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Sex determination of baleen whale artefacts: Implications for ancient DNA use in zooarchaeology

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ABSTRACT

Methods to determine the sex from tissue samples of mammals include the amplification of Y chromosome specific regions, which should only amplify from males, or amplification of homologous regions of the X and Y chromosome containing XY specific SNPs. A disadvantage of the first approach is that PCR failure can be misinterpreted as the identification of a female. The latter approach is proposed to identify PCR failure through non-amplification of the X homologue, which should be present in both sexes. This method is therefore potentially more suitable for molecular sexing of degraded DNA with a high probability of PCR failure, such as for example, ancient DNA samples. Here, we investigate the validity of this assumption regarding the use of XY homologue PCR assays for molecular sexing of ancient DNA. We tested a primer set targeting the ZFX/ZFY alleles using ancient DNA extracts from 100 to 4500 years old bowhead whale samples, and for comparison on dilution series from modern bowhead whales of known sex. DNA sequencing of PCR products obtained from the ancient material confirmed a higher proportion of successful PCR amplifications of the X homologue over the Y homologue. This potentially biased sex determination was further assessed by testing highly diluted DNA extracts of modern samples, for which a consistently higher success rate of PCR amplification and lower PCR cycle threshold was found for the X homologue from females than either homologue from males. This is most likely due to the higher copy number of the X homologue in females, although other yet unknown attributes of the protocol may also cause the observed bias. The current case study provides a valuable example of a potential pitfall in molecular sex determination of ancient mammal DNA in zooarchaeology. High-throughput sequencing methods, in which sufficiently large numbers of reads can be unambiguously mapped to X and Y regions, should overcome such biases and be the most robust approach for molecular sex determination using degraded DNA.

1. Introduction

Ancient DNA (aDNA) studies can provide insights into the history of extant and extinct populations. For example, aDNA has been successfully used to study changes in population dynamics and in particular effective population size in a variety of species by determining scaled mitochondrial DNA (mtDNA) diversity (Lorenzen et al., 2011; Mcleod et al., 2012; Foote et al., 2013) or heterozygosity in full genomes e.g. (Orlando et al., 2013; Palkopoulou et al., 2015). However, there have been relatively few studies that have used targeted sequencing of aDNA to study the social structure and demographic metrics other than effective population size (e.g. sex ratio) (but see Allentoft et al., 2010). Gowans et al. (2000) describe a method amplifying the SRY gene (sex-determining region Y gene) of the Y chromosome. However, this method is error-prone, as it cannot distinguish between non-amplification of the target DNA because of PCR failure or that the specimen is a female. Another approach targets specific regions of the ZFX/ZFY genes encoding for a zinc-finger protein domain (Aasen and Medrano, 1990). In placental mammals the ZFX/ZFY genes are homologous on the X and Y chromosome (Aasen and Medrano, 1990), with specific sequence differences between the X and Y copies (Morin et al., 2005). The method was first applied to cetaceans by targeting a 540 bp sequence of

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the respective genes (Bérubé and Palsbøll, 1996), but subsequently modified to a fluorescent 5′exonuclease assay amplifying 105 bp of the ZFX-Y genes by Q-PCR (Morin et al., 2005). The modified method employs one specific set of primers that allows the amplification of both the X and Y marker regions while emitting fluorescence with wavelengths specific for the X and Y targets. It is thus possible to determine the sex of the sample and to simultaneously rule out PCR failure for the X chromosome that is present in both males and females. The Y chromosome is present only in males and the ZFY marker should therefore only amplify if the specimen is a male. The advantage of this method is thought to be that it is less sensitive to misinterpretation of PCR failure, in particular when assuming that both homologues have the same probability of amplification. This method should therefore be suitable for sex determination of degraded DNA samples for which the possibility of PCR failure may be high. Here we assess this assumption by conducting molecular sex determination of aDNA extracted from 100 to 4500 years old and bowhead whale (*Balaena mysticetus*) samples from Disko Bay, Greenland.

