Preliminary microsatellite analyses of western North Pacific minke whales, *Balaenoptera acutorostrata*

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ABSTRACT

Four microsatellite loci were analyzed in a total of 228 minke whales collected from three areas in the western North Pacific: coastal Korea (sub-area 6, n= 26), coastal Japan (sub-area 7, n=104) and an offshore locality (sub-area 9, n=98). Homogeneity tests of allele frequency distributions revealed no significant differences in allele frequencies at any of the four loci between sub-areas 7 and 9. However, the combined samples from these two areas (7 and 9) differed significantly from the samples originating from coastal Korea at all four loci. These results support the previous view that two genetic stocks distribute in the western North Pacific, one around the Korean Peninsula and the other in the eastern side of Japan. Although no difference in allele frequencies were observed between areas 7 and 9 we did detect significant and close-to-significant deviations from the expected Hardy-Weinberg genotypic proportions under panmixis in the two sub-areas 7 and 9, respectively. Futher analyses considering more microsatellite loci are necessary before drawing definitive conclution of this phenomena.

INTRODUCTION

Previous genetic (Goto and Pastene, in press; Wada, 1983; Wada, 1984; Wada and Numachi, 1991) and morphological (Ohsumi, 1983) studies have supported the existence of two distinct populations of minke whales (*Balaenoptera acutorostrata*) in the western North Pacific. These two 'stocks' have been denominated as the 'J' stock (the Sea of Japan and East China Sea) and the 'O' stock (the Pacific coast of Japan and in the Sea of Okhotsk) by the International Whaling Commission (IWC). Furthermore, genetic analyses have demonstrated temporal mixing of these stocks in the southern part of the Sea of Okhotsk (Goto and Pastene, in press; Wada and Numachi, 1991).

In preparation of the implementation of the Revised Management Procedure (RMP), a Working Group on North Pacific Minke Whale Management Trials discussed in 1993 the issue of stock identity and structure in the western North Pacific. The group proposed the existence of three stocks ('J' stock, 'O' stock as well as an additional new stock, the 'W' stock). In addition the areas surrounding Japan were divided into several sub-stocks (IWC, 1994).

Later, in 1996, the North Pacific Minke Whale Trials Working Group met again to discuss the problem of the stock structure in the western North Pacific, incorporating new data obtained from the Japanese research catches in the western North Pacific (namely JARPN cruises). The group

concluded that the data from sub-areas 7, 8, 9, 11 and 12 were consistent with the assumption of a single stock (with a temporal component of 'J' stock animals in sub-areas 7, 11 and 12) as well as the distinction between the 'J' and 'O' stocks. The Group found that the new data were generally inconsistent with the existence of several sub-stocks characterized by different levels of latitudinal migration around Japan (IWC, in press).

With regard to the previous proposed stock 'W' , the Group found no evidence to support the existence of such a separate 'W' stock. However, some members of the Group felt that the information presented did not exclude this possibility, mainly due to power in the presented genetic analyses to detect low levels of genetic differentiation. Two different kinds of genetic analyses (mitochondrial DNA and allozyme analysis) had been used to compare minke whales from coastal Japan (sub-area 7) with minke whales from offshore areas (sub-area 9, equal to the hypothesized 'W' stock (IWC, in press).

Because of the lack of agreement on the possible occurrence of a hypothesized 'W' stock in offshore areas of the western North Pacific, the application of new and more sensitive molecular techniques was initiated to further investigate the results obtained using mtDNA and allozymes.

Here we present the preliminary results of an analysis of four microsatellite loci in western North Pacific minke whales focusing mainly on the comparison between minke whales caught in inshore (sub-area 7) and offshore (sub-area 9) waters. Microsatellites are a class of hyper-variable nuclear DNA (Tautz, 1989; Weber and May, 1989). They consist of tandem arrays of short (1 - 5 base pairs) DNA sequences. Relative to allozymes (also a nuclear marker) microsatellites generally display much higher levels of mutation and heterozygosity which can yield a higher level of power in genetic analyses (although not always) (e.g., Bruford and Wayne, 1993; Schlötterer and Pemberton, 1994). Microsatellite analyses have successfully been employed in behavioral and population genetic studies of cetaceans (Amos *et al.*, 1993; Andersen *et al.*, 1997; Bérubé *et al.*, in press; Buchanan *et al.*, 1996; Clapham and Palsbøll, 1997; Larsen *et al.*, 1996; Palsbøll *et al.*, 1997a; Richard *et al.*, 1996; Valsecchi *et al.*, 1997).

