assumed (Roman and Palumbi, 2003). The effective population sizes were thus set as 1/3 and 1/4 of the census population sizes.

A census population size of 16,000 was used. For each generation, the simulation produced genotype data for 17 independent microsatellite loci for each individual. The number of the loci simulated and maximum number of the allelic states (18) was set based on the observed data. The bidirectional migration model was assumed with an equal migration rate (m). Migration rates ranged from 0.01 to 0.5. A range of F_{ST} between the two assumed stocks was obtained, assuming island model. Mutation rate of $5x10^{-4}$ was chosen to represent microsatellite loci. A total of 100 replicates were made for each simulation parameter set. A total of 5,000 generations for each replicate before collecting data was run. In the final generation of each replicate, sample of 120 individuals were taken from each population for genetic analysis. The sample size of 120 in this study was approximately equals to the sum of the samples size from sub-area 1E, which is considered conservative given that larger sample size was actually used for 1W. Homogeneity tests were conducted for the generated data set using pairwise tests of differentiation option in the FSTAT2.9.3 (Goudet, 1995). In this option, for each pair of samples, multi-loci genotypes are randomized between the two samples. The overall loci G-statistic is given and statistical significance was decided with a table wide level of significance at 5%.

Mitochondrial DNA

Sequencing analysis of the 299bp 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 377™ or ABI3100 Automated DNA Sequencer (Applied Biosystems, Inc), following the protocols of the manufacturer. For each sample both strands were sequenced.

Data analysis

Level of polymorphisms

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).

Homogeneity test

Conventional hypothesis testing procedure was conducted using heterogeneity test in mtDNA haplotype frequencies among the samples. The randomized chi-square Test of Independence (Roff and Bentzen, 1989) and the conventional F_{ST} were 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 males, females and both sexes combined. A p-value smaller than 0.05, was used as a criterion to reject the null hypothesis of panmixia. The FDR approach was used for adjustment of *p*-value

in case of multiple comparisons. F_{ST} for mtDNA was calculated based on the analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992).

Phylogenetic analysis

The genealogy of the mtDNA haplotypes was estimated using the Neighbor-Joining method (Saitou and Nei 1987) as implemented in the program PHYLIP (Felsenstein 1993). Genetic distances among haplotypes were estimated using the program DNADIST of PHYLIP, based on Kimura's 2-parameter model (Kimura 1980). A transition-transversion ratio of 5:1was used. The genealogy was rooted using the homologous sequence from sei and Omura's whales. To estimate support for each node a total of 1,000 bootstrap simulations were conducted and the majority-rule consensus genealogy estimated.

RESULTS

Microsatellites

Level of polymorphisms

All 17 loci analyzed were polymorphic (Table 3). The total number of alleles per locus ranged from two at the DlrFCB to 18 at the GATA28 with an average of 8.76. Expected heterozygosity at each of the loci ranged from 0.35 at TAA31 to 0.91 at GATA28 with an average of 0.68. No significant departure from the expected Hardy-Weinberg genotypic proportions was found. However the F_{IS} for some loci were relatively large suggesting the possibility of homozygote excess.

Homogeneity test for samples from different sources

No significant genetic heterogeneity was observed in the test in sub-area 1W (Table 4) and sub-area 1E (Table 5) for sample from different sources and period. Consequently all samples in each of these sub-areas were pooled for the next analyses.

Homogeneity test for sub-areas

No significant genetic heterogeneity was found between sub-areas 1W and 1E (Table 6). Consequently all samples from sub-area 1 were pooled for the next analysis. In the overall test involving the 17 loci, significant statistical differences were found in the comparison between sub-areas 1 and 2 for males, females and both sexes combined (Table 7).

Assessment of the statistical power

Table 8 shows the input parameters used and the results of simulation analysis to assess the statistical power for the tests homogeneity. The simulation attempted to test the statistical power for very small genetic divergence between two samples. For instance, estimated F_{ST} values were all smaller than 0.01. For the homogeneity tests the input parameters for simulating 1W and 1E were all same. High statistical power was detected with m=0.01 and 0.02.

mtDNA

Level of polymorphisms

Levels of mtDNA diversity were relatively high in the North Pacific Bryde's whale (Table 9).

Homogeneity test for samples from different sources

No significant genetic heterogeneity was observed in the test in sub-area 1W (Table 10A) and sub-area 1E (Table 10B) for sample from different sources and period. Consequently all samples in each of these sub-areas were pooled for the next analyses.

Homogeneity test for sub-areas

No significant genetic heterogeneity was found between sub-areas 1W and 1E (Table 11A). Consequently all samples from sub-area 1 were pooled for the next analysis. Significant statistical differences were found in the comparison between sub-areas 1 and 2 for males and both sexes combined (Table 11B). In the case of females, the *p*-value was marginally significant (0.056).

Phylogenetic analysis
Figure 4 shows the phylogenetic relationship among mtDNA haplotypes. The figure also shows the haplotype frequencies in sub-areas 1W, 1E and 2. Several clades were observed in the figure but none was supported by high bootstrap values. There was no subarea-specific clade.

DISCUSSION

As noted earlier, the main objective of the present genetic analyses was the evaluation of the plausibility of the four stock structure hypotheses of North Pacific Bryde's whale used during the 2007 RMP *Implementation*. A number of different surveys allowed the collection of additional genetic samples in sub-area 1, and importantly, the collection of genetic samples from sub-area 2. As noted earlier, the plausibility of the four stock structure hypotheses in 2007 was evaluated in absence of any DNA data from sub-area 2. The total available samples were analyzed with two genetic markers, which are commonly used in most of the genetic studies on stock structure presented and discussed at the IWC SC meetings. It is believed that the combined use of mtDNA control region sequences and microsatellite DNA at 17 loci is a strong tool to investigate genetic differences in weakly differentiated stocks.

