Status of the Japanese DNA Register for Large Whales

Luis A. Pastene and Mutsuo Goto

The Institute of Cetacean Research, Toyomi-cho 4-5, Chuo-ku, Tokyo 104-0055, Japan

ABSTRACT

In contribution to the work of the Working Group on DNA Testing an update of the status of the Japanese DNA register for large whales is provided. This register is based on technical specifications similar to those of the Norwegian register. On the basis of the information summarized here it is concluded that the Japanese register contains almost all the genetic profiles of whales taken legally in Japan and therefore is close to be fully diagnostic.

INTRODUCTION

The basic position of the Government of Japan (GOJ) concerning DNA identification and tracking of whale products is that matters related to market or international trade of whale products are outside the jurisdiction of the IWC. At the same time the GOJ recognizes the usefulness and importance of DNA technology and it has been using this technology in its domestic management of whale products. Furthermore Japanese scientists have been contributing to the IWC/SC in discussions on the scientific aspects involved (e.g. genetic techniques).

Domestic management of whale products in Japan involves two main components: establishment and maintenance of a diagnostic DNA register for large whales and market monitoring through systematic DNA surveys in the retail market.

In contribution to the work of the Working Group on DNA Testing an update of the status of the Japanese DNA register is provided. This register is based on technical specifications similar to those of the Norwegian register (Dupuy and Olaisen, 1998).

BRIEF DESCRIPTION OF THE JAPANESE DNA REGISTER

Source of tissue samples

In Japan there are two main sources of tissues samples: 1) scientific whaling conducted under special permit in the Antarctic-JARPA (Antarctic minke whale since 1987/88) and western North Pacific JARPN II (common minke whale since 1994, Bryde's whale since 2000, sei whale since 2002 and sperm whales since 2000) and 2) by-catches, involving common minke whales mainly, since 1 July 2001.

In the case of scientific whaling, samples for genetic analysis are collected by cetacean researchers. These involve skin samples (two pieces of 5×5×5mm kept in 99% ethanol); liver, heart, kidney, skin and muscle (one piece of 20g each kept frozen). A large amount of information on each whale sampled is collected using established protocols of JARPA and JARPN II including date and locality (longitude, latitude) of sampling, species, body length and sex.

The Japanese regulation on by-catches (established from 1 July 2001) requires that all animals should be DNA-registered before whale meat can be sold in the market. Details of the regulation and procedure can be found in the following web page: http://www.icrwhale.org/pdf/higekujira.pdf. Skin or muscle samples (5×5×5cm) are taken by the fisherman and send to the laboratory at ICR (as frozen samples). Fisherman should provide several kind of information (on an established format) on the by-caught animal. Among these, the following relevant information is provided: date and locality of the by-catch, type of set net, species, body length and sex.

Laboratory analysis
At the laboratory, a single typing procedure is employed. Typing procedure is repeated only in the case of samples not providing satisfactory results in the first attempt. Total-cell DNA is extracted from tissues by standard phenol/chloroform extractions (Sambrook *et al*., 1989).

Specification of markers
In the Japanese register the whale DNA is composed of three parts:

a) Species identification: An approximately 500bp fragment of the 5'-end of the mitochondrial DNA control region.

The first 490 nucleotides at the 5' end of the mitochondrial control region is amplified by the polymerase chain reaction. The oligo-nucleotides employed in the PCR amplification are usually MT4 (Arnason *et al*. 1993) and Dlp5R (5'-CCATCGAGATGTCTTATTTAAGGGGAAC-3'). Reactions are carried out in 50 uL volumes containing 100 mM KCl, 20 mM Tris-HCl, 0.1 mM EDTA, 1 mM DTT; 0.5% Tween 20, 0.5% Nonidet P-40, 200 uM dNTPs, 2.5 pM of each oligo-nucleotide and one unit of *Taq* DNA polymerase. After an initial denaturation step at 95° C for 5 minutes, a PCR amplification cycle of 30 seconds at 94°C, followed by 30 seconds at 50°C and 30 seconds at 72°C is repeated 30 times. The amplification is completed with a final extension step of 10 minutes at 72°C. PCR products are purified by MicroSpin S-400HR columns (Pharmacia Biotech). Subsequent cycle sequencing reactions are performed with 100ng of products generated in the above PCR amplifications using the Prism™ dRhodamine Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.). The oligo-nucleotides used to prime the cycle sequencing reaction were the same as employed in the initial PCR amplification listed above. A total of 25 cycles with 10 seconds at 96°C, 20 seconds at 56°C and four minutes at 60°C are performed. The cycle sequencing products are purified by Auto Seq G-50 Spin Columns (Pharmacia Biotech). The nucleotide sequence of each cycle sequencing reaction is determined by electrophoresis through a 5% Long Ranger™ (FMC, Inc.) denaturing polyacrylamide matrix on a DNA Prism™ 377 DNA Sequencer (Applied Biosystems, Inc.) under standard conditions. Both strand samples are sequenced in their entirety for all samples.

