PCR-based sex determination of cetaceans and dugong from the Indian seas

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A sex-determination technique based on PCR amplification of genomic DNA extracted from the skin tissue has been standardized in cetaceans and dugong sampled from the Indian seas. A Y-chromosome-specific region (*SRY* or Sex-determining Y-chromosome gene) of 210–224 bp size in the genome has been amplified (only in males) using specific PCR primers. A fragment of the *ZFX/ZFY* (zinc finger protein genes located both on the X and Y chromosomes respectively) region in the size range 442–445 bp is also amplified (in both sexes) using another pair of primers simultaneously as positive controls for confirmation of sex. Molecular sexing was standardized in spinner dolphin (*Stenella longirostris*), bridled dolphin (*Stenella attenuata*), bottlenose dolphin (*Tursiops aduncus*), Indo-Pacific humpbacked dolphin (*Sousa chinensis*), Risso's dolphin (*Grampus griseus*), finless porpoise (*Neophocaena phocaenoides*), sperm whale (*Physeter macrocephalus*), blue whale (*Balaenoptera musculus*), Bryde's whale (*Balaenoptera edeni*) and dugong (*Dugong dugon*), which are all vulnerable/endangered species protected under the Indian Wildlife Act.

Keywords: Conservation, marine mammals, PCR-based sex determination, vulnerable/endangered species.

SEX identification is of fundamental importance in studies on population structure, social organization, distribution, behaviour and heavy metal accumulation in marine mammals[1]. However, distinguishing the males and females among these animals is difficult due to the poor sexual dimorphism, especially during their free-ranging state[2]. Direct anatomical evidence of an individual's sex comes only from a full-ventral inspection of its genital region and such opportunities are limited during field observations; unless the animal rolls and remains inverted at the surface[3], its genital region is visible only to an underwater observer, and that too only at a close range[4]. Non-molecular methods of sex identification have proved difficult and often unreliable. By examining the carcass remains of stranded/beach-cast cetaceans, which are often at decomposition levels, accurate assessment of reproductive organs is not possible. Though the individual may be inferred to be a female if it is observed near a calf[5,6], such inferences are troublesome for some species like the sperm whale, in which 'babysitting' males are possible[7].

Karyological identification of sex, though successful in some species of marine mammals[8,9], requires obtaining viable fibroblast tissue for cell culture, restricting its usefulness under many field conditions. The early molecular sexing techniques applied to cetaceans required Southern hybridization using probe derived from the human Y-chromosome[10] and amplification and then restriction digests of a fairly long fragment of the *ZFX/ZFY* genes[11]. Other techniques based on PCR amplification of sex-specific DNA fragments used genes *Amg*[12], *ZFX/ZFY*[2,13–16] and *Sry*[17].

In the present study, Y-chromosome-specific *SRY* locus was amplified simultaneously with the homologous *ZFX/ZFY* genes on the X-chromosome of females (*ZFX*) and XY chromosomes of males (*ZFX/ZFY*) as positive control, for the absolute confirmation of sex. Females lack a Y-chromosome and the test is based on the absence of a *SRY* product in females. This method was successful in identifying the sex of several mammalian groups[13,18]. One set of primers amplifies a fragment from the *ZFX/ZFY* genes in males and females[13]. The other set amplifies a much shorter fragment from the *SRY* gene in males only. Because the test with the *SRY* primers is based on the absence of the shorter *SRY* product in females, the *ZFX/ZFY* primers act as internal positive control to show that the lack of the former is not due an amplification failure and demonstrate the presence of amplifiable DNA in the PCR reaction.

Skin samples of the animals were obtained from either incidental fishery kills or from stranding (Table 1), and were stored in 70% ethanol for subsequent analysis. Total genomic DNA was extracted using the standard phenol–chloroform method[19], with slight modifications. Briefly, about 25 mg of skin tissue was extracted with a buffer (0.1M NaCl, 10 mM Tris HCL and 1 mM EDTA) and 10% SDS and digested by proteinase K (10 mg/ml) at 65°C for 3 h followed by phenol, chloroform : isoamyl alcohol extractions and alcohol precipitation. The genomic DNA was amplified by a duplex PCR reaction using two sets of primers to detect sex-specific markers. The technique used in the present study was adapted from Gilson *et al.*[20], with some modifications. The annealing temperature of 60°C proposed in the original method was reduced to 58°C. Each primer concentration was 60 pmol in the original method, while it was 25 pmol each in the present study.