2. Material and methods

2.1. Ancient bowhead whale samples

All ancient material was sampled from 89 archaeological paleo- and prehistoric Inuit artefacts made from bowhead whale (*Balaena mysticetus*) baleen. The artefacts are part of the Ethnographic Collections at The National Museum of Denmark, including material belonging to Qasigiannguit Museum and the Greenland National Museum and Archive. The samples originally stem from nine separate locations, two in Southeast Greenland and seven in West Greenland (one near Uummannaq, one near Uupernavik, and five in Disko Bay). Permits to sample the 89 baleen pieces or artefacts have been granted by the involved museums. Details of the ancient samples and a map of sampling locations are given in Supporting information file S1 and file S2 respectively. The samples were dated in relation to the Greenlandic Inuit cultures at each respective excavation site; i.e. the Saqqaq culture, which spanned from approximately 4500 to 2500 yrs B.P.; the Dorset culture of ca. 2500 to 2000 yrs B.P.; and the Thule culture spanning from 800 yrs B.P. up to the 19th century (Grønnow et al., 2006). The time spans indicate when the respective cultures were present in Greenland; in other Arctic regions different estimates may apply. This study faced the persisting challenge to identify individuals and to discriminate between them, as we used genetic material from animals with an archaeological context (Larson et al., 2002). We amplified and sequenced a fragment of the mitochondrial DNA (mtDNA) control region to ensure that only a single DNA sample per individual was used in downstream genetic analyses. Samples with identical mtDNA control region haplotypes from the same settlement that were aged within the same century and found to be the same sex, were considered as potential duplicates of the same of a single individual. All extractions of aDNA and downstream PCR set up were performed and conducted in a dedicated clean lab as detailed in (Sinding et al., 2012). The 453 bp region of the mitochondrial control region spanning from position 15,473 to 15,925 of the 16,390 bp bowhead whale mitochondrial genome (GenBank Accession no. AP006472; (Sasaki et al., 2005)) was targeted in six overlapping fragments (Table 1). For every nine samples from which DNA was extracted and/or amplified, we included at least one blank control (an additional sample without DNA added) to monitor for contamination. DNA sequences were subsequently edited and aligned by eye and checked against reference DNA sequences in GenBank using the BLAST algorithm in MEGA 5 (Tamura et al., 2011) or Geneious Pro 5.5.7. The obtained sequences were deposited in GenBank, accession numbers KY053806-KY053829. Sampling aimed to be minimally destructive given the cultural historical value of the specific artefacts. Sampling was authorized by the Ethnographic Collections, SILA, Arctic Centre at the Department of Modern History and World Cultures, The National Museum of Denmark.

2.2. Modern samples

Three modern bowhead whale skin biopsies collected from one female and two males from Disko Bay, West Greenland, were included. Molecular sexing was independently performed at the Biology Department, Saint Mary's University, Canada, or at the Natural History Museum University of Oslo, Norway, following the protocol described in Morin et al. (2005). We acquired 1.5 cm cylinder shaped skin biopsies with a diameter of 0.5 cm from bowhead whales by darting the whales non-lethally with a biopsy dart launched from a crossbow. The method for biopsy collections was reviewed by the Greenland Government, Department for Hunting, and dispensation from the legal order nr. 10 from 13 April 2009 from the Greenland Parliament were provided. The biopsies were collected under permission from the Ministry of Fisheries, Hunting and Agriculture, Greenland in accordance with § 24 in Greenland government executive order nr. 9 of 17th of April 2009 on protection and hunting of great whales. No IACUC permits are required in Greenland as bowhead whales in West Greenland are not endangered and under strict hunting quota regulation by the International Whaling Commission.

Table 1
Primer and probe sets used.

