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TECHNICAL NOTE

An efficient noninvasive method for discriminating among faeces of sympatric North American canids

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Abstract Molecular technology can identify species noninvasively from faeces found in the field. We describe a fast and reliable genetic method that differentiates faeces of five potentially sympatric North American canids without using multiple primer sets or restriction enzyme digestion. Our primer set amplifies a short fragment (237–288 bp) of the mitochondrial d-loop that is a different length in each species: kit fox (*Vulpes macrotis*), red fox (*Vulpes vulpes*), gray fox (*Urocyon cinereoargenteus*), coyote (*Canis latrans*), and dog (*Canis familiaris*). We extensively tested our technique using published and novel d-loop sequences and then applied it to two large faecal data sets collected in California and Virginia. It provides an efficient tool for noninvasively distinguishing sympatric canids in diverse regions of North America.

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Existing molecular methods to identify faeces of sympatric carnivores use either multiple primer systems, real-time PCR, or restriction enzyme digestion of a PCR product (e.g. Krausman et al. 2006; Bidlack et al. 2007; O'Reilly et al. 2007). Building on the work of Paxinos et al. (1997), we developed and tested a fast and reliable method for distinguishing faeces of five potentially sympatric North American canids—kit fox (*Vulpes macrotis),* red fox (*Vulpes vulpes*), gray fox (*Urocyon cinereoargenteus*), coyote (*Canis latrans*), and domestic dog (*Canis familiaris*)—based on sequencing part of the mitochondrial d-loop.

We designed primers from ear tissue biopsies from kit, red, and gray foxes, coyote, and dog from the San Joaquin Valley, California. Tissues were stored in 95% ethanol at room temperature and extracted using the DNeasy® Tissue kit (QIAGEN®). Universal primers (THRL-15926 and DLH-16340, Kocher et al. 1989) were used to amplify a 394 bp fragment of the d-loop region of mitochondrial DNA. Each 22 µl PCR reaction contained 4.0 µl template DNA, 0.23 mM each primer, 2.3 mM $MgCl_2$, 1 unit *Taq Gold* polymerase, 1.1X *Taq* buffer (Applied Biosystems), 0.91 mg/ml BSA, and 0.23 mM each dNTPs. The PCR profile included 10 min at 96°C, followed by 34 cycles of 1 min at 94°C, 1 min at 53°C, and 1.5 min at 72°C, and an extension of 5 min at 72°C. PCR products were cleaned using QIAquick® PCR purification kit (QIAGEN®). Sequence reactions were done with Big Dye v3.1 Terminator cycle sequencing kits (Applied Biosystems) and cleaned via Sephadex G-50 columns (GE Healthcare). Sequences were analyzed using an ABI PRISM 377 or a 3100 automatic sequencer and aligned by eye using

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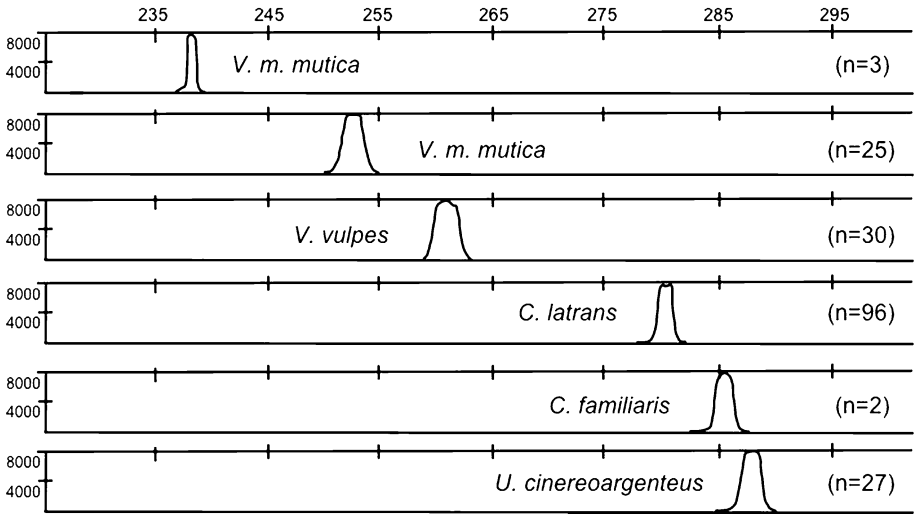