The present study addressed all 'simple issues' recommended by the 2009 JARPNII review workshop. For example the workshop recommended description of procedures to ensure data quality. In response to this recommendation and other one from the JARPAII review workshop, a document was prepared and presented to the IWC SC in 2014 (Kanda *et al*., 2014). The IWC SC welcomed this document and agreed that it responded appropriately to the recommendation (IWC, 2015). Another example is the 2009 workshop recommendation to use the False Discovery Rate approach instead of the Bonferroni correction for the adjustment of *p*-values in cases of multiple comparisons. This approach was used in the present study.

Other medium and long-term recommendations from the 2009 JARPNII review workshop were also addressed in this study as well as in previous documents presented to the IWC SC and other documents presented to this workshop. See more details on the responses to previous recommendations in Annex 5 of Tamura *et al*. (2016: SC/F16/JR1).

Hypothesis testing results and interpretation

Despite the increase in the number of samples used in the analysis from approximately 585 in the previous study to 973 in the present study, no significant genetic heterogeneity was found in sub-area 1 suggesting that whales in this sub-area belong to a same stock. This result is similar to that found previously by Kanda *et al*. (2007; 2009). In the present study this result was supported by high statistical power.

In contrast both genetic markers showed significant differences between whales in sub-areas 1 and 2, and this pattern was similar for males and females. In interpreting this result, the large difference in sample sizes between sub-area 1 (966 and 973 for microsatellite and mtDNA, respectively) and sub-area 2 (53 and 53 for microsatellites and mtDNA, respectively) was considered. There was the possibility that the large difference in sample sizes could have derived in the significant statistical differences between the sub-areas. We tested this through a simple simulation exercise based on mtDNA data for male and female samples combined.

For this simulation exercise, the smaller sample size of sub-area 2 (n=53) was kept fix and randomly re-sampling of individuals was carried out from sub-area 1 (n=973, with replacement) for n=50, n=100 and n=150. A total of 100 sets of haplotype frequencies were created for each of these sample sizes in sub-area 1, and these were compared statistically to haplotype frequencies in sub-area 2 (using the randomized chi-square test). Results indicated that for n=50, 86% of the 100 tests rejected panmixia. For n=100 and n=150, 100% of the tests resulted in rejection to panmixia.

The results of the simple simulation above suggested that the difference in sample sizes between sub-areas 1 and 2 was not the reason for the statistical significant differences found between the two sub-areas and that such result corresponded to a real biological event. Therefore this result suggested additional stock structure in the western North Pacific Bryde's whale, with the possibility that two stocks occur in

this oceanic basin separated by a boundary around longitude 180°. There is the possibility that a sector around 180°E represent a transition area where the two stock mix. This should be examined further in future.

Correspondence to patterns of movement of whales

An analysis of Discovery-type marks was carried out by Kishiro (1996). According to the authors a total of 537 Bryde's whales were effectively marked by the Japanese marking programme during the year 1972 to 1985, and a total of 52 marks had been recovered by the end of 1987. All of the mark-recaptures occurred west of 180° (sub-area 1). The main conclusion of the study was that Bryde's whales summering in the whaling grounds, winter over a wide latitudinal range (1°S-25°N). The authors did not find evidence of more than one stock of Bryde's whales in the western North Pacific. As noted in Table 1, mark-recapture analysis revealed movement of animals within sub-area 1, and a very limited number of marks were placed in sub-area 2.

More recently the movement of two Bryde's whales was recorded in summer using satellite-monitored radio tags in the western North Pacific (see details in Murase *et al*., 2016: SC/F16/JR45). These whales were recorded for 13 and 20 days, respectively. Both whales exhibited a north-south from approximately 40°N to 35°N within sub-area 1.

In summary the available information on movement is not inconsistent with the results of the present genetic analysis that suggested two stocks separated around longitude 180°.

Conclusions

Based on these results, it is suggested that the plausibility of the stock structure hypotheses for western North Pacific Bryde's whale used in the 2007 *Implementation* whale should be re-examined. The results of this study suggest that the two-stock hypotheses (Hypotheses 2 and 3) could be more plausible than the one-stock hypothesis (Hypothesis 1) and the three-stock hypothesis (Hypothesis 4).

ACKNOWLEDGEMENTS

We gratefully acknowledge the researchers and crew members that participated in JARPN/JARPNII, IWC/POWER and Japanese dedicated sighting surveys for the collection of genetic samples. We thank H. Oikawa and S. Azumi (ICR) who collaborated in the process of DNA extraction. We also thank N. Kanda (JANUS-Japan) for preparing the microsatellite data set used in this study, and to T. Hakamada for his assistance in the simulation on sample sizes.

REFERENCES

Árnason, Ú., Gullberg, A., and Widegten, B. 1993. Cetacean mitochondrial DNA control region: Sequences of all extant baleen whales and two sperm whale species. *Mol. Biol. Evol.* 10:960-970.

Balloux, F. 2001. EASYPOP (version 1.7): a computer program for the simulation of population genetics. *J. Hered.* 92:301-302.

