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GENOTYPING OF PSEUDOHERMAPHRODITE POLAR BEARS IN NUNAVUT AND ADVANCES IN DNA SEXING TECHNIQUES

LINDSEY E. CARMICHAEL,* PETER KRIZAN, SUSAN POLISCHUK BLUM, AND CURTIS STROBECK

Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada (LEC, CS)
Department of Sustainable Development, Nunavut Wildlife Service, P.O. Box 1000 Station 1170, Iqaluit, Nunavut Territory X0A 0H0, Canada (PK, SPB)
Present address of PK: P.O. Box 2294, Inuvik, Northwest Territories X0E 0T0, Canada
Present address of SPB: Office of Research Services (Vice President Research Offices), University of Saskatchewan, Room 3402, 110 Gymnasium Place, Box 500 Railway Post Office, Saskatoon, Saskatchewan S7N 4J8, Canada

Female pseudohermaphroditism is characterized by gonads consistent with chromosomal sex combined with ambiguous, masculinized external genitalia. Recognized in many mammals, this condition results from fetal exposure to androgens that can be embryonic, maternal, or environmental in origin. Female pseudohermaphrodite black and brown bears (*Ursus americanus* and *U. arctos*) from Alberta, Canada, and polar bears (*U. maritimus*) from Svalbard, Norway, have been identified. Recent population surveys in Nunavut, Canada, led to the discovery of 11 additional female pseudohermaphrodite polar bears. Each bear was screened for the presence of sex-determining region-*Y* (*Sry*) and amelogenin-*Y* (*AMELY*) genes as indicators of Y-chromosome DNA. One bear possessed both genes, implying that trisomy or a chromosomal rearrangement may account for her virilized phenotype. Preliminary data suggested that *Sry* was also present in the other 10 bears; further testing disproved that result, revealed an important source of error when using polymerase chain reaction (PCR) to screen for the *Sry* gene, and led to the development of new amelogenin primers that provide superior sex information for bears. Ultimately, these extensive screens also supported the conclusion that 10 of 11 morphologically abnormal individuals may possess no genuine male-specific DNA. Therefore, nongenetic mechanisms such as maternal tumors, freemartinism, or endocrinological effects of environmental contaminants may also influence the development of the female pseudohermaphrodite phenotype in Nunavut polar bears.

Key words: amelogenin, female pseudohermaphrodite, Gulf of Boothia, M'Clintock Channel, molecular sex test, Nunavut, polar bear, *Ursus maritimus*, *Sry*, Y chromosome

Female pseudohermaphroditism is characterized by gonads consistent with chromosomal sex (i.e., ovaries and an XX genotype) combined with ambiguous, masculinized external genitalia (Polani 1981). In humans, male pseudohermaphroditism results from mutation in any of several single genes, whole chromosomal abnormalities or rearrangements, or the influence of environmental agents. Female pseudohermaphroditism is less common and is usually attributed to fetal exposure to embryonic, maternal, or environmental androgens (Polani 1981).

Although less studied, pseudohermaphroditism has also been observed in other mammals, including rhesus monkeys (*Macaca mulatta*—Phoenix and Chambers 1982; Wilen et al. 1977); rats and mice (*Rattus norvegicus* and *Mus musculus*—Ikadai et al. 1988; Murphy and O'Shaughnessy 1991); sheep (*Ovis aries*—Smith et al. 2000; Verberckmoes et al. 2002); goats (*Capra hircus*—Just et al. 1994; Pailhoux et al. 1994; Yadav et al. 1993); horses (*Equus caballus*—Bouters et al. 1975; Milliken et al. 1995); bison (*Bison bison*—Lott et al. 1993); cattle (*Bos taurus*—Cribiu et al. 1989); dogs (*Canis lupus familiaris*—Nemzek et al. 1992; Peter et al. 1993); hyenas (*Crocuta crocuta*—Glickman et al. 1992; Yalcinkaya et al. 1993); and black, brown, and polar bears (*Ursus americanus*, *U. arctos*, and *U. maritimus*—Cattet 1988; Wiig et al. 1998). Among 4 female pseudohermaphrodite black bears, virilism (development of male secondary sex characteristics) ranged from mild clitoral hypertrophy to replacement of the clitoris and vulva with a large, penis-like structure containing urethra and baculum. This latter individual was accompanied by cubs, was lactating, and bore placental scars. Mating and parturition were

* *Correspondent: lec@ualberta.ca*

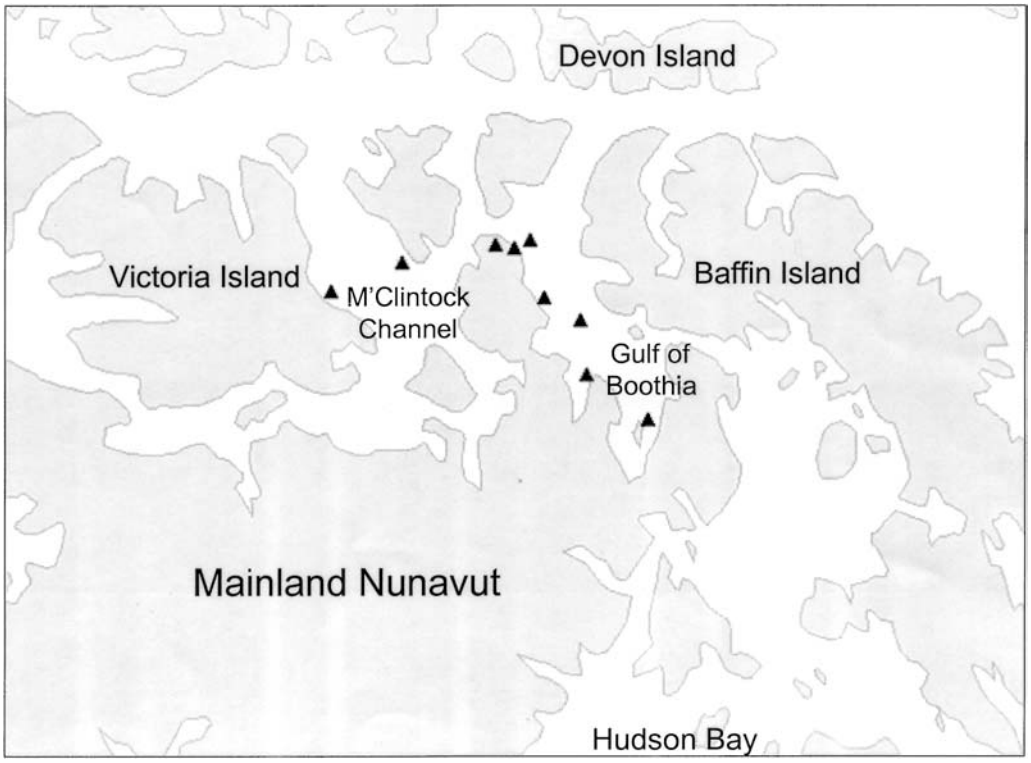

Fig. 1.—Capture locations of female pseudohermaphrodite polar bears in the Gulf of Boothia (70°N, 91°W) and M'Clintock Channel (72°N, 102°W) populations of Nunavut, Canada.

therefore assumed to have occurred through the urethra, the only external opening, which was internally connected to the vagina (Cattet 1988).

Female pseudohermaphrodite polar bears were first recognized in Svalbard, Norway, by Wiig et al. (1998). In 1999 and 2000, we identified 11 females with similar phenotypes in the Gulf of Boothia and M'Clintock Channel populations of Nunavut, Canada (Fig. 1). Wiig et al. (1998) did not find male DNA in Svalbard, Norway, pseudohermaphrodites; however, chromosomal abnormalities have been observed in virilized females of other species (Bouters et al. 1975; Cattet 1988; Verberckmoes et al. 2002).

