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SHORT COMMUNICATION

A noninvasive method for distinguishing among canid species: amplification and enzyme restriction of DNA from dung

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Abstract

Endangered San Joaquin kit foxes *Vulpes macrotis mutica* can be sympatrically distributed with as many as four other canids: red fox, gray fox, coyote and domestic dog. Canid scats are often found during routine fieldwork, but cannot be reliably identified to species. To detect and study the endangered kit fox, we developed mitochondrial DNA markers that can be amplified from small amounts of DNA extracted from scats. We amplified a 412-bp fragment of the mitochondrial cytochrome-*b* gene from scat samples and digested it with three restriction enzymes. The resulting restriction profiles discriminated among all five canid species and correctly identified 10 'unknown' fox scats to species in blind tests. We have applied our technique to identify canids species for an environmental management study and a conservation study. We envision that our protocol, and similar ones developed for other endangered species will be greatly used for conservation management in the future.

Keywords: kit fox, canid, endangered species, PCR amplification, noninvasive DNA sampling

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Introduction

Molecular genetics show great potential for use in conservation biology and wildlife management. One such application involves the development of species-specific genetic markers that can be detected noninvasively from shed integument, regurgitated items, or defecation products (Höss *et al*. 1992; Taberlet & Bouvet 1992; Constable *et al*. 1995; Gerloff *et al*. 1995). These markers can then be used to identify and survey elusive species or individuals. Endangered San Joaquin kit foxes *Vulpes macrotis mutica* in California's San Joaquin Valley are often sympatrically distributed with the native gray fox *Urocyon cinereoargenteus*, the introduced red fox *Vulpes vulpes*, the coyote *Canis latrans*, and the domestic dog *Canis familiaris*. The fox species may be secretive and difficult to detect with standard census methods (Orloff 1992), especially at low population densities. In addition, fox scats found in the field cannot be reliably identified to species on the basis of appearance or morphometrics (Halfpenny 1986; Clifton 1992). In order to improve detection of endangered kit fox and therefore enhance conservation and management strategies, we developed a system of restriction enzyme digestion of PCR-amplified mitochondrial DNA that can be easily and reliably amplified from scats found during routine fieldwork.

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Materials and Methods

Fox tissue or DNA samples ($n = 21$) and scats ($n = 10$) were obtained from field sites, museum tissue collections, and zoos. The identities of the tissue and DNA samples were known (four gray fox, four red fox and 13 kit fox), but the 10 scat samples were provided to the laboratory as 'unknowns' in order to test the reliability of our extraction and diagnosis techniques in a blind trial, and were sent to the lab in numbered paper bags. Collection localities were known from all samples. San Joaquin kit foxes were from 11 different groups within the San Joaquin Valley, California. Red and gray foxes were collected in both Los Angeles and Orange counties, California.

DNA from tissue samples was isolated by standard methods of Proteinase K digestion, phenol–chloroform extraction, and DNA precipitation in ethanol (Sambrook *et al*. 1989). DNA from scats was isolated with two different methods under quasi-clean room conditions to prevent contamination of potentially suboptimal material with the more robust DNA of fresh tissue samples (Pääbo 1990; Cooper 1994). Although there is DNA throughout the scat in the form of shed epithelial cells, scats remain unprotected from sun and rain for unknown lengths of time prior to collection, and may sustain oxidative and hydrolytic damage (Lindahl 1993). In the first method, after removal of rodent bones and visible plant material, about 0.5–1.0 g of fox scat was minced with a sterile razor blade and washed twice with 0.5 M EDTA (pH 8.0) via inversion followed by centrifugation. The samples, including extraction controls (reagents without template), were incubated with rotation for 12–24 h at 55 °C in 12 mL extraction buffer each [0.01 M NaCl, 0.01 M Tris-HCl, 0.1 M EDTA (pH 8.0), 1 mg/mL Proteinase K, 10 mg/mL DDT, and 1% SDS], then extracted three times with phenol and once with Chloroform : Isoamyl alcohol (24 : 1). The aqueous phase was filtered through Centriprep 30™ (Amicon) concentrators and rinsed twice via centrifugal dialyses with 2 mL deionized sterile water. Retentate was brought to 100 μL, and divided into aliquots to provide backup stocks in the event of post-extraction contamination of 'working' stocks. Two microlitres retentate was used for each subsequent PCR amplification reaction.