Benjamini, Y. and Yekutieli, D. 2001. The control of the false discovery rate in multiple testing under dependency. *The Annals of Statistics*. 29, No. 4, 1165–1188

Bérubé, M., Aguilar, A., Dendanto, D., Larsen, F., Notarbartolo-di-Sciara, G., Sears, R., Sigurjónsson, J., Urban-Ramirez, J., and Palsbøll, P.J. 1998. Population genetic structure of North Atlantic, Mediterranean Sea and Sea of Cortez fin whales, *Balaenoptera physalus* (Linnaeus, 1758); analysis of mitochondrial and nuclear loci. *Mol. Ecol.* 7:585-599.

Bérubé, M., Jørgensen, H., Mcewing, R., and Palsbøll, P.J. 2000. Polymorphic di-nucleotide microsatellite loci isolated from the humpback whale, *Megaptera novaeanglliae*. *Mol. Ecol.* 9:2181-2183.

Buchanan, F.C., Friesen, M.K., Littlejohn, R.P., and Clayton, J.A. 1996. Microsatellites from beluga whale *Delphinapterus leucas*. *Mol. Ecol.* 5:571-575.

Excoffier, L., Smouse, P.E., and Quattro, J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131:479-491.

Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) 3.5c. University of Washington, Seattle, USA.
Goudet, J. 1995. FSTAT, version 1.2: a computer program to calculate F-statistics. *J. Hered.* 86:485-486.
International Whaling Commission. 2007a. Report of the Scientific Committee. *J. Cetacean Res. Manage. 9 (Suppl.)*: 1-73.
International Whaling Commission. 2007b. Report of the Scientific Committee. Annex D. Report of the Sub-Committee on the Revised Management Procedure (RMP). *J. Cetacean Res. Manage. 9 (Suppl.)*: 88-128.
International Whaling Commission. 2008. Report of the Scientific Committee. *J. Cetacean Res. Manage. 10 (Suppl.)*: 1-74.
International Whaling Commission. 2009a. Report of the Scientific Committee. *J. Cetacean Res. Manage. 11 (Suppl.)*: 1-74.
International Whaling Commission. 2009b. Report of the Scientific Committee. Annex I. Report of the working group on stock definition. Appendix 2. Guidelines for DNA data quality control for genetic studies relevant to IWC management advice. *J. Cetacean Res. Manage. (Suppl.)* 11:252-256.
International Whaling Commission. 2010. Report of the Expert Workshop to Review the Ongoing JARPN II Programme. *J. Cetacean Res. Manage. 11 (suppl. 2)*:405-449.
International Whaling Commission. 2015. Report of the Scientific Committee, Annex N. Report of the Working Group on DNA. *J. Cetacean Res. Manage. 15 (suppl.)*: 329-334.
Kanda, N., Goto, M., Kato, H., McPhee, M.V., and Pastene, L.A. 2007. Population genetic structure of Bryde's whales (*Balaenoptera brydei*) at the inter-oceanic and trans-equatorial levels. *Cons. Genet.* 8:853-864.
Kanda, N., Goto, M., Yoshida, H. and Pastene, L.A. 2009. Stock structure of Bryde's whales in the western North Pacific as revealed by microsatellite and mitochondrial DNA analyses. Paper SC/J09/JR31 presented to the JARPN II Review Workshop, Tokyo, January 2009 (unpublished). 8pp.
Kanda, N., Goto, M., Oikawa, H. and Pastene, L.A. 2014. Update of note on sampling and laboratory procedure protocols of the genetic work at the Institute of Cetacean Research (SC/F14/J27rev). Paper SC/65b/DNA1 presented to the IWC Scientific Committee, May 2014 (unpublished). 6pp.
Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16:111-120.
Kishiro, T. 1996. Movements of Marked Bryde's Whales in the Western North Pacific. *Rep. int. Whal. Commn.* 46:421-428.
Murase, H., Tamura, T., Otani, S. and Nishiwaki, S. 2016. Satellite tracking of Bryde's whales *Balaenoptera edeni* in the offshore western North Pacific in summer 2006 and 2008. *Fish. Sci*. (in press).
Nei, M. 1987. *Molecular Evolutionary Genetics.* New York: Columbia University Press.
Palsbøll, P.J., Bérubé, M., Larsen, A.H., and Jørgensen, H. 1997. Primers for the amplification of tri-and tetramer microsatellite loci in baleen whales. *Mol. Ecol.* 6:893-895.
Roff, D.A. and Bentzen, P. 1989. The statistical analysis of mtDNA polymorphisms: χ^2 and the problem of small samples. *Mol. Biol. Evol.* 6**:**539-545
Roman, J, and Palumbi, S.R. 2003. Whales before whaling in the North Atlantic. Science 301:508-510.
Rousset, F. 2008. Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molec. Ecol. Resources* 8:103-106.
Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. 4(4):406-25.
Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular cloning: A laboratory manual. 2nd Ed*., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
Sokal, R.R., and Rohlf, F.J. 1995. *Biometry: the Principles of Statistics in Biological Research.* Freeman and Company, New York.
Tamura, T., Kishiro, T., Yasunaga, G., Murase, H., Kitakado, T and Pastene, L.A. 2016. The Japanese Whale Research Program under Special Permit in the western North Pacific Phase-II (JARPN II): results and conclusions in the context of the three main objectives, and scientific considerations for future research. Paper SC/F16/JR1 presented to the JARPNII special permit expert panel review workshop, Tokyo, February 2016 (unpublished). 68pp.

Valsecchi, E., and Amos, W. 1996. Microsatellite markers for the study of cetacean populations. *Mol. Ecol.* 5:151-156.

Waples, R.S., and Gaggiotti, O. 2006. What is a population? An empirical evaluation of some genetic methods for identifying the number of gene pools and their degree of connectivity. *Mol. Ecol.* 15:1419-1439.

