Aquatic Mammals 2017, *43*(6), 655-660, DOI 10.1578/AM.43.6.2017.655

Short Note

A Sexing Technique for Highly Degraded Cetacean DNA

Christine M. Konrad,[1] Alysha Dupuis,[2] Shane Gero,[3] and Timothy Frasier[2]

[1]*Department of Biology, Dalhousie University, 1355 Oxford Street, Halifax, Nova Scotia, B3H 4J1, Canada*
E-mail: cmkonrad@dal.ca
[2]*Department of Biology, Saint Mary's University, 923 Robie Street, Halifax, Nova Scotia, B3H 3C3, Canada*
[3]*Department of Zoophysiology, Institute for Bioscience, Aarhus University, C.F. Møllers Allé 3, Aarhus 8000, Denmark*

Determining the sex of individuals in wild populations is important to many areas of study from social structure and behavioral studies to population genetics. Most cetacean species lack obvious sexual dimorphism, however, and are difficult to sex based on visual observations. Even species that do exhibit sexual dimorphism can be hard to sex with confidence in the wild, particularly if not fully grown. Genetic methods provide an alternate approach for sex determination if tissue samples can be collected from target animals. Numerous molecular sexing methods have been developed for cetaceans (Bérubé & Palsbøll, 1996; Rosel, 2003; Shaw et al., 2003), but most have limitations when it comes to samples of low quality. With increasing interest in non-invasive sampling (Hunt et al., 2013), such as sloughed skin (Amos et al., 1992; Mesnick et al., 2011), fecal samples (Parsons et al., 1999; Gillett et al., 2010), and exhaled "blow" (Frère et al., 2010), working with low-quality DNA is becoming a reality for many cetacean researchers. Museum specimens are another resource that is increasingly being used in genetic studies of cetaceans (Rosenbaum et al., 1997; McLeod et al., 2008), and this "ancient" DNA is also degraded. Most current sexing methods are problematic with degraded DNA because they rely upon amplification of relatively large fragments (approx. 300 bp or larger), and samples containing degraded DNA will often fail to amplify fragments of this size. Additionally, many molecular sexing methods use one primer set to target a Y-chromosome marker and use a second primer set to target an X-chromosome or autosomal marker of a different length as an internal positive control (Bérubé & Palsbøll, 1996; Rosel, 2003). Multiplexed polymerase chain reaction (PCR) may be of concern for low copy number DNA due to amplification bias resulting from preferential amplification of shorter fragments or variance in primer efficiency, potentially leading to incorrect sex assignment.

Currently, only one molecular sexing method for cetaceans addresses the issues of fragment length and multiplexed primer sets. Morin et al. (2005) described a real-time PCR method that uses a single set of primers to target a 105-bp region of the ZFX/ZFY gene (containing two fixed nucleotide differences) and uses probes with different reporter dyes for the ZFY and ZFX sequences. However, real-time PCR requires specialized equipment and is expensive relative to methods based on detecting length differences in PCR products. As an alternative approach, Morin et al. mentioned the presence of a fixed difference between the X- and Y-chromosomes within a *Taq1* restriction site (TCGA), but the resulting amplicon from their suggested primers is relatively long (604 bp) and, thus, is unlikely to be successful with extensively degraded DNA.

We set out to design a molecular genetic assay that targets the sex-specific restriction site identified by Morin et al. (2005) and also amplifies reliably from poor quality, degraded DNA. Primers CetSex94-F (5'-AGAGCCACAAGCTGACC-3') and CetSex94-R (5'-CATTTTGTGAGTAAAC AAAGCC-3') were designed to target a 94-bp fragment containing the *Taq1* restriction site. We used *Clustal X*, Version 2.0 (Larkin et al., 2007) to align sequences of this region (from Morin et al., 2005) for seven cetacean species representing six families: (1) bowhead whale (*Balaena mysticetus*), (2) grey whale (*Eschrichtius robustus*), (3) sperm whale (*Physeter macrocephalus*), (4) pygmy sperm whale (*Kogia breviceps*), (5) beluga whale (*Delphinapterus leucas*), (6) eastern spinner dolphin (*Stenella longirostris orientalis*), and (7) harbour porpoise (*Phocoena phocoena*). We noted no variable sites adjacent to the restriction site that could otherwise interfere with the restriction patterns, and we designed the primers for conserved regions. After restriction by *Taq1*, the ZFX fragment is digested to a 37- and a 57-bp fragment,

while the ZFY fragment is left uncut. Therefore, females are expected to have two bands of 37 and 57 bp, whereas males should have three bands of 37, 57, and 94 bp.