MATERIALS AND METHODS

Samples and localities

A total of 228 individual minke whales were analyzed. Samples were available from three general areas (Fig. 1): coastal Japan (sub-area 7; n= 104), offshore area (sub-area 9; n= 98) and coastal Korea (sub-area 6; n= 26). All samples were collected from whaling operations, either small-scale coastal operations (sub-areas 6 and 7, in 1982-87) or during JARPN (Japanese Whale Reseach Program under Special Permit in the North Pacific areas; sub-areas 7 and 9, in 1994-1996.). The sex ratio was at parity in samples obtained from inshore Japan, whereas the offshore samples (sub-area 9) had a highly and significant skewed sex ratio towards males (8 males:1 female, X^2 test; $\chi^2 = 17.3$, 1 df, $P<0.001$). No information regarding sex was available for the Korean samples, but will be determined at by molecular analysis at a later stage (Bérubé and Palsbøll, 1996a; Bérubé and Palsbøll, 1996b).

Tissue and DNA extraction

Tissue samples were preserved by freezing at -20 degrees Celsius for a period of a few months up to several years. Total-cell DNA was isolated from liver or muscle following standard procedures

(Sambrook *et al.*, 1989). The extracted DNA was dissolved in 500 uL TE buffer and stored at -20 degrees Celsius until use.

PCR amplifications

Four oligonucleotide primers (three tetramer (Palsbøll *et al.*, 1997b) and one dimer (Palsbøll, unpublished data) microsatellites loci) were used to amplify regions containing simple sequence repeats within the minke whale genomic DNA. Primers for each locus were synthesized commercially with a fluorescent dye attached to the 5' end of one primer of each pair (Table 1). Reverse primers of GATA 417 and GATA 028 were end-labeled with 6-carboxyfluorescein (6-FAM) and GATA 098 with 4,7,2',4',5',7',-hexachloro-6-carboxyfluorescein (HEX). Forward primer of GT 023 was also end-labeled with 4,7,2',7',-tetrachloro-6-carboxyfluorescein (TET). PCR™ amplifications (Saiki *et al.*, 1988) were carried out in 15 uL reactions containing 5 pmol of each labeled and unlabeled primer, 0.625 units of Ex™ Taq polymerase (Takara Shuzo Co, Kyoto), 2mM of each dNTP, reaction reagent (reaction buffer is 100mM Tris-HCl [pH 8.3] , 500mM KCl, 2mM $MgCl_2$, 0.01%[W/v] gelatin) and 10ng of total DNA. PCR reaction was performed using a Perkin Elmer 9600 machine (Applied Biosystems, Inc. Foster City, CA) with annealing temperatures shown in Table 1. Each product was electrophoresed with internal size standard (N,N,N',N',-tetrametyl-6-carboxyrhodamine; TAMRA 500) through 5% polyacrylamide denaturing gel (Long Ranger™) using an ABI 377 DNA Prism™ sequencer.

Data analysis

Microsatellite data were retrieved using the computer program GeneScan™ (Applied Biosystems, Inc), following the manufacturer instructions. All peaks were checked by eye and, in few cases, excluded from analysis when they could not be resolved unambiguously. Allele frequencies, the expected heterozygosity as well as the probability of identity (Paetkau and Strobeck, 1994) were estimated for each and all loci. The 95% confidence interval of the expected heterozygosity at each sub-area was estimated from 1,000 bootstrap samples, each of an equal sample size (2n) as the original sample. The probability of identity (I) is the probability that two unrelated individuals have the same genotype at a single locus, or across all loci (obtained by simple multiplication of the estimated I's for all loci). Tests for linkage dis-equilibrium, deviations from the Hardy-Weinberg proportions as well as population differentiation (from allele frequencies) were conducted using the program GenePop (Raymond and Rousset, 1995). For each locus, an unbiased estimate of the P value (either Fisher exact test or by Markov-chain Monte-Carlo simulations) was performed following the procedure implanted in GenePop. P-values from the four loci was combined into a single P-value as described by Sokal and Rohlf (1995, p. 795). The level of differentiation was estimated as θ (Weir, 1990) using the DIPLOID computer program supplied with GenePop.

