

Molecular Method for Determining Sex of Walruses

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ABSTRACT We evaluated the ability of a set of published trans-species molecular sexing primers and a set of walrus-specific primers, which we developed, to accurately identify sex of 235 Pacific walruses (*Odobenus rosmarus divergens*). The trans-species primers were developed for mammals and targeted the X- and Y-gametologs of the zinc finger protein genes (ZFX, ZFY). We extended this method by using these primers to obtain sequence from Pacific and Atlantic walrus (*O. r. rosmarus*) ZFX and ZFY genes to develop new walrus-specific primers, which yield polymerase chain reaction products of distinct lengths (327 and 288 base pairs from the X- and Y-chromosome, respectively), allowing them to be used for sex determination. Both methods yielded a determination of sex in all but 1–2% of samples with an accuracy of 99.6–100%. Our walrus-specific primers offer the advantage of small fragment size and facile application to automated electrophoresis and visualization. (JOURNAL OF WILDLIFE MANAGEMENT 72(8):1808–1812; 2008)

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KEY WORDS molecular sexing, *Odobenus rosmarus*, polymerase chain reaction, validation, walrus, ZFX, ZFY, zinc finger.

Developments in projectile-based methods for remotely deploying radiotags and collecting tissue biopsies from walruses (*Odobenus rosmarus*) have opened new opportunities for telemetry and genetic studies (Wiig et al. 2000, Jay et al. 2006). Clearly, the sex of targeted walruses is of keen interest in these studies but is not always readily identifiable because targeted walruses usually flee quickly from a recumbent position, often making it difficult for the observer to view sexually diagnostic features. We desire a method to molecularly determine the sex of walruses from remotely collected tissue biopsies.

A few trans-species molecular sex-determination methods exist that have the desirable qualities of not requiring large amounts of DNA, a nested polymerase chain reaction (PCR) amplification, or use of restriction enzymes (Ennis and Gallagher 1994, Shaw et al. 2003, Curtis et al. 2007). However, the primers developed by Ennis and Gallagher (1994), which target X- and Y-gametologs of the amelogenin gene, fail to discern differences between male and female walruses (J. V. Jackson and S. L. Talbot, United States Geological Survey Alaska Science Center, unpublished data). Molecular sex-determination methods developed by Shaw et al. (2003) and Curtis et al. (2007), which target the ZFX and ZFY genes (Page et al. 1987), produce large PCR products (approx. 1,000 base pairs [bp]). Large PCR products increase likelihood of failure of amplification or misidentification of sex and require time-consuming electrophoresis techniques, most commonly agarose gels, rather than efficient automated electrophoretic procedures (Taberlet et al. 1997, Gowans et al. 2000, Murphy et al.

2000, Sefc et al. 2003, Teltchea et al. 2005). Furthermore, although Shaw et al. (2003) and Curtis et al. (2007) tested their methods on specimens from a range of mammals, applicability of these methods to walruses is unknown. We assessed the ability of the Shaw et al. (2003) method to accurately determine the sex of walruses, and we extend the method of Shaw et al. (2003) by developing novel PCR primers that target smaller sexually diagnostic PCR products from walrus ZFX and ZFY genes.

STUDY AREA

We obtained walrus tissues from several regions. We obtained tissues from known-sex walruses from subsistence-harvesting villages in the northern Bering Sea (Gambell [63°47'N, 171°45'W], Savoonga [63°41'N, 170°25'W], and Inaluk [65°45'N, 168°57'W]) and from a haulout in the southeastern Bering Sea (Cape Seniavin [56°23'N, 160°9'W]). We obtained tissues from Atlantic walruses (*O. r. rosmarus*) from subsistence-harvesting villages on the shores of northern Baffin Bay in northwestern Greenland (Municipality of Qaanaaq [77°29'N, 69°20'W]). We collected tissues from live Pacific walruses (*O. r. divergens*) in United States waters of the Bering and Chukchi Seas.

METHODS

To assess molecular sex-determination methods, we collected small tissue samples (approx. 1 cubic cm [cc]) from 235 dead Pacific walruses (71 M and 164 F). We sampled muscle ($n = 45$) and reproductive tissues ($n = 183$) from walruses harvested by Alaska Native hunters and we sampled skin from beach-cast carcasses ($n = 7$). We positively identified sex by either inspection of the whole

