FECAL DNA METHODS FOR DIFFERENTIATING GRIZZLY BEARS FROM AMERICAN BLACK BEARS

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Abstract: Cost effective methods to rapidly confirm the presence of grizzly bears (*Ursus arctos*) could contribute to their conservation. Although methods to differentiate mitochondrial DNA (mtDNA) of grizzly bears from those of American black bears (*U. americanus*) have already been published, these require automated sequencing equipment. We present a method using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis of mtDNA from fecal samples to differentiate these 2 bear species without the use of automated equipment.

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Brown bears have historically had a wide Holarctic distribution, ranging from Europe and Asia to North America. In the contiguous lower 48 states, 6 threatened grizzly bear sub-populations exist in Idaho, Montana, Wyoming, and Washington (Servheen 1990). American black bears are also present in these areas, and differentiating sign left by the 2 species can be difficult. Confirming the presence of grizzly bears is critical to the designation and monitoring of grizzly bear recovery zones.

Based on DNA sequence variations observed by Shields and Kocher (1991; see also Taberlet and Bouvet 1994; Taberlet et al. 1995; Paetkau and Strobeck 1996; Waits et al. 1998, 1999; Woods et al. 1999) we developed a PCR–RFLP method for species identification using DNA from scat. The relative ease of fecal sample collections should enhance grizzly bear confirmation probabilities. This approach was first validated with 2 matched blood and fecal samples each from grizzly and black bears, and then used to determine the species origin of 11 grizzly bear and one black bear from their feces. Geographic differences in RFLP patterns were also examined in 16 grizzly bears and 9 black bears.

STUDY AREA AND METHODS

Fecal samples of unknown species of bears were collected from the Yellowhead Region, Alberta, Canada; Glacier National Park, Montana; and the Alpine Lakes Wilderness Area, King County, Washington. Samples from Yellowhead ($n = 7$) were mixed, and a 30 g portion was wrapped in a coffee filter and placed in a zip-lock bag containing 120 g of silica as a preservative. Samples were stored frozen in this manner until freeze-dried, ground, and sifted into a powder for extraction. The Alpine Lakes sample was stored in a plastic bag on ice for transport and then frozen. Five 15-g subsamples were freeze-dried, ground, and sifted through a 3.2 mm nylon mesh screen. Two hundred mg of the powdered samples (Alpine Lakes 1A–1E) were extracted as in Wasser et al. (1997), and eluted in 120 µl of elution buffer (AE; Qiagen, Chatsworth, California, USA). Unknown Glacier National Park scat samples ($n = 4$) collected on silica or oven-dried were ground to powder, sifted, and extracted as above. We extracted control black ($n = 2$) and grizzly bear ($n = 2$) DNA from blood using a QIAamp kit (Qiagen, Chatsworth, California, USA) with the supplied reagents and procedures.

Control and unknown samples were PCR–amplified with the primers HSF21 and LTPROBB13 (Wasser et al. 1997), specific to a conserved region in the transfer RNA (tRNA) proline gene and a portion of the control region of mtDNA. All PCR reactions included mock extraction blanks and positive and negative DNA controls; all were physically separated from DNA extraction areas. The samples (2–5 µl of blood or fecal DNA extracts) were amplified in 25 µl volumes using 2.0 millimolar (mM) magnesium chloride ($MgCl_2$), 1X PCR buffer (Promega, Madison, Wisconsin, USA), 200 micromolar (µM) deoxynucleotide triphosphates (dNTPs; New England Biolabs, Beverly, Massachusetts, USA), 0.4 µM each primer, 2.5 units (U) Taq Polymerase (Promega), and 0.8 micrograms (µg) bovine serum albumin (BSA; New En-

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gland Biolabs). The samples were amplified in a MJ Research PTC-100 Thermocycler (MJ Research, Inc., Waltham, Massachusetts, USA) using the following cycling parameters: 94°C for 2 min; 35 repetitions of cycle (92°C for 30 sec, 53°C for 30 sec, 72°C for 40 sec); and 72°C for 3 minutes.

