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Sex Determination in the Near Threatened Guadalupe Fur Seal: Molecular Markers and Their Potential Applications

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Abstract

The determination of sex by simple observation is often difficult in marine mammals, even in sexually dimorphic species. Moreover, there is often the practical necessity to determine sex in samples that have been collected without handling or observing the subjects. In these cases, a molecular assay for sex determination is required. We adapted an assay that targets the zinc-finger region and is based on a single primer pair, to the Guadalupe fur seal, a near threatened species that has a very limited breeding range. First, we validated the assay with a sample of pups in which we determined the sex by direct observation during handling; second, we compared it to a more recent assay, based on two different primer pairs and, finally, we evaluated the effect of DNA quantity on its reliability. The assay that we tested produced excellent results and was more reliable than the other one based on two primers. Reliable results were also obtained when only remarkably small quantities of DNA were amplified. These results show the potential use of this molecular assay in case of non-invasive sampling, an overly common situation when dealing with species of problematic conservation status.

Keywords

1. Introduction

Guadalupe fur seals (*Arctocephalus townsendi*) show the smallest estimated total population size among fur

seals [1], and their breeding range is limited to almost a single island, Isla Guadalupe (Baja California, Mexico), as the only other breeding colony, Islas San Benito (Baja California), has a very low productivity (estimated production in 2010 was 18 pups, unpublished data, see also [2]). They were subject to intensive commercial sealing by fur traders from the late 1700s to late 1800s and they were repeatedly declared extinct [3], until few individuals were again spotted on Isla Guadalupe [4]. The Guadalupe fur seal is one of the least known pinniped species, and published literature on the species is very limited. It is considered "near threatened" by IUCN, "threatened" under the US Endangered Species Act, and "depleted" under the US Marine Mammal Protection Act [5]. Therefore, notwithstanding the sustained increasing trend of the Isla Guadalupe population [6], the species is still considered of great conservation concern.

Sex determination of live marine mammals in the field is often problematic when the subjects are not directly handled and the sexes are not morphologically dimorphic [7]. Likewise, sexing juveniles can be particularly difficult even in sexually dimorphic species, as dimorphism in size and secondary sexual traits is often not expressed until adulthood. Recently, due to practical and ethical concerns, there has been an increasing interest in non-invasive techniques to study natural populations [8], in particular when species status is uncertain and presents special conservation concern [9] [10]. Moreover, research in areas like conservation genetics and wildlife forensic is increasingly based on non-invasive samples, whose origins are completely unknown [11]. In fact, non-invasive methods allow studying animals without direct handling, and often also without direct observation of the subjects. In these situations, molecular methods of sex determination proved to be very useful [7], if properly applied [12]. Nevertheless, non-invasive samples, like feces or urine collected from the ground, often generate low quantity of DNA and, therefore, present specific problems for the development of molecular sexing protocols [13].

In this article, 1) we applied the molecular sex determination assay developed by [7] to Guadalupe fur seals; 2) we validated the assay on a sample of individuals for which the sex was determined during handling operations; 3) we compared the assay by [7] to a more recent one developed by [14], and 4) we examined the effect of DNA quantity on the efficiency of the assay.

2. Methods

Samples were collected at Isla Guadalupe (29˚02'N 118˚17'W, IG hereafter) and Islas San Benito (28˚18'N 115˚34'W, ISB hereafter), Baja California, Mexico. At IG, samples were taken from most of the breeding areas of the East coast, where the vast majority of fur seals breed. At ISB, samples were taken only at Isla del Oeste and Isla del Este, as no Guadalupe fur seals were observed on the third island, Isla del Medio.

Field work was carried out during the Guadalupe fur seal breeding season in July 2009 and 2010, at IG and ISB. Skin samples were collected from the flippers of live fur seals using ear notchers, either a) by briefly handling pups or b) by approaching resting non-pup-individuals and taking the sample by surprise. The handling of pups involved either a) a single operator, taking only a skin sample during a very short handling time (<10 s) or b) two or more operators, taking a skin sample and carrying out other procedures during a longer handling time (1 - 3 min). Skin samples (1 - 2 g) were stored in 2.0 mL screw-cap tubes with 95% ethanol, after cutting each sample in two or three pieces, to increase the surface exposed to ethanol. Samples were then kept for a maximum of two weeks in the field, in a cool box and in the shade, without refrigeration (10˚C - 30˚C). Once in the laboratory, samples were kept refrigerated at 4˚C until DNA extraction (more details of sampling and preservation protocols in [15]). We collected skin samples from 342 individuals, including 303 pups (88.6%) and 39 non pups; 299 samples (87.4%) were collected at IG and 43 at ISB.