Table 1 (reproduced from IWC, 2007b pp95).

General summary of the information useful to assess plausibility of alternative stock-structure hypotheses. A '+' indicates evidence in favour of a hypothesis, '-' indicates evidence against a hypothesis, '(+)' indicates weak evidence in favour of a hypothesis, '(-)' indicate weak evidence against a hypothesis, a (()) indicates very weak evidence, and 'NIW' indicates that the evidence is not inconsistent with the hypothesis. Note that the designation NIW often reflects the asymmetrical nature of information on stock structure (i.e., existence of differences can be viewed as positive evidence for multiple stocks, but absence of differences provides no information, and cannot be viewed as positive evidence for a single stock). Notations at the bottom of this table were extracted in part from the report of the 'First Intersessional Workshop' (SC/58/Rep1 published in this volume).

Evidence	Hypothesis 1	Hypothesis 2	Hypothesis 3	Hypothesis 4
Allozymes	(+) [a]	(-)[a]	(-)[a]	(-)[a]
mtDNA	((+))[b]	NIW[c]	NIW[c]	-[d]
Microsatellites	NIW[b]	NIW[c]	NIW[c]	-[d]
Sighting distribution	NIW[e]	NIW[e]	NIW[e]	NIW[e]
Catch distribution	NIW[e]	NIW[e]	NIW[e]	NIW[e]
External body proportion	NIW[f]	NIW[f]	NIW[f]	NIW[f]
Biological parameters	NIW[f]	NIW[f]	NIW[f]	NIW[f]
Mark-recapture	NIW[g]	NIW[h]	NIW[h]	NIW[g]
Age distribution	NIW[i]	NIW[i]	NIW[i]	(+)/NIW[i,j]

[a]The sub-committee agreed that while the power to detect genetic structure might be considered to be low as only a single locus was analysed, the fact that this had been sufficient to detect structure in the Pacific Ocean suggest that if there are multiple stocks differentiated at a level similar to that between the western North stock and other stocks, examination of this single locus should be sufficient to detect this.

[b, c]Clustering and hypothesis testing analyses of mtDNA and microsatellites were conducted, and as noted by the sub-committee, none of these analyses revealed any significant heterogeneity within sub-area 1. Only 6 mtDNA samples are available from sub-area 2 – these provide very real evidence for hypothesis 1, but this is not sufficiently strong to be considered inconsistent with hypotheses 2 and 3.

[d]*Mixing of two sub-stocks*: There are no genetic data for the breeding grounds so the possibility of multiple sub-stocks cannot be excluded. Furthermore if two breeding stocks mix almost completely, it will be difficult to detect differences using, for example, genetics tests based on comparisons between data for the west and east of sub-area 1. However while complete mixing may lead to all methods of detecting stock structure having low power, the plausibility of this was considered fairly low given the behaviour of most large whales. Hypothesis tests based on comparisons for approximately the same area in sub-area 1 found no significant differences among years which suggest that if two sub-stocks mix in sub-area 1, there is little difference in the distribution proportion among years. Although the data set encompasses only four years, such a lack of variation in distribution proportions among years seems unlikely given the known behaviour of whales. In principle evidence for hypotheses 4 could be obtained by testing for deviation from Hardy-Weinberg equilibrium within sub-areas 1W and 1E because such deviations provide evidence of non-random mating as well as selection or migration, i.e. when genetically two different populations are being sampled. Analyses of nuclear markers for Bryde's whales in sub-area 1 have been conducted and these analyses provide no evidence for the significant deviations in Hardy-Weinberg equilibrium within sub-areas 1W and 1E which would provide support for multiple sub-stocks.

Statistical power: The Workshop received a paper, which evaluated the power to detect population structure using the chi-square test and Fisher Exact test under an island model in which population differentiation is controlled using a single parameter, F_{st}. Statistical power of the genetic analysis in sub-area 1 was found to be high for moderate sample sizes and quite small values for Fst, while it was higher for microsatellite data than for mtDNA.

[e]The sub-committee examined the sightings data and agreed that it revealed no evidence of a discontinuity in distribution within sub-areas 1 and 2. A similar conclusion was reached with respect to catch distribution. Discontinuity in the commercial catches identified in earlier meetings merely reflected operational constraints. Evidence of spatial discontinuity would be viewed as positive evidence for multiple stocks, but absence of discontinuity is viewed as neutral information, as there is no expectation that their prey would have a discontinuous distribution. Therefore, multiple stocks could exist without spatial discontinuity.

[f]External body proportion data (three features) and several biological parameters (body length, pregnancy rate, length at sexual maturity, seasonality in breeding) had been examined using data from past commercial whaling. Although operational differences (e.g. different minimum length limits for coastal and pelagic whaling) meant that some comparisons could not be made, the authors of these analyses concluded that there were no differences that could not be attributed to operational factors. Evidence of differences in biological parameters would be viewed as positive evidence for multiple stocks, but absence of differences does not provide positive evidence for a single stock, because it is not necessary for separate populations to diverge in their biological parameters, as these are often constrained by their ecology.

[g]Mark-recapture analysis revealed movement of animals within sub-area 1. A very limited number of marks were placed in sub-area 2 and while none has been recovered in sub-area 1, the sample sizes for this sub-area are sufficiently small that even if there is mixing between sub-areas 1 and 2, zero recaptures would not be highly unlikely.

[h]Mark-recapture data are available mainly for sub-area 1.

[i]One of the possible explanation for the differences observed in age distribution between sub-area 1W and 1E+2 is that these differences are real. Other possible explanations were geographical segregation by age or non-representativeness of the samples, perhaps as the result of unreadability of certain earplugs, inaccuracy in length determination, or inter-reader differences in ageing.

