Determination of gender in cetaceans by the polymerase chain reaction

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We determined the gender of a variety of cetacean species, including both ondotocetes and mysticetes, using the polymerase chain reaction for amplification of the sex chromosome specific regions ZFY/ZFX and SRY. This quick and simple method requires extremely small amounts of tissue, and therefore allows gender to be determined from skin biopsies taken from free-ranging specimens. In the fin whale, *Balaenoptera physalus*, no gender-specific bands were observed when the ZFY/ZFX system was used, but when the SRY system was used, sex was accurately determined. Previous studies in other mammals have also shown the SRY system to be more reliable in sex determination. We therefore recommend amplification of the SRY region alone or in parallel with the ZFY/ZFX regions, as described here, as a test for gender in cetaceans and other mammals.

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Nous avons déterminé le sexe chez un bon nombre d'espèces de cétacés en utilisant la réaction de la chaîne des polymérases pour mettre en évidence les régions spécifiques ZFY/ZFX et SRY des chromosomes sexuels. Les espèces examinées appartenaient aux odontocètes aussi bien qu'aux mysticètes. Cette méthode rapide et simple ne requiert que des quantités minimes de tissus et permet donc la détermination du sexe à partir de biopsies de la peau prélevées chez des individus libres. Chez le Rorqual commun, *Balaenoptera physalus*, aucune bande spécifique au sexe n'a été observée à l'utilisation du système ZFY/ZFX, mais le système SRY a permis de déterminer le sexe avec précision. Des études antérieures d'autres mammifères avaient déjà démontré que le système SRY était plus exact. Nous recommandons donc l'amplification de la région SRY, ou son utilisation conjointe avec l'amplification des régions ZFY/ZFX, comme méthode de détermination du sexe chez les cétacés et chez d'autres mammifères.

[Traduit par la rédaction]

Introduction

Determining the gender of living wild cetaceans has always been difficult or impossible, and this has frequently hampered investigations of their biology and behavior. Sex determination based on photography of sexually dimorphic characters is possible for some species such as humpback whales, *Megaptera novaeangliae* (Glockner 1983), sperm whales, *Physeter macrocephalus* (Kasuya and Ohsumi 1966; Gordon 1987), and killer whales, *Orcinus orca* (Bigg *et al.* 1987). However, these techniques are not always dependable and often cannot be used for all animals in different situations. Previous studies have demonstrated that the gender of cetaceans can be determined from tissue samples, either by direct karyotyping of skin biopsies (Winn *et al.* 1973) or of cell cultures established from such biopsies (Lambertsen *et al.* 1988). In the last few years, new techniques based upon sex-specific DNA sequences have been used in gender determination. Two such systems have principally been used: ZFY and ZFX, which are similar DNA sequences located on the human Y and X chromosomes, respectively (Page *et al.* 1987; Schenider-Gädicke *et al.* 1989), and the SRY sequence, which is specific for the Y chromosome in mammals (Sinclair *et al.* 1990). Recently, Baker *et al.* (1991) demonstrated that humpback and fin whales display the same sex-specific pattern of restriction-fragment lengths as humans when hybridized with ZFY. The same seems to apply to other species of cetaceans (L. W. Anderson and G. Gradl, unpublished data).

In this study we utilized the polymerase chain reaction (PCR; Saiki *et al.* 1988) to analyze the ZFY/ZFX and SRY regions in six cetacean species, including both odontocetes (toothed whales) and mysticetes (baleen whales), from three taxonomic families. Our results suggest that the target regions are conserved in all cetaceans, and therefore that the technique has broad applicability. While this study involved cetaceans only, it seems likely that the method could be used for other mammalian species.

Materials and methods

Samples

The samples used in this study were selected from a variety of sources, including stranded animals, carcasses from aboriginal kills and commercial operations, and skin biopsies of living animals (the latter were collected as described in Palsbøll *et al.* 1991). Information about species and number of individuals tested, together with the sex of the tested individuals as determined by nonmolecular methods, is given in Table 1.

DNA extraction and amplification

DNA was extracted using standard protocols with cell lysis in 1.0% sodioum dodecyl sulfate (SDS), 0.15 M sodium chloride, 10 mM Tris–HCl (pH 8.0), and 1 mM ethylenediamine tetraacetic acid (EDTA), and digestion with proteinase K (100 μg/mL) at 65°C for 3 h, followed by phenol–chloroform extractions and precipitation with ethanol (Maniatis *et al.* 1982).