Primer name	Oligonucleotide sequence (5′ − 3′)	Position	Amplicon (bp)	Reference
134F	CCCAAAGCTGAAATTCTACATTAAACT	15,439–15,465	220	(Borge et al., 2007)
dip3.3R	CGTRGTGAARATAATTGAATGCAC	15,635–15,658		(Borge et al., 2007)
BmyUp098	AACCACAGTACTATGTCAG	15,499–15,517	160	(Rastogi et al., 2004)
dip3.3R	CGTRGTGAARATAATTGAATGCAC	15,635–15,658		(Borge et al., 2007)
109F	TGGCCGATACTAGTCCCAAC	15,581–15,600	243	(Borge et al., 2007)
351R	GCGGGTTGCTGGTTTCAC	15,806–15,823		(Borge et al., 2007)
109F	TGGCCGATACTAGTCCCAAC	15,581–15,600	139	(Borge et al., 2007)
BmyLP282	GATCAATGATTATTATGTACG	15,699–15,719		(Rastogi et al., 2004)
177F	TTCACTACGGGAAGTTAAAGCTCG	15,650–15,673	174	(Borge et al., 2007)
351R	GCGGGTTGCTGGTTTCAC	15,806–15,823		(Borge et al., 2007)
297F	CCGCTCCATTAGATCACGAG	15,769–15,788	201	(Borge et al., 2007)
dip5R	CCATCGWGATGTCTTATTTAAGRGGAA	15,943–15,969		(Borge et al., 2007)
CetZFXY_F1	AGTTTAAGTCGAGAGGTTTTTTGAAA	*41–66	105	(Morin et al., 2005)
CetZFXY_R1	TCTTGTTGGTAGTGTAATCACAGTCAGT	*118–145		(Morin et al., 2005)
CetZFXY_F2	AGTTTAAGTCGAGAGGGTTTTTGAAA	*41–66		This study
CETZFXprobe	AAAACCATCCTGAACACCTTACCAAGAA	*77–104		(Morin et al., 2005)
CETZFYprobe	AACCACCCTGAACACCTCACCAA	*79–101		(Morin et al., 2005)

Positions correspond to the complete bowhead whale mitochondrial DNA (Sasaki et al., 2005), GenBank Accession no. AP006472. Positions with * correspond to bowhead whale ZFX-chromosome gene and the ZFY-chromosome gene (Morin et al., 2005), ZFX GenBank Accession no. AF260783, ZFY GenBank Accession no. AF260785.

2.3. Molecular sexing

Quantitative PCR (Q-PCR) amplifications were performed using a Roche Lightcycler 480. Cy5 was used as fluorophore for the ZFX and Hex for the ZFY probe (the probe sequences are listed in Table 1), BHQ1 was used as quencher for both probes. Each 30 μl PCR contained 5 μl of DNA extract, 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.04 mg/ml BSA, 533 nM of each primer, 200 nM of each probe, 200 μM mixed dNTPs and 1.2 u AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA). The amplification protocol started with a 5 min activation step at 95 °C, followed by 60 cycles of 95 °C for 10 s, 60 °C for 60 s, followed by a cool off at 40 °C. PCR success was monitored by probe light emission at a certain cycle threshold illustrating exponential amplification of the target DNA.

Contamination of samples with foreign DNA is known to be a common issue in museum specimens that on their own contain DNA of low quality and amount. The presence of human DNA on the samples is quite likely as a result of their excavation and handling history. To evaluate the issue for the samples used in this study, at least one PCR product from each sample in which we successfully amplified the target region was purified using MinElute PCR purification kits (Qiagen), and was subsequently cloned to individualize PCR products using the Topo TA system (Invitrogen, Carlsbad, CA). Bacterial colonies were picked and the cloned DNA was amplified following the manufacturers' guidelines. Each 25 μl PCR contained 1 μl of DNA extract, 1 × PCR buffer, 2.5 mM $MgCl_2$, 1 μM of each primer, 25 mM mixed dNTPs and 0.1 μl AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA). Primers were "universal primers" M13-F (5′-GTAAAACGACGGCCAG-3′) and M13-PucR (5′-CAGGAAACAGCTATGAC-3′). PCR amplifications were performed using an MJ Thermocycler with a 1 min activation step at 94 °C, followed by 40 cycles of 94 °C for 20 s, 54 °C for 20 s, 72 °C for 30 s, followed by a final extension period of 72 °C for 7 min. 8–16 (in one case plasmid DNA from only 2 colonies were amplified) clones were sent for both purification and sequencing by the Macrogen Europe (Amsterdam) commercial Sanger sequencing service. Purified PCR products were only sequenced in reverse directions using the M13-PucR primer and BigDye (Applied Biosystems) sequencing chemistry.