[j]There was no consensus on this verdict. The different views hinged on different interpretations on an appropriate entry given that there were alternative explanations for the observation in question.

Table 2. Sample sizes of Bryde's whales used in the present study in the microsatellite and mtDNA (number in parenthesis) analyses, by sub-area, sex, and source of samples. Only the samples genotyped for the complete microsatellite loci set were included in the analysis.

Sub-area		Sex	JARPNII	Commerc.	POWER	Jap. dedic.	Total
1	West	Male	274 (269)	105 (116)		20 (20)	399 (405)
		Female	346 (342)	66 (73)		36 (36)	448 (451)
	East	Male	14 (14)	16 (16)	10 (10)	1 (1)	41 (41)
		Female	45 (45)	12 (10)	20 (20)	1 (1)	78 (76)
2		Male			29 (29)		29 (29)
		Female		1 (1)	23 (23)		24 (24)
Total			679 (670)	200 (216)	82 (82)	58 (58)	1019 (1026)

Table 3. The number of alleles (A), expected heterozygosity (He), test results for expected Hardy-Weinberg genotypic proportion (HW) and inbreeding coefficient (F_{IS}) in North Pacific Bryde's whale, at 17 microsatellite loci.

Microsatellite loci	A	H_E	HW	F_{IS}
GATA98	8	0,80	0,632	0,020
EV104	7	0,76	0,868	-0,009
GT011	3	0,50	0,303	-0,014
GATA53	7	0,63	0,426	0,027
GATA41	10	0,75	0,272	0,008
DlrFCB	2	0,49	0,279	0,034
DlrFCB	15	0,88	0,755	-0,005
GT23	9	0,74	0,446	0,018
EV14	9	0,78	0,851	0,003
GT310	5	0,62	0,678	-0,003
EV1	13	0,74	0,019	0,033
EV94	9	0,52	0,357	0,031
GGAA52	8	0,77	0,026	-0,001
EV21	7	0,66	0,338	0,020
GT575	11	0,66	0,457	-0,014
GATA28	18	0,91	0,371	0,000
TAA31	8	0,35	0,634	0,021
Overall	8,76	0,68	0,305	0,009

Table 4. Results of the microsatellite DNA heterogeneity test for different sample sources in sub-area 1W, by locus and sex. Bold indicate significant differences after FDR correction.

JARPNII x Commercial x Sighting in sub-area 1W						
Microsatellite loci	Sex combined		Male		Female	
	P -value	F_{ST}	*P* -value	F_{ST}	*P* -value	F_{ST}
GATA98	0,154	0,002	0,403	0,000	0,151	0,004
EV104	0,578	0,000	0,814	-0,004	0,759	0,000
GT011	0,559	-0,002	0,904	-0,004	0,217	0,004
GATA53	0,897	-0,002	0,624	-0,003	0,200	-0,004
GATA417	0,412	0,002	0,510	0,000	0,269	0,002
DlrFCB14	0,464	-0,001	0,659	-0,003	0,593	-0,003
DlrFCB17	0,511	0,000	0,122	0,003	0,351	0,000
GT23	0,571	0,000	0,194	0,002	0,923	-0,004
EV14	0,214	0,000	0,542	-0,002	0,066	0,000
GT310	0,354	0,000	0,799	-0,002	0,393	-0,001
EV1	0,202	-0,001	0,197	0,005	0,299	-0,003
EV94	0,017	0,000	0,126	-0,002	0,561	0,001
GGAA520	0,693	-0,001	0,957	-0,004	0,735	-0,001
EV21	0,458	0,001	0,436	0,002	0,838	-0,003
GT575	0,019	0,001	0,023	0,001	0,210	0,001
GATA28	0,762	-0,001	0,942	-0,002	0,563	0,000
TAA31	0,011	0,000	0,055	-0,003	0,040	0,005
Overall	0,053	0,000	0,318	-0,001	0,252	0,000

Table 5. Results of the microsatellite DNA heterogeneity test for different sample sources in sub-area 1E, by locus and sex. Bold indicate significant differences after FDR correction.

JARPNII x Commercial x POWER in sub-area 1E						
Microsatellite loci	Sex combined		Male		Female	
	P -value	F_{ST}	*P* -value	F_{ST}	*P* -value	F_{ST}
GATA98	0,086	0,000	0,604	-0,011	0,060	0,002
EV104	0,100	0,005	0,484	-0,002	0,256	0,002
GT011	0,352	0,002	0,882	-0,030	0,116	0,035
GATA53	0,137	-0,002	0,318	0,009	0,362	-0,002
GATA417	0,763	-0,002	0,634	-0,005	0,269	0,011
DlrFCB14	0,361	-0,002	0,424	-0,015	0,895	-0,023
DlrFCB17	0,407	-0,002	0,635	0,006	0,123	0,005
GT23	0,271	0,007	0,526	-0,013	0,078	0,041
EV14	0,784	-0,007	0,786	-0,017	0,699	-0,010
GT310	0,741	-0,007	0,818	-0,027	0,960	-0,018
EV1	0,604	-0,007	0,312	-0,009	0,963	-0,013
EV94	0,713	-0,004	0,079	0,048	0,584	-0,013
GGAA520	0,351	0,004	0,428	-0,010	0,697	0,001
EV21	0,322	0,012	0,017	0,079	0,921	-0,017
GT575	0,749	0,002	0,441	-0,009	0,930	-0,010
GATA28	0,441	-0,001	0,869	-0,008	0,916	-0,010
TAA31	0,675	-0,010	0,959	-0,026	0,589	-0,014
Overall	0,531	0,000	0,666	-0,002	0,612	-0,001

Table 6. Results of the microsatellite DNA heterogeneity test for Western and Eastern sectors in sub-area 1, by locus and sex. Bold indicate significant differences after FDR correction.

