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

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**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 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.

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.

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<tr>
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<th>Cytochrome b</th>
<th>Microsatellites</th>
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<tr>
<td><strong>Primers</strong></td>
<td>GLUDG-L and CB2 (Palumbi 1996).</td>
<td>Slo 1, 2, 3, 4, 9, 11, 13, 14, 15, 16 and Slo17 (Farro 2006).</td>
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<td><strong>Reagents</strong></td>
<td>25 ng of DNA, Buffer 10X,</td>
<td>1 µL of DNA, Buffer 10X,</td>
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<td></td>
<td>1.5 mM MgCl₂ (50 mM),</td>
<td>1.25 mM MgCl₂ (50 mM),</td>
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<td>0.2 mM dNTP (10 mM),</td>
<td>0.83 mM dNTP (10 mM),</td>
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<td></td>
<td>1 µL of <em>Taq</em> DNApolymerase (Invitrogen)</td>
<td>0.75 µL of <em>Taq</em> polimerase (Invitrogen)</td>
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<td></td>
<td>0.4 µM of each primer (10 µM)</td>
<td>0.42 µM of each primer (10 µM)</td>
</tr>
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<td><strong>Cycles</strong></td>
<td>95°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 60°C</td>
<td>95°C for 5 min, followed by 32 cycles at 94°C for 30 sec and 72°C for 20 s, the extension was at 72°C for 10 minutes.</td>
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<td>for 1 min and 72°C for 30 sec; the extension was at 72°C for 5 minutes.</td>
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</tr>
<tr>
<td><strong>Gel</strong></td>
<td>1% agarose</td>
<td>8 % polyacrilamide</td>
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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 Byosistems). 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 Byosystems). The obtained sequences were analyzed with the program SequencherTM, 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.
A simple protocol for a low invasive DNA accessing. 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*.

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