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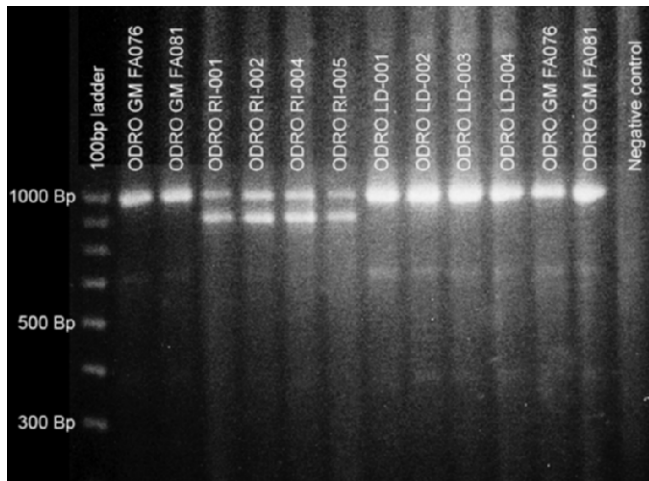


Figure 1. Electrophoresis banding pattern from the product of Pacific walrus samples, collected from Alaskan waters, USA, 2002–2003, and one negative control amplified in a polymerase chain reaction with the Shaw et al. (2003) genetic sexing primers and electrophoresed on a 2.0% agarose gel. Samples in lanes with 2 bands are males and with 1 band are females. Bp = base pair.

animal or excised reproductive organ from which we took the tissue sample.

In a field application of our method, we collected small (<0.5-cc) skin biopsies coincident with a remote radio-tagging effort in mixed-sex herds. We collected biopsies with stainless steel cutting heads (25-mm length, 10-mm diam, 1.4-mm wall thickness), with backward-projecting internal tines, mounted on crossbow bolts fit with floatation (CETA-dart, Virum, Denmark). The bolts were projected with crossbows (0.71 J work; models Wildcat III and Ranger; Barnett International, Odessa, FL), some of which we fit with a retrieval line (model 2500; Game Tracker, Flushing, MI). We placed retrieved cutting heads in individual polyethylene bags.

Within 12 hours of collection, we placed all tissue samples in a high-urea tissue-preservation buffer (4M Urea, 0.2M NaCl, 100 mM Tris-HCl pH 8.0, 0.5% n-lauroyl-sarcosine, 10 mM ethylenediaminetetraacetic acid [EDTA]), which allows storage of tissue samples at ambient temperatures for extended periods (up to several months). In the laboratory, we stored samples at -80°C until analysis.

We extracted genomic DNA using a salting-out protocol (Medrano et al. 1990), modified by substituting 0.7 volumes of 2-propanol instead of 2 volumes of ethanol. We quantified genomic DNA extractions using fluorometry, as per Handel et al. (2006), and diluted to 50 ng/ μL working solutions.

For molecular sexing by the Shaw et al. (2003) method, we conducted PCR amplifications in a final reaction volume of 10 μL that contained 1 μL (approx. 50 ng) genomic DNA extract, 0.4 mM dNTPs, 0.2 μM of each primer (LGL 331 and LGL 335; Amstrup et al. 1993, Cathey et al. 1998), 1 μL of buffer (0.5 M KCl, 0.1 M Tris-HCl, pH 8.5, 0.015 M MgCl_2), 50 mM MgCl_2 , 0.025% (vol/vol) Tween 20 (Chemical Abstracts Service Registry no. 9005-64-5), 10 μg bovine serum albumin (deoxyribonuclease- and ribonu-

lease-free) and 0.75 units *Amplitaq* DNA polymerase (USB[®], Cleveland, OH). The PCR reactions began with denaturation at 94°C for 2 minutes, followed by 35 cycles consisting of 15 seconds at 94°C , 15 seconds at 50°C , and 90 seconds at 72°C . We included a negative control (all PCR conditions identical to those described above but without added DNA) in each reaction.

We electrophoresed the PCR-amplified products on a 2.0% agarose gel containing 8 μL of ethidium bromide ($9.0 \times 10^{-7}\text{ M}$), at 150 V for 2 hours in a Tris-borate-EDTA buffer. Each gel included a negative control lane and 2 lanes of 100–1,000 bp ladders (50 ng; BioRad[®] Technologies, Hercules, CA). Ultraviolet transillumination visualized the product. We recorded results on Polaroid film (Polaroid Inc., Watham, MA). The Y-chromosome intron fragment was smaller (approx. 920 bp) than the X-chromosome fragment (approx. 1,000 bp; Fig. 1).

We assigned sex based on banding pattern of the products: 2 bands of different sizes (920 bp and 1,000 bp products) indicated male and 1 band (1,000 bp co-migrating products) indicated female (Shaw et al. 2003; Fig. 1.). For assessment of the sexing techniques, we scored samples without reference to the verified field sex. If we could not molecularly determine the sex of a sample for any reason, we repeated the extraction, amplification, and processing. To ensure quality control, we randomly assigned 15–50% (depending on the technician's experience) of samples for repeated extraction, amplification, and processing.