Standard phylogenetic analyses of 'test' and 'type' sequences are conducted to investigate the species of origin of unknown whale products.

b) Individual identification: a set of nuclear DNA markers (microsatellites or STR), which together identify each individual whale.

In the case of the Japanese register the microsatellite set used for population study and individual identification is composed of 17 loci, with the exception of Antarctic minke whale (10) and humpback whale (6) (see Table 1). New primers are being investigated and tested for optimizing their use in the case of the humpback whale. In the case of the Antarctic minke whale the number of loci is smaller but enough for individual identification purposes because of very high genetic variability.

Laboratory procedure for microsatellite analyses is as follow:

Microsatellite polymorphisms are analyzed using published primers (see Table 1). Although primer sequences follow those of the original authors, an annealing temperature of each of the loci is optimized for each of the whale species in the DNA register. PCR amplifications are 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) are run on a 6% polyacrylamide denaturating gel (Long Ranger) using BaseStation100 DNA fragment analyzer (MJ Research). Although alleles are visualized using Cartographer software specifically designed for the BaseStation, allelic sizes are determined manually in relation to the internal size standard and DNA of known size from 'control' individual that is rerun on each gel.

The number and degree of variability of loci used in both registers are enough for the objective of individual identification.

c) Sex determination: data from Y chromosome DNA

In the case of Japan the method of Abe *et al*. (2001) is used, which involves co-amplification of SRY gene on Y chromosome and a microsatellite locus.

Error rates estimations for microsatellite data sets

Hoffman and Amos (2005) estimated the error rate using a large data set (n=2000) of the Antarctic fur seal. The authors used several approaches (repeat-genotyping, deliberately re-sampled individuals, unintentionally re-sampled individuals, mother-offspring pairs, mismatches between pups and putative fathers) to estimate error rates. They found good concordance among the approaches used to error rate estimation with the range being 0.0013 to 0.0074 per single locus PCR. The most common errors involved the misinterpretation of allele banding patterns.

No attempts have been made to estimate statistically error rates for the microsatellite data set in the Japanese register. However side by side running to check genotypes is commonly conducted when there is some uncertainty in the pattern found. We believe that such errors are minimal in the Japanese register.

Format of individual records
In the case of the Japanese register, each registered whale has the following code:

Scientific whaling
'00NPM001': this specifies the year (2000), oceanic region (NP=North Pacific), species (M=minke) and individual number (001). This code allows for cross reference with the comprehensive data base on JARPA, JARPN II in ICR.

By-catches
'ICRY-05-028' (by-catch before DNA analysis); '30041016MI395' (by-catch after DNA analysis). In the latter code '30' is for prefecture, date (16 October 2004), species (MI=minke) and sequential number. Both codes allow for cross reference with the comprehensive data base of by-catch animals in ICR.

Database structure
Two Excel files are generated, one for microsatellites and gender profiles and the other for the mtDNA sequence. In each of these, consecutive whales are numbered.

In the STR/gender file, each whale is given in one row, with one column for each allele (two columns for each STR marker and for the gender locus). In the case of the Japanese register each species is given a different Excel sheet.

In the mtDNA file, each profile has one row. Again in the case of the Japanese register each species is given a different Excel sheet.

Status of the registry
The status of the Japanese register is shown in Table 2.

All whales taken under special permit in the western North Pacific (till 2003) and most of those taken in the Antarctic (till 2003/04) have been incorporated into the register. Sex information for whale taken for scientific permit are provided by JARPA or JARPN II researchers (no molecular sexing is conducted for these samples). In the case of by-catches sex is determined using molecular approach.

A similar regulation/procedure to that of the by-catches was adopted in October 2004 for stranding whales, in case of animals are utilized either for commercial or scientific purposes. DNA data from some few stranding occurred after that date (whales being utilized for scientific purposes) are being incorporated into the register. There was also some investigation of frozen stockpiles of whale meat (originating in past commercial whaling), and the amount still existing is very small. Notwithstanding genetic analysis on these products is being conducted for incorporation into the register.

FINAL REMARKS

The IWC/SC has agreed that registers should be diagnostic, i.e. that they should contain DNA profiles of any animals which products might legally appear in the market (e.g. from legal catches, bycatches, ship strikes) on the understanding that products from animals not included in the register would be considered infractions.

We conclude that the Japanese register contains almost all the genetic profiles of whales taken legally in Japan and therefore is close to be fully diagnostic.