To determine whether Nunavut, Canada, pseudohermaphrodite polar bears possess Y-chromosome DNA, we tested for the presence of male-specific markers *Sry* (the testis determining factor—Berta et al. 1990; Koopman et al. 1991; Sinclair et al. 1990) and *AMELY* (Y-chromosome variant of the pseudoautosomal tooth enamel gene amelogenin—Ennis and Gallagher 1994; Gibson et al. 1992). Amelogenin results were always consistent on replication, whereas recovery of *Sry*-sized fragments varied between trials. This variability prompted further investigation into the reliability of DNA-based sex-testing methods and refinement of techniques for assessing sex in bears with unknown or ambiguous genders.

Materials and Methods

Field methods.—During ongoing field studies of polar bear populations in Nunavut, Canada, in the Gulf of Boothia (70°N, 91°W) and M'Clintock Channel (72°N, 102°W), we observed a number of females displaying apparent virilism in the form of hypertrophic clitorides. This phenotype was first seen in 1999; in 2000, all females handled were checked for genital abnormalities. Polar bears were anesthetized by darting from a helicopter with tiletamine/zolazepam as described elsewhere (Zoletil, Virbac Australia, Peakhurst, Australia—Stirling et al. 1989). Capture location, standard morphometric body measurements, and blood and tissue samples were collected for all bears. New captures were assigned unique identifying numbers on lip tattoos and ear tags. Experienced field workers recognized pseudohermaphroditism in bears displaying an external vaginal opening and an obviously enlarged clitoris, as originally described by Wiig et al. (1998).

Screens using polymerase chain reaction (PCR).—Tissue or blood samples from pseudohermaphrodite and normal individuals were collected during field studies of wild, live bears. Eleven female pseudohermaphrodites were observed, all *U. maritimus*. Samples from 2 normal male and 5 normal female Canadian black, brown, and polar bears were used as controls. These 21 normal individuals were originally sampled by other workers for independent population genetic studies; for some bears, only extracted DNA was available because tissue samples had been previously exhausted.

All samples were stored at −80°C before extraction (DNeasy tissue kits, QIAGEN, Hilden, Germany). Amplifications were performed in a total volume of 15 µl using a thermocycler (Perkin Elmer 9600, Wellesley, Massachusetts); fluorescently labeled products were diluted and mixed with an in-lane size standard for isolation and visualization on a DNA sequencer (377 DNA sequencer, Applied Biosystems Inc., Foster City, California; 4.5% denaturing polyacrylamide gels, run at 2.3 kV for 3 h). Genescan 3.1 and Genotyper 2.0 software was used to determine fragment sizes and to measure peak heights (Applied Biosystems Inc). Detailed descriptions of reaction mix and cycling conditions for all PCR-based screens are provided in Appendix I.

PCR-based screens: pseudohermaphrodites.—Presence of *AMELY* was assessed in all 11 pseudohermaphrodites using PCR with primers SE47 and SE48 (Gibson et al. 1992). These primers were designed to amplify exon 5 of *AMELY* and *AMELX* (the longer X chromosome variant of the amelogenin gene) from domestic cattle (Ennis and Gallagher 1994) but are also informative for most bears (Yamamoto et al. 2002; M. Proctor, pers. comm.).

We tested for the presence of the *Sry* gene using SRY41F and SRY121R primers designed to amplify its conserved high-mobility group (HMG) box (Taberlet et al. 1993). Reactions were multiplexed with primers for the pseudoautosomal, zinc finger protein genes, *Zfx–Zfy*, as a control for PCR failure, following the method described by Woods et al. (1999).

During initial screens, a fragment appearing to be *Sry* was observed in all female pseudohermaphrodites. When samples were retested under more stringent conditions, this band was observed in only 1 individual. We therefore suspected that the *Sry* primers were cross-amplifying a different gene in females, hereafter called pseudo*Sry*.

PCR-based screens: characterization of pseudoSry amplification in normal bears.—To determine when primers SRY41F and SRY121R cross-amplify an *Sry*-sized fragment from females (pseudo*Sry*), we performed series of PCR reactions under varying conditions (Appendix I). The 21 known, phenotypically normal individuals described above and the pseudohermaphrodite Bear 1 were included in these screens. Due to limited material, some bears could not be tested under all conditions. We also tested for the presence of *AMELY* in all phenotypically normal controls.

Sequencing: Sry and pseudoSry.—We sequenced *Sry*-sized products from all pseudohermaphrodites and normal control bears (32 bears total). Template (a PCR product derived from genomic DNA for direct sequencing) was amplified using conditions that most consistently produced pseudo*Sry* in females. Reaction products were electrophoresed on 12% nondenaturing polyacrylamide gels at 80 V for 22 h. Gels were stained with ethidium bromide and target bands excised with a razor. Sufficient material for sequencing could not be recovered from these gel slices when the Qiaex II kit (QIAGEN) was used according to manufacturer's directions. Higher yields were obtained by combining the Qiaex II protocol with the Qiaquick protocol (QIAGEN, see Appendix I).

Both strands of each fragment were sequenced using the amplifying primers and a dRhodamine sequencing kit (Applied Biosystems Inc.), then visualized on a 377 DNA sequencer. Data were analyzed using Sequencing Analysis 2.1.1 and Sequence Navigator 1.0 software (Applied Biosystems Inc.).

Primer design: amelogenin-based sexing of carnivores.—Samples were obtained from 2 known male and 2 known female black bears, brown bears, polar bears, wolves (*Canis lupus*), arctic foxes (*Alopex lagopus*), Canada lynx (*Lynx canadensis*), and Weddell seals (*Leptonychotes weddellii*). Samples of 1 known male and 1 known female were obtained from badgers (*Taxidea taxus*), otters (*Lontra canadensis*), and wolverines (*Gulo gulo*).

Amelogenin exon 5 was amplified using primers SE47 and SE48 with 5′ M13 universal extensions. We observed a single band in all female bears, 2 bands in all male bears, and single bands in males and females of all other species. Purification, sequencing, and analysis of all fragments followed methods described above with 1 exception: dRhodamine sequencing reactions used M13 universal primers.

Sequences for all species were aligned using the bovine *AMELX* sequence as a reference (Genbank accession number S44231—Gibson et al. 1992). We observed conserved sequence blocks flanking the ursine *AMELY* deletion; Oligo 4.0 software (Molecular Biology Insights, Cascade, Colorado) was used to design several sets of primers that would anneal to these regions. We tested various combinations of new primers and new primers paired with bovine primers SE47 or SE48 for PCR amplification of sex-specific products. Best results in determining sex for bears were obtained using primers CST1834, CCGTCCAGCCACAGCCTCACCAG; CST1836, TTCCAGCCCCAGCCCGTCCAG; CST1837, GCTTCCAGAGG CAGGTCAGGA; and the following PCR conditions for DNA from tissue: 0.16 μmol each primer, 0.2 mmol deoxynucleotide triphosphates (dNTPs), 1.5 mmol magnesium chloride ($MgCl_2$), 1× PCR buffer (50 mmol KCl, 10 mmol Tris-HCl, pH 8.8, 0.1% Triton X100), 0.144 U Taq, and 0.6 ng genomic DNA (total volume 15 μl). Cycling conditions were 94°C, 1 min (94°C, 30 s; 58°C, 20 s; 72°C, 5 s) for 3 cycles; (94°C, 15 s; 58°C, 20 s; 72°C, 1 s) for 27 cycles; 72°C, 30 min.

Hair samples from 40 bears (collected previously by M. Proctor for an independent, noninvasive remote-censusing project) that could not be sexed using bovine primers were screened under the following conditions: 0.16 μmol primer CST1834, 0.24 μmol each of primers CST1836 and CST1837, 0.2 mmol dNTPs, 1.5 mmol $MgCl_2$, 1× PCR buffer, 1.44 U Taq (i.e., a 10-fold increase), and 0.25 ng genomic DNA. Three additional cycles (30 compared with 27 cycles above) were performed.

