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PRIMER NOTE

A PCR assay for gender assignment in dugong (*Dugong dugon*) and West Indian manatee (*Trichechus manatus*)

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Abstract

Gender assignment for some aquatic mammals in the field is difficult. Molecular sexing from tissue biopsies is possible as males are heterogametic. Here we describe a multiplex PCR assay that amplifies the male specific SRY gene and differentiates ZFX and ZFY gametologues in two sirenian species, dugong (*Dugong dugon*) and West Indian manatee (*Trichechus manatus*). The assay was validated with animals of known gender and proved accurate and robust to experimental failure.

Keywords: dugong, manatee, molecular sexing, SRY, ZFX, ZFY

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Dugongs (*Dugong dugon*) and West Indian manatees (*Trichechus manatus*) are large herbivorous marine mammals listed as vulnerable to extinction (IUCN 2006). Dugongs and West Indian manatees are found, respectively, in the tropical and subtropical west Indo-Pacific and from Florida (USA) to the north coast of Brazil (Vianna *et al*. 2006). Recent population and ecological studies of these species require gender information to determine important parameters (Lanyon *et al*. 2002; Vianna *et al*. 2006).

Gender assignment for some aquatic mammals in the field is difficult but molecular sexing from tissue biopsies is possible as males are heterogametic. Gender assignment by detection of male markers requires controls to detect experimental failure which may falsely assign female sex. Co-amplification of gametologues (highly homologous non-recombining regions on the sex chromosomes) overcomes this problem by targeting both sex chromosomes (Berube & Palsboll 1996). We have developed primers to detect the male-specific SRY gene and differentiate the male-specific ZFY gene from its gametologue ZFX. The multiplex polymerase chain reaction (PCR) assay is robust to experimental failure and accurately assigns gender of these two sirenian species.

DNA was isolated from ~10 mg of skin by salting out (Miller *et al*. 1988). Sequencing template was prepared in 20 or 50 μL PCRs containing 10–20 ng of DNA, 0.5x Q-Solution, 0.2 μM primers and 1× QIAGEN Multiplex Master Mix containing 3 mM $MgCl_2$. PCR cycling conditions were 94 °C for 15 min and 35 cycles of 94 °C for 30 s, 58 °C for 45 s, 72 °C for 60 s with a final extension at 72 °C for 10 min. Amplicons were purified using QIAGEN's QIAquick PCR Purification Kit. Cycle sequencing was conducted in 10 μL reactions using ABI's BigDye Terminator version 3.1. Cycle sequencing conditions were 94 °C for 60 s and 25 cycles of 94 °C for 10 s, 58 °C for 5 s, 60 °C for 60 s with a final extension at 60 °C for 5 min. All capillary electrophoresis tests were performed on an ABI 3130xl Genetic Analyser and sequence traces were analysed using Staden package (Staden *et al*. 2000). Primers were designed using PRIMER 3 (Rozen & Skaletsky 2000).

Sequence was obtained for 1057 bp of ZFX in dugong (no. EU078399) and West Indian manatee (no. EU078401) with primers ZFYX0097F and ZFYX1204R (Palsboll *et al*. 1992). Sequence from eight males and females of each species were compared to identify heteroplasmic sites and determine ZFY sequence in dugong (no. EU078400) and West Indian manatee (no. EU078402). Primers were designed to anneal to each gametologue and 3′ mismatching ensured gametologue specific amplification (Table 1).

Sequence was also obtained for 161 bp of *SRY* in dugong (no. EU078403) and West Indian manatee (no. EU078404) with primers ESRY3-F (GTCAAGCGACCCATGAA, derived from no. AF180946) and ESRY-R (GTTCGGG-TATTTCTCTCGGTGCA (Gupta *et al*. 2006)). Due to poor

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Table 1 ZFX and ZFY sequence polymorphism in 1057 bp of dugong and manatee showing the locations of primers used for gender assignment. From eight individuals of each species and each gender, we observed four inter-specific variations in ZFX and 16 in ZFY with no intraspecific polymorphism. The PCR assay uses two forward gametologue specific primers (ZFX-F and ZFY-F) and one reverse primer (ZFXY — ATGTCACACTTGTGGGGTA, shaded) to assign gender. 3′ mismatching of the forward primers ensures gametologue specific amplification. An M13 sequence tag (GAGCGGATAACAATTTCACACAGG) was appended to the 5′ -end of ZFXY so that amplicons could be detected with a fluorescently labelled M13 primer

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                                    Primer ZFX-F: AAGCATAGTAAAGAGATGCCGT
Dugong ZFX    GAATAACATAACGCTACATGTGAAAACTAAGCATAGTAAAGAGATGCCGTCTAGTACCCCCACAAGTGTGACATTGTGAAT
Manatee ZFX   A..........T........................................C........................A...
Dugong ZFY    .GGGGCTGCGGTAT........................GG....C...ACG..........................ACC.
Manatee ZFY   .GGGGGTGCGG...CG.....A...........C....GG....C...A..C.A....................CACACCC
                                   Primer ZFY-F: CATGTRAAAACTAAGCAYAGTAGG
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amplification, 1 μL of the initial PCR was re-amplified and agarose gel-purified with QIAGEN's QIAquick Gel Extraction Kit prior to cycle sequencing. A novel forward primer, DSRY-F (CAACTGCGAAACTCAGAGAT) was designed with the M13 sequence tag also appended to primer ZFXY (GAGCGGATAACAATTTCACACAGG).

Our sexing assay targeted SRY, ZFX and ZFY in 6 μL multiplex PCR amplifications comprising six primers (0.01 μM ZFX-F, 0.05 μM ZFY-F, 0.01 μM ZFXY, 0.02 μM DSRY-F, 0.2 μM ESRY-R, 0.11 μM TET-labelled M13), 10 ng of genomic DNA, 0.5x Q-solution and 3 μL of QIAGEN's multiplex master mix containing 3 mM $MgCl_2$. Cycling conditions were 94 °C for 15 min and 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s with a final extension at 72 °C for 10 min. Amplicons were diluted 25-fold with ultrapure Milli-Q water and six-fold with ABI Hi-Di Formamide before capillary electrophoresis. Alleles were sized against an internal size standard (GENESCAN-500 LIZ) and scored using GENEMAPPER software (version 3.7, ABI). Males were readily distinguishable from females by presence of three amplicons (153 bp from SRY, 230 bp from ZFX and 242 bp from ZFY) compared to a single product in females (230 bp from ZFX). This assay has potential to assign gender in other sirenians and closely related species. Gender assignment primers have been incorporated into microsatellite panels (Broderick *et al*. 2007) that genetically identify individuals in a long-term mark–recapture programme (Lanyon *et al*. 2002, 2006).

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