Fig. 1 Chromatograms showing variation in fragment size for five canid species. Sample sizes (*n*) include only faecal samples for which species assignment was verified by sequencing

Sequencher® 5.0 (Gene Codes Corporation). Primers (KFSPID-F 5′-TCAGCACCCAAAGCTGAAAT-3′ labeled with FAM on the 5′end and KFSPID-R 5′-GTTTCTCGAGG CATGGTGAT-3′) were designed for both flanking ends of a variable region. This set of primers amplified fragments ranging from 237 to 288 bp using the above PCR protocol. To measure base-pair size differences, samples were run with GS-500 ROX size standard on an ABI PRISM 3100 automatic sequencer and analyzed in Genotyper® 2.5 (Applied Biosystems). All five canid species could be distinguished based on variation in the size of the amplified fragment (Fig. 1).

To test the reliability of the method in other parts of North America, we examined the variable region in sequences obtained from GenBank. We downloaded 8 kit fox, 69 red fox, 39 dog, and 185 coyote sequences (Online Resource 1). Because there were no d-loop sequences for gray fox, we sequenced the variable region of 290 gray foxes from tissues collected across the Eastern U.S. (Bozarth et al., in preparation) and found 32 unique haplotypes. Sequences for all species were aligned by eye in Sequencher® 4.8 (Gene Codes Corporation) and examined for length polymorphism. Kit fox sequences ranged from 252 to 253 bp, red fox sequences from 260 to 264 bp, and coyote sequences from 279 to 283 bp. All dog sequences were 286 bp and all gray fox sequences were 288 bp.

We then tested our primers on samples from two large studies; one targeting kit foxes in the San Joaquin Valley, California (e.g. Smith et al. 2006), and one targeting coyotes in Northern Virginia. The kit fox is broadly sympatric with coyotes and both target species are potentially sympatric with red and gray foxes, and dogs. Faecal samples were stored either at −20°C or at room temperature with silica beads. We extracted DNA using the QIAamp DNA stool kit (QIAGEN®) and sequenced a subset of the samples using the methods described above. In 28 California samples, we found 3 kit fox haplotypes at 253 bp and one with a significant deletion of 16 bp. In 155 Virginia samples, we found 5 red fox, 8 gray fox, 7 coyote, and 2 dog haplotypes, all within previously reported size ranges. We validated these species identifications based on fragment length by sequencing. We then used the method on all of the samples in these two data sets. We identified 1224 kit fox and 8 coyote samples from the 1,232 California samples and 111 red fox, 98 gray fox, 114 coyote, and 4 dogs from the 327 Virginia samples.

Application of our method to published and novel haplotypes and two large faecal data sets revealed a high degree of intraspecific size polymorphism in the region of interest that proved very efficient for the identification of these five canid species in diverse regions of North America. Our method is being used successfully in ongoing studies in California and Virginia as well as by Kays et al. (2008) in New York. However, swift fox (*Vulpes velox*) and kit fox as well as red wolf (*Canis rufus*) and coyote cannot be distinguished using this method. Swift foxes are closely related to kit foxes and have the same fragment length (Maldonado et al. 1997). Additionally, both the single red wolf tissue we sequenced and the only published d-loop haplotype (Adams et al. 2003) are the same length as a common coyote haplotype (282 bp). Methods for differentiating coyotes, red wolves, and their hybrid offspring exist (Adams et al. 2007; Adams and Waits 2007).

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