DNA was extracted from skin samples using the DNeasy Blood and Tissue kit (Qiagen), which uses silica-gel-membrane technology for purification of total cellular DNA without organic extraction or ethanol precipitation. Tissue lysis is followed by selective binding of DNA to the DNeasy membrane. We followed the manufacturer mouse-tail protocol with minor changes to improve tissue digestion and DNA extraction. The changes applied respect to the manufacturer protocol were a longer (overnight) digestion of the tissues in lysis buffer and an additional step of centrifugation of the digested tissues followed by removal of the solid matter to avoid clotting of the spin columns. Extracted DNA was checked for degradation by electrophoresis on 1.0% agarose gels and by NanoDrop ND-1000 (ThermoScientific) measurements of absorbance. Most samples showed a good DNA concentration (100 - 300 ng/µL) and no signs of degradation or contamination.

The first molecular sex determination assay (LGL hereafter) was carried out using the primers presented in

Shaw *et al.* (2003): LGL331 and LGL335. In various species of mammals, this pair of primers amplifies fragments of the Zfx and Zfy introns, with size ranges that go from 912 to 1013 bp for the X fragment, and from 868 to 1278 bp for the Y fragment [7]. The PCR mix was as follows: 15 µL total volume, 1x Promega PCR buffer, 2 mM MgCl2, 0.20 mM dNTPs, 0.02 U/µL Promega Go-Flexi Taq, 0.30 mM forward primer, 0.30 mM reverse primer, 2.0 µL template DNA. Before amplification, the DNA was diluted 1:3 to 1:5 depending on the intensity of the bands produced on the agarose gels, to achieve a DNA concentration of approx 20 - 60 µg/µL. The thermal cycler (Apollo 401, NyxTechnik) program was as follows: 3 min at 94˚C; 35 cycles: 30 s at 94˚C, 30 s at Tm, 40 s at 72˚C; 10 min at 72˚C; hold at 4˚C. To verify the efficiency of the protocol, each sample was amplified by PCR at least three times. If the first three amplifications did not give three valid PCR products, the sample was amplified more times to generate a minimum number of three valid PCR products. PCR products were visualized on 2% agarose gels with ethidium bromide, run at 150 V for a total of 120 min, and pictures of the gels were taken under UV lighting at 60, 90, and 120 min. In most cases, sex was determined on the 120 min picture. Sex determination was as follows (**Figure 1**): 1) a single clear amplification band of about 950 bp = female; 2) two clear amplification bands of about 850 bp and 950 bp = male; 3) no band, or one or two light bands, or non specific amplification with multiple bands = failed samples, to be re-amplified.

