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Pvc74 is sex linked in elephant seals (genus *Mirounga*)

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The identification of sex-linked loci has a theoretical and an applied value (Hurles and Jobling 2001), and sex-linked markers can be more useful than autosomal markers to address sex-specific aspects of behavioral and molecular ecology (Erler *et al.* 2004). Sex-linked loci permit identifying sex in samples collected noninvasively and in species in which the sex cannot be easily determined in the field (Shaw *et al*. 2003), facilitate parentage analysis in species with large clutches (Walker *et al.* 2005), and help in confirming sex-biased dispersal and determining male effective population size (Eriksson *et al.* 2006). One of the first sex-linked loci identified in pinnipeds was Pvc74, a microsatellite locus originally cloned by Coltman *et al*. (1996) in the harbor seal (*Phoca vitulina concolor*). These authors found no heterozygous males in 25 males and 10 heterozygous females in 25 females, concluding that the locus

was sex-linked. In a recent note, Herreman *et al.* (2008) presented a study of harbor seal (*Phoca vitulina richardii*), in which they genotyped Pvc74 for 171 individuals, finding only three alleles, two of which were present at very low frequency (frequency of the most frequent allele = 0.9795). A total of five heterozygous individuals were observed, two of which were males. Despite the weak evidence, the authors concluded that Pvc74 is, in fact, not sex linked in the harbor seal, and cast doubts on the viability of Pvc74 as a sex-linked marker in pinnipeds at large. In this short note, we show that Pvc74 is sex linked in both species of elephant seals (genus *Mirounga*).

Northern elephant seals (*M. angustirostris*, NES hereafter) were sampled during the 2004/2005 and 2005/2006 breeding seasons in the San Benito Islands (Baja California, Mexico). We sampled a total of 426 NES, 103 males (24.2%) and 323 females, all marked and individually identified. Southern elephant seals (*M. leonina*, SES hereafter) were sampled at Sea Lion Island (Falkland Islands) between 1996 and 2004. We sampled 222 SES, 120 males (54.1%) and 102 females, again all identified. We restricted our analysis to (1) adult breeders, because sex can be attributed with 100% reliability due to the clear sexual dimorphism (NES and SES), and (2) weaned pups that were sexed at least three times during weighing operations (SES only). Skin samples were collected from unrestrained adults and physically restrained weanlings by piercing the interdigital membrane of the rear flippers using ear notchers. Samples were preserved in 2.0 mL microtubes with 95% ethanol at room temperature until processing (Fabiani *et al.* 2004). DNA was extracted from skin samples using the DNEasy Blood and Tissue kit (Qiagen, Valencia, CA), following the manufacturer mouse-tail protocol with minor changes to improve tissue digestion (overnight digestion in lysis buffer) and DNA extraction (centrifugation of the digested tissue and removal of the solid matter to avoid clotting of the spin columns). Extracted DNA was checked for degradation by electrophoresis in 1.5% agarose gels and by NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA) measurements of absorbance. All samples showed a good DNA concentration (mean = 190 ng/μL) and no signs of degradation or contamination (as from NanoDrop absorbance graphs). Amplification by PCR was carried out using the "universal tag" method of Schuelke (2000). The sequences of the Pvc74 PCR primers were as follows: Forward = 5′—CCATCTGTGTCCTCTGATA–3′, Reverse = 5′—TGTAAAACGACGGCCAGTCTGATATTCCATGTCTGAGATA–3′ (reverse modified from Coltman *et al.* (1996) by adding a M13 5′ tail, as from Shuelke 2000). We used a standard PCR mix (15 μL total volume, 1× PCR buffer (Promega, Madison, WI), 2 mM magnesium, 0.2 mM dNTPs, 0.023 U/μL Go-Flexi Taq (Promega), 0.30 mM forward primer, 0.075 mM reverse primer with M13 universal tag tail, 0.30 mM NED labeled M13 universal tag, 2.5 μL template DNA) and PCR program (3 min 94°C, 30 cycles: 30 s 94°C, 30 s 53°C, 40 s 72°C, followed by eight cycles: 30 s 94°C, 30 s 47°C, 40 s 72°C; 10 min 72°C, hold 4°C) on an Apollo ATC401 thermal cycler (NyxTechnik, San Diego, CA). PCR products were resolved on an ABI 3730XL Automated Sequencer (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) using a GeneFlow 625 size standard (ChimerX, Milwaukee, WI), and genotyped using GeneMarker software (version 1.85, SoftGenetics, State College, PA). Genotypes were validated using FLEXIBIN (Amos *et al.* 2007) and MICROCHECKER (Van Oosterhout *et al.* 2004). To assess the

presence of genotyping errors, we randomly selected 57 NES samples (13.4% of the original samples) and 23 SES samples (10.4% of the original samples) and repeated the whole genotyping procedure (PCR amplification, resolution of PCR products on automated sequencer, determination of genotypes in GeneMarker, validation of genotypes). Repetition of amplification and scoring in a 10%–15% subset of the original samples has been suggested as an effective way to estimate genotyping error rate (Selkoe and Toonen 2006). The genotypes determined during repetition were in full agreement with the original genotypes in all cases (genotyping error = 0%). Deviation from expected Hardy–Weinberg genotype frequencies and heterozygote deficiency were tested in GenePop4 (Rousset 2008) using the exact option for NES, and the Markov Chain option (with default parameters) for SES, because it was not computationally feasible to use the exact option in case of SES, due to the large number of alleles found.