Results

Pseudohermaphrodite polar bear phenotype.—Eleven polar bears with unusual genitals were captured in M'Clintock Channel and the Gulf of Boothia, Nunavut, Canada, in 1999 and 2000 (Fig. 1). Phenotype consisted of an unusually large clitoris, similar to that observed by Wiig et al. (1998). In the most extreme case, Bear 1, the clitoris was approximately 10 mm long by 7.5 mm wide, resembling a small penis. Field data were insufficient for statistical size comparisons of normal and pseudohermaphrodite clitorides.

Bears ranged in age from yearlings to approximately 28 years. Three pseudohermaphrodites were lactating and captured with 2 cubs each. One set of twins was male; 2 were multisex, with females displaying the pseudohermaphrodite phenotype. Bear 1 (field number X2818, 28 years of age) was captured in 1975, 1999, and 2000. She was accompanied by an adult male in 1975 but was never observed with cubs.

Pseudohermaphrodite genotype.—Pseudohermaphrodites carried gene *AMELX* alone, as expected for normal females, except for Bear 1, which carried both *AMELX* and *AMELY*. Initial tests for *Sry* genotype suggested that all pseudohermaphrodites carried *Sry* because a fragment approximately the size of the *Sry* target band was observed for each individual (Fig. 2). However, the fragment amplified from pseudohermaphrodites was, on average, estimated to be 0.4 bases smaller than the *Sry* band from known males. Using a 377 DNA sequencer, normal variation in estimated size among identical amplification products on a single gel is within 0.2–0.3 bases. PCR product from pseudohermaphrodite Bear 1 appeared the same size as product from known male controls (Fig. 2).

When the pseudohermaphrodites were screened under more stringent conditions (Appendix I), all bears tested as normal females except Bear 1, which was consistently positive for an *Sry*-sized fragment. Known males tested under the same conditions were also consistently positive for *Sry*. We therefore suspected that, excepting Bear 1, the pseudohermaphrodites did

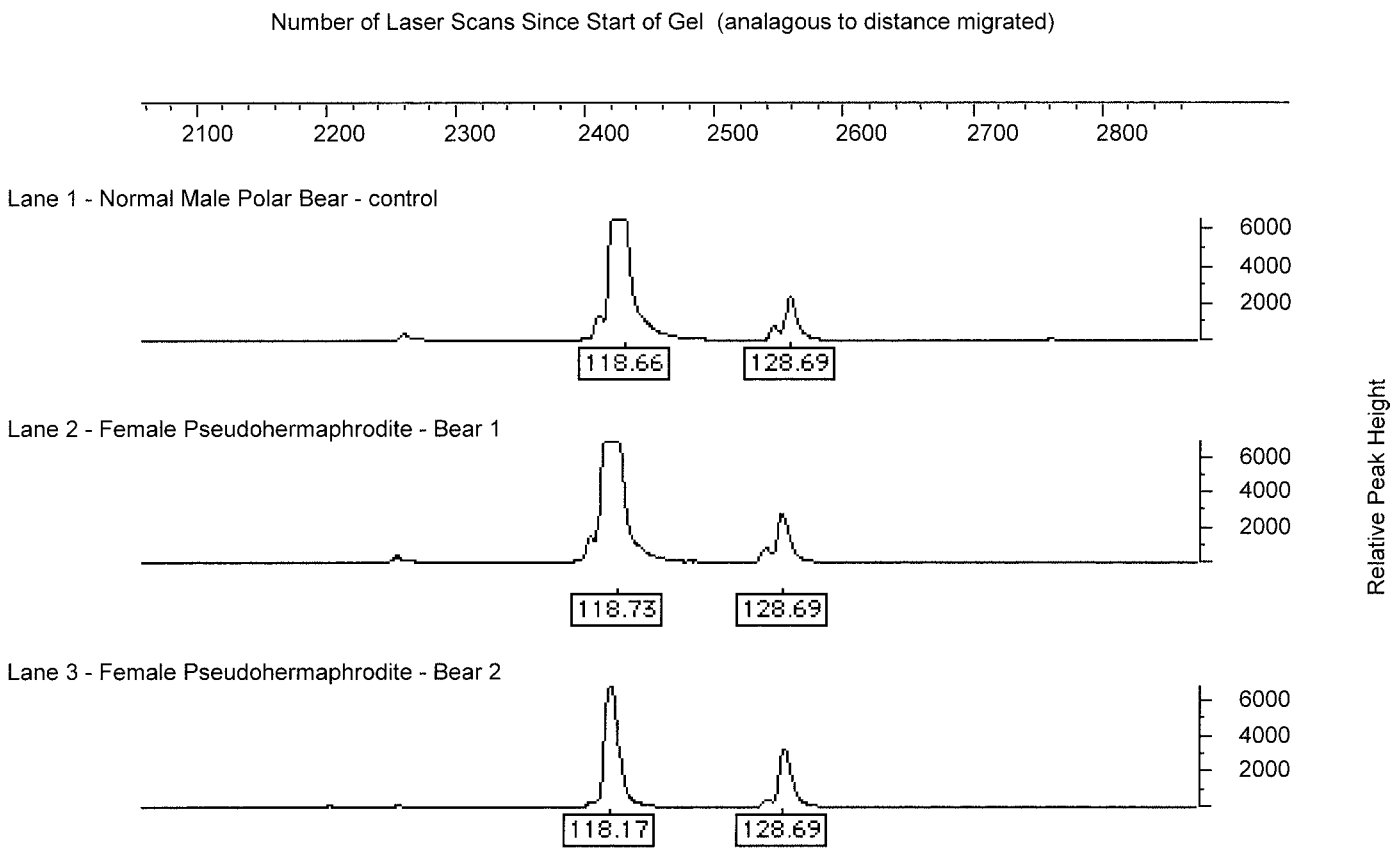

Fig. 2.—Initial sex determination of pseudohermaphrodite polar bears from Nunavut, Canada, using sex-determining region Y (*Sry*) gene and an automated DNA sequencer (Applied Biosystems Inc., Foster City, California). Size of zinc finger protein, (*Zfx–Zfy*) gene control bands (128.69 bases, indicated beneath each peak) were always consistent between bears; size of the putative *Sry* fragment (peaks approximately 118 bases) varied between individuals. Size variation is detectable by differing positions on the gel (using an automated sequencer, this is indicated by number of laser scans, an analogue of distance migrated when using agarose gels) and by fragment size, assigned based on relative position of within-lane standard bands (not shown). Variation in fragment size might not be detected under normal laboratory conditions but likely reflects sequence variations between *Sry* and pseudo*Sry*.

not contain Y-chromosome DNA and that primers SRY41F and SRY121R were cross-amplifying another gene fragment of similar length under permissive reaction conditions (presumably due to mispriming at a comparable binding site). This fragment was designated pseudo*Sry*.

Characterization of pseudoSry amplification.—*Sry* and Pseudo*Sry* amplification in normal bears and in Bear 1 was assessed under a wide range of conditions (Appendix I). In all cases, results for Bear 1 were identical to those of phenotypically normal males. All control bears possessed amelogenin genotypes consistent with phenotypic sex; however, due to limited material, some normal bears could not be tested for *Sry* or pseudo*Sry* amplification under all conditions.

Results for normal bears were most accurate when combining 25 cycles with 0.288 U of Taq. Under these conditions, *Zfx–Zfy* (controls for PCR failure, see ''Materials and Methods'') was amplified from all individuals, *Sry* was amplified from all known males, and pseudo*Sry* amplification was observed in only 1 female. In this normal black bear, relative peak height of pseudo*Sry* was approximately 90, whereas relative peak height of the *Zfx–Zfy* control fragment was 1640. Given this imbalance, the pseudo*Sry* fragment might not be recognized as a product, and the individual would likely be scored correctly in general sex-testing situations. (Detailed results for all tests are given in Appendix I.)