The second molecular sex determination assay (ZF hereafter) also targets the zinc-finger region, but is based on two different PCR, one to amplify the X chromosome fragment (ZFX), and one to amplify the Y chromosome fragment (ZFY). The amplification was carried out using the pairs of primers ZFYF/ZFYR and ZFXF/ZFX2RA presented in [14], respectively for the X and Y chromosomes. The PCR mix was: 15 µL total volume, 1x Promega PCR buffer, 0.20 mM dNTPs, 0.02 U/µL Promega Go-Flexi Taq, 0.30 mM forward primer, 0.30 mM reverse primer, 2.0 µL template DNA. The MgCl$_2$ concentration was 1.5 mM for ZFX, and 2.0 mM for ZFY. The thermal cycler program was: 3 min at 94˚C; 35 cycles: 30 s at 94˚C, 30 s at Tm, 40 s at 72˚C; 10 min at 72˚C; hold at 4˚C. The annealing temperature was 55˚C for ZFX, and 57˚C for ZFY. PCR products were visualized on 2% agarose gels with ethidium bromide, run at 150 V for a total of 60 min, and pictures of the gels were taken under UV lighting at 30 and 60 min. In most cases sex was determined on the 60 min picture. The sex determination was as follows: 1) samples showing a clear amplification band for ZFX but not for the ZFY were classified as females; 2) samples showing a clear amplification band for both fragments were classified as males; 3) samples showing lack of amplification for the ZFX, or dubious amplification for one or both frag-
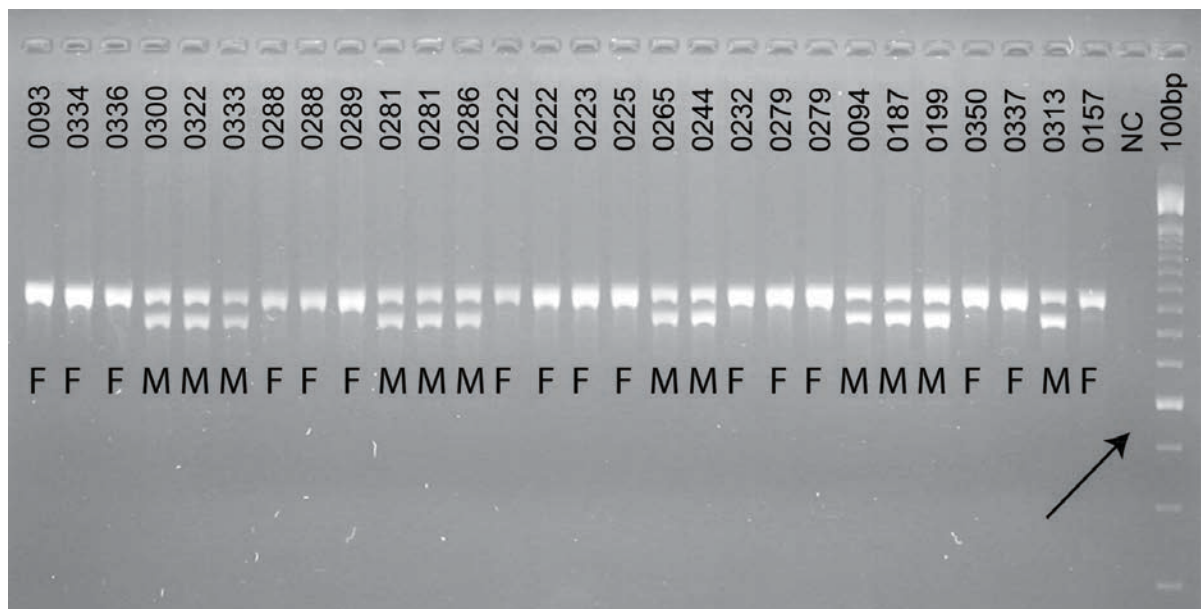

Figure 1. Electrophoresis gel of fragments amplified with primers from Shaw *et al.* (2003), using DNA extracted from Guadalupe fur seal skin samples. Sex of the individuals is indicated at the bottom of each gel lane (F = female, M = male). 2% agarose gel, run at 150 V for 120 min. Individual IDs are reported below the wells; last lane on the right is a 100 bp size standard (Track It 100 bp DNA Ladder, Invitrogen); the most intense fragment (indicated by the arrow) is 600 bp and fragments are spaced by 100 bp; NC = negative control.

ments, or non-specific amplification bands, were not sexed.

Amplification quality (good/average/bad) and sex were attributed to all samples by three experienced operators that scored the products of the PCRs of each sample upon visual inspection of the gel pictures. Each operator scored the gel pictures separately to guarantee between-operator independence and without knowing the identity and sex assigned to each fur seal in the field. The large number of samples, the temporal spacing between scoring sessions of each PCR belonging to the same sample and the recording of sex in a separate spread sheet for each scoring session, guaranteed within-operator independence of the scoring. For the LGL method, since we obtained three sex scorings, one for each of the three PCRs, for each operator and each sample, we calculated an "operator sex" for each sample, combining the three operator's sex assignments. If the scores were not in agreement, the "operator sex" was that attributed on two of the three scores. Finally, for both methods, for each sample we obtained a consensus sex by combining the "operator sex" of the three operators. As above, if the operators were not in agreement, the consensus sex was the sex attributed by two of them. To assess the agreement between the two sexing methods (LGL and ZF), we calculated the kappa coefficient of agreement and its 95% confidence limits [16]. Statistical tests were carried out with the exact option in StatXact 4 (Cytel Corporation).