PCR products were electrophoresed on horizontal GenePhor acrylamide gels (GeneGel Clean 15/24, Amersham-Pharmacia Biotech, Uppsala, Sweden) and stained with DNA silver stain reagents (Amersham-Pharmacia Biotech). To detect RFLPs, 5 µl of the PCR products were digested in 15 µl volumes using 4 units of restriction enzymes *MseI* or *DraI* (New England Biolabs), and 6 µls were loaded on a GenePhor gel.

RESULTS

Ursid Identification from Unknown Samples

Four scat samples with matched blood from 2 known brown and 2 black bears were PCR amplified with the mtDNA 5' control region primers. All scat and blood sample replicates amplified, with product sizes of approximately 220 base pairs (bp) for black bears and approximately 200 bp for grizzly bears (Fig. 1). The PCR product sizes differ due to an insertion–deletion region of variable length in black and grizzly bears (Paetkau and Strobeck 1996, Wooding and Ward 1997, Woods et al. 1999).

The PCR products were digested with restriction enzymes *RsaI*, *AluI*, *Sau3A*, and *MseI* and electrophoresed on acrylamide gels. Although RFLPs were observed with all the enzymes used (not shown), *MseI*—a 4-base cutter with the recognition sequence 5' TTAA (T = thymine, A = adenine)—gave the most distinctive patterns for distinguishing black and grizzly bears.

Seven samples were identified as grizzly bear from the Yellowhead Region and 4 fecal samples were identified as grizzly bear from Glacier National Park (Figs. 1 and 2). The unknown scat sample collected in the Alpine Lakes Wilderness Area was conclusively identified as black bear (Fig. 1, lanes 8–10), although the paw prints and the diameter of the scat (6.5 cm) had suggested grizzly bear.

Intraspecific Variation

Matched blood and fecal DNA from 5 grizzly bears at Woodland Park Zoo (Canada, $n = 2$; Montana, $n = 3$) gave identical *MseI* RFLPs, as did the previously unidentified samples from Glacier National Park. Grizzly bear samples from Yellowhead, Alberta ($n = 7$), also exhibited the same *MseI* restriction profile (Fig. 2). We did not detect any localized geographical mtDNA differences resolvable by PCR–RFLPs in the 16 samples we analyzed, although nucleotide differences resolvable by DNA sequencing may be present nonetheless.

Black bear samples from representative geographic locations in Washington state (Olympic Peninsula, $n = 3$; West Cascades, $n = 3$; East Cascades, $n = 3$) were simi-

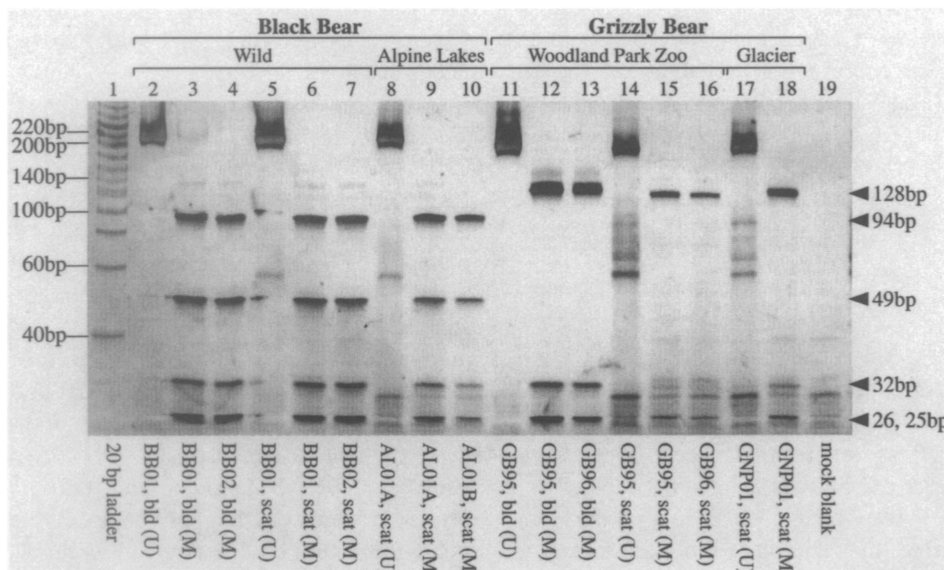

Fig. 1. DNA restriction fragment length polymorphisms (RFLPs) from matched blood (bld) and fecal samples following PCR amplification and digestion with *MseI (M)* for black (BB) or grizzly bear (GB) controls and unknown fecal samples. "U" indicates undigested PCR products. AL = Alpine Lakes Wilderness Area, GNP = Glacier National Park.