Identification of pseudoSry.—Despite repeated attempts, we were unable to obtain reliable gene sequences for every individual. However, sequence data were obtained for at least 1 representative of each sex and species class (Genbank accession numbers AY179736–AY179745) but should be considered preliminary (improved sequences may be attainable using cloning techniques).

Sequences from male bears and Bear 1 were most similar to *Sry* of giant panda (*Ailuropoda melanoleuca*, Genbank accession number AF461117—Zhou et al. 1998). Average pairwise divergence between sequences from males and females was 18%. Several pseudo*Sry* variants were observed among normal and pseudohermaphrodite females and showed greatest homology to rat *Sox11* (Genbank accession number NM053349—Kuhlbrodt et al. 1998), giant panda *Sox11* (Genbank accession number AF016375—Zhou et al. 1998), or giant panda *Sry*. This variation may be due to truncation of some sequences or may imply that several different genes in the *Sox* family were amplified from different individuals.

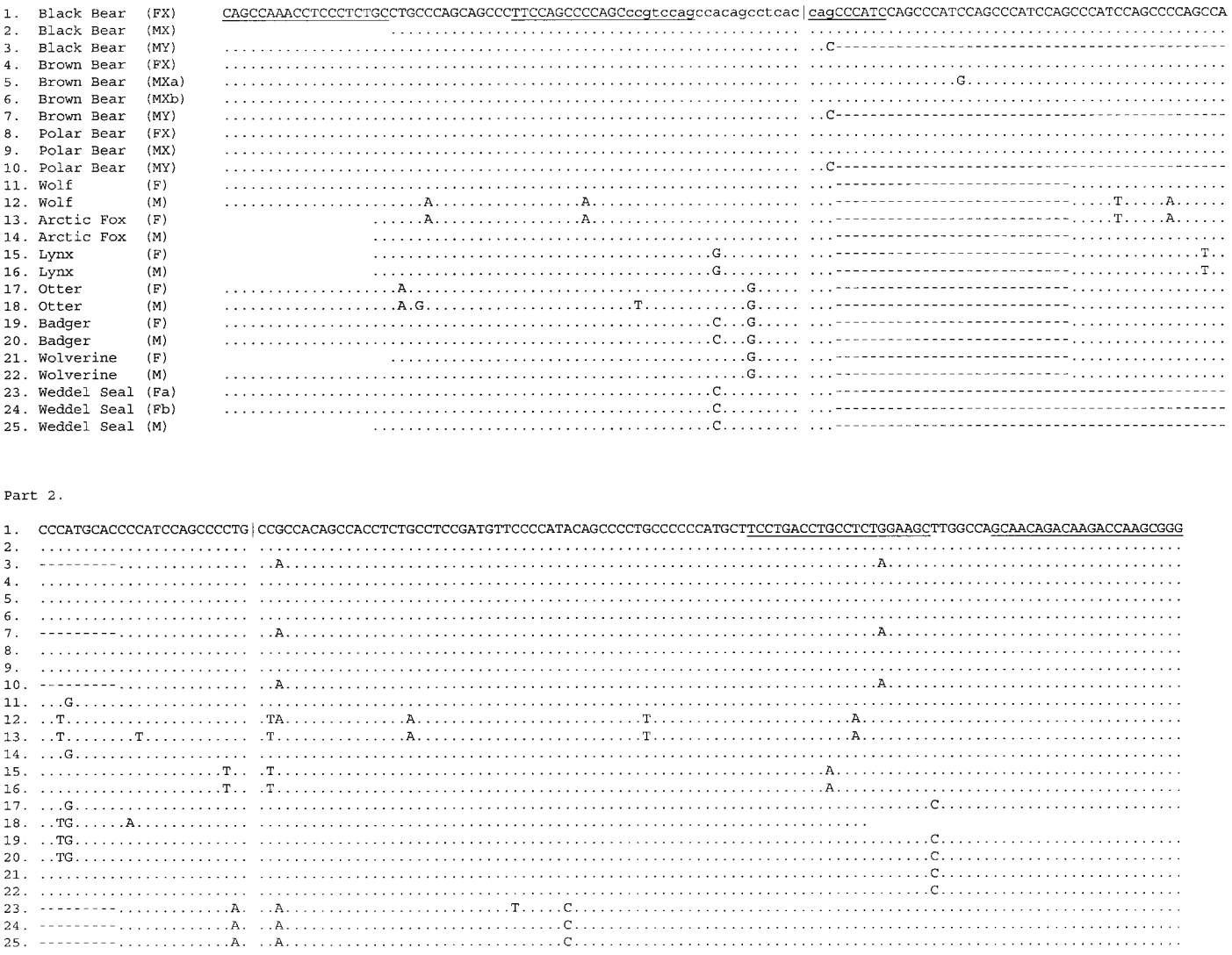

FIG. 3.—Amelogenin exon 5 sequence in carnivores. Sex is indicated in brackets, M denoting male, F denoting female. For bears, sex is followed by X or Y to indicate chromosomal origin of the gene. Multiple sequences recovered from different individuals in a sex and species category are indicated by lowercase letters following sex. Dots equal identical bases, dashes represent indels. Vertical lines flank the sequence repeat region, and the repeat motif is underlined. Underlining at the ends of the sequence indicates the original bovine primers. Ursid primers CST 1836 and CST 1834 overlap upstream of the *AMELY* deletion; CST 1836 is underlined; CST 1834 is indicated in lowercase letters. Annealing position of CST 1837 is underlined downstream of the *AMELY* deletion.

Use of amelogenin as a molecular sex test.—Primers designed from domestic cattle will amplify the amelogenin gene of Japanese black bears (*U. thibetanus japonicus*—Yamamoto et al. 2002) and most North American bears. However, in approximately 10% of brown and polar bears, no product can be obtained (e.g., in 1 data set, 60 of 800 unknown brown bears could not be sexed with these primers—M. Proctor, pers. comm.). Furthermore, in all other carnivores tested using these primers, a single, sex-unspecific fragment was observed. Carnivore-specific amelogenin primers would therefore be useful for sexing a wider range of species and individuals.

We sequenced *AMELX* and *AMELY* from black, brown, and polar bears and single bands amplified from 7 other carnivore species (Fig. 3). Average pairwise sequence divergence across all carnivores was 3%. Sex-specific substitutions indicated for wolves, arctic foxes, and otters in Fig. 3 reflect apparent sequence polymorphisms, suggesting amplification of both *AMELX* and *AMELY* sequences from males of these species. Our sequences showed greatest identity with bovine *AMELX*; average pairwise divergence between carnivore and bovine sequences was 8%.

We designed several sets of primers in conserved regions flanking the ursine *AMELY* deletion and the β-helix sequence repeat region (Fig. 3). No combination of primers or amplification conditions resulted in size differentiated, sex-specific products in nonursine carnivores.

