



A simple protocol for a low invasive DNA accessing in *Stenella longirostris* (Cetacea: Delphinidae)

ANA PAULA C. FARRO¹, MARIO M. ROLLO JR.², JOSÉ M. SILVA JR.³ &
CELSO L. MARINO⁴

¹Universidade Federal do Espírito Santo (UFES), Centro de Ciências Humanas e Naturais, Av Fernando Ferrari, 514, Vitória, ES, 29075-010, Brazil. E-mail: anafarro@yahoo.com.br

²Campus Experimental do Litoral Paulista, UNESP, Pça Infante D. Henrique, s/n, São Vicente, SP, 11330-205, Brasil.

³Centro Mamíferos Aquáticos - ICMBio, Cx Postal 49, Vila do Boldró, s/n, Fernando de Noronha, PE, Brasil.

⁴Instituto de Biociências, Departamento de Genética, UNESP, Distrito de Rubião Jr., s/n, Botucatu, SP, 18618-000, Brasil.

Abstract: The most significant studies about the spinner dolphin (*Stenella longirostris*) in the Southwestern Atlantic Ocean were conducted in Fernando de Noronha Archipelago, off Northeastern Brazil. The continuity of these studies depends upon the development of non-invasive methods. In this work, we present results from the skin swabbing sampling procedure for this species. We tested the performance of this method for nuclear and mitochondrial DNA analysis, unknown for this population. A total of skin 161 samples were collected during two expeditions. After the contacts the most of the dolphins remained close to the boat. Microsatellites markers and cytochrome b region primers were evaluated and the respective fragments were successfully amplified. Thus, skin swabbing may be considered an efficient strategy to obtain tissue samples for spinner dolphin genetic analysis in Fernando de Noronha Archipelago.

Key words: Cytochrome b, Fernando de Noronha Archipelago, microsatellites, spinner dolphin.

Resumo. Um protocolo simples e pouco invasivo para acesso de DNA de *Stenella longirostris* (Cetacea: Delphinidae). Os principais estudos sobre golfinhos rotadores (*Stenella longirostris*) no Atlântico Sul têm sido desenvolvidos no Arquipélago de Fernando de Noronha, Brasil. A continuidade desses estudos requer a aplicação de métodos cada vez menos invasivos. Nesse trabalho apresentamos resultados obtidos a partir do procedimento de raspagem de pele para esta espécie. Nós testamos o desempenho deste método de coleta para análises nucleares e mitocondriais, ainda inéditos para essa população. Um total de 161 amostras de pele foi coletado durante duas expedições. A maioria dos golfinhos permaneceu próxima à embarcação após o contato. Marcadores microssatélites e *primers* da região do citocromo b foram avaliados e os respectivos fragmentos foram amplificados com sucesso. Assim, concluímos que o método de raspagem de pele pode ser considerado uma eficiente estratégia para obtenção de amostras de tecido para análises genéticas de golfinhos rotadores no Arquipélago de Fernando de Noronha.

Palavras-chave: Citocromo b, Fernando de Noronha, golfinho-rotador, microssatélites.

Since the onset of molecular studies, a myriad of different methods have been used to improve the acquisition of biological material in order to perform DNA analysis and protect animals' health and well being. For large cetaceans, efficient and non-invasive methods include the collection of sloughed skin (Whitehead *et al.* 1990, Amos *et al.* 1992, Valsecchi *et al.* 1998, Gendron & Mesnick

