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Short Communication

Molecular sexing of tucuxi dolphins (*Sotalia guianensis* and *Sotalia fluviatilis*) using samples from biopsy darting and decomposed carcasses

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Abstract

We tested the zinc-finger sex chromosome-linked genes *Zfx/Zfy* and the sex-determining region Y (*Sry*) genes for gender determination of biopsy samples from marine and riverine tucuxi dolphins (*Sotalia guianensis* and *S. fluviatilis*). We also evaluated the performance of these genes with decomposed carcasses, for which sexing cannot rely on the direct examination of the reproductive tract. Both systems proved reliable for sexing 46 fresh and decomposed samples, making them especially useful when biopsy darting is coupled with photo-identification studies.

Key words: cetaceans, remote sampling, sex determination, *Sry*, *Zfx/Zfy*.

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Few cetaceans exhibit easily observable sexual dimorphism. For most species, sex determination relies on the observation of the ventral region, which is a rare event *in situ*. The impracticality of morphological sex determination hinders a thorough understanding of important biological aspects. Molecular sexing can close this gap and can be coupled with low impact sampling such as biopsy darting, which has proved to be safe and minimally invasive for free-ranging cetaceans (IWC, 1991).

Two techniques, based on the polymerase chain reaction (PCR) amplification of the sex-determining region Y (*Sry*) and the sex-specific regions of the zinc-finger sex chromosome-linked *Zfx* and *Zfy* genes, have proved to be fast, cheap and reliable for sex determination (Palsbøll *et al.*, 1992; Bérubé and Palsbøll, 1996). Moreover, PCR techniques work well with degraded DNA, which is typical of samples from stranded carcasses, a major source of cetacean samples.

In this study we tested both techniques for the first time in tucuxi dolphins (genus *Sotalia*). This genus was considered monotypic until morphological and genetic studies demonstrated that the riverine (*Sotalia fluviatilis*) and the marine (*Sotalia guianensis*) dolphins were distinct species (Monteiro-Filho *et al.*, 2002; Cunha *et al.*, 2005).

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Many tucuxi populations are under human threat, but they are still considered "data deficient" by the International Union for the Conservation of Nature (2006) and the Brazilian Environmental Agency (Instituto Brasileiro de Meio Ambiente e Recursos Naturais Renováveis - IBAMA) (IBAMA, 2001).

We analyzed 36 *S. guianensis* samples of skin, muscle or liver, collected from stranded carcasses, by-caught specimens or through biopsy darting along the Brazilian coast (1°20' S and 25°31' S) as well as 10 *S. fluviatilis* skin biopsy samples obtained during a capture procedure in the Brazilian state of Amazonas (2°57' S, 64°48' W), during which the dolphins were sexed by examination of their genital openings. The condition of the samples was graded according to the codes proposed by Geraci and Lounsbury (1993), with all biopsy samples qualifying as Code 1, while the condition of the remaining samples varied from Code 2 (fresh) to 4 (poor). We also analyzed six *S. guianensis* sun-dried genitals obtained from the Ver-o-Peso market, in the Brazilian State of Pará, which were assessed as Code 5 (mummified).

Total genomic DNA of all samples was extracted by the phenol-chloroform procedure of Sambrook *et al.* (1989). We initially optimized and validated the *Zfx/Zfy* and *Sry* sexing techniques in 10 specimens each of *S. guianensis* and *S. fluviatilis* whose gender was known. The nuclear DNA *Zfx/Zfy* and *Sry* regions were PCR-amplified in separate reactions using the ZFXY582, ZFX923 and ZFY767 primers (Bérubé and Palsbøll 1996) and the

SRY593 and SRY764 primers (Palsbøll *et al.* 1992), and the PCR products mixed before electrophoresis in 2% (w/v) agarose gels. Two positive controls were used for sexing the dolphins with the *Sry* system: (1) PCR amplification of the mitochondrial control region, as described by Palsbøll *et al.* (1992), using the primers Dlp 1.5 (modified from Pichler *et al.*, 1998) and H00034 (Rosel *et al.*, 1994); (2) amplification of the *Zfx* or *Zfy* band in the sample. Negative controls were included in all experiments. Amplifications were carried out in 20 µL reactions containing 0.20 mM dNTPs, 2 mM $MgCl_2$, 0.5 µM of each primer and 1 unit of *Taq* polymerase (Amersham Pharmacia). The PCR cycling conditions were 3 min at 94 °C, followed by 38 cycles of 1 min at 92 °C, 1 min at 50 °C and 1 min at 72 °C; plus a 5 min extension at 72 °C.

Nucleotide sequences of the *Zfx* and *Zfy* regions of three *S. fluviatilis* and seven *S. guianensis* males were deposited in the GenBank under accession numbers DQ409819 and DQ409820.

Our results show that the *Zfx/Zfy* and *Sry* regions can be reliably used for sexing both *Sotalia* species, reproducing exactly the expected patterns (Figure 1) and unambiguously identifying the sex of all dolphins tested in the control runs.

