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TECHNICAL NOTE

Development of a reliable method for determining sex for a primitive rodent, the Point Arena mountain beaver (*Aplodontia rufa nigra*)

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Abstract The mountain beaver (*Aplodontia rufa*) is a primitive species of rodent, often considered a living fossil. The Point Arena mountain beaver (*Aplodontia rufa nigra*) is an endangered subspecies that occurs in a very restricted range in northern California. Efforts to recover this taxon have been limited by the lack of knowledge on their demography, particularly sex and age-specific vital rates. Recent studies have employed non-invasive genetic sampling to conduct capture-mark-recaptures to estimate abundance, survival and recruitment. Here we report on the development of a method using restriction fragment length polymorphisms to determine sex from tissues, bone and non-invasively collected hair samples for the Point Arena mountain beaver.

Keywords *Aplodontia rufa nigra* · Sex determination · Mountain beaver · Non-invasive genetic sampling · ZFX · ZFY

The mountain beaver (*Aplodontia rufa*) is the sole genus and species within the family Aplodontiidae, and represents the oldest known group of living rodents (Wilson and Reeder 2005). One of seven recognized subspecies, the Point Arena mountain beaver (*Aplodontia rufa nigra*) was listed as endangered under the US Endangered Species Act in 1991 (50 FR 64716) and occupies a small range in Mendocino County in northern California (Steele and Litman 1998).

In 2007 we initiated a survey to obtain information on population size, movements and genetic connectivity in several sites within the Point Arena mountain beaver geographic range, using non-invasive hair sampling. In our initial work, we developed nine variable microsatellite loci for this species (Pilgrim et al. 2006) combined with three additional loci (*ArE04F*, *ArA08F*, and *ArG05F*; Piaggio et al. 2009) that presented enough power to distinguish samples at the individual level (Zielinski et al. in press), and was therefore useful for noninvasive genetic analysis. In addition to identifying individuals, an understanding of differential survival, abundance, spatial patterning, and movement rates between males and females is critical. However, the monotypic nature of this species reduced the likelihood of success using methods developed for other rodents and current methods use amplification of the sex determining region Y-*SRY* (Bryja and Konecny 2003) which are not ideal for noninvasive applications.

Restriction fragment length polymorphism (RFLP) analysis of the conserved zinc-finger region of the X and Y-chromosomes (ZFX and ZFY) has been used previously to successfully sex many species (Aasen and Medrano 1990; Garcia-Muro et al. 1997; Fernando and Melnick 2001). The ability to determine sex from non-invasive samples using this region is preferable to other approaches such as the sex-determining region of the Y-chromosome (Y-*SRY*) because it provides a positive result for both sexes. In this note we report our use of restriction enzymes in combination with primers for the ZFX and ZFY to reliably distinguish male and female mountain beavers on a variety of samples.

Genomic DNA was extracted from Point Arena mountain beaver tissues from 52 individuals of known sex (25

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Table 1 Total number of tissue, hair and bone samples analyzed using the ZFX/ZFY method

Sample type	Total samples tested	Unique individuals	Male samples	Female samples	Failed	Individuals sexed	Sample % success
Tissue	55	55	27	28	0	55	100
Hair	206	84	90	81	35	71	83
Bone	2	2	1	0	1	1	50

Unique individuals represented by the hair samples were determined by microsatellite analysis

females and 27 males) and 3 juveniles that could not be sexed in the field, using the DNeasy Blood and Tissue kit, (Qiagen Inc.). Tissues for molecular sex determination were collected from live-trapped mountain beavers by snipping a small amount of skin (approximately 1 × 2 mm) from the edge of the external ear; these samples were stored in a vial with desiccant until DNA extraction. The sex of adult animals was determined by the presence or absence of a baculum. However, because the baculum is undeveloped in sexually-immature rodents we were unable to definitively determine the sex of 3 subadult individuals. Twenty tissues (2 males and 2 females) from adults of additional species were also extracted: Columbian ground squirrel (*Urocitellus columbianus*), deer mouse (*Peromyscus maniculatus*), snowshoe hare (*Lepus americanas*), yellow-pine chipmunk (*Tamias amoenus*) and coyote (*Canis latrans*). The coyote tissues were obtained from carcasses associated with predator-control actions while the other species were from mortalities during live-trapping efforts.

Additionally, DNA from 206 hair samples was also extracted using the DNeasy Blood and Tissue kit, (Qiagen Inc.) with modifications suggested by Mills et al. 2000. Optimally, 10 hairs with follicles were used for each DNA extraction from a sample although if there were fewer than 10 hairs, we used what was available for each sample. These hair samples were previously determined to be from mountain beaver using DNA sequencing analysis of the cytochrome b region and/or by amplification with species-specific microsatellites (Zielinski et al. In press). DNA from two bone samples was extracted using phenol–chloroform protocols according to Wisely et al. 2004. ZFX and ZFY fragments were amplified using the primers P1-5EZ and P2-3EZ (Aasen and Medrano 1990).

Polymerase chain reaction was performed in 50 µl reactions containing 1× reaction buffer (*Applied Biosystems*), 3.5 mM $MgCl_2$, 0.2 mM each dNTP's, 1 µM of each primer, 1 U Titanium *Taq* polymerase (*Clontech*) and ~20 ng genomic DNA. Samples were amplified using an initial 5 min of denaturation at 94 °C, followed by 35 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 1 min), with final extension time of 4 min at 72 °C.

Following DNA amplification, we applied three common restriction enzymes: *Alu*I, *Hpa*II and *Mbo*I. The reaction volume (10 µl) contained 5.0 µl PCR product, 0.6 µl restriction enzyme, 1.0 µl and 3.4 µl sterile water. Products were digested for a minimum of 3 h at 37 °C and run in a 2.6 % agarose gel containing ethidium bromide and gel star (Lonza Inc., Rockland, ME, USA).

Samples from mountain beaver males and females both amplified an approximately 500 bp product. Sex-specific PCR/RFLPs were obtained with the application of the *Mbo*I enzyme. Males and females consistently produced unique patterns, with both having the 500 bp product and males producing an additional digested band of 450 bp. PCR products from the five other species tested also were approximately 500 bp, however sex-specific PCR/RFLPs were not observed.

This test successfully sexed tissues from 3 juveniles that were captured of unknown sex and 1 of 2 bone samples in our study. Amplification success of hair samples was high (83 %) with 171 of 206 hair samples successfully sexed (representing 71 of 84 unique individuals noninvasively identified; Table 1). Hair and bone samples that weren't successfully sexed with this method were due to failure to amplify the ZFX/ZFY region.

We found a reliable DNA based method to determine sex for the Point Arena mountain beaver. This method is cost effective and is useful for tissue, hair and bone samples. Our observed high success in sex determination of hair samples is similar to those reported for other noninvasively sampled species based on different sex-typing protocols (Bellemain et al. 2004; Pilgrim et al. 2005; Schwartz and Monfort 2008). Development of this sex-typing method will allow us to test more sophisticated hypotheses to explain survival and abundance of the endangered Point Arena mountain beaver in California.

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