Using PCR on low quantity and quality DNA can suffer from allelic dropout, i.e. only amplifying one allele (Taberlet et al., 1996), which in this study may lead to misidentify the sex of the specimen, i.e. in the case that the ZFY gene is affected. While both alleles in this assay are of the same length, which may reduce the risk of dropout, there is a single mismatch between the ZFY region and the primer sequence that might bias the relative amplification of the ZFX over the ZFY region (Morin et al., 2005). We therefore followed a multiple PCR procedure as described in (Taberlet et al., 1996) to increase the confidence level of successfully amplifying both the ZFX and ZFY targets in males to 99% after 7 successful amplifications. Eighty-nine aDNA extracts were screened twice for the ZFX and ZFY targets. Samples that amplified the ZFX/ZFY as well as the mtDNA target was subjected to a further five replicate PCRs, totalling 476 PCR reactions including 60 blank reactions.

To evaluate the amplification success and potential PCR biases of low copy number DNA templates, four separate DNA extracts from a modern male bowhead whale skin sample were amplified four times in eight different dilutions. The dilution series were as follows from the purified extractions 1:1, 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000, 1:1,000,000, and 1:10,000,000. In total 160 reactions were assessed including 32 blank reactions.

To assess the impact of ZFY primer sequence mismatch on sex determination reliability, a new primer was designed with the respective base changed in that the primer matched 100% to the ZFY template, but now with a single mismatch to the ZFX target (Table 1). This may shift the PCR bias towards the amplification of the Y-chromosomal product. One male and one female genomic DNA extract were serially diluted eight times according to concentration from, 1 to 1×10^{-7} ng/μl. Each dilution was tested 24 times, and in total 432 reactions was assessed including 48 blank reactions.

3. Results and discussion

3.1. Genotyping of ancient samples

The analyses of the PCR amplifications targeting the mitochondrial control region of 89 ancient bowhead whale samples, delivered nucleotide sequences for 27 samples that represented 19 different haplotypes. In the two attempts to amplify the ZFX/ZFY homologues at least one PCR was successful for at least one homologue for 17 different samples. These were then further assayed in order to increase the confidence of correctly identifying ZFX and ZFY homologues in any male samples. From a total of seven replicate PCR assays the ZFX marker was successfully amplified for 16 samples, and both the ZFX and ZFY homologues were amplified for one sample. The cloning and sequencing of at least one PCR product of these 17 samples indicated no contamination with foreign DNA for 14 samples, while two samples yielded human sequences; for one sample this affected only the ZFY homologue and for the other sample both human homologues were detected. For one sample, however, no species-identifiable sequences were observed. None of the blank PCR assays yielded any product. The low representation of males detected by the PCR assays on the ancient samples may indicate a long-term biased sex ratio in the bowhead whales in Disko Bay, West Greenland. A recent study showed that the majority of individuals aggregating during the spring in Disko Bay, West Greenland, are females, which was interpreted as reflecting different seasonal geographic distribution patterns of males and females of the East Canadian and West Greenland bowhead whales (Heide-Jørgensen et al., 2010). However, our results may also reflect a bias in the success rate of PCR amplification of the ZFX and ZFY homologues for the aDNA extracts. To further discriminate between these two contingencies, the PCR amplification success of each homologue was assessed for a dilution series of modern male bowhead whale DNA.

3.2. Genotyping of diluted modern samples

For the initial dilution series of four extracts from one modern male bowhead whale, there was a slight bias of amplification of the ZFX allele in earlier cycles than the ZFY allele, this was even more prominent in the more dilute samples (Fig. 1) (CT values for dilutions are given in Supporting information file S3 and S4.). The results suggest that due to dilution of template DNA there is an increased stochastic bias towards amplification of the ZFX region (Fig. 1). No sample diluted below 1:10,000 yielded any PCR product. One PCR assay on a 1:1,000 dilution and two PCR assays on the 1:10,000 dilution of the DNA extract from the modern male individual only successfully amplified the ZFX homologue. It therefore appears that there is a biased amplification of the ZFX region over the ZFY region for diluted samples of genomic DNA from known male bowhead whales. This bias may also cause an increased risk of misidentification of males as females when using DNA extracts from ancient bowhead whales of unknown sex. In the current study, sample L8.1021 may be affected, and may illustrate the problem. The ZFX homologue was amplified in four of the seven PCR assays from the ancient sample L8.1021, whereas the ZFY homologue was amplified in only one of the seven PCR assays (in which the ZFX homologue was also amplified); with a lower number of replicates the ZFY homologue could have been easily missed, leading to an erroneous sex determination. In a degraded ancient or diluted sample, the overall sex-chromosome DNA survival should be equal for both sexes. However, as the copy number of DNA templates is decreased in such samples, the stochastic probability of amplifying the target from only one of the two chromosomes may increase. The one base mismatch in the forward primer with respect to the ZFY homologue may give the ZFX homologue detection the small advantage that made it outcompete the ZFY homologue in