To understand if the LGL method could be applied also to samples with scarce quantity of DNA, we validated the method using different concentrations of DNA. We started with two samples with similar good DNA concentration (100 µg/µL), one of a sure female and one of a sure male, made serial dilutions from 2:1 to 1:8192, amplified all dilutions, and scored the sex as described above.

The research strictly adhered to the Society for Marine Mammalogy guidelines for treatment of marine mammals in field research [17] and to the American Society of Mammalogists guidelines for the use of wild mammals in research [18]. The research was carried out under research license granted by the Mexican government, and in respect of the local legislation for research on protected species and marine mammals.

3. Results

3.1. Performance and Validation of the LGL Assay

The number of PCRs required to get three amplified products suitable for sex attribution was variable. Out of 342 samples, 80.1% required three PCRs, 8.8% four, and 11.1% five. All operators were able to score at least three good PCRs for each sample. When the PCR products were analyzed for sex assignment, two operators showed 100% within-operator concordance. The third operator showed 99.4% concordance, as in two cases he attributed a different sex to different PCRs of the same sample. Both cases regarded male samples: the first fur seal was assigned female in one and male in three PCR, respectively; the second fur seal was assigned female in two PCR and male in other two. This failure in recognizing the band of the Y chromosome was possibly due to the quality of the PCR, as the amplifications were scored as scarce by all three operators. The overall concordance among the three operators was 99.7%, as the "operator sex" was in agreement in all cases except one. Sex was successfully assigned to 341 fur seals (53.7% males and 46.3 females), and only one sample remained unassigned (0.3%). For 331 of the samples (97%) the first PCR was enough to attribute the sex correctly. The sex of 214 pups scored with the LGL assay was determined in the field during handling operations carried out by two or more operators. Almost always (98.1% of the time) observations were consistent with the LGL assignment (kappa = 0.963, 95% confidence interval = 0.926 - 0.999) and discrepancy was found in only four seals: three classified as females in the field were classified as males with LGL, and one classified as male in the field was classified as female with LGL. When only one operator collected the skin sample and recorded the animal sex in the field (n = 73 pups), observations and LGL assignment gave different results in 13.7% of the cases (kappa = 0.725, 95% confidence interval = 0.567 - 0.883), and the mismatches regarded six LGL females and four LGL males. The proportion of sex mismatches was significantly higher for one operator handlings (Barnard unconditional test for the difference in two proportions: exact P = 0.0001).

3.2. Comparison with the ZF Assay

The amplification with the ZF assay proved to be more difficult than with the LGL assay. To achieve a single sex scoring, a minimum of two PCRs are required (one for the X chromosome fragment and one for the Y chromosome) but, in practice, more PCRs were required. For ZFX, 68.4% of the samples required one PCR,

24.9% two, and 6.7% three. For ZFY 81.3% of the samples required one PCR, 18.1% two, and 0.6% three. Moreover, the results from the assay were sometimes ambiguous, as we were not sure if we had lack of amplification of ZFY because the fur seal was a female, or because of problems with the PCR itself. All together, we assigned sex to 321 fur seals, while 21 samples (6.1%) could not be safely assigned. Although small (5.8%), the difference in the proportion of non sexed samples between the LGL and the ZF assays was statistically significant (Barnard unconditional test for the difference in two proportions: exact P = 0.0001; 95% exact confidence interval of the difference = 2.6% - 9.6%). The ZF assay confirmed that four of the sexes attributed in the field that were identified as non correct by the LGL assay were in fact not correct (see previous results section). In 321 samples that were sexed both by LGL and ZF, the two methods agreed in 320 of the cases (99.7%). In only one case, the ZF assay assigned female to a fur seal that was classified as male in the field, and that gave two clear amplification bands in the LGL.