The ZFY/ZFX and SRY DNA regions were amplified by PCR (Saiki *et al.* 1988). Approximately 10 ng of total DNA was amplified in 20 μl reaction volume (67 mM Tris–HCl, pH 8.8, 2 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 200 μM dNTP, 0.5 μM of each primer, and 0.4 units of Taq™ DNA polymerase. Commercially available PCR kits also work well. The oligonucleotide primers and reaction conditions used are given in Table 2.

TABLE 1. Numbers and sources of specimens used in this study and the number of each species for which gender determination had previously been made by nonmolecular methods

	Location	Number	Source	Number of species for which sex was known[a] Males	Females
Balaenoptera acutorostrata	West Greenland	6	Whaling	3	3
B. musculus	West Greenland	4	Skin biopsies	—	—
	Eastern Canada	5	Skin biopsies	—	—
B. physalus	West Greenland	11	Skin biopsies	4	3
	Eastern Canada	35	Skin biopsies	—	—
Megaptera novaeangliae	Dominican Republic	170	Skin biopsies	4	3
	West Greenland	18	Skin biopsies	—	2
	Eastern Canada	24	Skin biopsies	1	2
	Gulf of Maine	14	Sloughed skin	4	3
Delphinapterus leucas	West Greenland	31	Aboriginal catch	5	6
P. phocoena	Denmark	17	Stranding	3	3

[a]Gender was determined by physical examination or photographs of the genital region (humpbacks only), or (in some females) on the basis of close association with a calf.

TABLE 2. Sequences of the oligonucleotides used in the polymerase chain reaction and amplification parameters

Designation	Nucleotide sequence	Annealing temp. (°C)	Extension time (min)
ZFY/X system[a]			
ZFY1204	5′CAT TAT GTG CTG GTT CTT TTC TG 3′	60	4
ZFY0097	5′CAT CCT TTG ACT GTC TAT CCT TG 3′	60	4
SRY system[b]			
SRY593	5′AAG CGA CCC ATG AAC GCA TT 3′	55	1
SRY764	5′GTA TTT CTC TCT GTG CAT GG 3′	55	1

NOTE: The numbers in the primer codes designate the position of the 3′ end of the primer in the original published sequence. All amplifications were initiated by a 2-min denaturation step at 94°C, followed by 35 cycles with denaturing at 94°C for 1 min and annealing for 1 min, and an extension temperature of 72°C.

[a]From Schneider-Gädicke *et al.* 1989.

[b]Sinclair *et al.* 1990.

Analysis of the amplification products

The ZFY/ZFX system

Ten microlitres of the PCR product was digested with 3 units of *Taq*I restriction endonuclease (New England BioLabs Inc.) for 1 h at 60°C, following the manufacturer's instructions. The restriction fragments were separated and visualized by agarose gel electrophoresis in 4% NuSieve™ agarose (with 0.5 μg/mL ethidium bromide) at 200 V in 1 × TBE (0.089 M Tris–HCl, 0.089 M boric acid, and 1 mM EDTA, pH 8.3).

The SRY system

The amplification products were visualized by agarose gel electrophoresis as described for the ZFY/ZFX system. A positive control should be included in the amplification process. Here a part of the mitochondrial cytochrome *b* sequence was co-amplified.

Results

The ZFY/ZFX system

The results of the restriction-enzyme analysis of the ZFY/ZFX amplification product from the human and minke whale are shown in Fig. 1. The restriction-fragment patterns observed for the amplified ZFY/ZFX sequences from humans are as predicted from the published sequences (Schneider-Gädicke *et al.* 1989). The restriction-fragment patterns observed for the corresponding sequences from cetaceans are similarly sex-specific, but an extra *Taq*I recognition site is observed in both the ZFY and ZFX sequences.

All six species included in this study display the same gender-specific restriction-fragment pattern except the fin whale. In this species, both sexes have the 'male' cetacean pattern, owing to the loss of a *Taq*I site in the ZFX sequence. Figure 1B is a map of the approximate locations of the *Taq*I recognition sites and fragment sizes.

The ZFX/ZFY amplification products of fin whales of known gender were digested with the restriction endonucleases *Alu*I and *Msp*I, which in humans give sex-specific restriction-fragment patterns. Neither of these gave sex-specific patterns in the fin whale.

The SRY system

Amplification of the SRY region resulted in a male-specific fragment of approximately 170 base pairs (Fig. 1A). This fragment was not observed in any of the tested females. In all cases where tests were performed on specimens whose sex was already known (see Table 1), the results were in agreement with the known gender.