2001). A potentially alternative sampling method that does not require puncturing the skin, used with some species of cetaceans is the skin swabbing (Harlin *et al.* 1999, Gales *et al.* 2002). This procedure can be challenging for some species that tend not to approach boats (Bearzi 2001). The spinner dolphin *Stenella longirostris* lives in deep waters of tropical and subtropical seas

and is commonly observed close to banks or islands (Perrin 2002), approaching boats of different sizes (Norris *et al.* 1994). A large number of spinner dolphins are found in Fernando de Noronha, an Oceanic Archipelago off Northeastern Brazil. Their typical bow-riding behavior allows easy approximation (Silva Jr. *et al.* 2005a). However, no previous attempts for a DNA analysis using skin swabbing methods have been reported for the species. One important issue with this method is related to sample adequacy, function of the total skin amount collected. In this work we show the results of nuclear and mitochondrial DNA analysis performed on *Stenella longirostris* skin samples collected in Fernando de Noronha, Brazil. Considering the conservation status of the area and the lack of knowledge for the species in Brazilian waters, standardization of a less invasive method to spinner dolphin's genetic analyses is an important task.

Skin samples of 161 spinner dolphins were collected along Fernando de Noronha Archipelago, off Northeastern Brazil (Fig. 1), using a small inflatable boat. Two expeditions were conducted, in August 2004 and February 2006, during ten and six days, respectively. Contrasting meteorological and

oceanographic typical conditions characterize these sampling periods. Trade winds predominate in August, generating high amplitude waves. February is characterized as a dry period with calm waters. A moderately abrasive, 4 X 4-cm synthetic fiber scrub pad, made of synthetic fiber, attached with plastic fasteners to the tip of 130-cm long wooden sticks, was used to collect the skin samples, following the skin-swabbing method described by Harlin *et al.* (1999). Samples were removed by friction of the scrub pad against the back of an approaching dolphin. Skin samples were transferred to a flask containing 20% dimethylsulfoxide (DMSO) or 70% alcohol solution. Samples were ranked taking the amount of skin collected into account. Care was taken to avoid keeping the same individuals moving along with the boat, by frequently changing both the course and speed of the boat. In the laboratory, the skin adhered in the scrub pad was removed and DNA were extracted using the Chelex resin. The samples were quantified by spectrophotometer (Pharmacia Biotech GeneQuant). DNA amplification was performed using primers of cytochrome b and nuclear DNA through microsatellite markers (Table I).