Subareas 1W x 1E						
Microsatellite loci	Sex combined		Male		Female	
	P -value	F_{ST}	*P* -value	F_{ST}	*P* -value	F_{ST}
GATA98	0,614	-0,001	0,540	-0,002	0,598	-0,002
EV104	0,440	0,002	0,843	-0,003	0,577	0,002
GT011	0,115	0,000	0,606	-0,003	0,209	-0,002
GATA53	0,100	0,002	0,037	-0,005	0,199	0,007
GATA417	0,343	-0,001	0,335	0,000	0,481	-0,002
DlrFCB14	0,549	-0,001	0,298	0,002	0,864	-0,004
DlrFCB17	0,992	-0,001	0,849	0,000	0,481	0,001
GT23	0,570	0,000	0,351	-0,002	0,647	-0,001
EV14	0,368	0,001	0,433	0,000	0,339	0,002
GT310	0,149	0,005	0,061	0,021	0,775	-0,002
EV1	0,149	0,002	0,712	0,003	0,174	0,000
EV94	0,482	-0,001	0,554	0,001	0,306	0,003
GGAA520	0,931	-0,002	0,935	-0,006	0,917	-0,002
EV21	0,160	0,004	0,664	0,002	0,051	0,007
GT575	0,291	-0,001	0,757	-0,004	0,121	-0,001
GATA28	0,360	0,000	0,771	-0,001	0,167	0,001
TAA31	0,071	0,004	0,090	0,003	0,510	0,002
Overall	0,199	0,000	0,556	0,000	0,357	0,001

Table 7. Results of the microsatellite DNA heterogeneity test for sub-areas 1 and 2, by locus and sex. Bold indicate significant differences after FDR correction.

Subareas 1 x 2						
Microsatellite loci	Sex combined		Male		Female	
	P -value	F_{ST}	*P* -value	F_{ST}	*P* -value	F_{ST}
GATA98	0,202	0,007	0,372	0,003	0,336	0,011
EV104	0,089	0,000	0,008	0,002	0,532	0,003
GT011	0,199	0,010	0,392	0,000	0,333	0,011
GATA53	0,607	0,001	0,340	0,007	0,637	-0,009
GATA417	0,009	0,005	0,013	0,024	0,155	-0,006
DlrFCB14	0,327	0,000	0,689	-0,007	0,386	-0,002
DlrFCB17	0,037	-0,001	0,124	0,000	0,226	-0,004
GT23	0,174	0,002	0,611	0,001	0,086	0,006
EV14	0,128	0,005	0,110	0,007	0,197	0,016
GT310	0,225	0,003	0,539	-0,005	0,243	0,017
EV1	0,140	0,003	0,342	0,005	0,054	0,011
EV94	0,016	0,009	0,274	0,008	0,084	0,002
GGAA520	0,142	0,002	0,040	0,010	0,739	-0,008
EV21	0,517	-0,003	0,250	-0,005	0,685	0,003
GT575	0,143	-0,001	0,792	-0,006	0,422	-0,004
GATA28	0,002	0,004	0,067	0,005	0,069	0,000
TAA31	0,001	0,031	**<0,001**	0,038	0,064	0,025
Overall	**<0,001**	0,004	**<0,001**	0,005	**0,031**	0,004

Table 8. Input parameter sets used for generating simulated data set using EASYPOP to assess statistical power in our samples and results of the homogeneity tests with the simulated data. The following were fixed in all sets other than shown in the table: diploid, random mating, equal sex ratio, subpopulations of constant Ne, mutation rate of 0.0005, and 100 replicates each with 5000 generations.

	n	N	Ne	m	Nem	*Fst*	S	L	A	% rejecting panmixia
N=3Ne	2	16000	5300	0.01	53	0.0047	120	17	18	100
	2	16000	5300	0.02	106	0.0024	120	17	18	84
	2	16000	5300	0.05	265	0.0009	120	17	18	30
	2	16000	5300	0.1	530	0.0005	120	17	18	9
	2	16000	5300	0.2	1060	0.0002	120	17	18	9
	2	16000	5300	0.5	2650	0.0001	120	17	18	7
N=4Ne	2	16000	4000	0.01	40	0.0062	120	17	18	100
	2	16000	4000	0.02	80	0.0031	120	17	18	86
	2	16000	4000	0.05	200	0.0012	120	17	18	33
	2	16000	4000	0.1	400	0.0006	120	17	18	12
	2	16000	4000	0.2	800	0.0003	120	17	18	7
	2	16000	4000	0.5	2000	0.0001	120	17	18	7

N=census population size, Ne=effective population size, m=migration rate, Nem=number of migrants per generation, S= number of sample size, L = number of loci analyzed, A=possible number of allelic states.

Table 9. Results of mtDNA diversity for North Pacific Bryde's whale.

Number of haplotypes	Nucleotide diversity (SE)	Haplotype diversity
51	0.01086 (0.00036)	0.82797

Table 10. Results of the mtDNA heterogeneity test for different sample source in sub-area 1W (A) and 1E (B), by sex. Bold indicate significant differences after FDR correction.