We extended the Shaw et al. (2003) method by developing walrus-specific sexing primers. We amplified and sequenced fragments containing the X- and Y-chromosome homologues of the zinc finger intron, described above, from male and female Atlantic and Pacific walrus using the primers LGL 331 and LGL 335 (Amstrup et al. 1993, Cathey et al. 1998). We synthesized primers with universal sequences SP6 Promoter (5'-GATTTAGGTGACACTATAG-3') and M13Rev (5'-GGATAACAATTTTCACACAGG-3') added to primers LGL 331 and LGL 335, respectively, allowing for subsequent simultaneous bidirectional sequencing (SBS; LI-COR, Inc. 1999), using labeled universal tailed primers (Oetting et al. 1995). We electrophoresed PCR products in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) against a 100-bp DNA ladder on a 1.5% agarose gel stained with ethidium bromide and visualized using ultraviolet transillumination. We isolated PCR products from the X- and Y-chromosome, separately, by slicing them from agarose gel using a commercial purification kit (Amicon Ultrafree-DA; Millipore, Bedford, MA; Catalog no. 42600). We cycle-sequenced purified products via SBS using a commercial kit (Sequitherm LCII 2.0[®]; Epicentre Technologies, Madison, WI). We used fluorescently labeled universal primers (LI-COR, Inc. Lincoln, NE; SP6 Promoter, M13Rev) to prime the SBS reaction. We electrophoresed the SBS products on a 64-lane 41-cm 5.5% polyacrylamide gel on a LI-COR 4200L automated sequencer (LI-COR, Inc. 1999). We analyzed the sequences using eSeq[™] imaging software (LI-COR, Inc.) and aligned

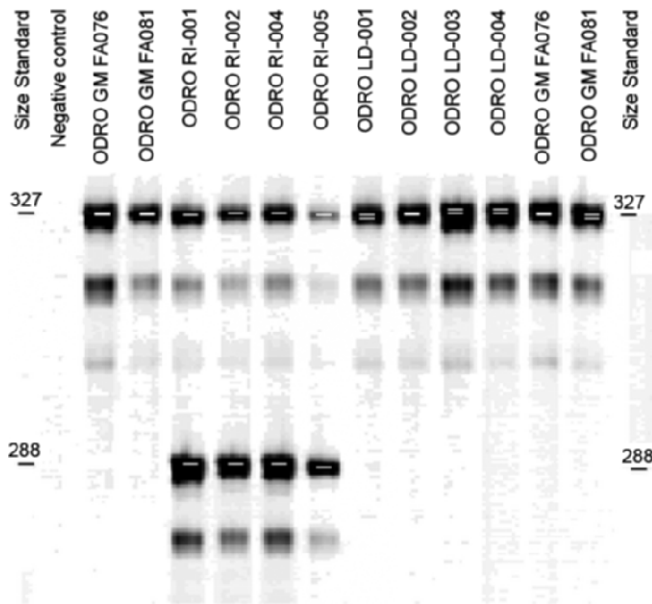


Figure 2. Electrophoresis banding pattern from the product of Pacific walrus samples, collected from Alaskan waters, USA, 2002–2003, and one negative control amplified in a polymerase chain reaction with walrus zinc-finger intron sexing primers and electrophoresed on a 6% polyacrylamide gel. Samples in lanes with 2 bands are males and with 1 band are females.

them using AlignIR 2.0™ (LI-COR, Inc.). Based on the aligned sequence (Genbank accession no: EU840178–EU840181), we designed the walrus-specific sexing primers (ORXY[F]:GCATGAGTGATCAAACCAAGT; ORXY[R]:RAACTTTGTTTTATGAA).

The Y-chromosome intron fragment targeted by these primers was smaller (approx. 288 bp) than the X-chromosome fragment (approx. 327 bp; Fig. 2). The size difference allowed identification of sex by producing differential banding patterns following a non-nested PCR amplification and subsequent electrophoresis.