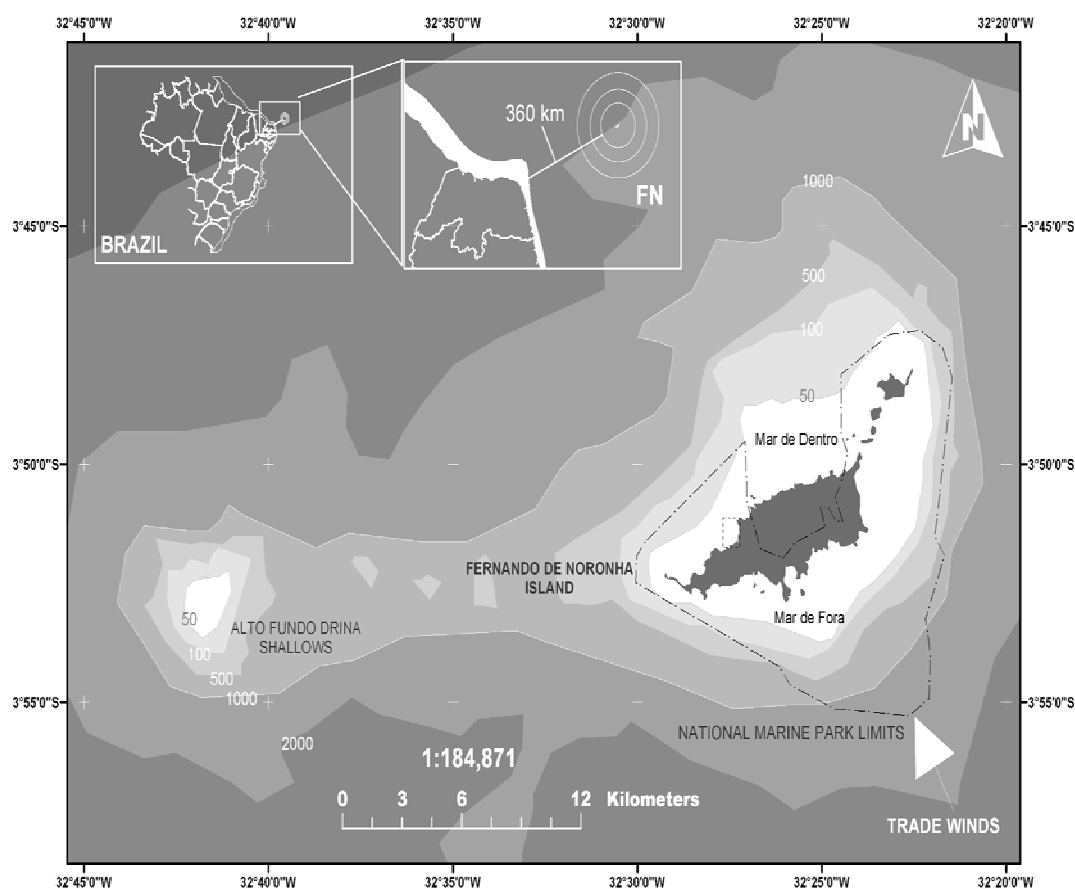


Figure 1. Map of the study area, the Fernando de Noronha Archipelago, showing the main island that bears the same name, the limits of the Fernando de Noronha National Marine Park and the “Mar de Dentro” portion of the waters surrounding the islands, where all data were collected.

Table I. PCR conditions in cytochrome b and microsatellite analysis.

	Cytochrome b	Microsatellites
Primers	GLUDG-L and CB2 (Palumbi 1996).	Slo 1, 2, 3, 4, 9, 11, 13, 14, 15, 16 and Slo17 (Farro 2006).
Reagents	25 ng of DNA, Buffer 10X, 1.5 mM MgCl ₂ (50 mM), 0.2 mM dNTP (10 mM), 1u of <i>Taq</i> DNA polymerase (Invitrogen) 0.4 μM of each primer (10 μM)	1 μL of DNA, Buffer 10X, 1.25 mM MgCl ₂ (50 mM), 0.83 mM dNTP (10 mM), 0.75 u of <i>Taq</i> polimerase (Invitrogen) 0.42 μM of each primer (10 μM)
Cycles	95°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 1 min and 72°C for 30 sec; the extension was at 72°C for 5 minutes.	95°C for 5 min, followed by 32 cycles at 94°C for 30 sec, annealing (t°C = X) for 20 sec and 72°C for 20 s, the extension was at 72°C for 10 minutes.
Gel	1% agarose	8 % polyacrilamide

In the cytochrome analysis a spinner dolphin's liver sample was used for comparison. After the PCR and the fragment's verification, other PCR reaction was performed with the Big Dye Terminator® Kit (Applied Biosystems). In this procedure 0.5 μL of the amplified product was mixed, 1.8 μL of *Big Dye*, v.3.01, 1 μL of *Save money* and 0.5 μL of each primer (GLUDG-L ou CB2, both for 10μM) for a total volume of 10μL. Amplification was realized in the following conditions: 96°C for 2 min and 25 cycles at 96°C for 45 sec, 52°C for 30 sec and at 60°C for 4 minutes. Sequencing was performed in an automatic sequencer ABI 3100 (Applied Biosystems). The obtained sequences were analyzed with the program Sequencher™, v.3.1 version. After that, a consensus sequence was generated and compared with other sequences deposited in GenBank. Besides GenBank comparison, the sequences were submitted to the site DNA Surveillance (<http://www.cebl.auckland.ac.nz:9000>). This site compares the submitted sequences with those obtained from other cetacean species, and informs the taxon that has the highest homology with them. The microsatellite fragments were visualized using a silver-staining protocol, under white light and the picture was taken with EagleSight. The sizes were determined with the program Kodak Digital Science 1D, 3.0.1 version.