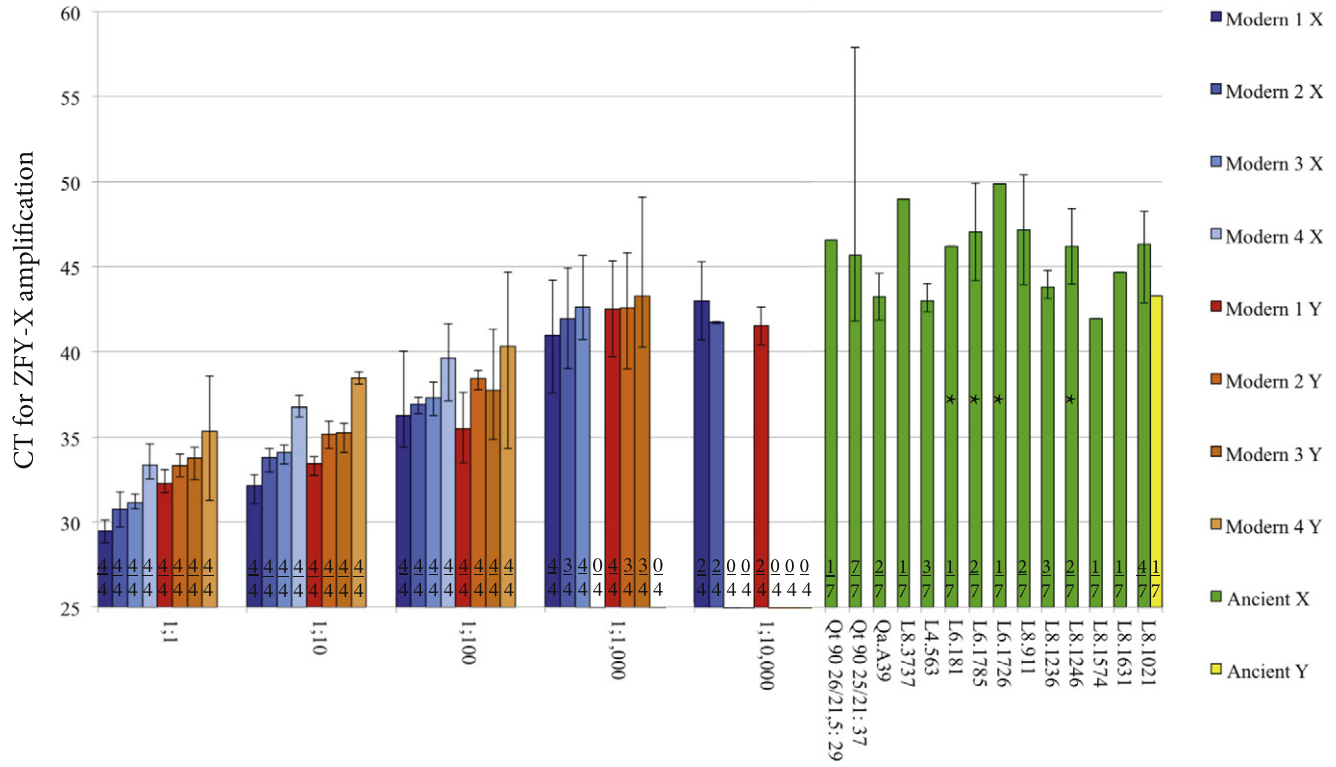

Fig. 1. Plot of amplification success and cycle threshold (CT) values on the Y-axis of the individual samples and dilutions on the X-axis. Each column is an average of the CTs in repeated amplifications, the fraction in each column describes each number of amplification attempts (denominator) and successes (nominator). The bars on top of each column are the interval between the highest and lowest CT value in multiple amplifications of the sample. Ancient samples without mtPCR amplification success are marked with a star. The list at right assigns the colour of a bar to the X or Y of a specific dilution.

the dilution series of the PCR assays on the modern male bowhead whale DNA extract. Comparisons of the cycle threshold (CT) at which the amplification profile of DNA becomes exponential between ancient and modern diluted samples, suggested an extremely low copy number in the aDNA extracts. The CT differences are possibly also affected by a different molecular composition of modern and aDNA extracts, which have been made with different chemicals on different tissue types and hence will have different PCR inhibition characteristics.