Further, it should be noted that genetic data contained in the Japanese register was used by members of the IWC/SC Committee during the RMP *implementation assessment* of North Pacific minke whale and RMP *pre-implementation assessment* of North Pacific Bryde's whale.

ACKNOWLEDGEMENTS

We thank N. Kanda (ICR) for providing information on the microsatellite part of the Japanese DNA register, H. Ishikawa (ICR) for providing information on the by-catches and stranding regulation/procedures and J. Morishita (GOJ) for valuable comments on this manuscript.

REFERENCES

Abe, H., Goto, M., Pastene, L.A., Dewa, K. and Naito, E. 2001. Practical use of multiplex fluorescent PCR for cetacean sex identification. *Marine Mammal Science* 17 (3):657-664.

Arnason, U., A. Gullberg, and B. Widegren. 1993. Cetacean mitochondrial DNA control region: sequences of all extant baleen whales and two sperm whale species. Journal of Molecular Biology and Evolution 70:960-970.

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.

Dupuy, B.M. and Olaisen, B. 1998. Typing procedure for the Norwegian minke whale DNA register. Department of Forensic Medicine, University of Oslo, Norway. 32pp.

Hoffman, J.I. and Amos, W. 2005. Microsatellite genotyping errors: detection approaches, common sources and consequences for paternal exclusion. *Mol Ecol* 14:599-612.

Palsbøll, P.J., Bérubé, M., Larsen, A.H., Jørgensen, H. 1997. Primers for the amplification of tri-and tetramer microsatellite loci in baleen whales. *Mol Ecol* 6:893-895.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular cloning: A laboratory manual. 2nd Ed.*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

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

Wada, S., Oishi, M. and Yamada, T.K. 2003. A newly discovered species of living baleen whale. *Nature* 426 (6964):278-281.

Table 1: Microsatellite loci used for different whale species in the Japanese register

	Species	Whales						
Primer	designed for	SH minke	NP minke	Bryde's	Sei	Sperm	Humpback	Ref.
DlrFCB14	Beluga whale	x	x	x		x		a
DlrFCB17	Beluga whale			x	x	x		a
EV1	Sperm whale	x	x	x	x	x		b
EV5	Sperm whale					x		b
EV14	Sperm whale		x	x	x			b
EV21	Sperm whale		x	x	x			b
EV30	Sperm whale					x		b
EV37	Humpback whale		x			x		b
EV94	Humpback whale		x	x	x	x		b
EV104	Humpback whale	x	x	x	x	x		b
GATA28	Humpback whale	*	x	x	x	x	x	c
GATA53	Humpback whale			x	x		x	c
GATA98	Humpback whale	*	x	x	x	x	x	c
GATA417	Humpback whale	*	x	x	x	x	x	c
GGAA520	Humpback whale			x	x			c
GT011	Humpback whale			x	x	x		d
GT023	Humpback whale	x	x	x	x	x	x	e
GT195	Humpback whale	x	x					e
GT211	Humpback whale	x	x		x	x		e
GT271	Humpback whale				x	x		e
GT310	Humpback whale		x	x	x			e
GT509	Humpback whale	*	x			x		e
GT575	Humpback whale	*	x	x	x	x		e
TAA031	Humpback whale		x	x			x	c
Total number of loci used		6 (10-11)	17	17	17	17	6	

* indicates the loci used for only individual identification.

a=Buchanan et al. (1996)
b=Valsecchi and Amos (1996)
c=Palsbøll et al. (1997)
d=Bérubé et al. (1998)
e=Bérubé et al. (2000)

Table 2: Status of the Japanese DNA register for large whales

Source/Species	Period	Catch/ Stranding	mtDNA	STRs	Sex
Scientific whaling					
NP minke whale	94-03	938	938	938	938
NP Bryde's whale	00-03	193	193	193	193
NP Sei whale	02-03	89	89	89	89
NP Sperm whale	00-03	28	28	28	28
SH minke whale (Antarctic)	89/90-03/04	5,841	992	5,808	5,841
SH minke whale (Common)	87/88-93/94	16	16	16	16
By-catches					
NP minke whale	01-04*	403	403	403	403
NP humpback whale	01-04*	9	9	9	9
NP right whale	01-04*	1	1	1	1
Bryde's whale**	01-04*	3	3	3	3
Stranding***					
NP humpback whale	04	1	1	-	-
NP minke whale	04	1	1	-	-
NP fin whale	04	1	1	-	-

* From 1 July 2001 to 31 December 2004
** Including two animals identified genetically as *B. omurai (*Wada *et al. 2003)*
*** Nov.-Dec. 2004