Discussion

Determination of gender on the basis of small tissue samples enables free-ranging whales and dolphins to be sexed from skin biopsies. These samples can be collected in a directed and controlled manner. Other methods of gender determination, such as photography and visualization, are highly opportunis-

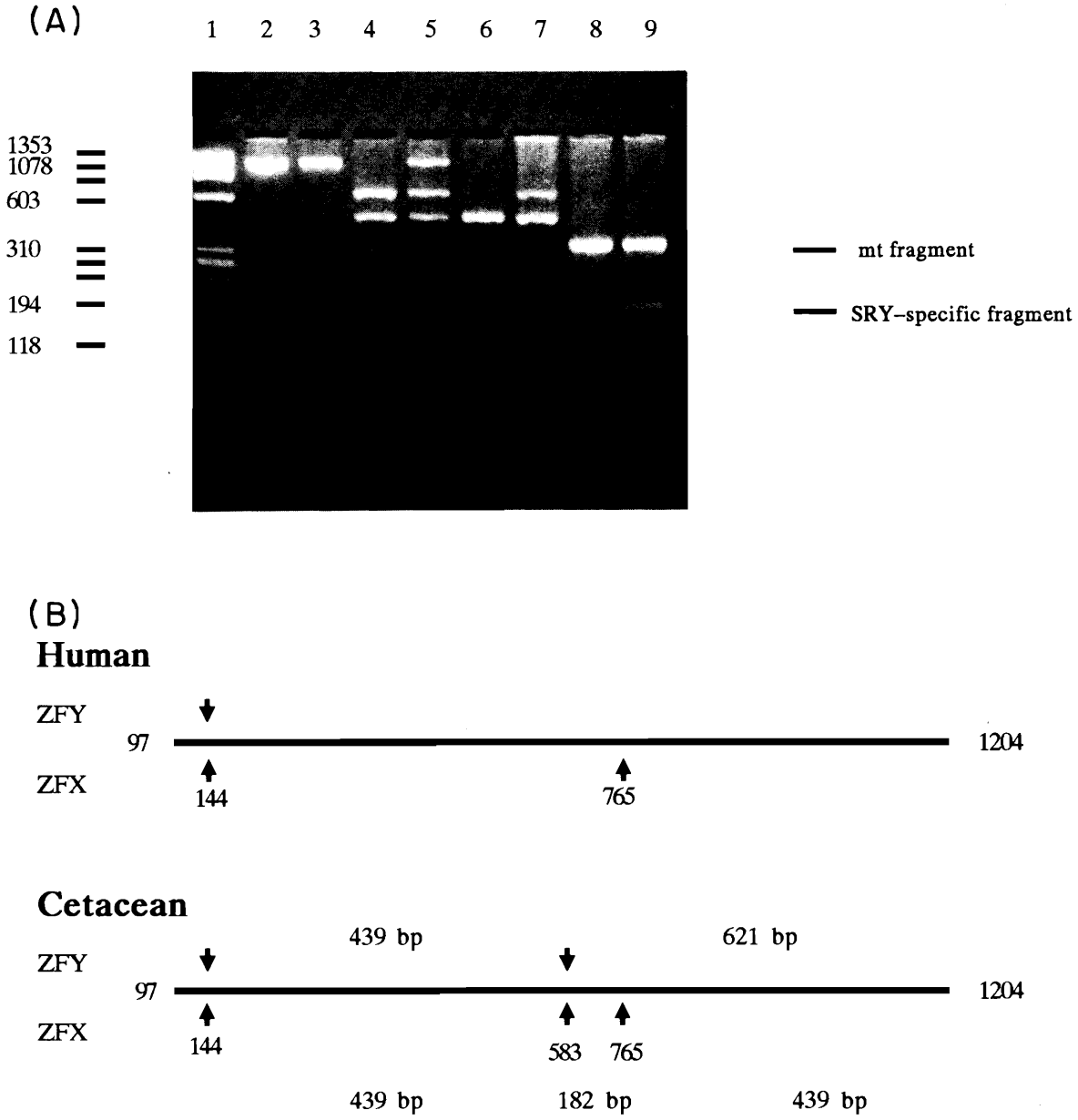

FIG. 1. (A) Amplification products and *Taq*I restriction-fragment patterns. Lane 1: ΦX174 double-stranded DNA digested with Hae III. Sizes of bands are given in base pairs. Lanes 2 and 3: undigested DNA o the human and cetacean (minke whale) after amplification with the ZFY/ZFX primers. Lanes 4 and 5: ZFY/ZFX amplified human female and male DNA, respectively, after digestion with *Taq*I. Lanes 6 and 7: XFY/XFX amplified minke whale female and male DNA after digestion with *Taq*I. Lanes 8 and 9: SRY amplified minke whale female and male DNA. The fragment of approximately 308 base pairs is the co-amplified mitochondrial sequence used as the positive control. All species included in this study display exactly the same restriction-fragment pattern (ZFY/ZFX system) or amplification-product pattern (SRY system). (B) *Taq*I restriction sites in ZFY/ZFX sequences. The arrows and numbers indicate the positions of *Taq*I restrictions sites in the ZFY and ZFX sequences of humans (Schneider-Gädicke *et al.* 1989), and their possible corresponding sites in cetaceans. The positions of the sites are based on the observed fragment lengths (see Fig. 1A) and are therefore very approximate. The fragment lengths are listed in base pairs (bp). Please note that the fin whale only displays the male cetacean restriction-fragment pattern (2FY/2FX system) (see text).