Although organic extraction of DNA is reliable, it is unattractive for forensic applications due to long turnaround time, number of steps and extra sample transfers which increase the probability of cross-contamination. We therefore modified a Chelex® 100 resin (Bio-rad) method (Walsh *et al*. 1991) for DNA isolation from scat samples. Scat material (≈ 0.1–0.3 μg) was suspended in 500 μL 5% Chelex® 100 in sterile water in screw-cap tubes. Samples were then boiled in a waterbath for 7 min, vortexed at full speed, boiled another 7 min, and finally centrifuged 5 min at 14 000 *g*. Extraction controls (all reagents, no scat material) were made concurrently throughout the procedure with the samples. The supernatant was removed as a source of template DNA and aliquoted into three sterile Eppendorf tubes. Four microlitres was used for each subsequent PCR amplification.

Using published mitochondrial cytochrome-*b* sequences from three fox species (Geffen *et al*. 1992), and the coyote (Gotelli *et al*. 1994), we designed a canid specific primer, CanidL1 (5'-AATGACCAACATTCGAAA-3') as well as a previously described primer, H15149 (Kocher *et al*. 1989) to amplify a 412-bp fragment of this gene. Final amplification reagents in 100 μL volumes were: 1X reaction buffer (Perkin-Elmer), 2.5 mM $MgCl_2$, 200 μM each dNTP, 1.7 mg/mL Fraction-V BSA, 2 units *Taq* polymerase (Perkin-Elmer), and 1 μM each primer. The reactions for scat extracts as well as extract and PCR controls (reaction components without template) were cycled 35 times following an initial hot start using the following profile: 94 °C for 1 min, 52 °C for 1 min, and 72° for 1.5 min. There was no evidence of contamination of extraction or amplification reagents. Products amplified from the DNA isolated from the 10 scats, as well as the 21 known tissue samples were gel purified via electrophoresis in TAE buffered 2% NuSieve™ agarose, then excised, and eluted (Wizard Prep™, Promega). PCR products were sequenced on both strands using an ABI 373 automated DNA sequencer (using PRISM™ Ready Reaction DyeDeoxy Terminator Cycle Sequencing chemistry: ABI). Sequences from tissues with known identity were aligned to reveal diagnostic restriction endonuclease cut sites and percentage sequence divergence between species.

Although sequencing is the most reliable method, it is expensive and time consuming. We therefore developed a system of restriction endonuclease digestion of the amplified region that easily distinguishes between canid species. Cytochrome-*b* amplification products from DNA isolated from scats were subjected directly to restriction digestion by three endonucleases following manufacturers' recommended conditions. Digest products were visualized with ethidium bromide staining following electrophoresis in TBE buffered 2% NuSieve agarose.

Results

Chelex® extractions of scat samples were comparable in quality and reliability in producing PCR amplification products to more expensive organic methods. The Chelex® method was preferred because it eliminated the need of laborious centrifugal dialyses to remove detergents, salts and enzymes from extracts, and involved fewer steps and sample transfers, thereby minimizing the potential for contamination.

Alignment of 378-bp of cytochrome-*b* sequence of the three fox species (total $n = 13$) revealed uncorrected sequence differences of 12.0% for kit vs. red, 14.4% for red vs. gray, and 14.7% for kit vs. gray foxes (Fig. 1). Restriction sites diagnostic for each fox species were present in the aligned sequences. Restriction endonuclease digestion of the PCR products with two enzymes (*Alu*I and *Hin*fI) resulted in profiles that distinguished each of the fox species from each other, and all of the foxes from domestic dogs and coyotes (Fig. 2a and b). A third enzyme (*Taq*I) also distinguished the three fox species from each other (Fig. 2c). However, the kit fox, coyote, and domestic dog could not be distinguished with this enzyme, as these three species do not have a *Taq*I restriction site. 'Correct' species-specific digest profiles were found for every individual amplified (seven gray, eight red, and 16 kit foxes)

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Vulpes macrotis           GACTCACCCACTAGCTAAAATCGTTAACGACTCATTCATTGACCTCCC [48]
Vulpes vulpes             ..........AluI.....C..........AluI..T..T..
Urocyon cinereoargenteus  ..AC.....CG.....C...AG..TaqI.....G.....A..

Vulpes macrotis           CGCACCATCAAACATTTCTGCCTGATGAAACTTCGGATCCCTCCTAGG [96]
Vulpes vulpes             ..........T.....G.....G.....G.....
Urocyon cinereoargenteus  T.....G.T.....A.....G.....G.....GT....
                             AluI

Vulpes macrotis           TATCTGCCTCATTCTACAGATTATAACAGGCCTATTTCTAGCCATACA [144]
Vulpes vulpes             .G.A.....T.....GC......T......T.........
Urocyon cinereoargenteus  A........T.....T....CT.G........