A

1W					
Chi-square *p*-value			F_{ST}		
Sex combined	Male	Female	Sex combined	Male	Female
0.4886	0.9228	0.2525	-0.00069	-0.00590	0.00448

B

1E					
Chi-square *p*-value			F_{ST}		
Sex combined	Male	Female	Sex combined	Male	Female
0.2115	0.0700	0.2707	-0.00547	0.01667	0.01068

Table 11. Results of the mtDNA heterogeneity test for sectors in sub-area 1(A) and between sub-areas 1 and 2 (B), by sex. Bold indicate significant differences after FDR correction.

A

1W × 1E					
Chi-square *p*-value			F_{ST}		
Sex combined	Male	Female	Sex combined	Male	Female
0.2223	0.5264	0.2260	0.00218	-0.00184	0.00062

B

1W-1E × 2					
Chi-square *p*-value			F_{ST}		
Sex combined	Male	Female	Sex combined	Male	Female
0.0001	**0.0022**	0.0558	**0.02227**	**0.02234**	0.01424

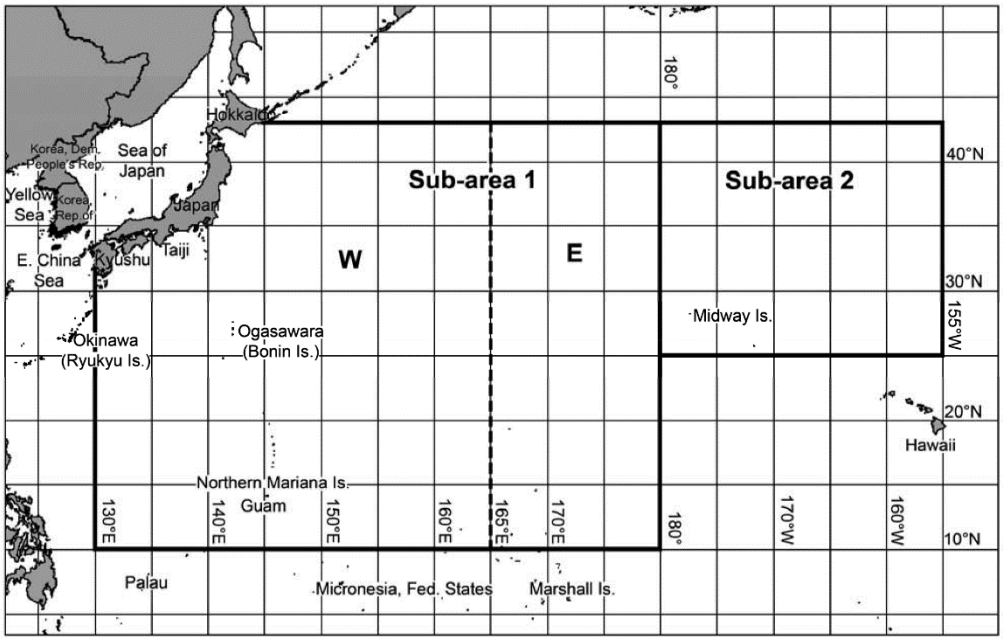


Figure 1. Sub-areas used for the management of Bryde's whale under the RMP (IWC, 2009 pp7).

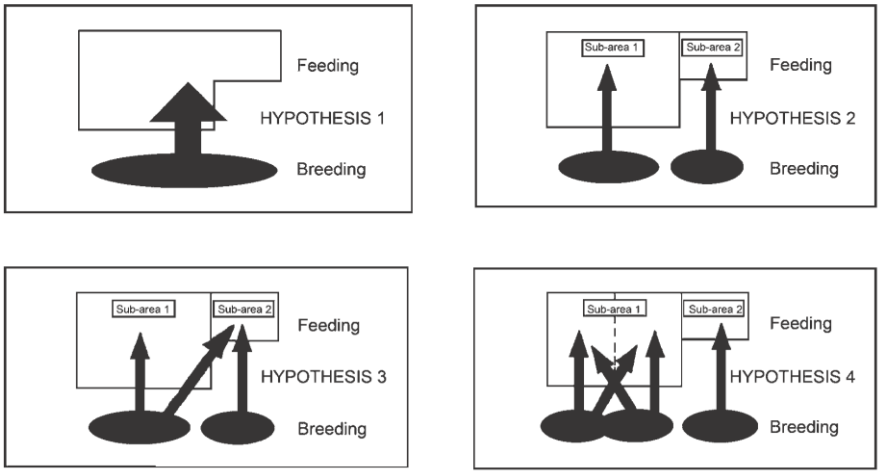


Figure 2. Hypotheses on stock structure of Bryde's whale in the western North Pacific used during the RMP *Implementation* (IWC, 2007 pp8).