Best results for bears were obtained with a multiplex of 3 primers, CST1834, 1836, and 1837; CST1834 and 1837 amplified *AMELX*, preferentially; and CST1836 and 1837 amplified *AMELY* (data not shown). Amplification patterns

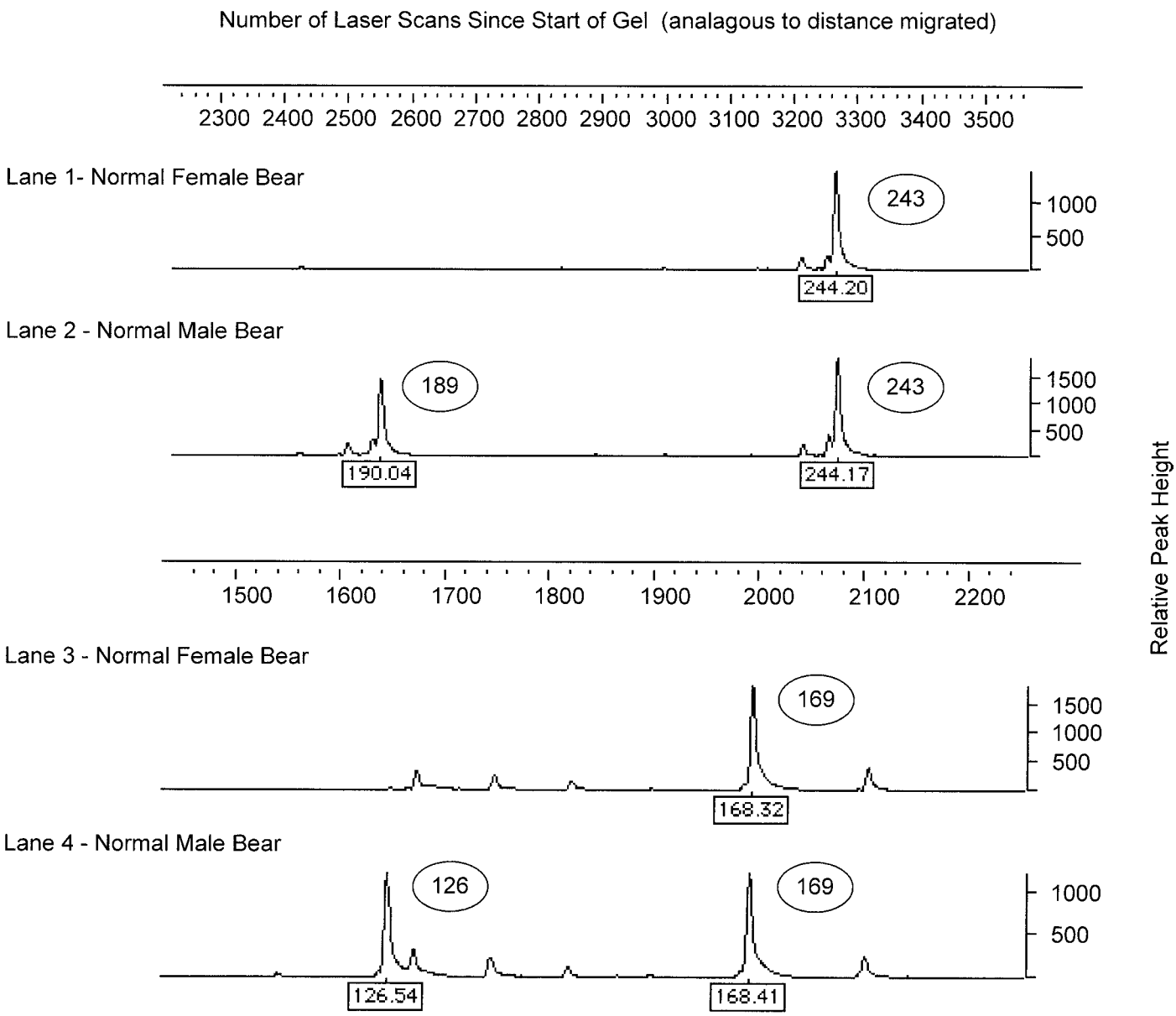

FIG. 4.—Use of *AMELX* and *AMELY* for sex testing in polar bears from Nunavut, Canada. Lanes 1–2 are fragments obtained using Ennis and Gallagher's cattle primers (1994); lanes 3–4 show products from the same individuals using bear-specific primers developed here. Results shown are also representative of amplification patterns for North American black and brown bears. Apparent fragment size relative to within-lane standard is indicated below each peak; circled values to the right of each peak indicate absolute length in bases (see Table 1).

obtained with bovine and ursine primers are shown in Fig. 4 (Table 1 indicates peak sizes across carnivores). Additional low-intensity peaks amplified with ursine primers most likely reflect nonspecific binding to the β-helix repeat region of the gene. We could not eliminate these peaks without compromising amplification of the target bands, and we could not obtain sufficient template to verify their identity through sequencing. Because relative peak height was low and fragment size did not overlap with the target bands, this cross-amplification did not interfere with sexing. Furthermore, the ursine primers were equally applicable to all 3 North American bear species, allowed accurate sexing of known bears, and provided sex information for bears that did not respond to bovine primers. They were also effective for sexing of hair samples collected using noninvasive techniques (data not shown).

DISCUSSION

Reliability of molecular sex-testing methods.—Molecular sex-identification techniques provide invaluable information for a range of applications: determining the sex of a fetus before morphological differentiation (Lambert et al. 2000; Pollevick et al. 1992); clarifying sex in species that are morphologically cryptic or difficult to observe (Gowans et al. 2000); assessing sex ratios in wild populations via remote-censusing population surveys (Woods et al. 1999); identifying donors of forensic samples (Stacks and White 1996; Wilson and White 1998); and

verifying harvest reports in managed populations (Amstrup et al. 1993; Schliebe et al. 1999). All of these applications assume that molecular methods are highly accurate; data presented here suggest this assumption may not always be justified.

Sry is a natural target for DNA-based sexing techniques. It is Y chromosome–specific and the single locus that initiates development of male sex in mammals (Koopman et al. 1991; Sinclair et al. 1990). The Y chromosome in general and *Sry* specifically are highly variable among species; primers capable of amplifying *Sry* from a number of species must be targeted towards its conserved HMG-box (Graves 2002; Taberlet et al. 1993). However, the HMG-box is also conserved between *Sry* and other members of the *Sox* gene family (*Sry*-like HMG-box containing genes—Graves 2002).

At least 1 of these *Sox* genes can be amplified using primers designed for *Sry*. Cross-amplification has been observed by other researchers but was not considered problematic in previously published studies (mountain lions—Ernest et al. 2000; brown bears—Taberlet et al. 1993; Woods et al. 1999). Our data indicate that cross-amplification could be a significant source of error and may be undetectable by previously published sex-screening protocols (Fig. 2).

In large-scale population genetic studies or noninvasive population inventories (e.g., Paetkau 2003), verifying the identity of each PCR product by Southern blotting or sequencing rapidly becomes impractical. Therefore, it is important to determine the conditions under which PCR primers are specific for the fragment of interest. For *Sry*-based sex testing, we suggest a maximum of 25 amplification cycles, combined with the lowest Taq concentration that produces consistently detectable PCR control bands (in this study, *Zfx–Zfy*). Labs employing *Sry* are also advised to validate their procedures by screening a statistically defensible number of known individuals. However, because incidence and strength of pseudo*Sry* amplification were affected by use of 2 different, identical model thermocyclers (Appendix I), we encourage development of other, more accurate methods of molecular sex testing.

As a genetic marker for sex, the amelogenin locus has several advantages over *Sry*. It is pseudoautosomal, providing an inherent internal control, but with size variation that is male-specific (Gibson et al. 1992; Lau et al. 1989). Furthermore, amelogenin does not belong to a gene family (Lau et al. 1989), thus reducing the risk of cross-amplification to other loci. Bear-specific primers developed here were accurate on all known bears tested and supplied sex information for samples, including hairs, that could not be assessed using the original method.

Unfortunately, sex-specific size variation may not be present in the amelogenin gene of other carnivore species. In mice, amelogenin is found on the X chromosome alone (Chapman et al. 1991); in marsupials and monotremes, it is autosomal (Watson et al. 1992); chromosomal location has not been determined for any carnivore. If the gene is pseudoautosomal in these species, our data suggest that sex-specific size variation of the 5th exon (Ennis and Gallagher 1994) does not exist (Fig. 3). Given that deletions characterizing *AMELY* lie within the β-helix repeat region of the gene, they may have arisen independently in several lineages. Cloning amelogenin from several carnivore species would clarify this issue and, perhaps, suggest other regions of the gene that would facilitate size-based sex identification.

