Stock structure of humpback whales in the Antarctic feeding grounds as revealed by microsatellite DNA data

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ABSTRACT
A total of 581 humpback whale biopsy samples obtained from Areas III to VI during surveys of the JARPA and JARPAII up to 2010/11 season as well as International Decade for Cetacean Research/Southern Ocean Whale and Ecosystem Research (IDCR/SOWER) were analyzed using 14 microsatellite DNA loci in order to describe their stock structure in the Antarctic feeding ground. The number of the loci used was increased from six in the paper submitted to the JARPA Review workshop in 2006. In three of 37 cases of duplicate sampling, the second samples were collected at least a day apart (1 day, 9 years, and 11 years). Paternity analysis of 13 calf and mother pairs failed to detect any potential fathers in the samples for the calves. After exclusion of some of these samples, 528 were used for further analyses at stock level. These individuals were divided into four groups based on the IWC management areas: IIIE (N=93), IV (N=218), V (N=153) and VIW (N=64). Heterogeneity tests were conducted for the samples of females only, males only, and both sexes combined, respectively. Although a few cases of small temporal genetic differences were detected within the areas, major genetic differences were observed among the samples from the different areas. As similar to the previous report, stronger differentiation was seen in females than in males. These results corresponded to those of the previous studies. Despite the increase of the number of loci, the level of the stock differentiation (F_{ST} = 0.003) was still too low to conduct a clustering analysis at the individual level.

With substantial increases in the numbers of the analyzed microsatellite loci and the biopsy samples, our genetic study again showed that humpback whales from the different stocks occupied the research areas with higher differentiation in females than in males. The level of the genetic differentiation among the areas was still so low that further analysis would require using samples from their breeding areas to better understand the stock structure in the feeding grounds. This study demonstrated one of the significant contributions of non-lethal part of the comprehensive large-scale JARPAII to acquire valuable information for effective management of large whales in the Antarctic.

KEYWORDS: ANTARCTIC, BIOPSY SAMPLING, FEEDING GROUNDS, GENETICS, HUMPBACK WHALE

INTRODUCTION

Humpback whales conduct seasonal migration between mid to high latitudinal waters in summer for feeding and low latitudinal waters in winter for breeding. In the Antarctic, humpback whales appear to congregate into five or six distinct feeding groups during the austral summer season that roughly match to IWC Management Areas I-VI (Mackintosh, 1965).

At the JARPA Review workshop in 2006, Pastene *et al*., (2006) conducted genetic analyses of humpback whales from the JARPA research area (Areas III to VI) using mitochondrial DNA and microsatellite DNA markers, and demonstrated that humpback whales from the different areas belonged to genetically different groups because of the observed genetic differences among them. In addition, it showed that the level of genetic differentiation was larger in females than in males, strongly suggesting higher female phylopatry and large male dispersion in the feeding grounds. The next question to be addressed was whether or not these feeding groups were genetically distinct enough from each other that they could correspond to breeding stocks. Pastene *et al*. (2013) conducted mtDNA analysis using samples from both the feeding and breeding grounds, and showed the genetic differences observed among the areas were a consequence of the different proportions of the stocks from single stock occupancy in some areas to multi-stock occupancy in other areas.

This study used microsatellite DNA markers to analyze the same humpback whale samples from Areas III to VI used in Pastene *et al*. (2013). The main purpose of the study is to better understand their stock structure by increasing the number of the loci from Pastene *et al*. (2006). In that study, the use of the only six loci might have resulted in the observed low level of the genetic differentiation among the samples. The estimated F_{ST} value was about 0.005. At that level of differentiation, it was difficult to conduct clustering analysis at the individual base. In this paper, we increased the number of the microsatellite loci to 14. In addition, sample size was also substantially increased from the previous study because of the effort of JARPAII.

MATERIALS AND METHODS

Samples

Table 1 shows the number of humpback whale biopsy samples used in this study separated by IWC management areas. A total of 581 samples were obtained from the JARPA and JARPAII surveys up to 2010/11 season as well as International Decade for Cetacean Research/Southern Ocean Whale and Ecosystem Research (IDCR/SOWER).