The amplifications were performed under the following conditions: A reaction volume of 20 µl containing 10–100 ng of extracted genomic DNA template, 10 mM of Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 240 µM each of dATP, dTTP, dCTP and dGTP, 1 U of HotStart *Taq* DNA polymerase and 25 pmol each of forward and reverse primers for both sets. The primer sequences for *SRY*[20] were F 5′ CCC ATG AAC GCA TTC ATT GTG TGG 3′ and R 5′ ATT TTA GCC TTC CGA

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Table 1. Particulars of gender identification carried out by external examination and PCR method in cetaceans and dugong during the present study

Species	Place of sample collection	Sample code assigned	Identified sex[1]	
			By E.I[2]	By PCR
Tursiops aduncus	Vizhinjam	Viz1	Female	Female
T. aduncus	Kakinada	VRC/Dol/01	Female	–
T. aduncus	Chennai	CHO4	Male	Male
T. aduncus	Chennai	CHO8	Male	Male
Stenella longirostris	Kakinada	VRC/Dol/05	Male	Male
S. longirostris	Kakinada	VRC/Dol/04	Female	–
S. longirostris	Kakinada	VRC/Dol/07	Female	–
S. longirostris	Kakinada	VRC/Dol/06	Female	–
S. longirostris	Chennai	CHO2	Male	–
S. longirostris	Chennai	CHO3	Male	–
S. longirostris	Mangalore	MNG3	–	Male
S. longirostris	Chennai	CH6	Male	Male
S. longirostris	Chennai	CHO7	Female	Female
S. longirostris	Chennai	CH9	Female	Female
S. longirostris	Chennai	CH10	Female	Female
S. longirostris	Chennai	CH11	Male	Male
S. longirostris	Chennai	CH12	Male	Male
S. longirostris	Chennai	CH13	Male	Male
S. longirostris	Chennai	CH17	Male	Male
S. longirostris	Chennai	CH18	Male	Male
S. longirostris	Chennai	CH19	Male	Female
Stenella attenuata	Chennai	CH5	Male	Male
Delphinus capensis	Kakinada	VRC/Dol/03	Female	–
D. capensis	Malpe	MNG18	Female	–
Sousa chinensis	Gangoli	MNG 4	Male	Male
S. chinensis	Mangalore	MNG16	Female	Female
Grampus griseus	Chennai	CHO1	Male	Male
G. griseus	Chennai	CH15	Female	Female
Neophocaena phocaenoides	Gangoli	MNG5	Male	Male
N. phocaenoides	Gangoli	MNG6	Male	Male
N. phocaenoides	Gangoli	MNG7	Male	Female
N. phocaenoides	Gangoli	MNG8	Male	Male
N. phocaenoides	Gangoli	MNG9	Male	Male
N. phocaenoides	Gangoli	MNG10	Male	Male
N. phocaenoides	Gangoli	MNG11	Male	Male
N. phocaenoides	Gangoli	MNG12	Female	Female
N. phocaenoides	Malpe	MNG13	Female	Female
N. phocaenoides	Malpe	MNG14	Female	Female
N. phocaenoides	Mangalore	MNG15	Female	Female
N. phocaenoides	Mangalore	MNG17	Male	Male
Physeter macrocephalus	Chennai	CHWI	–	Female
Balaenoptera musculus	Mandapam	M5	Male	Male
B. edeni	Mandapam	M6	–	Male
Dugong dugon	Mandapam	M4	Female	Female

[1]Dash indicates either absence of external identification or failure of PCR to identify the sex.
[2]External identification.

CGA GGT CGA TA 3′ and those for *ZFX/ZFY*[13] were F 5′ ATA ATC ACA TGG AGA GCC ACA AGC T 3′ and R 5′ GCA CTT CTT TGG TAT CTG AGA AAG T 3′. The temperature profile for the amplifications was 94°C for 1 min followed by 35 cycles of 94°C for 45 s, 58°C for 45 s, 72°C for 1 min and finally 72°C for 7 min. The amplification products were resolved in 1.2% agarose gel and stained with ethidium bromide.