All *S. fluviatilis* and 67% of *S. guianensis* male samples produced two bands of about 380 bp and 230 bp during the amplification of the *Zfx/Zfy* regions, corresponding, respectively, to the fragments present in the X and Y chromosomes, and a single 170 bp band in the amplification of the *Sry* region (Figure 1). In the two remaining *S. guianensis* male samples, the *Zfx* bands were not observed, but the presence of the *Zfy* and *Sry* fragments allowed unambiguous sex determination. The female expected pattern, a single 380 bp *Zfx* band and no *Zfy* or *Sry* product, was observed in all female samples used in the test phase. All samples successfully amplified the mitochondrial control region.

Once the technique was proven valid and reliable for both *Sotalia* species, we analyzed samples from 26 *S. guianensis* dolphins of unknown gender. The biopsies and the Ver-o-Peso samples were easily sexed, revealing six males and three females. Of the 17 carcass samples, 13 (76%) could be directly sexed (six males and seven females), and four samples amplified the mtDNA but not the sex genes. We conducted two series of experiments to verify whether PCR failure was due to a low number of single-copy DNA molecules of adequate size for amplification, or the presence of PCR inhibitors. To verify if PCR failure was due to DNA degradation, we performed serial dilutions of the DNA template of a male and a female positive control. The PCR amplifications using template dilutions of up to 1:10000 were successful for the mitochondrial control region, the *Zfy* and the *Sry* genes, whereas the *Zfx* band could not be amplified in template dilutions greater than 1:200 (Table 1).

The inhibition hypothesis was initially investigated using a tenfold dilution of template DNA so that any contaminants would become too diluted to inhibit the PCR. No PCR bands were observed using any of the diluted templates. Next, we mixed 1 µL of the DNA from a male that amplified the *Zfx* and *Zfy* genes with 9 µL of each of the four samples that failed to amplify. A 1:10 dilution of the DNA of the positive male in DNA from another positive male and in water were used as positive controls. Although

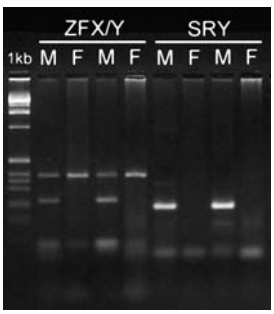

Figure 1 - Sex determination patterns for male (M) and female (F) *Sotalia* samples, using the *Zfx/Zfy* and *Sry* primer systems.

Table 1 - Results of dilution and inhibition experiments. Key: A, B, C and D, problematic samples; F, positive female; M, positive male.

Treatments	*Zfx*	*Zfy*	*Sry*	mtDNA
Dilution experiment				
M + water (1:100)	+	+	+	+
M + water (1:500)	-	+	+	+
M + water (1:1,000)	-	+	+	+
M + water (1:5,000)	-	+	+	+
M + water (1:6,000)	-	+	+	+
M + water (1:10,000)	-	+	+	+
M + water (1:20,000)	-	-	-	-
F + water (1:100)	+			+
F + water (1:200)	+			+
F + water (1:400)	-			+
F + water (1:1,000)	-			+
F + water (1:10,000)	-			+
Inhibition experiment				
M + *M* (1:10)	+	+	+	+
M + *F* (1:10)	-	+	+	+
M + water (1:10)	-	+	+	+
M + A*	-	+	+	+
M + B*	-	+	+	+
M + C*	-	+	+	+
M + D*	-	+	+	+
A, B, C or D + water (1:10)	-	-	-	+

*Each tested with three different dilutions: 0.5 µL of M diluted in i) 4.5 µL problematic sample; ii) 2.3 µL problematic sample + 2.3 µL water; and iii) 1 µL problematic sample + 3 µL water. All dilutions gave the same result.

only the positive male/positive male treatment amplified the *Zfx* band, the *Zfy*, *Sry* and control region bands were observed in all other treatments (Table 1). Therefore, we conclude that the lack of amplification in those four samples was DNA degradation. Hence, the most parsimonious explanation is that the four samples that failed to amplify the sex linked markers were females, since *Zfx* amplification was much more sensitive to template concentration than both the mtDNA and the Y-specific genes. This indicates that the mtDNA control region can be consistently used as a positive control for the *Sry* system in *Sotalia* species. It is advisable, however, that a dilution experiment be conducted prior to choosing this marker as a positive control in other species.

The *Zfx/Zfy* and *Sry* systems have been used by a large number of workers carrying out research on cetacean species, *e.g.*, *Sry* by Rosenbaum *et al.* (2002), *Zfx+Sry* by Harlin *et al.* (2003), and Möller and Beheregaray (2004), *Zfy+Sry* by Gowans *et al.* (2000), and *Zfx+Zfy* by Pichler *et al.* (1998) and Escorza-Treviño and Dizon (2000). Their applicability across the taxonomic spectrum is suggested by the high evolutionary conservativeness of *Zfx/Zfy* (Morin *et al.*, 2005). Indeed, all *Sotalia* sequences were identical and 99.4% similar to those of *Phocoena phocoena*, which belongs to a different Family.

In summary, the technique described in this paper is fast, reliable and works on most degraded samples, including sun-dried tissues. This is particularly useful since decaying carcasses are a major source of material for *Sotalia* and other cetaceans, especially in tropical regions. Undoubtedly, molecular sexing will offer new insights into *Sotalia* social structure and improve conservation and management decisions.

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