RESULTS

Degree of polymorphism

The four microsatellite loci were all highly polymorphic in minke whales from the three localities. We observed between 3 - 14 alleles within each population at different loci. The number of different alleles, and the expected heterozygosity amongst the Korean coastal samples was lower at all four loci than those estimates observed in the combined sub-areas 7 and 9 (Table 2). The estimated expected heterozygosity in the Korean sample was significantly lower than that observed in the sub-areas 7 and 9 at all four loci.

Linkage dis-equilibrium and Hardy-Weinberg proportions

Only a single instance of significant levels of linkage disequilibrium was observed between locus GT023 and GATA028 among the Korean samples ($P < 0.00001$). The lack of a similar observation in the much larger sample from the combined sub-areas 7 and 9 indicate that the deviation from linkage dis-equilibrium probably doesn't reflect physical linkage, but maybe was a product of previous population history and non-equilibrium conditions or simply a random incidence (in a total of 17 pair-wise comparisons). No significant deviation from the expected Hardy-Weinberg genotypic was observed within the Korean samples, whereas the P-value for all loci for the combined sub-areas 7 and 9 was marginally significant ($P=0.039$). A significant deviation ($P=0.028$) was observed within sub-area 7 and a close-to-significant deviation ($P=0.064$) in sub-area 9 .

Genetic differentiation among sub-areas

The results of the homogeneity tests revealed no significant differences in allele frequencies between sub-areas 7 and 9 at any of the four loci. Hence sub-areas 7 and 9 were combined and tested against the Korean samples, which yielded a highly significant level of differentiation ($P<0.0001$, see Table 3). The level of divergence (as Weir's θ) between the Korean samples and the combined sub-areas 7 and 9 was estimated at 0.0429 (95% CI: 0.0339 - 0.0539). Weir's θ (1990) is an estimator of the proportion of genetic variance that is due to the partitioning of the samples, and thus equivalent of Excoffier's Φ_{ST} (1992).

DISCUSSION

The results of the preliminary microsatellite analysis of western North Pacific minke whales presented here were in general agreement with the results obtained earlier, either from analysis of other genetic loci, such as mtDNA and allozymes (Goto and Pastene, in press; Wada, 1983; Wada, 1984; Wada and Numachi, 1991), or morphological studies (Ohsumi, 1983). A clear cut differentiation between samples collected off the Korean Peninsula (sub-area 6) and the Pacific side of Japan (sub-areas 7 and 9) was evident at all four loci and supported by a relatively high θ of 0.0429 (95% CI: 0.0339 - 0.0539). We did not detect any differences in allele frequency distributions at any of the four loci between sub-area 7 and 9, indicating that samples from these two areas were collected from the same stock.

However, the finding of significant or close to significant deviations from the expected Hardy-Weinberg genotypic proportions under panmixis in sub-areas 7 and 9 should be futher investigated. There is a possibility that samples from these areas were not drawn from a single panmictic population. In such cases the lack of differentiation between these two samples (considering the relatively large sample sizes) suggests that sub-structure within these two sub-areas does not have a simple longitudinal explanation. Thus further analysis of more loci as well as additional samples collected from different point in time and space is required. Such deviations from the expected Hardy-Weinberg proportions could also be due to null-alleles generating an excess of homozygotes, but as the significant deviations were at different loci in the two sub-areas, null-alleles seem an unlikely cause of these deviations. At this stage no difinitive conclution can be reached.

In sub-area 6, the levels of heterozygosity was significantly lower at all four loci in comparison to sub-areas 7 and 9, consistent with the estimates of abundance (IWC, in press) which yielded a estimate of abundance for the Korean stock that was ten times lower than the combined estimates of sub-areas 7 and 9.

