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Teeth and Bones as Sources of DNA for Genetic Diversity and Sex Identification of Commerson's Dolphins (*Cephalorhynchus commersonii*) from Tierra del Fuego, Argentina

Lida E. Pimper,[1, 2, 3] Maria I. Remis,[1] R. Natalie P. Goodall,[2, 3] and C. Scott Baker[4, 5]

[1]*Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Intendente Güiraldes 2160, Ciudad Universitaria, Capital Federal (1428), Argentina; E-mail: liditas@ege.fcen.uba.ar (Lida E. Pimper, corresponding author)*

[2]*Museo Acatushún de Aves y Mamíferos Marinos Australes (AMMA), Sarmiento 44, (9410) Ushuaia, Tierra del Fuego, Argentina*

[3]*Centro Austral de Investigaciones Científicas (CADIC), Bernardo Houssay 200, (9410) Ushuaia, Tierra del Fuego, Argentina*

[4]*School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand*

[5]*Marine Mammal Institute, Hatfield Marine Science Center, Oregon State University, 2030 SE Marine Science Drive, Newport, OR 97365, USA*

Abstract

Museum specimens are often the only source of genetic material for many species of cetaceans. Most of the protocols that have been developed to extract DNA from teeth and bone samples involve mechanical or chemical disintegration of the material. An alternative method to the mechanical reduction process is presented here for 212 Commerson's dolphin (*Cephalorhynchus commersonii*) samples collected in Tierra del Fuego, Argentina, from 1974 to 2004. From the total samples, 82.5% (n = 175) allow for amplification to occur of a minimum length of 200 pb for the mtDNA control region, 66.5% (n = 141) of 400 bp, and 51% (n = 108) of 500 bp. A total of 11 haplotypes were defined from 10 polymorphic sites. From the initial number of samples, 64.6% could be sexed via molecular markers. This method is effective for processing large quantities of degraded samples over a short time period, analysing a representative number of mtDNA haplotypes, and allowing robust estimation of historical genetic diversity and trends over time.

Key Words: teeth, bones, DNA extraction, population genetic analysis, Commerson's dolphin, *Cephalorhynchus commersonii*

Introduction

Knowledge of the population and social structure of marine mammals is essential for proper evaluation of their present and potential future distribution and abundance. Commerson's dolphins (*Cephalorhynchus commersonii*) (Lacépède, 1804) are found in the shallow waters of the continental shelf of the eastern coast of South America between about 41º 30' S and 56º S (Goodall et al., 1988). For this species, for which moderate levels of incidental exploitation have been documented (Goodall & Cameron, 1980; Goodall et al., 1988, 1994), such knowledge is fundamental for understanding the impact of this bycatch. A study at the genetic level can often be improved by the addition of historical samples from museum collections (Rosenbaum et al., 2000).

Museum specimens are often an important source of DNA for species identification and historical analyses of genetic diversity (Rosenbaum et al., 2000; Pichler & Olavarría, 2001). Depending on the treatment and storage of the specimen, bones, baleen plates, and teeth have been found to provide adequate DNA for genetic analyses (Rosenbaum et al., 1997; Pichler et al., 2001a; Dalebout et al., 2004). Most of the protocols that have been tested to extract DNA from teeth and bone samples involve mechanical and chemical disintegration of the material. Here, we describe an alternative method of extracting amplifiable DNA from bones and teeth based on a relatively simple chemical approach. This method is tested on Commerson's dolphin samples collected in Tierra del Fuego, Argentina; measures of genetic diversity from mitochondrial DNA sequences are estimated and presented.

Materials and Methods

A total of 212 samples of Commerson's dolphins from Tierra del Fuego, Argentina, were collected from teeth (n = 178) and bones (n = 34) of different

specimens provided by the Goodall Collection (RNP) at the Museo Acatushún de Aves y Mamíferos Marinos Australes. These were obtained from beachcast and bycatch specimens collected along the coast of Tierra del Fuego, Argentina, during the years 1974 to 2004. Post-cranial carcasses were first soaked in freshwater-filled containers, which accumulate bacteria that aid in flesh decomposition. After maceration, post-cranial skeletons from all specimens were cleaned in pots with warm water, bleach, and detergent. Skulls were cleaned by hand in dedicated reservoirs to avoid the loss of teeth. Final steps included rinsing them in fresh water and putting them on racks to dry. Specimens were stored in cardboard trays in designated cupboards.