DNA extraction

In regard to our DNA data quality control under the IWC guidelines, see Kanda *et al*. (2014). Total DNA was extracted from 0.05 g of biopsy skin tissue using either the protocol of Sambrook *et al*. (1989) or GENTRA PUREGENE DNA extraction kit (QIAGEN). Extracted DNA was stored in the TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Microsatellite analysis

Genetic variation at microsatellite loci was analyzed using 14 sets of primers: AC137, CA234 (Bérubé *et al*., 2005), EV1, EV14, EV37 (Valsecchi and Amos, 1996), GT23, GT195, GT271, GT310 (Bérubé *et al*., 2000), GATA28, GATA53, GATA98, GATA417, TAA31 (Palsbøll *et al*., 1997). All except EV1 and EV14 were designed specifically from humpback whales. Amplified products were run on a 6% polyacrylamide denaturating gel using a BaseStation 100 DNA fragment analyzer (Bio-Rad), and then sizes of visualized alleles were determined manually in relation to the internal size standard (Genescan 400HD, Life technologies) as well as the humpback whale's microsatellites of known size that were rerun on each gel. The sex of the whales was determined through co-amplification of SRY locus located on the Y chromosome and GT23, which is a slight modification from Abe *et al*. (2001). With this combination of loci, males show amplified products of both SRY and GT23 loci, while females show only GT23.

Data analysis

MICRO-CHECKER (van Oosterhout *et al*., 2004) was used to check for null alleles and reading/typing errors. The number of alleles per locus, allelic richness, and expected heterozygosity per locus 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). Paternity analysis for assignment of mother and calf pairs to their potential fathers using genetic markers was conducted using CERVUS (Marshall *et al*., 1998). In order to examine genetic differences among samples, conventional hypothesis testing procedure was conducted using heterogeneity test in microsatellite allele frequencies among samples. A probability test (or Fisher's exact test) implemented in GENEPOP 4.0 (Rousset, 2008) was used to conduct the heterogeneity tests. Our null hypothesis to be tested is whether or not the samples came from a genetically same group of humpback whales. If statistically significant allele frequency differences exist, it could indicate these samples came from genetically different stocks of humpback whales. Statistical significance was determined using the chi-square value obtained from summing the negative logarithm of p-values over the 14 microsatellite loci (Sokal & Rohlf 1995). F_{ST} value was calculated using FSTAT 2.9.3 (Goudet, 1995).

RESULTS AND DISCUSSION

Among 37 cases of the duplicate sampling, the second samples of three cases were collected at least a day apart (1 day, 9 years, and 11 years; Table 2). One case was first collected in Area IV and was recollected in Area III, while the other two cases were collected and re-collected in the same area. Because Pastene *et al*. (2013) showed the migration of whales from the same stock to Areas III and IV, our results were consistent to the feeding site fidelity of humpback whales in the Antarctic. Thirteen calf and mother pairs were used for paternity analysis, but no potential father was detected in the samples.

After exclusion of the second individuals from the duplicated samples and calves sampled with mothers, a total of 528 samples were used for further analyses. These individuals were divided into four groups based on the

management areas: IIIE (N=93), IV (N=218), V (N=153) and VIW (N=64). The levels of genetic diversity of the area samples were represented as an average number of alleles per locus, average allelic richness, and expected average heterozygosity (Table 3). These values were similar among them as well as to those based on the six loci previously reported in Pastene *et al*. (2006).

Heterogeneity tests for genetic differences among the samples from the different areas were conducted for samples of females+males, females only, and males only, respectively (Tables 4-9). For the females+males samples, first, genetic differences among the different year samples within the areas were examined (Table 4). No evidence of genetic differences was found among the samples from the different years in Areas IIIE, IV, and V, while small level of genetic differences was observed in Area VIW. Because the observed significance in Area VI was small and none of the pair-wise comparisons among the year samples was significant, the samples from the different years within each of the areas were combined for further analysis. Evidence of the genetic differences was then detected among the samples from the different areas (Table 5). Pair-wise comparisons showed evidence of statistically significant differences for all possible area-pairs (Table 5). The heterogeneity tests for the females only and males only samples showed similar results to each other with clearer structuring in females than in males (Tables 6-9). A yearly genetic difference was detected in both samples (Tables 6 and 8). In females, two (94/95x98/99 and 98/99x08/09) out of the 31 pair-wise comparisons were significant with close-to 5% p-values (0.035 and 0.049). In males, one (96/97x00/01) out of three pair-wise comparisons was significant with a 0.017 p-value. Because it was difficult to conclude whether or not the difference had a biological meaning, we combined the year samples within the areas for the next tests. Evidence of the genetic difference among the different areas was detected in both samples. In females, all of the pair-wise comparisons were significantly different, whereas in males, all except Areas IIIxV and Areas VxVI comparisons were significantly different (Tables 7 and 9). F_{ST} was 0.003 among the different area samples for all three sample cases. The results of this study were thus consistent to those previously reported based on six microsatellite loci as whale groups occupying the different areas were genetically defferent from each other with stronger differentiation in females than in males.