In first season ninety-two contacts were made in 10 days, an average effort of four hours per day, and 87 percent of them were effective. Samples

were ranked according to the amount of skin tissue observed, yielding in the first cruise: 10 samples with no skin fragment (--), 39 samples containing small amounts, and 40 containing larger amounts of skin (++). Laboratorial analysis verified that only (+) and (++) samples provided enough DNA, thus, only samples ranked as (+) and (++) samples were stored during the second cruise, resulting in 109 contacts, with 33 (+) and 49 (++) samples. Amplifications with nuclear and mitochondrial markers were successfully accomplished. The alleles were adequately identified in microsatellite analyses (Fig. 2). With the fragments and the individual genotypes we verified that nine dolphins were probably sampled twice. The doubled samples, discharged after its detection, demonstrated that the exfoliating procedure did not promote a trauma to the animals, since they still performed bowriding behaviour after been sampled.

In cytochrome b test the resulted bands showed the expected sizes, around 460 bp for the next analysis step (Fig. 3). After sequencing and also submitting to the GENBANK and DNA SURVEILLANCE site and it was confirmed that these samples corresponded to *Stenella longirostris* species, as it was expected. In GenBank, the submitted sequences showed e-values of zero. The samples showed score of 823 to the accession AF084101 (gi = 5870054), the accession number is the identification of a GenBank sequence, and score of 831 to the accession X92524 (gi = 1199854), to liver and skin respectively.

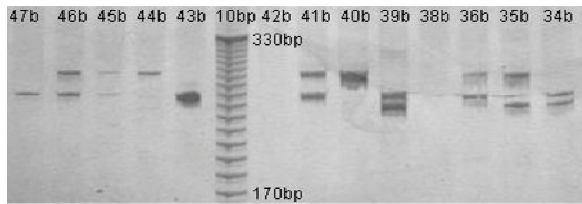


Figure 2. Microsatellite fragments amplified with the Slo15 marker from 13 spinner dolphin's skin samples collected in 2006. Column 6 = ladder 10bp.

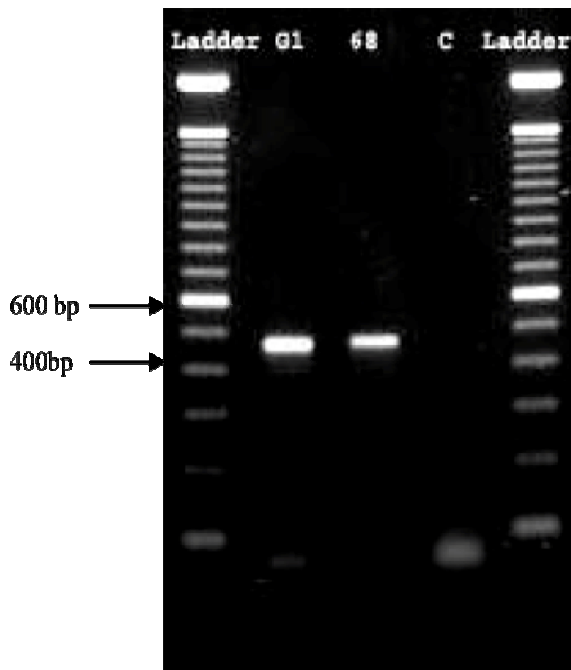


Figure 3. Fragments around 460 bp amplified in a spinner dolphin's muscle sample (G1) and skin sample (68: extracted by chelex) using cytochrome b primers. Agarose gel 1% stained with bromide ethidium. First and last columns are ladder 100bp. Control (C) = sample without DNA.

