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GENETIC METHODS IMPROVE ACCURACY OF GENDER DETERMINATION IN BEAVERS

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Gender identification of sexually monomorphic mammals can be difficult. We used analysis of zinc-finger protein (Zfx and Zfy) DNA regions to determine gender of 96 beavers (*Castor canadensis*) from 3 areas and used these results to verify gender determined in the field. Gender was correctly determined for 86 (89.6%) beavers. Incorrect assignments were not attributed to errors in any one age or sex class. Although methods that can be used in the field (such as morphological methods) can provide reasonably accurate gender assignments in beavers, the genetic method might be preferred in certain situations.

Key words: AGS, beaver, *Castor canadensis*, gender determination, Zfx, Zfy

Gender identification is a fundamental component of many wildlife studies and is pivotal in studies of kinship and social behavior (Ralls et al. 2001) and analysis of mating tactics (Hogg and Forbes 1997). In mammalian species exhibiting sexual dimorphism, gender assignments for mature adults based on size or secondary physical characteristics (antlers, horns, etc.), or by visually inspecting genitalia, are usually simple and accurate. However, not all mammals exhibit sexual dimorphism, which suggests that there is some ambiguity in gender assignments for some species. These ambiguities can be clarified by visual inspection of sexual organs by dissection (Parker et al. 2002), but biologists often require less invasive methods that can be prone to greater inaccuracy. Knowledge of gender in monogamous species is especially important because sex ratios can influence pregnancy rates, often a key component of population management.

Beavers (*Castor canadensis*) are monogamous, semi-aquatic mammals that lack sexual dimorphism and are difficult to sex based on external characteristics (Hill 1982). Specifically, beavers are one of the few mammalian species to lack external genitalia. They are economically important as furbearers (Novak 1987) and are considered a keystone species because their dam-building and foraging activities have profound effects on ecosystem structure and function (Baker and Hill 2003). Correct gender identification is important to understand beaver population biology and social structure (Brooks et al. 1980; Müller-Schwarze and Schulte 1999; Wheatley 1997), and during beaver reintroductions where males and females are released together at relocation sites. Thus, accurate gender identification is often necessary for this ecologically and economically important mammal.

In addition to visual inspection during necropsy, methods to determine gender in this species have included microscopic examination for sexual dimorphism in neutrophils (Larson and Knapp 1971), cloacal examination and palpation for the os penis (palpation—Allred 1986; Larson and Taber 1980), and examination of anal gland secretions (Schulte et al. 1995). Currently, the most reliable field method to identify gender in beaver is thought to be a combination of palpation and examination of anal gland secretions, although its accuracy has not been widely tested nor reported.

In contrast to the field method, genetic methods of gender identification typically use polymerase chain reaction (PCR) amplification and subsequent analysis to detect gender-specific differences in regions of the sex determining chromosomes. For example, several approaches focus on the zinc-finger protein (Zfx and Zfy) genes on the X and Y chromosomes. These zinc-finger protein genes are thought to be present in all eutherian mammals. This method was first used in mammalian wildlife species by Amstrup et al. (1993, 1995) and Cathey et al. (1998). Shaw et al. (2003) tested this method on several terrestrial and marine mammal species, but did not include any members of the order Rodentia in their analysis. Other researchers have focused on the Y-chromosome-specific *Sry* region (Meyers-Wallen et al. 1995), usually in combination with an internal positive control for correct identification of females (Gilson et al. 1998; Williams et al. 2003). No genetic method to determine gender has yet been applied to beavers.

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Here we applied the method developed by Amstrup et al. (1993) and Cathey et al. (1998) and extended by Shaw et al. (2003) for gender identification to verify the gender of 96 beavers which had previously been identified using both palpation and examination of anal gland secretions in the field. We choose this method because it appears to be robust and is useful in a wide range of species. Our goals were 1) to determine gender in beavers using molecular methods, 2) to determine how accurate the method of palpation and examining anal gland secretions is in the field, and 3) to determine if errors in gender assignments were associated with any particular age or sex class.