Pastene *et al.* (2013) analyzed mitochondrial DNA variations on humpback whales from both the feeding and breeding grounds to better describe their stock structure, and showed that IV from 80˚E to 120˚E was occupied by one stock (Western Australia stock) and V from 140˚E to 160˚E by another stock (Eastern Australia stock). The rest of the areas were mixing areas of the adjacent stocks. Despite the substantial increase in the number of the loci, the level of the stock differentiation (F_{ST} = 0.003) was still too low to conduct a clustering analysis at the individual level. In such a situation, it was difficult for this study to further distinguish between stock mixing and stock core areas without using the data from the breeding areas. Future microsatellite study should use the samples from the breeding areas. Nevertheless, substantial increases in the numbers of the analyzed microsatellite loci and the biopsy samples allowed us to confirm our previous conclusion on the humpback whale stock structure in the Antarctic. This study demonstrated one of the significant contributions of non-lethal part of the comprehensive large-scale JARPAII to acquire valuable information for effective management of large whales in the Antarctic.

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Table 1. The number of humpback whale biopsy samples usid in this study by surveys and areas.

Survey	Survey area			
	IIIE	IV	V	VIW
JARPA	56 (33, 23)	132 (58, 74)	106 (56, 50)	46 (18, 28)
JARPAII	14 (6, 8)	49 (26, 23)	61 (36, 25)	3 (0, 3)
IDCR/SOWER	36 (24, 12)	51 (30, 21)	7 (3, 4)	20 (12, 8)
All	106 (63, 43)	232 (114, 118)	174 (95, 79)	69 (30, 39)

Total (female, male)

Table 2. Sampling dates and lcoations of the matched samples.

Sex	Sampling	lat.	long.	Cap.→recap.
Female	1994/2/12	66.54	70.07E	IV → IIIE, 9 yrs later
	2003/12/14	60.31S	48.57E	
Female	1997/1/25	61.44S	151.42E	V → V, 11 yrs later
	2008/12/13	63.05S	138.26E	
Male	2002/2/12	64.08S	96.58E	IV → IV, next day
	2002/2/13	64.06S	97.24E	

Table 3. Genetic diversity indices estimated from all samples within each of survey areas.

Genetic indice	Survey area			
	IIIE	IV	V	VIW
No. alleles per locus	10.6	11.9	11.9	10.1
Allelic richness per locus	10.2	10.3	10.7	10.1
Heterozygosity	0.758	0.752	0.755	0.746
Hardy-Weinberg	n.s.	n.s.	n.s.	n.s.

n.s. = no significance

Table 4. Results (p-values) of heterogeneity tests for year differences within areas: Females+Males.

IIIE	IV	V	VIW
0.814	0.555	0.204	**0.049**

Bold p-values indicate statistically significance.

Table 5. Results (p-values) of heterogeneity tests among areas: Females+Males.

Among areas	IIIExIV	IIIExV	IVxV	IIIxVIW	IVxVIW	VxVIW
Highly sig.	**0.000043**	**0**	**0**	**0.000380**	**0.000176**	**0.0471**

Bold p-values indicate statistically significance.

Table 6. Results (p-values) of heterogeneity tests for year differences within areas: Females only.

IIIE	IV	V	VIW
0.595	0.510	**0.015**	

Bold p-values indicate statistically significance.

Table 7. Results (p-values) of heterogeneity tests among areas: Females only.

Among areas	IIIExIV	IIIExV	IVxV	IIIxVIW	IVxVIW	VxVIW
1.28E-06	**0.014**	**0.000118**	**0.000052**	**0.019**	**0.046**	**0.045**

Bold p-values indicate statistically significance.

Table 8. Results (p-values) of heterogeneity tests for year differences within areas: Males only.

IIIE	IV	V	VIW
0.414	0.641	0.284	**0.020**

Bold p-values indicate statistically significance.

Table 9. Results (p-values) of heterogeneity tests among areas: Males only.

Among areas	IIIExIV	IIIExV	IVxV	IIIxVIW	IVxVIW	VxVIW
8.75E-05	**0.009**	0.067	**0.007**	**0.022**	**0.001**	0.163

Bold p-values indicate statistically significance.