This new assay was designed primarily to optimize molecular sexing of sperm whales from sloughed skin samples, but we also wanted to develop a method that would be useful across multiple cetacean species. Therefore, in addition to including multiple species in the initial alignments, we tested amplification across seven cetacean species from six families and three non-cetacean mammal species to examine the taxonomic breadth of applicability of the newly designed primers (Table 1). DNA was extracted from all samples using standard phenol-chloroform procedures (Sambrook & Russell, 2001), except for the cow (*Bos taurus*) DNA, which was from calf thymus DNA purchased from Rockland Immunochemicals (Limerick, PA, USA). After extraction, DNA was quantified via spectrophotometry, using a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA), and DNA concentrations were standardized accordingly for use in PCRs.

For each cetacean species, and for the horse (*Equus ferus caballus*), we used samples from two males and two females. For the cow and grey seal (*Halichoerus grypus*) samples, a single male was available and tested. We preferentially selected samples for which sex had been previously determined based on physical examination of the individuals' genitalia or, if no such samples were available, sex was corroborated by an alternative molecular sexing method (Table 1).

All PCRs were carried out in 20 µl reactions in 1x PCR buffer, with 0.4 mg/mL bovine serum albumin (BSA), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 µM of each primer, 0.05 U/µl of GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA), and 20 ng of template DNA. Reactions were run on an ABI Veriti 96 well thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following parameters: initial denaturing for 5 min at 94° C, then 35 cycles of denaturation for 30 s at 94° C, annealing for 1 min at 55° C, and extension for 1 min at 72° C, followed by a final elongation step for 10 min at 72° C. We included a no-template negative control with all reactions. After amplification, the restriction digest was performed in 20 µL reactions, containing 10 µL of PCR product and 10 µL of a solution made up of three components: (1) *Taq1* buffer, (2) 0.02% BSA, and (3) 5 U *Taq1* (Thermo Scientific, Waltham, MA, USA), with an incubation at 65° C for 1 h.

We explicitly compared the success of our method on degraded DNA to an existing PCR fragment-based method (Rosel, 2003) that employs two sets of primers and targets amplicons of a more typical length. The primers described by Rosel (2003) target fragments of the ZFX and SRY genes that are 339 and 382 bp, respectively. For this comparison, we selected sperm whale DNA of varying levels of degradation, specifically DNA from two samples (one male and one female) from each of three categories: (1) high quality from biopsy samples, (2) moderately degraded from sloughed skin, and (3) highly degraded from sloughed skin. Relative level of degradation of DNA from sloughed skin samples was qualitatively assessed based on visual inspection of total genomic DNA via agarose gel electrophoresis and staining with Sybr green (Cambrex, Rockland, ME, USA; see Figure 1). All samples were used as templates in reactions with Rosel's primers and with CetSex94-F and CetSex94-R

Table 1. Success of the new sexing method across taxa. Sex of samples was corroborated, as listed in the Method column, by at least one of the following methods: (1) physical examination of the genitals, (2) Gilson & Syvanen (1998), or (3) Rosel (2003). For each cetacean species and for the horse, we used samples from at least two males and two females. For the cow and grey seal samples, a single male was tested.

Family	Species	Common name	Success	Method
Physeteridae	*Physeter macrocephalus*	Sperm whale	Yes	1, 3
Delphinidae	*Globicephala melas*	Long-finned pilot whale	Yes	2, 3
Monodontidae	*Delphinapterus leucas*	Beluga whale	Yes	1, 3
Eschrichtiidae	*Eschrichtius robustus*	Grey whale	Yes	2, 3
Balaenidae	*Eubalaena glacialis*	North Atlantic right whale	Yes	2, 3
Balaenopteridae	*Megaptera novaeangliae*	Humpback whale	Yes	3
	Balaenoptera physalus	Fin whale	No	2, 3
Non-cetaceans	*Bos taurus*	Cow	No	2
	Equus ferus caballus	Horse	No	2
	Halichoerus grypus	Grey seal	No	2

primers (this study). We used the same reagent concentrations and cycling parameters as outlined above for reactions with Rosel's primers, except with an annealing temperature of 51° C, and only 0.06 μM of the reverse SRY primer, as recommended in the published protocol for this method (Rosel, 2003), and 0.3 mg/ml BSA.