Materials and Methods

We studied beavers from 3 areas in the United States: Dinosaur National Monument in Colorado ($n = 35$), Rocky Mountain National Park in Colorado ($n = 34$), and various locations in South Carolina ($n = 27$). Beavers from Dinosaur National Monument and Rocky Mountain National Park were livetrapped during research projects (Breck et al. 2001, 2003a, 2003b), and South Carolina beavers were trapped in the course of United States Department of Agriculture Wildlife Services damage control. Beavers were sexed in the field by palpating for an os penis and then qualitatively examining anal gland secretions for both color and viscosity (Schulte et al. 1995). Secretions from males were darker and more viscous than those from females (Schulte et al. 1995). If the gender determination from the 2 methods did not match, the procedure was repeated (for Dinosaur National Monument and Rocky Mountain National Park). We refer to combined palpation and examination of anal gland secretions in the field as the field method. Personnel had varying levels of experience in using the field method, from novice to several years, and were either federal or university biologists or their assistants.

Beavers at each site were aged as young (<12 months), yearlings (12–24 months), or adults (>24 months) using mass-based criteria. Weight ranges for each age class varied by site but in general followed previously published weight trends (Patric and Webb 1960). The number, age, and genetically determined gender of beavers from each site was, for Dinosaur National Monument, 13 young (7 male [M], 6 female [F]), 7 yearlings (2 M, 5 F), 15 adults (5 M, 10 F—see Breck et al. 2001 for details of aging); for Rocky Mountain National Park, 13 young (8 M, 5 F), 1 yearling (M), 20 adults (9 M, 11 F); and for South Carolina, 1 young (M), 1 adult (F), and 25 age unknown (13 M, 12 F). Blood or tissue (a small section of ear or tail) was taken from each beaver after livetrapping (Dinosaur National Monument, Rocky Mountian National Park) or removal trapping (South Carolina).

Blood was placed in tubes containing EDTA (Fisher Scientific, Pittsburgh, Pennsylvania). Samples were frozen at −20°C until DNA was isolated. Tissue samples from Dinosaur National Monument were stored at room temperature in ethanol for >18 months prior to being stored at −20°C. DNA was isolated using a commercial kit and the manufacturer's protocols (DNeasy Tissue Kit, Qiagen, Inc., Valencia, California) and quantified by fluorimetry. Gender was determined by PCR amplification of regions of the zinc-finger protein (Zfx and Zfy) genes. Reactions consisted of 2 µl template DNA (typically 30–250 ng), 2.5 µg BSA (Fisher Scientific, Pittsburgh, Pennsylvania), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of primers LGL331 and LGL335 (Cathey et al. 1998), and 1 U Amplitaq Gold in 1 × Amplitaq Gold amplification buffer (Applied Biosystems, Foster City, California). The amplification profile was 7 min at 95°C, then 37 cycles of 30 s at 94°C, 30 s at 47°C, and 30 s at 72°C. A final 2-min elongation step was performed at 72°C, and reactions were then held at 4°C. Amplified fragments were visualized by electrophoresis through 2% 1:3 Genepure (ISC BioExpress, Kaysville, Utah), using a 100 base pair (bp) ladder (Promega, Madison, Wisconsin) for size comparison and a negative control without template DNA.

Results

Male and female beavers were clearly and unambiguously distinguished using Zfx and Zfy analysis. Female beavers yielded a single DNA fragment from both copies of the X chromosome (Zfx, approximately 1,350 bp); males yielded 2 DNA fragments, 1 from the X and 1 from the Y chromosome (Zfx, approximately 1,350 bp, and Zfy, approximately 1,200 bp).

Using this genetic method as a known comparison, we found the field method was correct in determining gender for 86 of 96 beavers (89.6%). Irrespective of their experience levels, biologists at all 3 sites made incorrect gender assignments for both sexes. The errors were not confined to any age class. Average accuracy of gender determinations was similar among the 3 sites (Rocky Mountain National Park 88.2%, South Carolina 88.9%, Dinosaur National Monument 91.4%). Of the 10 beavers whose gender was misidentified using the field method, 5 were young (3 M, 2 F), 3 were adults (1 M, 2 F), and 2 misclassified females were of unknown age. All 8 beavers identified as yearlings were correctly gender classified using the field method (3 M, 5 F).