ACKNOWLEDGMENTS

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Table 1. Primer sequences and other characteristics of four microsatellite loci used in this study. Temperature shown is the annealing temperature.

Locus	Primer sequences (5' ..... 3')	Source	Label	T(°C)	Allele size (bp)
GT 023	GTTCCCAGGCTCTGCACTCTG				
	CATTTCCTACCCACCTGTCAT	Palsbøll (unpublished data)	TET	59.5	88 - 120
GATA 028	CGCTGATAGATTAGTCTAGG				
	AAAGACTGAGATCTATAGTTA	Palsbøll *et al.* (1997b)	6-FAM	47.0	194 - 230
GATA 098	TGTACCCTGGATGGATAGATT				
	ATGTCTCTCTCACACCTCACC	Palsbøll *et al.* (1997b)	HEX	59.5	100 - 120
GATA 417	CTGAGATAGCAGTTACATGGG				
	TCTGCTCAGGAAATTTTCAAG	Palsbøll *et al.* (1997b)	6-FAM	55.0	200 - 228

Table 2. Summary of the microsatellite data for all four loci at the three western North Pacific sub-areas 6,7 and 9.

Locus		GT023				GATA028				GATA098				GATA417		
Sub-area		*6*	*7*	*9*		*6*	*7*	*9*		*6*	*7*	*9*		*6*	*7*	*9*
	Allele				Allele				Allele				Allele			
	88	-	.006	.005	**194**	-	.024	.005	**100**	-	.005	.005	**200**	-	.014	.026
	94	.115	.006	.015	**198**	-	.053	.026	**104**	.058	.091	.066	**204**	-	.067	.056
	96	.096	.005	.005	**202**	.250	.192	.153	**108**	.750	.587	.490	**208**	.038	.072	.092
	98	-	.115	.077	**206**	-	.154	.133	**112**	.192	.216	.306	**212**	.500	.351	.357
	100	.019	.014	.026	**210**	.269	.154	.209	**116**	-	.096	.128	**216**	.442	.313	.311
	102	-	.039	.041	**214**	.481	.303	.291	**120**	-	.005	.005	**220**	.019	.168	.158
	104	.077	.130	.148	**218**	-	.082	.122					**224**	-	.005	-
	106	.173	.091	.107	**222**	-	.034	.051					**228**	-	.010	-
	108	.038	.063	.071	**226**	-	.005	.01								
	110	.058	.115	.122												
	112	.423	.178	.179												
	114	-	.111	.128												
	116	-	.120	.071												
	118	-	.010	-												
	120	-	-	.005												
Expected heterozygosity		.75	.88	.88		.62	.81	.81		.37	.59	.64		.53	.74	.74
Upper 95% CI		.81	.89	.89		.65	.83	.83		.49	.64	.68		.58	.77	.76
Lower 95% CI		.64	.86	.87		.52	.78	.78		.21	.52	.59		.46	.70	.70
Probability of identity		.084	.025	.024		.206	.060	.060		.409	.212	.184		.300	.108	.111

Table 3. Results of the homogeneity test comparing allele frequency of minke whales from sub-areas 6, 7 and 9. Figures shown are the probabilities of drawing the observed allele frequency distributions in each of two samples from the same stock.

Sampling areas	GT023	GATA028	GATA098	GATA417	All loci
Sub-area 6 and sub-areas 7 & 9	<.00001	.00004(SE .00004)	.023(SE .0025)	.0059(SE .0021)	<.00001
Sub-area 6 and sub-area 7	<.00001	<.00001	.093(SE .0084)	.0073(SE .0024)	<.00001
Sub-area 6 and sub-area 9	<.00001	.00052(SE .00033)	.00060(SE .00024)	.0052(SE .0016)	<.00001
Sub-areas 7 and 9	.81(SE .015)	.24(SE .017)	.18(SE .015)	.89(SE 0.010)	<.7

For estimation of probabilities please refer to the Materials and Methods section. Estimates without standard errors were calculated using Fisher's exact test.