Table 1.—Amelogenin fragment length (bases) amplified from carnivores using cattle and bear-specific primers. Total sample size (*n*) includes equal numbers of males and females.

	Cattle primers		Bear primers	
Species (*n*)	Male	Female	Male	Female
Black bear (4)	189, 243	243	126, 169	169
Brown bear (4)	189, 243	243	126, 169	169
Polar bear (4)	189, 243	243	126, 169	169
Wolf (4)	216	216		
Arctic fox (4)	216	216		
Lynx (4)	216	216		
Badger (2)	216	216		
Otter (2)	216	216		
Wolverine (2)	216	216		
Weddell seal (4)	189	189		

The bear-specific amelogenin primers presented here may represent the most convenient and reliable molecular sex test currently available for ursids. Another method recently proposed by Shaw et al. (2003) exploits size variation in an intron of the *Zfx–Zfy* genes; their primers bind to conserved flanking sequences and are, therefore, applicable to a range of species. PCR products thus obtained vary from 800 to 1,300 bp and may not be recoverable from low-quantity or poor-quality DNA samples. However, sequencing of this intron and design of species-specific primers that target smaller fragments may be more efficient than de novo cloning of the amelogenin gene for the same purpose.

Pseudohermaphroditism in Nunavut, Canada, polar bears: genetic mechanisms.—Female pseudohermaphroditism arises from fetal exposure to androgens (Polani 1981; Yalcinkaya et al. 1993). The source of the androgens, whether genetic or environmentally induced, fetal or maternal, is less understood. We will explore a number of possibilities with respect to female pseudohermaphrodite polar bears in Nunavut, Canada.

The presence of male DNA in a female fetus is the most obvious source of virilizing hormones and can lead to full sex reversal (Koopman et al. 1991). Pseudohermaphrodites in Svalbard, Norway, were *Sry* negative (Wiig et al. 1998). Ten of 11 pseudohermaphrodites in Nunavut, Canada, were also *Sry*- and *AMELY*-negative, suggesting the absence of Y-chromosome DNA in these individuals. However, amplification patterns for pseudohermaphrodite Bear 1 were identical to those of normal male bears under all testing conditions. It is unclear whether this result is due to translocation of Y-chromosome DNA to another chromosome or whether it reflects the XXY trisomy characteristic of Klinefelter's syndrome in humans. However, given that male development in Klinefelter's syndrome is normally greater than that observed here, a chromosomal rearrangement seems more probable. Close correspondence between the phenotype of Bear 1 and other pseudohermaphrodites also suggests that an alternative—feminization of a genetic male individual—is unlikely.

Bear 1, captured in 3 different years, was always in excellent physical condition but never found with cubs; this observation is very unusual. Furthermore, limited endocrine testing performed on Bear 1 suggests her androgen levels may be consistent with those of a normal male (E. Cummings, pers. comm.). The presence of a possibly infertile, and thus noncontributing, female in the M'Clintock Channel polar bear population may be particularly important because this population is considered seriously depleted due to recent, former harvesting. Significance of this result could be further addressed given an accurate estimate of the frequency of pseudohermaphroditism from chromosomal abnormality in this population.

The 10 Nunavut, Canada, pseudohermaphrodites negative for male DNA may possess genetic abnormalities undetectable by assays used here. The presence of a functional *Sry* gene initiates male development; however, most genes active later in the differentiation pathway (downstream genes), including many genes involved in spermatogenesis, are located on the X chromosome or the autosomes (Graves 2000; Graves and Delbridge 2001). A mutation affecting function or regulation of this type of gene could increase sensitivity of the developing fetus to maternal androgens or increase the concentration of maternal androgens during pregnancy (Yalcinkaya et al. 1993). As the phenotype does not seem to affect reproduction, such a predisposition may not cause strong adverse selection.

Pseudohermaphroditism in Nunavut, Canada, polar bears: alternative inherent mechanisms.—Adrenal or ovarian tumors may be a potential source of increased maternal androgens during pregnancy (Polani 1981). However, it seems unlikely that this condition would be frequent enough to account for the relatively large number of pseudohermaphrodites observed in Nunavut, Canada.

In contrast, freemartinism is a possible explanation. Freemartins arise when the placentas of mixed-sex twins fuse, allowing androgens from the male sibling to pass to the female (Benirschke 1981). Two mixed litters were captured here; the female of each displayed the pseudohermaphrodite phenotype. However, because both mothers were pseudohermaphrodites, this hypothesis requires us to assume that these females were also mixed-sex twins. Wiig et al. (1998) considered freemartinism improbable in Svalbard, Norway, pseudohermaphrodites, partially because freemartins are usually sterile (Benirschke, 1981). Although this effect may contribute to the incidence of Nunavut, Canada, pseudohermaphroditism, it seems likely that other mechanisms are also at work.

Pseudohermaphroditism in Nunavut, Canada, polar bears: environmentally induced mechanisms.—Cattet (1988) and Wiig et al. (1998) suggest that environmental pollutants acting via the mother may also virilize bear fetuses. Identity of such compounds and mechanism of their action is not currently clear. In polar bears, pseudohermaphroditism might result from endocrinological effects of organochlorines such as polychlorinated biphenyls (PCBs). Because the energy source for prenatal polar bears is maternal lipids, significant exposure to lipophilic contaminants occurs during fetal development (Polischuk 1999).

Unfortunately, it is difficult to assess the relationship between extent of organochlorine exposure and incidence of pseudohermaphroditism among distinct polar bear populations. Data on the frequency of pseudohermaphrodites in Nunavut, Canada, are inadequate for comparison to Svalbard, Norway, bears. Further, published accounts of organochlorine levels in polar bears from different regions must be evaluated carefully because factors such as season and nutritional status can dramatically influence results (Polischuk et al. 2002).

Pseudohermaphroditism has also been observed in 4 black bears and 1 brown bear with the most extreme phenotype of any ursid in a black bear from Alberta, Canada (Cattet 1988). Black and brown bears, due to differing geographic ranges and shorter food chains, should not be influenced by organochlorine contamination to the extent of their arctic cousins. However, it remains possible that a different type of contaminant, more ubiquitous in distribution and still unidentified, could contribute to abnormal phenotypes in these species.

Conclusions.—Ten of 11 female pseudohermaphrodite polar bears observed in Nunavut, Canada, did not possess *Sry* or *AMELY* and, hence, may not contain male-specific DNA. Therefore, mechanisms such as freemartinism and the potential androgenic effects of environmental pollution are likely to contribute to the development of this abnormality. However, in at least 1 case, a chromosomal abnormality has resulted in a similar pseudohermaphrodite phenotype. The application of reliable, molecular sex-testing methods to such individuals may yet increase our understanding of normal and abnormal sexual development in wild living mammals.

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Literature Cited

Amstrup, S. C., G. W. Garner, M. A. Cronin, and J. C. Patton. 1993. Sex identification of polar bears from blood and tissue samples. Canadian Journal of Zoology 71:2174–2177.

Animal Care and Use Committee. 1998. Guidelines for the capture, handling, and care of mammals as approved by the American Society of Mammalogists. Journal of Mammalogy 79:1416–1431.

Benirschke, K. 1981. Hermaphrodites, freemartins, mosaics, and chimaeras in animals. Pp. 421–463 in Mechanisms of sex differentiation in animals and man (C. R. Austin and R. G. Edwards, eds.). Academic Press, London, United Kingdom.

Berta, P., et al. 1990. Genetic evidence equating *Sry* and the testis-determining factor. Nature 348:448–450.

Bouters, S., M. Vanderplassche, and A. Demoor. 1975. Intersex (male pseudohermaphrodite) horse with 64XX–XXY mosaicism. Journal of Reproduction and Fertility Suppl. 23:375–376.

Cattet, M. 1988. Abnormal sexual differentiation in black bears (*Ursus americanus*) and brown bears (*Ursus arctos*). Journal of Mammalogy 69:849–852.