Statistics by species and sex of the Pvc74 alleles observed are presented in Table 1. In the 426 NES genotypes, we identified only three Pvc74 alleles (generating five possible genotypes—three homozygous and two heterozygous), with approximate sizes of 213 (38.4%), 219 (19.8%), and 225 (41.8%) base pairs (excluding the universal tag). In the whole NES sample, 49.8% of the individuals were heterozygous and there was a clear heterozygote deficiency (Exact test: $P = 0.0000$). In the 323 females, all genotypes were observed, 66.3% of the individuals were heterozygous, and there was no evidence of heterozygote deficiency (Exact test: $P = 0.7349$). On the contrary, in the 103 males just the three homozygous genotypes were observed, and no individual was heterozygous. In the 222 SES genotyped, we identified 16 alleles (base pair range = 187–221). In the whole sample, 64.4% of the individuals were homozygous, and there was a strong heterozygote deficiency (Markov chain test: $P = 0.0000$, $SE = 0.0000$). Although the 102 females showed heterozygote deficiency (Markov chain test: $P = 0.0017$, $SE = 0.0012$), 77.5% of the individual were heterozygous. On the contrary, all 120 males genotyped were homozygous.

In their study of the harbor seal, Herreman *et al.* (2008) concluded that "future studies should focus on identifying more appropriate sex-linked primers for seals." We have presented strong evidence that Pvc74 is a sex-linked locus in both species of elephant seals, and we have shown that their general conclusion is not warranted. Although our study is not directly relevant to the specific results obtained in harbor seals by Herreman *et al.* (2008), we would expect a marker that is sex linked in elephant seals to be sex linked also in the closely related harbor seal. Notwithstanding most previous studies of Pvc74 that proposed sex linkage involved a rather small number of individuals, their conclusions were rather clear-cut. In the original paper about harbor seals by Coltman *et al.* (1996), no heterozygous male was found in a sample of 25. The locus was also classified as sex linked in the Mediterranean monk seal (*Monachus monachus*) by Pastor *et al.* (2004), who found no heterozygous males in a sample of 23. Establishing whether Pvc74 is actually sex linked or not in harbor seals will require a replication of Herreman *et al.* (2008) study. We would like to note that harbor seals show a modest degree of sexual dimorphism (Lindenfors *et al.* 2002), and that detection of sex in the field is not always easy.[1] This, combined with

[1]Personal observation from demographic surveys carried out by the authors S. Sanvito, Y. Schramm and F. Galimberti.

Table 1. Statistics of the observed Pvc74 alleles. N = sample size, number of individuals genotyped; Size = size range of observed alleles, in base pairs; n = number of alleles; % het. = percentage of individuals with heterozygous genotype; H_o = observed heterozygosity; H_e = expected heterozygosity, calculated with the unbiased formula of Nei (1978) as $H_e = \frac{2n[1-\sum_{i-1}^{n} p_i]}{(2n-1)}$, where p_i is the relative frequency of allele i; AD = allele diversity, calculated as the Shannon index (Shannon 1948) of the relative frequencies of the alleles, $AD = -\sum_{i-1}^{n} p_i \, ln\,(p_i)$; n_e = number of effective alleles, calculated following Morgante *et al.* (1994) as $n_e = \frac{1}{\sum_{i-1}^{n} p_i^2}$.

Species	Class	N	n	Size	% het.	H_o	H_e	$H_o - H_e$	$\frac{H_e - H_o}{H_e}$	AD	n_e
NES	All	426	3	213–225	50.2	0.502	0.639	−0.137	0.214	1.053	2.767
	Females	323			66.3	0.663	0.636	0.027	−0.043	1.047	2.740
	Males	103			0.0	0.000	0.651	−0.651	1.000	1.069	2.843
SES	All	222	16	187–221	35.6	0.356	0.901	−0.545	0.605	2.450	9.934
	Females	102			77.5	0.775	0.894	−0.119	0.133	2.325	9.079
	Males	120			0.0	0.000	0.904	−0.904	1.000	2.480	10.018

possible data recording errors, can be a source of uncertainty that should be taken into account. Unfortunately, although Herreman *et al.* (2008) stated that samples were obtained from live-captured harbor seals, they did not mention how certain was the sexing of the sampled individuals.

An advantage of Pvc74 as a sex-linked marker is the small size of the PCR products. Many sex-linked markers in use for marine mammals involve larger PCR products (above ~850 bp; Shaw *et al.* 2003, Curtis *et al.* 2007) that may be more difficult to amplify by PCR than Pvc74. This aspect can be particularly relevant when dealing with degraded or low-quality samples (Fischbach *et al.* 2008), for example, when carrying out forensic examination of stranded seals, or when collecting samples in wild populations using noninvasive methods. The usefulness of each marker should be evaluated on a per-goal and per-species basis. Pvc74, by itself, is useful just for the identification of heterozygous females while the safe sexing of other individuals will require a second marker. The use of more than one sex-linked locus may greatly improve the confidence in the sexing (Robertson and Gemmell 2006), in particular when analyzing samples obtained from unknown source (*e.g.*, in wildlife forensic, Alacs *et al.* 2010), or samples that present specific PCR amplification problems (*e.g.*, in the case of fecal samples, Reed *et al.* 1997).

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