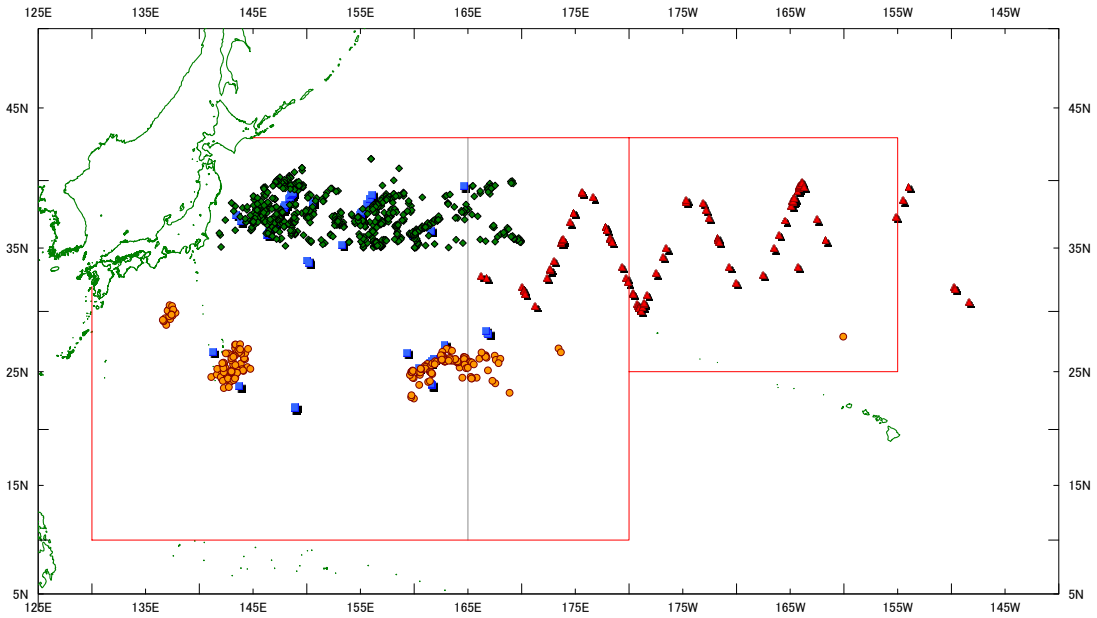
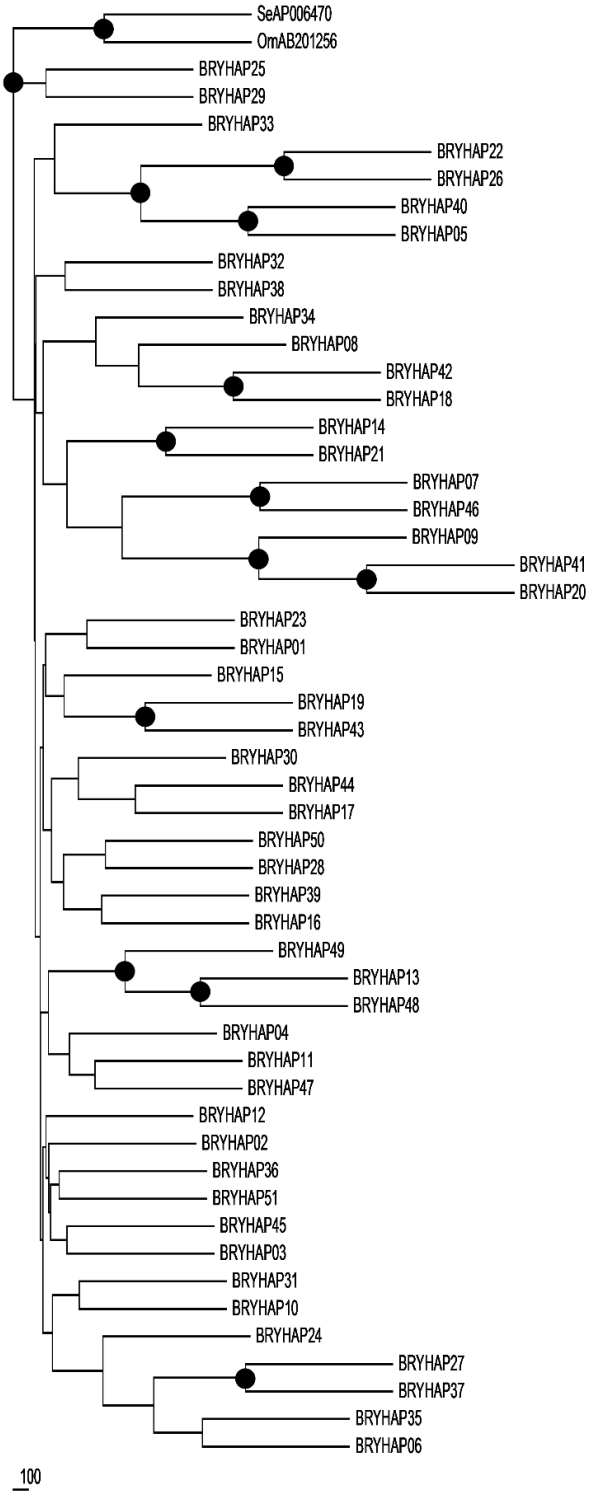


Figure 3. Geographical distribution of Bryde's whales examined in this genetic study. Green: JARPNII samples; Blue: Japanese dedicated sighting survey samples; Orange: past commercial whaling samples; Red: IWC/POWER survey samples.



Hap	Sub-area		
	1W	1E	2
25	5		1
29	6	1	
33	7	2	
22	2		1
26	4		1
40	1		
05	5	2	
32	3	1	
38		1	
34	1		
08	31	5	4
42	1		
18	16	2	
14	13	1	
21	9	4	3
07	37	7	5
46	2		
09	33	11	6
41	1		
20	4	1	
23	11		
01	10	2	
15	11	1	
19	18	2	3
43	1		
30	2		
44	1		
17	6		
50			2
28	18	1	3
39	2	1	5
16	14		
49		2	
13	13	1	2
48	1		
04	87	3	4
11	17	3	
47	2	1	
12	1		
02	20	3	
36	1		1
51	1		
45	2		
03	333	47	13
31	6		
10	1		
24	1		
27	10	1	
37	1		
35	1		
06	83	11	1
Total	856	117	55

Figure 4. Phylogenetic relationship among mtDNA haplotypes of North Pacific Bryde's whale and haplotype frequencies in management sub-areas.