CHAPMAN, V. M., B. T. KEITZ, C. M. DISTECHE, E. C. LAU, AND M. L. SNEAD. 1991. Linkage of amelogenin (*AMEL*) to the distal portion of the mouse X-chromosome. Genomics 10:23–28.

CRIBIU, E. P., S. CHAFFAUX, V. DURAND, AND P. HUMBLOT. 1989. Cytogenetic, anatomical, histological and hormonal study of a female pseudohermaphrodite cow. Zuchthygiene—Reproduction in Domestic Animals 24:265–273.

ENNIS, S., AND T. F. GALLAGHER. 1994. A PCR-based sex determination assay in cattle based on the bovine amelogenin locus. Animal Genetics 25:425–427.

ERNEST, H. B., M. C. T. PENEDO, B. P. MAY, M. SYVANEN, AND W. M. BOYCE. 2000. Molecular tracking of mountain lions in the Yosemite Valley region in California: genetic analysis using microsatellites and faecal DNA. Molecular Ecology 9:433–441.

GIBSON, C. W., E. E. COLUB, W. R. ABRAMS, G. SHEN, W. DING, AND J. ROSENBLOOM. 1992. Bovine amelogenin message heterogeneity: alternative splicing and Y–chromosomal gene transcription. Biochemistry 31:8384–8388.

GLICKMAN, S. E., L. G. FRANK, S. PAVGI, AND P. LICHT. 1992. Hormonal correlates of ''masculinization'' in female spotted hyaenas (*Crocuta crocuta*). 1. Infancy to sexual maturity. Journal of Reproduction and Fertility 95:451–462.

GOWANS, S., M. L. DALEBOUT, S. K. HOOKER, AND H. WHITEHEAD. 2000. Reliability of photographic and molecular techniques for sexing northern bottlenose whales (*Hyperoodon ampullatus*). Canadian Journal of Zoology 78:1224–1229.

GRAVES, J. A. M. 2000. Human Y chromosome, sex determination and spermatogenesis—a feminist view. Biology of Reproduction 63:667–676.

GRAVES, J. A. M. 2002. The rise and fall of *Sry*. Trends in Genetics 18:259–264.

GRAVES, J. A. M., AND M. L. DELBRIDGE. 2001. The X—a sexy chromosome. BioEssays 23:1091–1094.

IKADAI, H., C. AJISAWA, S. TSUJIMURA, G. WATANABE, K. TAYA, AND T. IMAMICHI. 1988. A new male pseudohermaphrodite rat mutant with androgen deficiency. Journal of Reproduction and Fertility 84: 303–312.

JUST, W., C. CABRAL DE ALMEIDA, B. GOLDSHMIDT, AND W. VOGEL. 1994. The male pseudohermaphrodite XX polled goat is *Zfy* and *Sry* negative. Hereditas 120:71–75.

KOOPMAN, P., J. GUBBAY, N. VIVIAN, P. GOODFELLOW, AND R. LOVELL-BADGE. 1991. Male development of chromosomally female mice transgenic for *Sry*. Nature 351:117–121.

KUHLBRODT, K., B. HERBARTH, E. SOCK, J. ENDERICH, I. HERMANS-BORGMEYER, AND M. WEGNER. 1998. Cooperative function of *POU* proteins and *Sox* proteins in glial cells. Journal of Biological Chemistry 273:16050–16057.

LAMBERT, J. F., B. O. BENOIT, G. A. COLVIN, J. CARLSON, Y. DELVILLE, AND P. K. J. QUESENBERRY. 2000. Quick sex determination of mouse fetuses. Journal of Neuroscience Medicine 95:127–132.

LAU, E. C., T. K. MOHANDAS, L. J. SHAPIRO, H. C. SLAVKIN, AND M. L. SNEAD. 1989. Human and mouse amelogenin gene loci are on the sex-chromosomes. Genomics 4:162–168.

LOTT, D. F., K. BENIRSCHKE, J. N. MCDONALD, C. STORMONT, AND T. NETT. 1993. Physical and behavioral findings in a pseudohermaphrodite American bison. Journal of Wildlife Diseases 29:360–363.

MILLIKEN, J. E., D. L. PACCAMONTI, R. S. SHOEMAKER, AND W. H. GREEN. 1995. XX male pseudohermaphroditism in a horse. Journal of the American Veterinary Medical Association 207:77–79.

MURPHY, L., AND P. J. O'SHAUGHNESSY. 1991. Testicular steroidogenesis in the testicular feminized (TFM) mouse—loss of 17-alpha-hydroxylase activity. Journal of Endocrinology 131:443–449.

NEMZEK, J. A., L. D. HOMCO, L. G. WHEATON, AND G. L. GRMAN. 1992. Cystic ovaries and hyperestrogenism in a canine female pseudohermaphrodite. Journal of the American Animal Hospital Association 28:402–406.

PAETKAU, P. 2003. An empirical exploration of data quality in DNA-based population inventories. Molecular Ecology 12:1375–1387.

PAILHOUX, E., E. P. CRIBIU, S. CHAFFAUX, R. DARRE, M. FELLOUS, AND C. COTINOT. 1994. Molecular analysis of 60,XX pseudohermaphrodite polled goats for the presence of *Sry* and *Zfy* genes. Journal of Reproduction and Fertility 100:491–496.

PETER, A. T., D. MARKWELDER, AND E. K. ASEM. 1993. Phenotypic feminization in a genetic male dog caused by nonfunctional androgen receptors. Theriogenology 40:1093–1105.

PHOENIX, C. H., AND K. C. CHAMBERS. 1982. Sexual behavior in adult gonadectomized female pseudohermaphrodite, female and male rhesus macaques (*Macaca mulatta*) treated with estradiol benzoate and testosterone propionate. Journal of Comparative and Physiological Psychology 96:823–833.

POLANI, P. E. 1981. Abnormal sex development in man II: anomalies of sex differentiating mechanisms. Pp. 449–590 in Mechanisms of sex differentiation in animals and man (C. R. Austin and R. G. Edwards, eds.). Academic Press, London, United Kingdom.

POLISCHUK, S. C. 1999. Organochlorine dynamics in free-ranging polar bears and their cubs. Ph.D. dissertation, University of Saskatechewan, Saskatoon, Saskatechewan, Canada.

POLISCHUK, S. C., R. J. NORSTROM, AND M. A. RAMSAY. 2002. Body burdens and tissue concentrations of organochlorines in polar bears (*Ursus maritimus*) vary during seasonal fasts. Environmental Pollution 118:29–39.

POLLEVICK, G. D., ET AL. 1992. Sex determination of bovine embryos by restriction fragment length polymorphisms of PCR amplified *Zfx*/*Zfy* loci. Biotechnology 10:805–807.

SCHLIEBE, S. L., T. J. EVANS, A. S. FISCHBACK, AND M. A. CRONIN. 1999. Using genetics to verify sex of harvested polar bears: management implications. Wildlife Society Bulletin 27:592–597.

SHAW, C. N., P. J. WILSON, AND B. N. WHITE. 2003. A reliable molecular method of sex determination for mammals. Journal of Mammalogy 84:123–128.

SINCLAIR, A. H., ET AL. 1990. A gene from the human sex determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 346:240–244.

SMITH, K. C., T. J. PARKINSON, S. E. LONG, AND F. J. BARR. 2000. Anatomical, cytogenetic and behavioral studies of freemartin ewes. Veterinary Record 146:574–578.

STACKS, B., AND M. M. WITTE. 1996. Sex determination of dried blood stains using the polymerase chain reaction (PCR) with homologous X–Y primers of the zinc finger protein gene. Journal of Forensic Sciences 41:287–290.

STIRLING, I., C. D. SPENCER, AND D. ANDRIASHEK. 1989. Immobilization of polar bears (*Ursus maritimus*) with Telazol in the Canadian Arctic. Journal of Wildlife Diseases 35:159–168.

