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A reliable, non-invasive method for sex determination in the endangered San Joaquin kit fox (*Vulpes macrotis mutica*) and other canids

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We describe a fast and reliable method to determine sex non-invasively from faeces. We designed a primer set that amplifies a short (195 bp) fragment of the zink finger (*Zfx* and *Zfy*) protein genes. This fragment contains a *Taq*I digestion site unique to the *Zfy* gene in the endangered San Joaquin kit fox (*Vulpes macrotis mutic*a). Successful PCR products showed a clear single band for females and a double band for males in 3% agarose gels. This protocol using the new set of primers also proved to be effective in tissue and faecal samples of other canid species.

Molecular genetics is a powerful tool with many potential applications for conservation biology and wildlife management. One such application involves the development of fast and reliable methods to determine sex non-invasively from shed integument and feces (Kohn et al. 1999). Analysis of zink finger (ZF) protein genes, that are found in both X and Y chromosomes, has been widely used in sexing studies (Aasen and Medrano 1990; Bérubé and Palsbøll 1996; García et al. 1997, Fernando and Melnick 2001; Lucchini et al. 2002). However, because the size of the fragments amplified with some of these previous methods is relatively large (868–1278 bp), they might be unsuitable for samples with degraded DNA (hair or scat; Shaw et al. 2003).

Other studies in mammals have demonstrated that degraded DNA material can be used to sex individuals (Kohn et al. 1999; Dallas et al. 2003; Eggert et al. 2003). In these studies, amplification of Y-specific fragments has been based on the Y chromosomal SRY gene, with positive amplification indicating male identity. Non-amplification of the target fragment does not equate with female identity as PCR amplification may fail for many reasons. Coamplification of a mitochondrial or single copy nuclear gene fragment(s) has been used as an external control to address this problem. However, the sensitivity and optimal conditions of different primer sets are unlikely to be identical and external controls may be unreliable, especially when amplifying from suboptimal sources of DNA such as hair or feces.

The objective of this study was to develop a method that is reliable for sex determination of samples with degraded DNA from the San Joaquin kit fox (*Vulpes macrotis mutica*). The endangered San Joaquin kit fox is a nocturnal and elusive species that is difficult to observe and capture at low population densities. Because of these characteristics, non-invasive genetic methods

are useful tools for providing valuable information (Taberlet and Luikart 1999).

Ear tissue biopsy from 31 individuals of known sex (15 males, 16 females) of the San Joaquin kit fox were used to amplify the *Zfy* and *Zfx* genes. We used the primers P1-5EZ and P2-3EZ described by Aasen and Medrano (1990), to amplify *Zfy* and *Zfx* fragments (~447 bp), and then search for internal primers. DNA extractions were performed using a DNeasy® kit (QIAGEN®). The final reactions were as follows: 1.0 μl of DNA, ddH_2O, 0.2 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems), 1.5 μl of each 10 μM primer, 25 mM of $MgCl_2$, 10× PCR Gold Buffer (Applied Biosystems), 2 mM of dNTPs, and 2 μl of 100 mg/ml BSA. PCR conditions were 35 cycles of 60 s at 94 °C, 60 s at 58 °C, 90 s at 72 °C, and a final extension step of 72 °C for 5 min.

PCR products were purified using the QIAquick® PCR Purification kit. Sequencing was carried out using the forward and reverse primers in an ABI model 377 automated sequencer. Results were analyzed using the program SEQUENCHER® (Gene Codes Corp.). Sequences of *Zfx* and *Zfy* from kit foxes were submitted to GeneBank (Accession Nos. AY310919–AY310920). In the analysis of the sequences for the male and female fragments, we determined that the males had a unique restriction enzyme site for *Taq*$^{\alpha}$I. Internal primers were designed using the program PRIMER3® (Rozen and Skaletsky 1996). With the new set of primers (forward ZFKF 203L 5′-CAAAAGGTGGCGATTCAATAA-3′, reverse ZFKF 195H 5′-ATGGAGAGCCACAAGCTR-AC-3′), amplification was narrowed to a 195 bp fragment that contains the *Taq*$^{\alpha}$I digestion site. We expected females to yield one band: one X band of 195 bp, while males would yield two visible bands: one X band of 195 bp and one Y band of 152 bp (Figure 1a).