Samples were handled in a laboratory facility that had not been used previously to analyze modern or ancient cetacean DNA. A laminar flow chamber, sterile conditions (UV irradiation of surfaces, cleaning with bleach), and disposable equipment were used to minimise risk of contamination. The reagents were made up in a "DNA-free" positive pressure room, isolated from the main laboratory. Small fragments of bone (approximately 5 mm × 5 mm) or single teeth (18 to 99 mg) were decalcified/demineralised with rotation in 950 µl EDTA 0.5M pH 8 at 55° C for 6 d (Hanaoka et al., 1995) without changing the solution. This was followed by a silica-based DNA extraction protocol of Boom et al. (1990) as modified by Höss & Pääbo (1993). A maximum of 15 samples were extracted at one time. Each set of extractions included one extraction control at the beginning of the digestion phase and one control every five samples. The first step was a protein digestion with 200 µl of 10% SDS, 100 µl DTT (10 mg/ml), and 100 µl Proteinase K (20 mg/ml) added to the tube used in the demineralization phase, with incubation at 37º C overnight, followed by one hour at 50º C. Samples were then subjected to centrifugation at 5,000 rpm for 5 min, and 750 µl of the supernatant were removed to a new tube, where 1 ml of extraction buffer (0.1M Tris-HCl, pH 6.4; 0.02M EDTA, pH 8.0; 1.3% Triton-X-100; 10M guanidinium thiocyanate [GuSCN]) was added. This was then incubated with rotation at 60º C for one to several hours. After that, 50 µl of silica suspension prepared as in Boom et al. (1990) was added, mixed, and incubated at room temperature for 10 min. The samples were briefly centrifuged for 15 s at 11,500 rpm, and the supernatant was discarded. Subsequently, the silica pellet was washed twice with a buffer consisting of 10M GuSCN; 0.1M Tris-HCl, pH 6.4; and 0.02M EDTA, pH 8.0, and twice with 70% ethanol. After drying the pellet for about 40 min in laminar flow, nucleic acids were eluted at 55º C in two aliquots of 75 µl 1X TE (10mM Tris-HCL, 1mM EDTA) and stored at -20º C.

The efficiency of the DNA extraction was tested through PCR amplifications of a mtDNA fragment of the control region and sex-specific molecular markers. Each set of amplifications included a no-template control and one positive control. The extraction controls were also included to be sure that there was no carryover contamination of PCR products or cross-contamination between samples. A 550 base pair (bp) region of the mitochondrial DNA control region was amplified by Polymerase Chain Reaction (PCR) with primers M13dlp1.5 (5'-TGTAAAACGACAGCCAGTTCACCCAAAGCTGRARTTCTA-3') and dlp5 (5'-CCATCGWGATGTCTTATTTAAGRGGAA-3') (Dalebout et al., 1998). The following fragment sizes were attempted from samples that failed to amplify the 550-bp fragment, 400-bp with dLp1.5–dLp4 (5'-GCGGGWTRYTGRTTTCACG-3') (Baker et al., 1998) and finally a 206-bp fragment with dlpFBP (5'-GTACATGCTATGTATTATTGTGC-3') (Pichler & Baker, 2000) and dlp4. All amplification reactions were carried out in a total volume of 50 µl. Five µl of the extract was added to 1X PCR buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl), 5 mM $MgCl_2$, 0.4 µM of each primer, 0.2 mM deoxynucleoside triphosphate (dNTP), and 0.5 U of Platinum® Taq DNA Polymerase (Invitrogen Life Technologies). To overcome inhibition of PCR, 1 mg/mL bovine serum albumin (BSA) was added. The PCR temperature profile was as follows: a preliminary denaturing period of 3 min at 94° C followed by 30 cycles of denaturation for 30 s at 94° C, primer annealing for 45 s at 55° C, polymerase extension for 60 s at 72° C followed by a final extension period for 10 min at 72° C. Amplicons were quantified by 1.6% agarose gel electrophoresis staining in ethidium bromide and UV visualization with DNA low-mass ladder. PCR products were purified for sequencing with SAPEX (Amersham), cleaned with Agencourt® CleanSEQ® Kit (Beckman Coulter), and sequenced with BigDye™ terminator chemistry, Version 3.1 (Applied Biosystems, Inc.) on an ABI Prism™ Sequencer 3130 xl Genetic Analyzer (Applied Biosystems, Inc.) from the service of the Centre for Genomics & Proteomics, University of Auckland, School of Biological Sciences.