To determine molecular sex of extracted genomic DNA with the walrus-specific primers, we ran PCR amplifications in a final reaction volume of 10 μ L containing 1 μ L (approx. 50 ng) genomic DNA extract, 0.2 mM dNTPs, 0.1 μ g BSA, 1X PCR buffer (Perkin Elmer Cetus I; PE Biosystems, Forest City, CA), and 1.0 units of *Taq* polymerase. We set primer concentrations at 1.5 pmoles fluorescently labeled universal forward primer (LI-COR, Inc.; SP6 Promoter), 0.5 pmoles of unlabeled forward primer (ORXYF), synthesized with an added universal tail (SP6 Promoter), and 0.5 pmoles of unlabelled, untailed reverse (ORXYR) primer. We began PCR reactions at 94° C for 2 minutes, followed by 40 cycles consisting of 30 seconds at 94° C, 30 seconds at 50° C, and 60 seconds at 72° C. We concluded each reaction with a final extension at 72° C for 30 minutes. We included a negative control (all PCR conditions identical to those described above but without added DNA) in each reaction. We electrophoresed the PCR products on a 48-well 25-cm 6% polyacrylamide gel on a LI-COR 4200LR automated sequencer (LI-COR, Inc.). We assigned sex based on the presence of 2 bands of different sizes (M: XY) or 2 co-migrating bands of the same

size (F: XX). If we could not determine the sex of a sample for any reason, we repeated the extraction, amplification, and processing. To ensure quality control, we randomly assigned 15–50% (depending on the technician's experience) of samples for repeated extraction, amplification, and processing.

We used logistic regression to assess whether successful molecular sex determination (response variable: yes or no) was dependent on the sex of the walrus (explanatory variable: M or F) or the molecular technique employed (explanatory variable: Shaw et al. [2003] method or walrus-specific primer). We maintained data in Access 2002 (Microsoft Corporation®, Redmond, WA) and conducted statistical analyses in SAS version 9.1 (SAS Institute®, Cary, NC).

RESULTS

The Shaw et al. (2003) method determined the sex of 230 (97.9%) of the 235 walrus samples, of which we re-examined 74 samples (31.5%) for quality control. Failure to determine sex of 5 samples was due to nonamplification. The method was equally effective determining sex of male (69 of 71) and female (161 of 164) samples (Wald $\chi^2 = 0.229$, $df = 1$, $P = 0.633$). It incorrectly assigned sex to one male sample of the 230 samples sexed, resulting in an estimated error rate of 0.4% (binomial 95% CI = 0.06–3.0%). Subsequent re-extraction, re-amplification, and electrophoresis produced the correct sex assignment.

The walrus-specific primers determined the sex of 232 (98.7%) of the 235 walrus samples examined, of which we re-examined 54 samples (23.0%) for quality control. Failure to determine sex of 3 samples was due to nonamplification. The method was equally effective determining sex of male (71 of 71) and female (161 of 164) samples (Wald $\chi^2 = 0.0044$, $df = 1$, $P = 0.947$). Walrus-specific primers determined the sex accurately for all sexed samples ($n = 232$). The 2 methods yielded comparable results. In terms of yielding a sex determination from a sample, the 2 methods performed equally well (Wald $\chi^2 = 0.641$, $df = 1$, $P = 0.424$). In terms of accuracy of sex determination when a determination could be made, the Shaw et al. (2003) method produced one erroneous assignment, whereas the walrus-specific method produced no erroneous assignments.

We applied the walrus-specific primer method to samples collected remotely from 128 live walruses on sea ice. We were unable to view sufficient diagnostic features to unequivocally verify the sex of 109 (85.1%) walruses. Of these 109 walruses, we retrieved biopsy darts from 64, of which 57 darts (89%) retained a tissue sample. Despite numerous repeated extractions and amplifications, no PCR product was produced from 2 of these samples, preventing sex determination. As a result, the walrus-specific primer method yielded a sex determination from 96.5% (55 of 57) of the retrieved tissue samples. Of note, in recent field work, we have greatly improved our biopsy dart-retrieval method with a retrieval line (Game Tracker Model 2500; Game

Tracker, Inc.) and achieved a greater biopsy dart retrieval rate (95%, $n = 20$).

DISCUSSION

We designed the new walrus-specific sex-determining primers to anneal to walrus consensus sequences and to yield smaller PCR products; thus, we expected superior performance relative to the Shaw et al. (2003) method. However, we found that both techniques yielded a sex-determination at equally high rates without bias by sex; and both made little to no errors (0.4% using the Shaw et al. [2003] method vs. <0.01% using our walrus-specific primers method).