3.3. Modified genotyping of diluted modern samples

Using redesigned primers, so that the 1 bp mismatch of the forward primer was now with the ZFX rather than the ZFY homologue, no extract diluted more than 1×10^{-3} ng/μl yielded any PCR product. However, there was now an overall slight bias towards amplifying the ZFY allele in the male sample (Fig. 2) (CT values for dilutions are given in Supporting information file S5), and for the ZFX allele there was

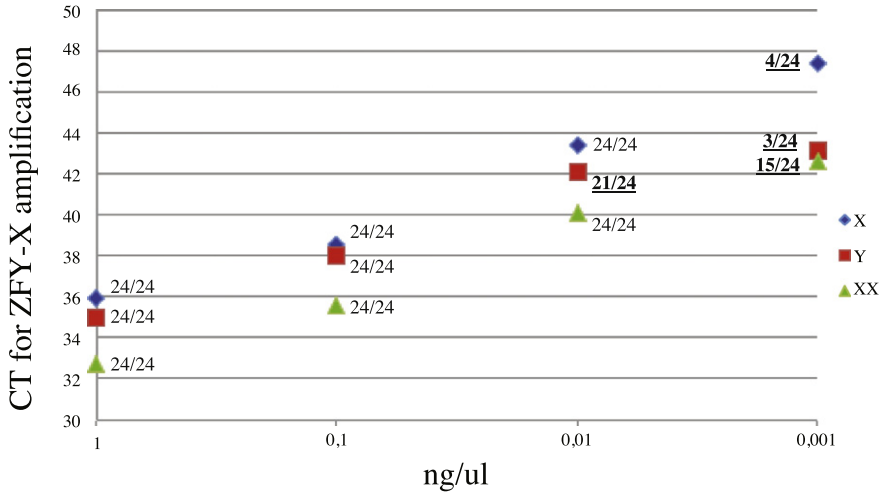

Fig. 2. Plot of cycle threshold (CT) values for ZFX and ZFY markers in male and female. CT values are on the Y-axis and DNA contents of extract on X-axis. Each mark is an average of the CT value of amplifications of the samples at the particular dilution. The fraction next to each marker is the number of amplifications attempts (denominator) and successes (nominator) in the particular sample and dilution. Fractions with bold marks show points with PCR dropouts.

consistently a lower cycle threshold in all dilutions of the female than in the male DNA extract (Fig. 2). This is likely due to the difference in copy number, with females having two copies of the ZFX homologue. This can be considered a pitfall in targeting homologous regions of both sex chromosomes for molecular sex determination of aDNA samples. However, it was of particular concern that in three 1×10^{-2} ng/μl and one 1×10^{-3} ng/μl dilutions of the male DNA extract, only the ZFX homologue was amplified. This suggests that there are additional further parameters affecting the amplification of the ZFX and ZFY alleles that we have not identified yet, rendering more frequent amplification failure of the ZFY as compared to the ZFX homologue.

4. Conclusions

The results presented here suggest that conventional PCR approaches for molecular sex determination of ancient bowhead whale samples are prone to biased amplification of the X homologues. This bias increases and becomes substantial in highly diluted DNA extracts, indicating that the low DNA concentration in aDNA extracts makes the investigated molecular sex determination approach unsuitable for assaying ancient material. However, there are several different approaches that may be more suitable for sex determination of mammals in zooarchaeology. The Q-PCR method used could potentially be optimised further towards degraded and ancient template DNA, for example by testing different reagents, master mix setups, and PCR amplification programs. Alternatively high-throughput sequencing methods, in which sufficiently large numbers of reads can be unambiguously mapped to either the X or the Y regions and thus overcome X-homologue-biased PCR amplification, may be a more robust approach to molecular sex determination of degraded DNA samples. Regrettably it has not been possible to successfully determine the sex of the chosen ancient bowhead whale samples, yet the findings here can be informative for other projects wishing to perform molecular sex determination of ancient mammalian samples.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jasrep.2016.11.001.

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