TABERLET, P., H. MATTOCK, C. DUBOIS-PAGANAON, AND J. BOUVET. 1993. Sexing free ranging brown bears (*Ursus arctos*) using hairs found in the field. Molecular Ecology 2:399–403.

VERBERCKMOES, S., A. VAN SOOM, I. DE PAUW, S. VAN CUCHTEN, AND A. DE KRUIF. 2002. Freemartinism in a sheep. Vlaams Diergeneeskundif Tijdschrift 71:211–215.

WATSON, J. M., J. A. SPENCER, J. A. M. GRAVES, M. L. SNEAD, AND E. C. LAU. 1992. Autosomal localization of the Amelogenin gene in monotremes and marsupials—implications for mammalian sex-chromosome evolution. Genomics 14:785–789.

WIIG, O., A. E. DEROCHER, M. M. CRONIN, AND J. U. SKAARE. 1998. Female pseudohermaphrodite polar bears at Svalbard. Journal of Wildlife Diseases 34:792–796.

WILEN, R., R. W. GOY, J. A. RESKO, AND F. NAFTOLIN. 1977. Pubertal body weight and growth in the female rhesus pseudohermaphrodite. Biology of Reproduction 16:470–473.

WILSON, P. K., AND B. N. WHITE. 1998. Sex identification of elk (*Cervus elaphus canadensis*), moose (*Alces alces*), and white-tailed deer (*Odocoileus virginianus*) using the polymerase chain reaction. Journal of Forensic Sciences 43:477–482.

WOODS, K. G., D. PAETKAU, D. LEWIS, B. N. MCLELLAN, M. PROCTOR, AND C. STROBECK. 1999. Genetic tagging of free-ranging black and brown bears. Wildlife Society Bulletin 27:616–627.

YADAV, B. R., C. SINGH, P. KUMAR, O. S. TOMER, AND J. S. YADAV. 1993. Morphological, anatomical, and cytogenetical investigations in sexually anomalous goats. Small Ruminant Research 11:331–342.

YALCINKAYA, T. M., ET AL. 1993. A mechanism for virilization of female spotted hyenas in utero. Science 260:1929–1931.

YAMAMOTO, K., ET AL. 2002. Sex identification of Japanese black bear, *Ursus thibetanus japonicus*, by PCR based on amelogenin gene. Journal of Veterinary Medical Science 64:505–508.

ZHOU, R., H. CHENG, Y. QIXING, AND Z. YAPING. 1998. *Sox* and *Zfx* genes in giant panda. Science in China (Series C) 41:623–627.

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APPENDIX I

Further molecular methods

(Please note that subheadings used here correspond to relevant sections of the ''Materials and Methods.'')

PCR-based screens: pseudohermaphrodites.—Reaction mixes for initial amelogenin screening contained 0.08 μmol each of primers SE47 and SE48; 0.2 mmol dNTPs; 2 mmol $MgCl_2$; 1× PCR buffer; 0.5 U Taq; and 1 ng DNA. Cycling conditions were 94°C, 1 min; (94°C, 30 s; 54°C, 20 s; 72°C, 5 s) for 3 cycles; (94°C, 15 s; 54°C, 20 s; 72°C, 1 s) for 33 cycles; 72°C, 30 min.

Initial reaction mixes for *Sry* included 0.21 μmol of each primer; 0.16 mmol dNTPs; 2 mmol $MgCl_2$; 1× PCR buffer; 1.44 U Taq; and 1 ng DNA. Cycling conditions were 94°C, 1 min; (94°C, 30 s; 54°C, 20 s; 72°C, 5 s) for 3 cycles; (94°C, 15 s; 54°C, 20 s; 72°C, 1 s) for 33 cycles. All samples were then retested using more stringent amplification (0.11 μmol of each primer; 0.16 mmol dNTPs; 2 mmol $MgCl_2$; 1× PCR buffer; 0.144 U Taq; and 1 ng DNA) and cycling conditions of 94°C, 1 min; (94°C, 30 s; 54°C, 20 s; 72°C, 5 s) for 3 cycles; (94°C, 15 s; 54°C, 20 s; 72°C, 1 s) for 25 cycles.

PCR-based screens: Sry series analysis.—Twenty-one normal bears (see ''Materials and Methods'') and pseudohermaphrodite Bear 1 were tested for *Sry*–pseudo*Sry* amplification under a wide range of conditions. We describe below only those factors that varied from initial *Sry* testing conditions outlined in the preceding subsection. Number of cycles refers to the number of times the 3rd phase of the PCR program (94°C, 15 s; 54°C, 20 s; 72°C, 1 s) was repeated. Unless otherwise noted, *Sry* was successfully amplified from all male bears.

DNA concentration tests were performed using 0.5, 1, or 2 ng of genomic DNA; 2 mmol $MgCl_2$; 1.44 U Taq; 25 cycles; and an annealing temperature of 54°C. No pseudo*Sry* amplification was observed under any conditions.

The role of magnesium concentration on pseudo*Sry* amplification was assessed using 0.5 ng DNA; 1, 1.5, 2, or 2.5 mmol $MgCl_2$; 0.288 or 1.44 U Taq; 33 cycles; and an annealing temperature of 54°C. Incidence of pseudo*Sry* increased with increasing Taq concentration and peak height with increasing magnesium concentration. Under the most stringent conditions, amplification was observed in 4 out of 13 females. Average peak height of pseudo*Sry* was similar to that of *Zfx–Zfy* in females but consistently less than *Sry* peak heights in males.

Cycle number and Taq concentration analysis included 0.5 ng DNA; 2 mmol $MgCl_2$; 0.228, 1.44, or 7.2 U Taq; 25, 27, 29, 31, or 33 cycles; and an annealing temperature of 54°C. Pseudo*Sry* amplified weakly from 1 female black bear under the most stringent conditions. Incidence and peak height of pseudo*Sry* increased with both cycle number and Taq concentration. With few exceptions, peak height of pseudo*Sry* was less than that of *Zfx–Zfy* in females and *Sry* in males.

We surveyed the effect of annealing temperature and Taq concentration using 0.5 ng DNA; 2 mmol $MgCl_2$; 0.288, 1.44, or 7.2 U Taq; 33 cycles; and annealing temperatures of 59°C, 61°C, 62°C, 64°C, or 69°C. No temperature permitted amplification of *Zfx–Zfy* in females and amplification of *Sry* in males without permitting pseudo*Sry* amplification in at least 1 female. Incidence of pseudo*Sry* also varied with the use of 2 different, identical model thermocyclers.

Finally, we tested whether pseudo*Sry* would be amplified by AmpliTaq Gold (Applied Biosystems Inc.) using the following conditions: 0.5 ng DNA; 2 mmol $MgCl_2$; 0.375, 0.75, or 1.875 U of AmpliTaq; 25 or 33 cycles; and an annealing temperature of 54°C (cycling program also included an initial 10 min, 59°C activation). Pseudo*Sry* was amplified using 33 cycles or with higher concentrations of AmpliTaq than recommended by the manufacturer.

Sequencing: Sry and pseudoSry.—Initial efforts to purify template DNA from acrylamide gels employed the Qiaex II purification system (QIAGEN); however, sufficient material for sequencing could not be recovered using the manufacturer's directions. Superior results were obtained by combining the Qiaex II protocol and the Qiaquick (QIAGEN) protocol for purification of DNA from agarose gels. Three gel volumes of diffusion buffer (1 mM EDTA, pH 8.0, 0.1% SDS, 0.5 M ammonium acetate, 10 mM magnesium acetate) were added to each whole gel slice for overnight incubation at 55°C. Supernatant was removed to a clean tube, and 3 volumes of Qiaquick buffer QG were added. The remainder of the Qiaquick protocol was performed without modification.