Discussion

Beaver gender identification by the field method was about 90% accurate in typical field situations with personnel of mixed experience levels. Incorrect gender assignments were not limited to any one age or sex class. Although a higher percentage of young were assigned incorrect genders, adults of both sexes were also misclassified. Misclassification of gender of young should not impact the assumed structure of beaver colonies, although it could be a source of error for studies on dispersal. Correct gender identification is especially important for adults because beavers are monogamous and typically occur in a family unit (an adult male and female with their young from the previous 2 years). Misclassifying adult gender might affect interpretations of beaver social structure or numbers of colonies. For example, based on field gender 2 adult females were trapped a week apart in close proximity within Rocky Mountain National Park. However the 2nd beaver was later genetically determined to be a male and the close proximity of these 2 indicated they likely were a mated pair (Allred 1986). Likewise, 3 days after the misclassified Dinosaur National Monument adult female was trapped, an adult male was trapped in the same area, indicating they also were mated.

The level of error with the field method is not surprising, given that the method can be difficult with a struggling animal in field situations and that there is some subjectivity in classifying anal gland secretions, due to color variation within sexes. However, ease of use and the relatively short time required to train personnel might make a 10% error rate acceptable for many studies or management objectives, particularly because misclassification seems sporadic. However, in-depth population studies (Müller-Schwarze and Schulte

1999) might benefit if the more objective and accurate genetic method is used. The genetic method would also be useful if insufficient anal gland secretion is collected to allow gender determination (Schulte et al. 1995). Genetic analyses should be particularly beneficial to relocation programs where misclassification of released beavers could interfere with pair bond formation.

Our analyses showed that the beaver Zfx fragment (1,350 bp) is larger than Zfy (1,200 bp). This pattern also occurs in white-tailed deer (*Odocoileus virginianus*) and moose (*Alces alces*) but Zfy is larger than Zfx in black bear (*Ursus americanus*) and canids (Shaw et al. 2003). Our size estimates, based on visual comparison with the size standard, indicate that in beavers both fragments are much larger than most of those reported for a variety of wildlife species (959 bp average Zfx, 1,009 bp average Zfy—Shaw et al. 2003). Comparative DNA sequence analysis could reveal the basis of those size differences, which appear real because similar gel conditions and standards were used in both studies.

We prefer using Zfx and Zfy amplification method to identify gender because both sexes produce amplification product, compared with only using smaller regions on the Y chromosome (Meyers-Wallen et al. 1995), which yield amplification product only from males (thus, unless an internal control is used, an absence of PCR product can indicate either a female or an amplification failure). However, we agree with Shaw et al. (2003) that the large size of Zfx and Zfy fragments might preclude their use with noninvasive samples, such as hair, which typically yield low amounts of degraded DNA (Taberlet et al. 1999). This is especially true for the large Zfx and Zfy we detected in North American beavers. Smaller Zfx and Zfy regions than the one we used have been used for gender determination in many mammal species (using primers P1-5EZ and P2-3EZ—Aasen and Medrano 1990). However those primers do not differentiate male and female beavers because amplification results in fragments of both identical length and DNA sequence in both genders (data not shown). Gender determination in beavers using noninvasive samples will likely require either use of the Sry region or further optimization of Zfx and Zfy primers to target a smaller, variable subregion (Gilson et al. 1998).

In conclusion, the field method of gender identification in beavers was about 90% accurate. We recommend the use of Zfx and Zfy amplification using blood or tissue samples where 100% accuracy is required. This genetic method, which can be performed in any modestly equipped genetic laboratory in about 2 days, will likely improve the accuracy of gender determinations in other difficult to sex species, including Eurasian beavers (*Castor fiber*).

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