Sequences were aligned and edited using *Sequencher 4.6* (Gene Codes Corporation), *BioEdit 7.0.5.3* (Hall, 1999), and *ClustalX*, Version 1.8 (Higgins & Sharp, 1988). Variable sites and unique haplotypes were identified using *MacClade*, Version 4.0 (Maddison & Maddison, 1992). Nucleotide (π) and haplotype (h) diversity were estimated according to Nei (1987) with *Arlequin*, Version 3.11 (Excoffier et al., 2005).

The sex of many specimens in the collection had been recorded at the time of collection and was

confirmed in the laboratory via molecular sexing. This protocol consisted of a multiplex amplification of a fragment of the male-specific sry gene (*ca*. 240 bp) and fragments of the ZFY/ZFX genes (*ca*. 450 bp) as positive control (Gilson et al., 1998). Male and female positive controls, and a negative PCR control were added to the reaction.

Results

From the total of 212 samples (178 teeth and 34 bones), mtDNA was successfully amplified (single amplification products) from 175 samples (83%): 164 samples from teeth (92.1%) and nine samples from bones (26.5%). Only very small teeth (e.g., 18 mg) were fully digested in the Proteinase K phase, and most of the larger teeth were recovered without obvious damage. Of the 175 samples that amplified for at least a minimum length of 200 pb for the mtDNA control region, 141 (80.5%) amplified a fragment of 400 bp, and 108 (62.0%) amplified a fragment of 500 bp. Sequencing of 425 bp of the amplified fragments from 108 samples revealed a total of 11 mtDNA haplotypes defined from 10 polymorphic sites (Table 1).

Five of the 11 haplotypes were consistent with those found in a previous survey (Pichler et al., 2001b). Sex was identified for 137 samples (64.6%): 81 males and 56 females. The overall nucleotide diversity ($\pi = 0.35\%$) and the haplotype diversity ($h = 0.77$) demonstrated low levels of genetic variability according to other Odontocete populations but was similar to that of other species of this genus (Pichler & Baker, 2000).

Table 1. Positions of the 10 variable sites within the 425-bp consensus fragment of the mtDNA control region that defines the 11 haplotypes; dots indicate no variation from the top sequence (haplotype *C.com*-A).

	3	42	51	124	173	207	216	237	271	425
C.com-A	C	G	T	G	C	G	A	C	G	T
C.com-C	.	.	.	.	.	A	.	.	A	.
C.com-D	.	.	C	.	.	A	.	.	A	.
C.com-F	.	.	.	.	.	.	.	.	.	C
C.com-H	.	.	.	.	.	A	.	.	A	C
Hap 1	.	.	.	.	.	A	.	.	.	.
Hap 2	.	.	.	.	.	A	G	.	A	.
Hap 3	.	.	.	.	T	A	.	.	A	.
Hap 4	.	.	.	A	.	.	.	.	.	.
Hap 5	.	A	.	.	.	.	.	T	.	.
Hap 6	T	.	.	.	.	A	.	.	A	.

Discussion

The present study confirmed that the teeth and bone samples of dolphins preserved in collections are a good source of mitochondrial DNA for population genetics and taxonomic studies. The DNA extraction efficiency in both teeth and bones was high considering that most of the samples were exposed to adverse environmental conditions and heavy cleaning methods (such as simmering in water with bleach) that might degrade the DNA (Kemp & Smith, 2005). In addition, DNA extracted from "ancient" samples allows sex identification, corroborating the sex composition obtained by morphological characters. The relatively high success of amplification for the sex marker suggests the potential for success with additional nuclear markers (e.g., introns, microsats, SNPs). The soaking extraction was easier and faster than crushing or drilling the samples, which requires bleaching, acid washing, and autoclaving the elements between each round. An alternative nondestructive method involves soaking the samples in GuSCN without the addition of substantial amounts of demineralizing reagents. Only the buffer is subsequently processed, thus preventing damage to the specimen. It is less efficient than extraction methods that include a demineralization/digestion step using EDTA and Proteinase K (Rohland et al., 2004).

The use of bone and tooth samples as a source of DNA to analyze the genetic variability of Commerson's dolphin is part of a study of the genetic structure of the species in populations of Tierra del Fuego, Argentina, and Chile. This approach enables us to analyze a large number of individuals in the region and to examine areas for which only museum specimens are available.

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