3.3. The Effect of DNA Template Quantity

When we diluted the DNA, the LGL assay produced viable and correct sex identification at a wide range of dilutions (**Figure 2**). PCR at 1:1 and 2:1 concentrations were not successful, possibly due to inhibition. In case of male samples, sex was clearly attributable at dilutions up to 1:2048 in all three gel pictures (60, 90, 120 min), although lighter PCR bands were obtained from 1:256 dilutions. The two bands were visible, although very light, up to dilution 1:8192 in the 60 min picture. In case of female samples, sex was clearly attributable up to 1:4096 in the 60 min picture, and up to 1:1024 in the following pictures, although amplification became lighter at dilutions higher than 1:512.
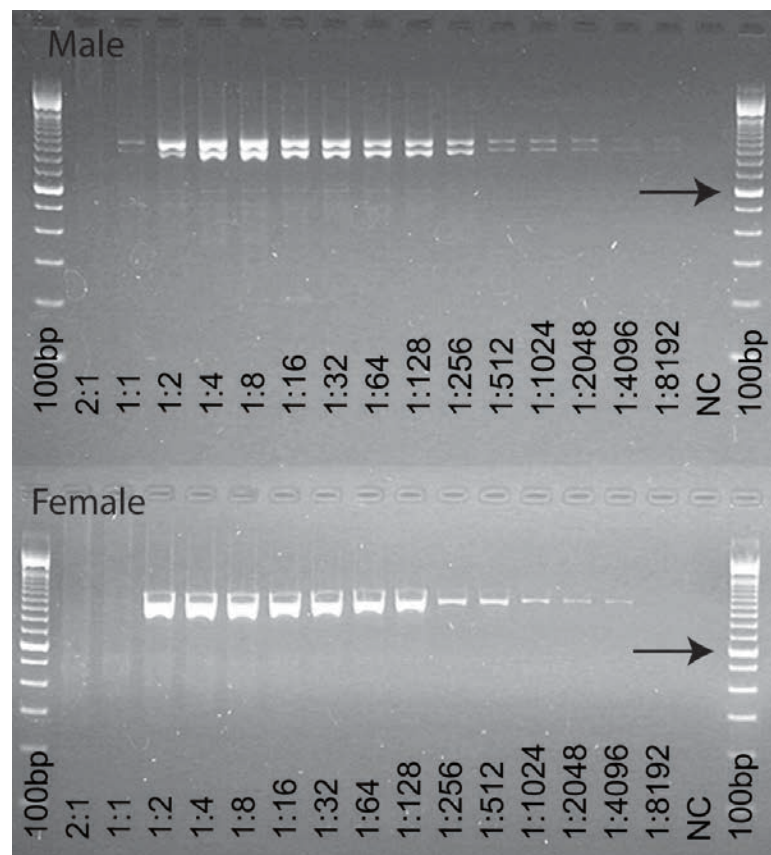

Figure 2. Electrophoresis gel of fragments amplified with the Shaw *et al.* (2003) primers using serial dilutions (from 2:1 to 1:8192 of the original sample) of DNA extracted from Guadalupe fur seal skin samples. Top panel: male. Bottom panel: female. Original DNA concentration was approximately 100 µg/µl for both samples. 2% agarose gel, run at 150 V for 60 min. First and last lanes on both panels are 100 bp size standards (TrackIt 100 bp DNA Ladder, Invitrogen); the more intense fragment is 600 bp (indicated by the arrows) and fragments are spaced by 100bp; NC = negative control.

4. Discussion

The attribution of sex in marine mammals is often complex, even when sexual dimorphism is present [7]. Many pinniped species show an absent or small sexual dimorphism, which is usually related to low polygyny in their mating system [19]. Even in species where the opportunity for sexual selection favored a strong sexual dimorphism, both in size and secondary sexual traits, the sexes are usually monomorphic during the first years of life [20], and the development of sexual secondary traits is slow and gradual (e.g., the elephant seal proboscis[21]). Therefore, in many cases the external morphology cannot be easily used to sex individuals belonging to the first age classes and capture is the only way to determine sex. Nevertheless, capture is often not possible, in particular when populations or species have a critical conservation status [22] and a non-invasive approach is required.