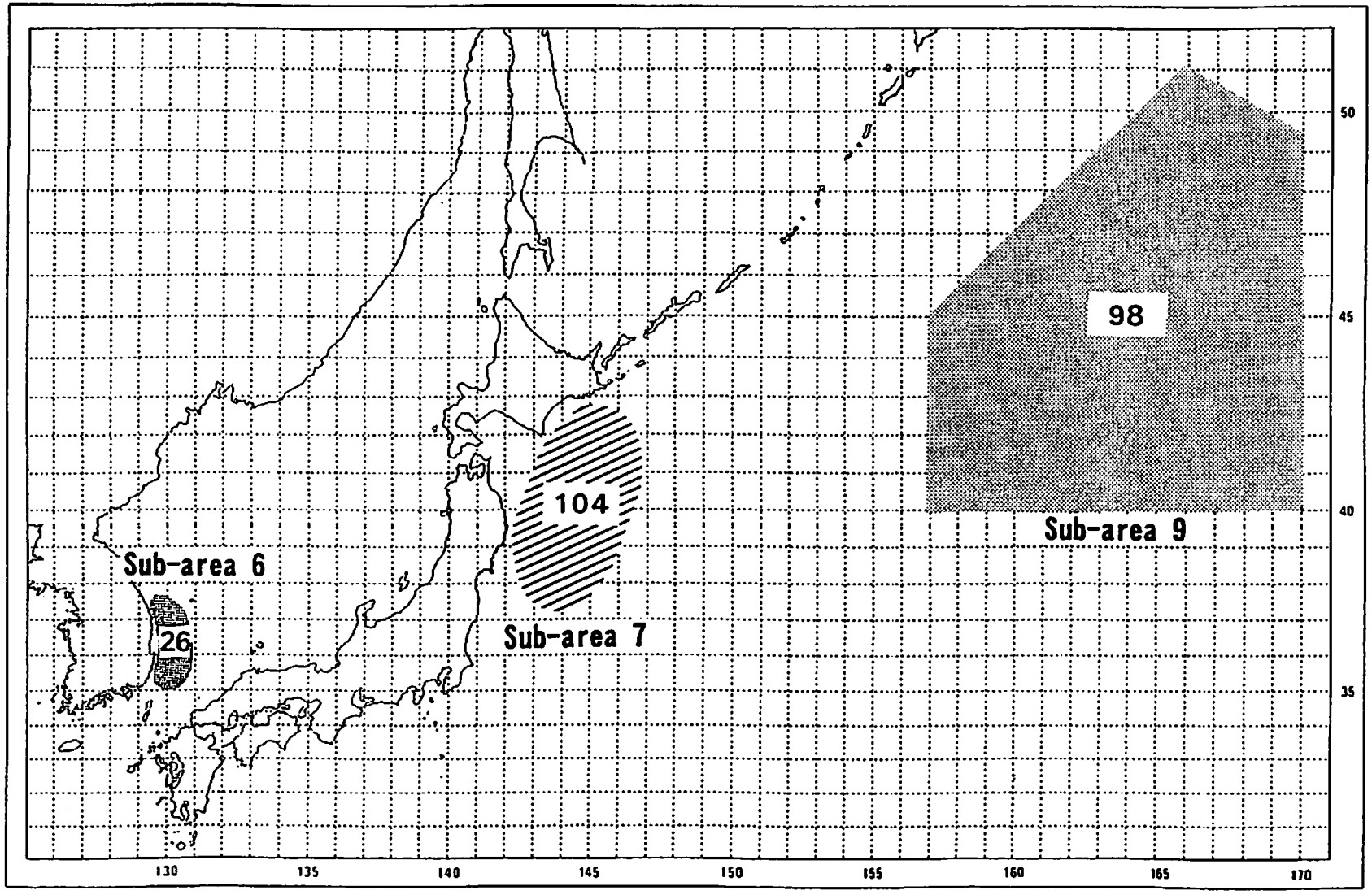

Fig. 1. Geographical origin of the samples. Numbers indicate sample sizes for each locality. Korea correspond to sub-area 6, the Japanese coastal waters to sub-area 7 and the offshore waters to sub-area 9 as defined by the Working Group on North Pacific Minke Whale Management Trials (IWC. 1994).