For both methods, PCR product was run on 3% agarose gels stained with GelRed (Biotium, Fremont, CA, USA). For the Rosel (2003) method, 10 μL of PCR product was loaded directly. For our new method, 10 μL of PCR product was digested, and the resulting 20 μL of post-digestion product was loaded. Although the total volumes loaded differed between the methods, equivalent volumes of the original PCR product were loaded in each case. Sex was determined based on the fragment sizes. For the new method, the undigested 94-bp ZFY fragment is expected to be displayed with twice the brightness of the 37- and 57-bp ZFX fragments, making incorrect classification of males as females very unlikely. Females should also be correctly classified so long as the restriction enzyme is given sufficient time to digest the ZFX fragment. To ensure that digestion is sufficient and that fragments are interpreted correctly, a known sample of each sex should be included as positive controls.

Our sexing method was consistently successful for sperm whales, long-finned pilot whales (*Globicephala melas*), beluga whales, grey whales, humpback whales (*Megaptera novaeangliae*), and North Atlantic right whales (*Eubalaena glacialis*; Table 1). For fin whale (*Balaenoptera physalus*) samples, however, we obtained a single fragment at 94 bp regardless of sex, suggesting that the *Taq1* restriction site in the ZFX sequence is not conserved in this species. This pattern was consistent despite testing an additional five fin whale samples of each sex. Conversely, for male and female horse samples and the male grey seal sample, only 37- and 57-bp fragments were observed, suggesting that either the priming sites were only conserved on the ZFX sequence or that the *Taq1* restriction enzyme site was present on both the ZFY and ZFX sequences for these species. The cow sample failed to amplify this fragment at all.