In fact, non-invasive methods are often the only possible approach to study rare and elusive species [9]. Moreover, in accordance to increased interest for research ethics issues [23] [24], they are now also applied to pinnipeds that are more common and often not elusive at all [25] [26]. Strict non-invasive molecular methods, in which research is conducted by recovering samples from the substrate, are enjoying an increasing momentum [8]. These methods are now applied also to pinnipeds that used to be invasively sampled in the past [27]. All together, effective non-observational sexing methods are strongly needed, and those based on amplification of sex-linked molecular markers are a viable option [7], although careful application and validation are required [12].

In fur seals, males grow faster than females, but the difference in growth rate becomes clearly evident only around puberty (about 4 years of age), when males start enjoying a growth spurt that greatly increases the size dimorphism [20]. Guadalupe fur seals, in fact, show a clear sexual dimorphism [28] and adults can be sexed from the distance. Nevertheless, up to age 3 or 4, males and females are very difficult to tell apart by simple observation. Guadalupe fur seals are somehow elusive, of uncertain conservation status, and with a scarce tolerance to human presence and disturbance. In spite of this, knowing the fur seal haul-out pattern, urine, feces or fur samples can be easily collected from the rookeries with minimal disturbance, when the fur seals are at sea. Non-invasive methods are, therefore, the best approach to study this species and an effective molecular assay to sex them represents a valuable addition to the conservation biologist toolbox.

We showed that the well established LGL molecular assay by [7] can be easily adapted to Guadalupe fur seals. The assay provides a safe determination of the individual sex, using a single primer pair and visualizing the product on agarose gels. The whole procedure requires only one PCR per sample, can be carried out in laboratories with limited resources, and does not require the knowledge of advanced molecular biology techniques. In fact, for validation purposes, we carried out at least three PCR per sample but 96% of the times, the first PCR was sufficient to identify the individual sex.

When compared with the more recent ZF assay proposed by [14], LGL was more successful, as it produced a significantly smaller proportion of cases of undetermined sex. Moreover, the ZF assay is based on two different amplifications, with the risk of generating some ambiguity. In fact, the lack of amplification in the Y chromosome could be either the product of a female amplification or a failure in the amplification itself. The LGL assay does not present this limitation, as the co-amplification of a fragment in both sex chromosomes allows a PCR failure to be detected when the X chromosome band is absent [7].

The reliability of the LGL assay has been questioned, as it could be subject to the larger allele dropout (due to competition) and wrongly assign female to a male [14]. In addition, the fragments amplified are rather large and, therefore, might present amplification problems when quantity and quality of the DNA template are low [7]. In general, an assay targeting smaller fragments should be, in fact, more effective when applied to non-invasive samples containing degraded DNA [29] [30]). However, we believe that these concerns are overemphasized. In our LGL assay, the amplifications of the two fragments, as visually evaluated on agarose gels, were rather similar; when different, the larger fragment showed a more intense band, giving no evidence of a competition against the larger allele. Moreover, the LGL assay is robust to operator and PCR mistakes, as the amplification of the X chromosome fragment is an intrinsic control of the PCR amplification success.

We applied the sex assay on skin samples collected with a low-invasive method, which involved no or short handling, but nonetheless required approaching the animals and produced short lasting pain. In addition, we showed that the assay worked well with very low DNA concentrations, even lower than the concentration usually found in Guadalupe fur seal urine samples collected from the ground (unpublished data). For this reason, although we have not tried the procedure on completely non-invasive samples, we believe that it would be also

suitable for low-quality samples, providing a useful method to assign sex to samples collected non-invasively. All together, we think that the LGL assay is a simple and effective method to sex Guadalupe fur seals and could be safely used when direct sexing of the individuals is impossible or uncertain. The proposed method is an important addition to the molecular toolbox needed to effectively study the population structure of Guadalupe fur seal, a near threatened species that has currently a very limited breeding range. The only significant breeding area of Guadalupe fur seal is Isla Guadalupe. The Islas San Benito shelter a rather large non breeding population of Guadalupe fur seal, but breeding is limited to a small number of pups produced each year [2]. Until now, the sex and age class composition of the non breeding individuals at Islas San Benito is unknown. Molecular sexing using the proposed method will help the determination of the structure of this incipient breeding population, greatly increasing our understanding of the migration of individuals from the main breeding colony of Isla Guadalupe.

Acknowledgements

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