Vulpes macrotis           CTACACATCCGATACAGCTACTGCTTTTTCATCAGTTACACACATTTG [192]
Vulpes vulpes             ...T.....T..C..........C..T.....C..
Urocyon cinereoargenteus  ...T.....G..C..C..C..A..C.....C.....C..T..C..
                                      AluI

Vulpes macrotis           CCGAGACGTAAACTACGGCTGAATCATTCGCTACATACATGCAAATGG [240]
Vulpes vulpes             .....T.....T.....T..C..T.....C..
Urocyon cinereoargenteus  T.....T.....T.....C.....T.....C..C..C..
                          TaqI          HinfI

Vulpes macrotis           AGCATCTATATTCTTCATTTGTCTGTTCATGCACGTGGGACGAGGCCT [288]
Vulpes vulpes             .....T..T..C..C..C.....A.....T.
Urocyon cinereoargenteus  ...C..A.....C..C..A...C.....A.....A..

Vulpes macrotis           ATATTATGGATCCTACGTATTCATAGAAACATGAAACGTCGGAATTGT [336]
Vulpes vulpes             ...C.....T..T.....TA.T.....A.
Urocyon cinereoargenteus  ...C.....TAC.....A.

Vulpes macrotis           CTTACTATTCGCAACCATAGCCACAGCATTTATAGGTTACGT       [378]
Vulpes vulpes             ....T.G.....G.....G..C.....
Urocyon cinereoargenteus  .C.....T..G.....C.....C..T..
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Fig. 1 378 bp of cytochrome-*b* sequence from three sympatric fox species. Enzyme-restriction sites are underlined and identified.

including the 10 scat samples (four gray, three red, three kit foxes). Restriction digestion proved highly reliable for the identification of fox species from their scats.

Discussion

We present above a simple method of isolating DNA from canid scats and a protocol for PCR amplifying a small fragment of DNA from the scat DNA. Further, we show that digestion with a set of three restriction enzymes provides clean and reliable markers for identification of five sympatric canids to species. The ability to identify canid species present at a given locality by mitochondrial DNA markers amplified from scats has been useful for biologists conducting surveys of San Joaquin kit foxes. In the last year, we successfully identified unknown canid scats to species for two separate conservation programmes: the San Joaquin Endangered Species Recovery Planning Program (Fresno, CA) and PRC, Environmental Management, Inc. (Denver, CO). Our results provided these organizations with species determination unavailable by other means. The Chelex® and restriction enzyme methods we adapted have proven much less time consuming and expensive but are equal in reliability to trapping programmes for the identification of canids.

Coyotes, red foxes and domestic dogs may prey upon kit foxes (Ralls & White 1995) and all canids may scavenge

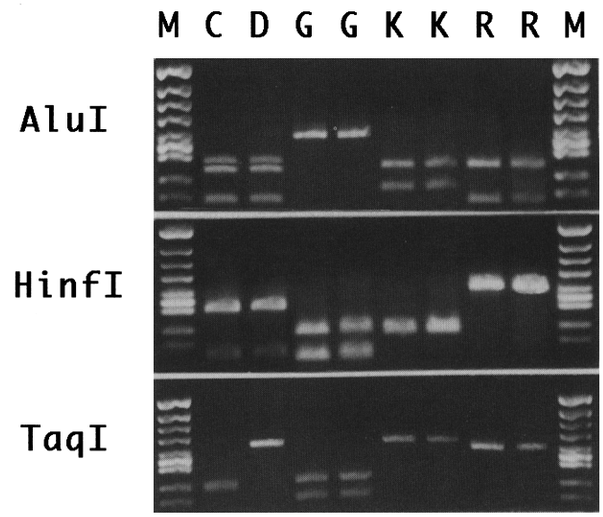

Fig. 2 Restriction enzyme digested cytochrome-*b* amplification products of five canid species electrophoresed in a 2.0% agarose gel: coyote (C), domestic dog (i.e. greyhound; D), gray fox (G), kit fox (K), and red fox (R). DNA size marker (M) is *Hinc*II-digested ϕx 174 [fragment sizes from the top are 1057 bp, 770 bp, 612 bp, 495 bp, 392 bp (345–341–335 bp combined) (297–291 bp combined), 210 bp, and 162 bp]. (a) *Alu*I digests. (b) *Hin*fI digests. (c) *Taq*I digests. The second lane of each fox species is a digestion of a product amplified from scat. Coyote and domestic dog had identical patterns for *Alu*I.

food from the carcass of another canid species. Thus DNA from more than one species could conceivably be amplified from a single scat, and would be evident as 'hybrid' restriction digest patterns or ambiguous sequence. Such a result would indicate that both species were present at the locality where the scat was collected. Their identities could be explicitly resolved using a restriction enzyme which cuts only one species' amplification product, leaving all others intact. When electrophoresed through agarose, the uncut product could be isolated from agarose and then identified using restriction enzymes, or sequencing.

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