Harlin *et al.* (1999) described a skin swabbing sampling method for *Lagenorhynchus obscurus* from Kaikoura, New Zealand, and we reproduced here for *Stenella longirostris*. The spinner dolphins reacted as dusky dolphins (*L. obscurus*) to the scrub pad contact. They swam faster, jumping or diving after being touched but, in several occasions, they returned to the boat prow. This suggests that the skin swabbing procedure did not cause a trauma for the animals and it can be used to collect tissue samples for different types of genetic analysis. Despite the different oceanographic conditions during both collection periods, a similar sampling success was observed in both. The number of very good samples (++) was higher in the second expedition. We believe that this was due to the changes in the sampling procedure (particularly the increase in dowel size). Our results

confirm the efficiency of the proposed procedure for any time period in Fernando de Noronha area. The cytochrome b analysis showed that the samples had enough DNA for mitochondrial studies, assuring positive results, as reported in Harlin *et al.* (1999). In addition, we verified if the samples could be used in nuclear analysis. In microsatellite tests, both heterozygote and homozygote individuals were identified adequately. Therefore, the visible samples attached in the scrub were enough to perform nuclear as well as mitochondrial DNA analyses. For this reason, the skin sampling method can be considered a reliable tool in genetic studies with *Stenella longirostris*.

Acknowledgements

We gratefully thank the professionals of BIOGEN laboratory for their help, G. A. Braga da Rosa, Instituto Estadual de Meio Ambiente e Recursos Hídricos, and A. M. Ciotti, Universidade Estadual Paulista, for the comments on the manuscript. We also thank the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) for the collection and manipulation licenses. CAPES and PETROBRAS provided financial support.

References

- Amos, W., Whitehead, H., Ferrari, M. J., Glockner-Ferrari, M. D., Payne, R. & Gordon, J. 1992. Restrictable DNA from sloughed cetacean skin – It's potential for use in population analysis. **Marine Mammal Science**, 8(3): 275-283.
- Bearzi, G. 2001. Biopsy sampling and intrusive research. <http://www.tethys.org/biopsy.htm>. Last access in 4/17/2007.
- Gales, N. J., Dalebout, M. L. & Bannister, J. L. 2002. Genetic identification and biological observation of two free-swimming beaked whales: Hector's beaked whale (*Mesoplodon hectori*, Gray, 1871), and Gray's beaked whale (*Mesoplodon grayi*) Von Haast, 1876. **Marine Mammal Science**, 18: 544-551.
- Gendron, D. & Mesnick, S. L. 2001. Sloughed skin: a method for the systematic collection of tissue samples from Baja California blue whales. **Journal of Cetacean Research and Management**, 3(1): 77-79.
- Harlin, A. D., Würsig, B., Baker, C. S. & Markowitz, T. M. 1999. Skin swabbing for genetic analysis: application to dusky dolphins (*Lagenorhynchus obscurus*). **Marine Mammal Science**, 15: 409-425.

- Norris, K. S., Würsig, B., Wells, R. S. & Würsig, M. 1994. **The Hawaiian Spinner Dolphin**. University of California Press, Berkeley and Los Angeles, 408 p.
- Perrin, W. F. 2002. Spinner dolphin. Pp. 1174-1178. *In*: Perrin W. F., Würsig B., Thewissen J. G. M. (Eds). **Encyclopedia of Marine Mammals**. Academic Press, San Diego.
- Silva Jr., J. M., Silva, F. J. L. & Sazima, I. 2005a. Rest, nurture, sex, release, and play: diurnal underwater behaviour of the spinner dolphin at Fernando de Noronha Archipelago, SW Atlantic. **Aqua**, 9(4): 161-176.
- Valsecchi, E., Glockner-Ferrari, D., Ferrari, M. & Amos, W. 1998. Molecular analysis of the efficiency of sloughed skin sampling in whale population genetics. **Molecular Ecology**, 17(10): 1419-1422.
- Whitehead, H., Gordon, J., Mathews, E. A. & Richard, K. R. 1990. Obtaining skin samples from living sperm whales. **Marine Mammal Science**, 6(4): 316-326.

Received June 2007

Accepted June 2008

Published online July 2008