It is unclear why one sample yielded an incorrect score using the Shaw et al. (2003) method. Inaccurate sex determination by molecular techniques can arise from errors associated with sample collection, storage, transportation, DNA extraction, molecular analysis, scoring, record keeping, and data analysis. Laboratory errors can include the amplification of artifacts (Polisky et al. 1975, Smith et al. 1995, Koonjul et al. 1999, Rodriguez et al. 2001), allelic dropout (Broquet and Petit 2004), variation in laboratory temperature (Davison and Chiba 2003), quality of materials and stringency of protocols used (Delmotte et al. 2001), and quality and quantity of DNA template (Goossens et al. 1998, Matthes et al. 1998, Bradley and Vigilant 2002). The incorrect determination of sex by the Shaw et al. (2003) method likely occurred during molecular analysis, because the new walrus-specific method correctly sexed the DNA extraction that had been incorrectly sexed by the Shaw et al. [2003] method, and the Shaw et al. (2003) method yielded the correct sex upon repeated extraction and amplification of the incorrectly sexed sample.

Application of the new walrus-specific primers on samples obtained by remote biopsy of live animals yielded an amplification success rate comparable to that achieved during testing of the method. Inefficiencies in remote biopsy collection lower the chance of successful genetic sex determination from wild walruses. We estimate the field success rate for obtaining a genetic sex determination from a walrus successfully struck with a biopsy dart to be 82% from the product of the biopsy retrieval rate (95% when using the recently improved retrieval method), the remote biopsy tissue retention rate (89%), and the rate of successfully amplifying a PCR product from a remote biopsy tissue sample (96.5%). Inefficiencies in striking a walrus with a biopsy dart will lower this rate.

Both sex-determination methods provide accurate sexing of individual walruses. A principal advantage of the walrus-specific primer method is the ability to perform the sex determination more easily and quickly using automated equipment, which is afforded by the smaller PCR product sizes. Automated electrophoresis of smaller fragments allows the processing of more samples in less time and with fewer manipulations. Reduced laboratory time translates to reduced operational costs. Reduced manipulations generally translate to reduced opportunities for human error. An

additional advantage of automated electrophoresis is that PCR products too weak to be visualized on agarose gels are more likely to be visualized using fluorescence. The principal advantage offered by the Shaw et al. (2003) method is the ability to accurately determine sex of walrus using less expensive electrophoresis equipment.

Our comparisons of the 2 methods relied on tissue samples collected under ideal conditions (e.g., ample clean tissue biopsies placed in preservation buffer in the field). We did not perform comparisons using DNA extracted from substandard tissue samples, such as feces or hair. DNA extracted from substandard tissue sources can yield PCR artifacts such as false alleles and allelic dropout. Sefc et al. (2003) demonstrated that, for degraded or low-quantity DNA, large fragment size increased the likelihood of allelic dropout. For both the Shaw et al. (2003) method and the walrus-specific primers method, allelic dropout could result in the misassignment of sex (of M as F). However, based on evidence from numerous other studies allelic dropout would more likely occur with the Shaw et al. (2003) method than with the walrus-specific primers method, due to the larger PCR product sizes from the Shaw et al. (2003) method (Taberlet et al. 1997, Gowans et al. 2000, Murphy et al. 2003, Sefc et al. 2003, Teletchea et al. 2005). Regardless, we concur with Robertson and Gemmel (2006) that statistical reliability and error rates associated with molecular sex determination should be assessed using large numbers of known-sex individuals whenever accurate assignment of sex is required.

MANAGEMENT IMPLICATIONS

We developed a robust and accurate molecular sex-determination method for walruses using primers that targeted short fragments of consensus sequences from walruses sampled across the species' range. Both our walrus-specific primers and the Shaw et al. (2003) method identified sex accurately and without bias and are suitable for walrus studies that obtain tissue samples from walruses lacking a verified field sex. Although we saw little evidence of spurious results from the Shaw et al. (2003) method applied to DNA extracted from biopsied samples, we suggest walrus-specific markers, which amplify a smaller product, be employed or used in addition to the Shaw et al. (2003) method when determining sex using DNA extracted from tissue sources known to give low yields or fragmented DNA. More importantly, however, we recommend that researchers make every effort to identify sex in the field, because it is not always possible to obtain a biopsy from walruses under field conditions and the techniques were unable to determine sex from a small proportion of samples.

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Savoonga graciously donated samples from harvested walrus. The United States Fish and Wildlife Service, Office of Marine Mammals Management, supported sample collections during harvest monitoring activities. The Togiak National Wildlife Refuge provided samples. The guidance, assistance, and curation efforts of C. R. Dial, J. Gust, B. Pierson, and J. Rearick at the Molecular Ecology Laboratory, Alaska Science Center, United States Geological Survey was instrumental to this study. We thank J. Gust and D. Derksen for reviewing the manuscript. Any use of trade names is for descriptive purposes only and does not imply endorsement of the United States Government. Biopsies and radio applications were authorized under United States Fish and Wildlife Service, Division of Management Authority, Permit Number MA801652-3.

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