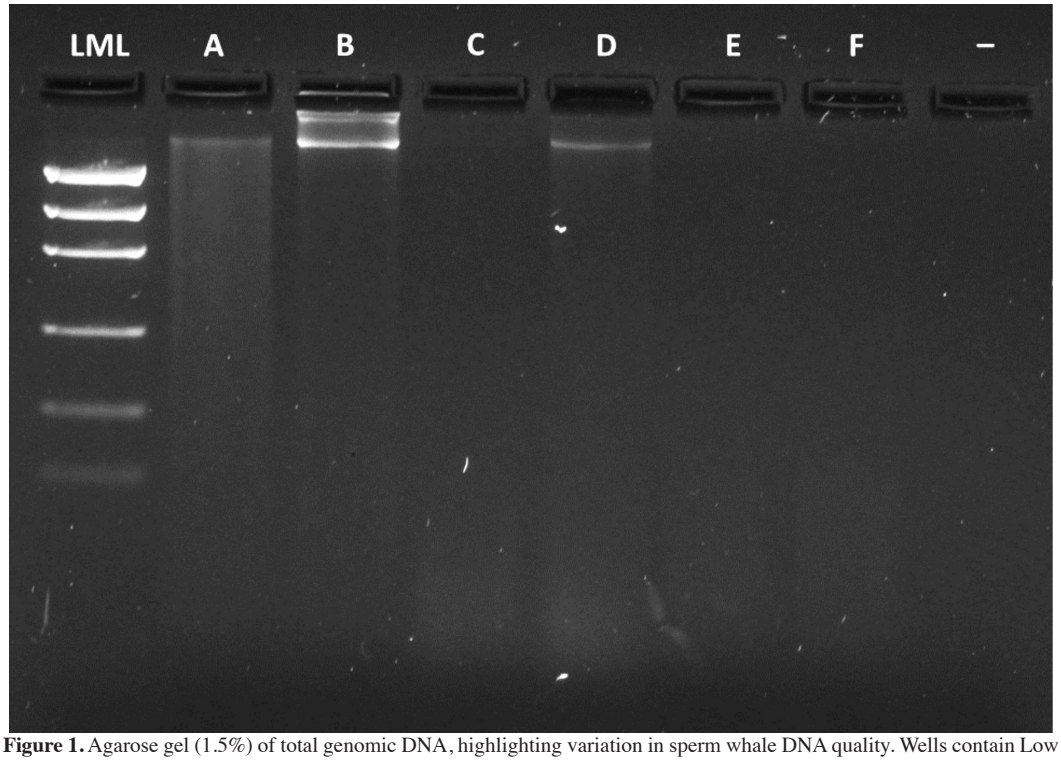

Figure 1. Agarose gel (1.5%) of total genomic DNA, highlighting variation in sperm whale DNA quality. Wells contain Low DNA Mass Ladder (LML; Invitrogen, Carlsbad, CA, USA), DNA from two biopsies (A & B) and four sloughed skin samples (C-F), and a no-DNA negative control (–). For each sample, 20 ng of DNA, as quantified by spectrophotometry, was loaded on the gel and stained with Sybr green (Cambrex, Rockland, ME, USA). Brightness of the bands in the ladder represent, from top to bottom, 20, 12, 8, 4, 2, and 1 ng of DNA.

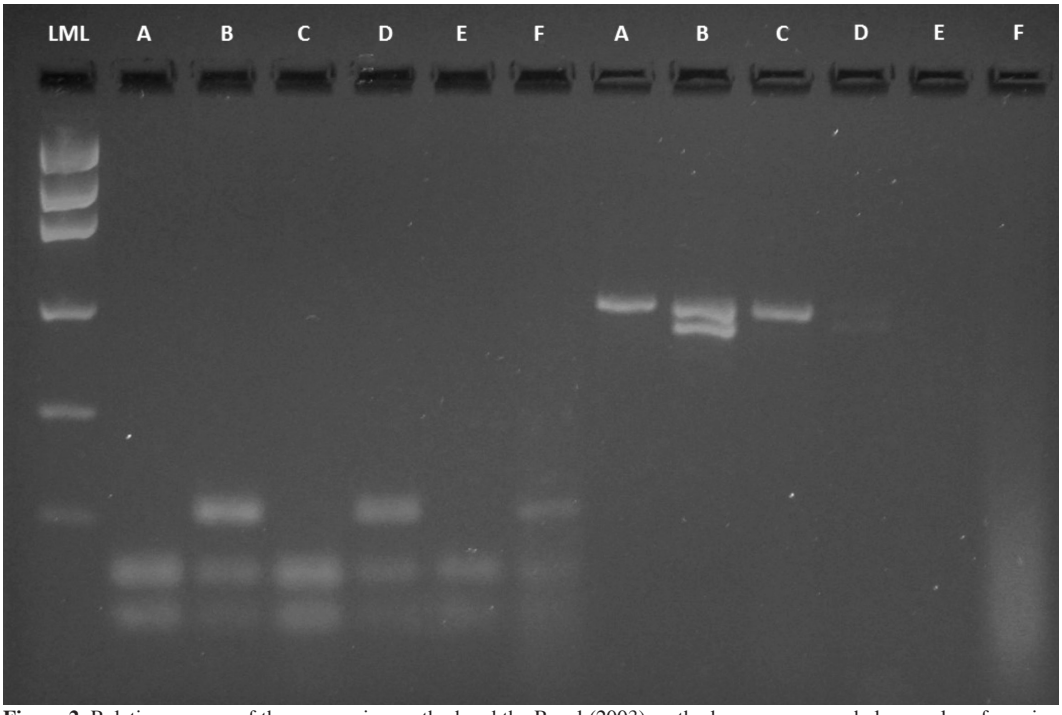

Figure 2. Relative success of the new sexing method and the Rosel (2003) method across sperm whale samples of varying degrees of degradation. The same six samples as in Figure 1 (A-F) were amplified using the method described in this note and using the Rosel method. On a 3% agarose gel stained with GelRed (Biotium, Fremont, CA, USA), LML was loaded in the first well, followed by 10 µL of PCR product in each subsequent well. Samples A, C, and E are female, while samples B, D, and F are male. Two highly degraded samples (E and F) failed to amplify using the Rosel method.