tic. In most cetacean species, determination of gender from skin biopsies is the only practical method available, since no dimorphic characters have been identified.

We have presented a technique that enables the gender of cetaceans to be quickly and reliably determined from minute amounts of tissue. As noted above, the methods are applicable to a broad variety, if not all, cetacean species, including baleen as well as toothed whales. Analyses of specimens of known gender, as well as a large number of specimens of one species (the humpback whale, see Table 1), have demonstrated the accuracy and consistency of the technique.

Baker *et al.* (1991) were able to demonstrate the determination of gender in a whale by Southern blotting (Southern 1975) using human ZFY (Page *et al.* 1987) as a probe. The technique presented here offers several advantages over the blotting technique. Amplification by PCR is technically simple and rapid. The whole process, from extraction of DNA to visualization of amplification products, taken approximately 4–9 h depending on the thermocycle system used, as opposed to several days. There is no use of radionucleotides, making the operation cheaper and less hazardous.

The practicality of this technique will simplify the analysis

of large samples as the practice of collecting skin biopsies from cetaceans becomes more common. Proposals for large-scale sampling (Beard *et al.* 1990) have been put forward. The limited quantity of tissue in a skin biopsy makes it crucial to limit the amount of tissue required for each analysis, thereby increasing the number of analyses.

In our study we applied PCR to amplify the DNA regions ZFY/ZFX and SRY for sex determination. The ZFY/ZFX system has the advantage that amplification gives rise to a product in both sexes, unlike the SRY system, where no product is detected for females. This lack of PCR product could be caused either by failure of the amplification or by the specimen not being from a female. This problem can be solved by adding another set of primers with an autosomal or mitochondrial target sequence, resulting in a second amplification product (see Fig. 1A), which thereby acts as a control for the amplification process. In the present study we co-amplified a mitochondrial sequence. Owing to the many mitochondrial genomes per cell as opposed to one SRY gene, there is a risk that lack of amplification of the SRY sequence is due to competition between the two sequences. Now we co-amplify the first intron in the lactalbumin gene, which is a single-copy sequence, thereby preventing the internal control sequence from outcompeting the amplification of SRY sequence. Alternatively, internal control can be achieved by lowering the stringency of the amplification process. Lowering the annealing temperature to 50°C gave a number of unspecific PCR products in the samples (data not shown). These were different from the specific male SRY amplification product.

The exponential nature of the amplification process makes it a very efficient technique, using as little as a single target DNA molecule as substrate (Jeffreys *et al.* 1988). Use of minimal amounts of DNA from very small samples, though, increases the possibility of contamination. The use of ample controls, as blind extractions and blind amplifications, should provide the necessary controls to detect contamination.

In the ZFY/ZFX system, sex is determined by restriction-pattern analysis. This is superfluous in the SRY system, in which only PCR amplification is needed. Also, it can be difficult to distinguish between the sexes by restriction-fragment pattern analysis, because the ZFY and ZFX sequences have a high degree of similarity. This is exemplified by the fin whale, in which no sex-specific pattern was found. This can be over-come by sequencing a male fin whale ZFY/ZFX sequence, which most likely will reveal sequence differences that can produce sex-specific restriction-fragment patterns. Studies of other mammals have also shown this to be a problem (Aasen and Medrano 1990). In marsupials ZFY homologous sequences have been mapped to the autosomes instead of the sex chromosomes (Sinclair *et al.* 1988). This is one of the strong arguments against ZFY being the testis-determining factor (TDF) (see review by Erickson and Verga 1989). Currently SRY seems to be the strongest candidate for being the TDF. It has recently been shown that the murine equivalent of SRY is sufficient to induce male development when introduced into chromosomally female embryos (Koopman *et al.* 1991). For this reason, SRY seems to be the most logical system to use for gender determination, since it provides the correct gender for all species tested so far. Alternatively, parallel use of both the SRY and the ZFY/ZFX system for determining the gender of rare species, when no stranded specimens of known gender are available as controls, would mutually validate the gender assigned by each of the two systems, provided the results correspond.

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