The perfect molecular sex marker would: (1) amplify diagnostic products in one step, in order to be fast, cheap and sample-conserving, (2) produce at least one product that exists in both sexes, to provide an internal positive control, (3) amplify small products (100–300 bp) likely to be present in degraded DNA and (4) be diagnostic in a range of species[21]. The markers visualized in the present study qualify all these conditions.

In the present study, the *SRY* gene had a size ranging from 210 to 224 bp, which was present only in males, whereas the *ZFX/ZFY* locus was longer with a size range of 442–445 bp and appeared in both sexes (Figure 1).

Table 2. Particulars of PCR-based gender identification in different species of marine mammals

Species	Number of individuals sexed by PCR		Size of *SRY* band (bp)		Size of *ZFX/ZFY* band (bp)	
	Male	Female	Mean	SD	Mean	SD
Tursiops aduncus	2	1	224	2.1	443	0.7
Stenella longirostris	8	4	222	1.7	444	0.8
S. attenuata	1	–	222	0	444	0
Sousa chinensis	1	1	220	0	443	0
Grampus griseus	1	1	215	0	444	0
Neophocaena phocaenoides	7	5	222	1.2	444	0.5
Physeter macrocephalus	–	1	–	–	445	0
Balaenoptera musculus	1	–	210	0	445	0
Balaenoptera edeni	1	–	210	0	445	0
Dugong dugon	–	1	–	–	442	0

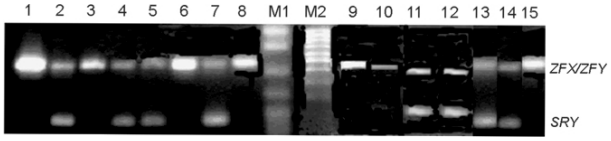

Figure 1. PCR amplification of *SRY* and *ZFX/ZFY*-specific fragments in different species of cetaceans and dugong. Lane 1, Female finless porpoise; lane 2, Male finless porpoise; lane 3, Female spinner dolphin; lane 4, Male spinner dolphin; lane 5, Male bottlenose dolphin; lane 6, Female bottlenose dolphin; lane 7, Male Indo-Pacific humpbacked dolphin; lane 8, Female humpbacked dolphin; lane 9, Female Risso's dolphin; lane 10, Female dugong; lane 11, Male blue whale; lane 12, Male Bryde's whale; lane 13, Male Risso's dolphin; lane 14, Male bridled dolphin and lane 15, Female sperm whale. M1, pBR322 DNA/*MSP*I digest and M2, 100-bp DNA ladder.

PCR appears to favour the amplification of the shorter fragment, i.e. the *SRY* fragment. Hence, false female positive tests never resulted from male DNA. This further supports the reliability of this system with two sets of primers[20]. The present results also indicate the presence of longer *SRY* fragment in dolphins and porpoise compared to that in sperm whale and baleen whales, whereas there was no size difference of the *ZFX/ZFY* fragment (Table 2).

The sex determination method used in this study for cetaceans and dugong is technically simple, requiring only PCR amplification and agarose gel electrophoresis. By using HotStart *taq* enzyme, the specificity of PCR could be improved as the process would discourage non-specific annealing.

Testing the technique using samples of known sexes (determined by physical examination of stranded/accidentally caught individuals) from ten cetaceans and dugong indicated that this sexing method was effective across a broad taxonomic range. The method can provide the secondary confirmation necessary for positive sex identification in marine mammal specimens, or a primary method where accurate field observation of sex is not possible. In three cases, one each of spinner dolphin, sperm whale and Bryde's whale specimens, where external sex determination was not possible, molecular sexing was done. In two cases, one each spinner dolphin and finless porpoise, PCR-based method revealed erroneous sexing by external examination. Upon re-examining these specimens it was found that external determination of sex was wrong due to partial mutilation of the body. PCR method could not be wrong because co-amplification of internal control ensures success of PCR reaction. However, in 8 individuals molecular sexing failed probably due to the highly deteriorated condition or non-availability of gDNA of the particular specimens. It is expected that the PCR-based sex determination method standardized in the present work would help in the studies of conservation, population structure and forensic issues of marine cetaceans and dugong.

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