We applied our method to 167 sperm whale samples (8 biopsy samples and 159 sloughed skin samples) collected during a longitudinal project off the Caribbean island of Dominica (Gero et al., 2014) and determined sex for 138 of these (33 male and 105 female), including numerous samples that had low-quality DNA. For eight samples, sex was known from field observations of mature males of a distinctly large size or photographs of the genitals or genital slits. Genetic sex determined by our new method was consistent with the field data. Further demonstrating the reliability of this method, a male used as a control was sexed 15 times, and 17 other individuals were sexed twice, providing consistent results each time.

For low-quality sperm whale samples, our method was more successful than that of Rosel (2003). The Rosel (2003) method successfully sexed the two biopsy samples and the two moderately degraded sloughed skin samples but failed to amplify the highly degraded sloughed skin samples, while our method successfully sexed all six samples (Figure 2).

The sexing method outlined in this note can also be used to address another challenge with degraded DNA—namely, the disconnect between DNA concentration and amplifiability. Quantification via spectrophotometry, for example, can overestimate the amount of amplifiable DNA because it provides a measure of DNA concentration that includes fragments that are too short to be amplified in PCR. Additionally, DNA from degraded sources, such as sperm whale sloughed skin, can vary substantially in its degree of degradation (Figure 1) and, thus, in its ratio of amplifiable DNA to total DNA. This, in turn, impacts the success of amplification. We have found that this sexing method can reliably be used to determine the relative amplifiability of degraded DNA, which then allows the success of PCRs to be optimized.

After standardizing all samples to the same DNA concentration, based on spectrophotometric quantification, we used this sexing method to amplify sperm whale sloughed skin samples alongside a biopsy sample. For each sloughed skin sample, we assessed its band brightness relative to the biopsy sample. If they differed by at least twofold, we adjusted the amount of template DNA used in subsequent reactions in proportion

to the difference in band brightness. This protocol of adjusting template DNA amounts led to successful amplification across a suite of 18 microsatellite loci for these samples. We qualitatively compared how well amplification success across this suite of microsatellites was predicted by successful amplification of the ZFX/ZFY fragment and each of the 18 microsatellite loci—the ZFX/ZFY fragment was visibly the best predictor. The method works well regardless of whether band brightness is compared before or after cutting the fragment at the restriction enzyme site. Increasing the number of cycles to 40 improved sexing success for lower quality samples, which is advantageous for maximizing sexing success but is not recommended if the sexing reaction is being used to assess sample quality.

This straightforward, quick, and inexpensive sexing method was successful across six cetacean species from six families, and across samples of varying levels of degradation. As such, it appears to be a promising option for researchers working on a variety of different cetacean species, and particularly those researchers using highly degraded DNA such as that originating from noninvasive sampling techniques and ancient DNA. Researchers who intend to use this method on species that were not assessed here, however, should first validate the method for their species of interest, considering that the assay was not universally successful across all species that we tested.

Acknowledgments

We thank the following collaborators for the use of their samples when testing this technique: John Ford (fin whales), Robert Michaud (beluga whales), Zoe Lucas (horse and grey seal), and Jim Darling (grey and humpback whales). Sperm whale sampling through The Dominica Sperm Whale Project was carried out under scientific research permits SCR 013/05-02, RP-2/12 IW-1, RP-09/014 IW-1, RP-01/079W-2, RP-03/059W-4, P-122/4W-2, P-40/2W-7, and RP-16-04/88-FIS-9, and transported through CITES permits for the import and export of animal parts issued by Environment Canada and the Environmental Coordinating Unit of Dominica. Sperm whale sampling was funded through a Carlsberg Foundation field expedition grant and an FNU fellowship for the Danish Council for Independent Research supplemented by a Sapere Aude Research Talent Award to SG, as well as by Discovery and Equipment grants to Hal Whitehead from the Natural Sciences and Engineering Research Council of Canada (NSERC). SG is supported by a technical and scientific research grant from the Villum Foundation. The authors declare that no conflicts of interest exist.

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