We tested the reliability of this new protocol in 323 scat samples collected for a study to evaluate detection and accuracy rates of dogs trained to find scats of endangered San Joaquin kit foxes (Smith et al. 2003). DNA was extracted from every scat sample using a QIAGEN DNeasy™ extraction kit following manufacturer's protocol. Each sample was isolated a minimum of two times and typed three times to test for errors and ambiguities in sex identification. Negative controls (no scat material added to the extraction) accompanied each set of extractions and were used to check for contamination. PCR amplifications were run using a PTC-100 Programmable Thermal Cycler (MJ Research Inc.). A reaction volume of 25 μl contained 1.5 μl of DNA, ddH_2O, 0.2 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems), 10 μM of each new primer, 25 mM of $MgCl_2$, 10× PCR Gold Buffer (Applied Biosystems), and 2 mM of dNTPs. We used the same PCR conditions as described above.

PCR products were digested by restriction enzyme *Taq*$^{\alpha}$I in a volume of 20 μL. This contained 10 μl of PCR product, ddH_2O, 1 μl of NEB buffer

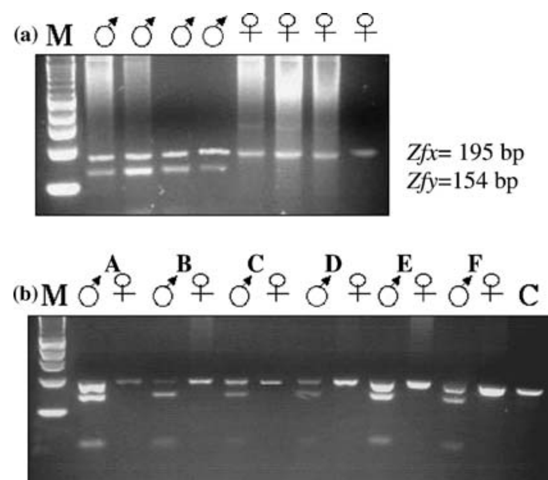

Figure 1. (a) Band patterns from PCR/RFLP analysis of four male and four female kit fox DNA extracts. DNA size marker (M) is a 100 bp ladder. (b) Band patterns for male and female DNA extracts from different canid species. The letters above the figures denote species: (A) domestic dog; (B) kit fox; (C) gray fox; (D) red fox; (E) coyote and (F) maned wolf.

Table 1. List of samples of five species of canids used to test the new ZF canid primers showing the different sample types and the number of samples successfully tested for each sex

Species	Tissue		Blood		Hair		Feces		Total
	M	F	M	F	M	F	M	F	
Coyote	4	3	–	–	–	–	–	–	7
Gray fox	1	2	–	–	–	–	–	–	3
Red Fox	1	1	–	–	–	–	–	–	2
Domestic dog	–	–	–	–	1	1	–	1	3
Maned wolf	1	2	6	2	14	11	5	13	54

Taq$^{\alpha}$I, 0.2 μl of BSA, and 5 U of restriction enzyme *Taq*$^{\alpha}$I (New England Biolabs). The digestion was incubated 3 h at 65 °C, and 20 min at 80 °C for heat inactivation. Products were run slowly (≈40 V) in 3% agarose gels stained with GelStar® (BioWhittaker Molecular Applications), photographed and examined to assess the sex of the samples.

In order to test the reliability of this protocol and to discard the possibility of PCR artifacts, we examined the sex of 75 scats from 16 individuals of known sex (8 females and 8 males). Of these 75 scats, 64 scats gave the predicted banding pattern for the correct sex, 4 did not yield PCR products and 7 amplified PCR products but did not yield reliable restriction enzyme patterns that we could score to identify the sex. For the unknown kit fox faecal samples, we were able to determine sex with 100% confidence for 86% of the faecal DNA extracts (n = 278). These samples showed consistent results for all three replicates. Twelve percent (n = 39) of the DNA extracts showed ambiguous results (i.e. one of the PCR did not work or the restriction enzyme analysis was ambiguous). Finally, 2% of our samples (n = 6) did not yield PCR amplifications for any of the three replicates.

The new primers were tested on other canid species using the same PCR and restriction enzymes conditions mentioned above. These primers were useful to sex the following 5 different canid species: coyote (*Canis latrans*), gray fox (*Urocyon cinereoargenteus*), red fox (*Vulpes vulpes*), domestic dog (*Canis familiaris*), and maned wolf (*Chrysocyon brachyurus*), tested for various tissue types (Table 1, Figure 1b). Our assessment of tissue types included faecal and hair samples where DNA degradation is likely or possible. Further testing is recommended in these other canid species, however, as our sample sizes are too small to confidently discard the possibility of polymorphism at the *Taq*$^{\alpha}$I restriction site.

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