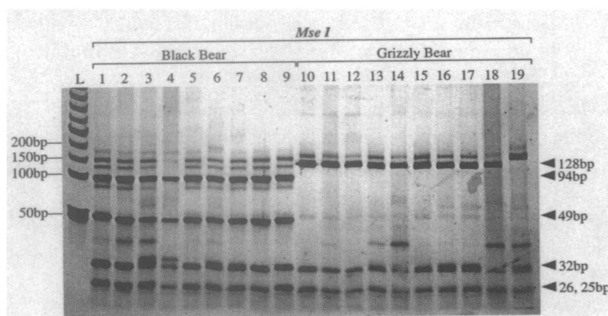

Fig. 2. Species assignment for black and grizzly bears using PCR-RFLPs with the restriction enzyme *MseI*. Black bear samples (lanes 1–9) were from Washington state. Grizzly bear samples were from Canada (WPZ GB95 exact location unknown, lane 10), Montana (lanes 11 and 12), and Yellowhead, Alberta (lanes 13–19). Faint bands not marked by arrows are partial restriction digestion products. Lane "L" is a 50 bp DNA ladder.

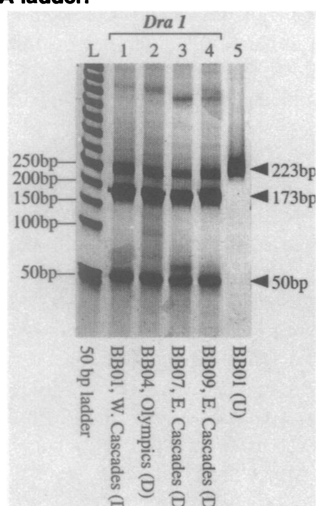

Fig. 3. Representative samples (4 shown) for clade assignment of Washington State black bears using PCR-RFLPs with the restriction enzyme DraI (D). Faint bands not marked by arrows are partial restriction digestion products. Lane "L" is a 50 bp DNA ladder; "U" indicates an undigested PCR product.

larly examined for mtDNA differences, but all displayed a uniform mtDNA haplotype using the restriction enzyme *DraI* (5' TTTAAA) (Fig. 3).

DISCUSSION

Species Identification from Scat

The use of scat, despite its ease of collection, has been limited in conservation applications primarily due to sample degradation and/or the presence of PCR inhibitors. These PCR inhibitors can be particularly problematic for fluorescent methods used in species identification, genotyping or sequencing, that have been successfully applied to DNA from hair (Woods et al. 1999, Mowat and Strobeck 2000, Clarke et al. 2001), tissue, or blood. Although new methods for the extraction and amplification of DNA from scat have been developed (Flagstad et al. 1999, Kohn et al. 1999, Clarke and Wasser, unpublished data), they may not be cost effective for all researchers. Recent studies using mtDNA PCR–RFLPS for canid identification from scat (Foran et al. 1997, Paxinos et al. 1997, Kohn et al. 1999) demonstrated the feasibility of using such an approach. We developed a bear species identification test using mtDNA PCR–RFLPs that could be used with DNA from scat. This method is fast, cost-effective, and does not rely on the use of fluorescent detection systems. The broad utility of PCR–RFLPs has been demonstrated for forensic or conservation purposes in a number of species (Taberlet et al. 1995, Foran et al. 1997, Paxinos et al. 1997, Wooding and Ward 1997, Pilgrim et al. 1998, Kohn et al. 1999, Steve Fain, USFWS, Ashland, Ore., USA, personal communications, 1995–2000).

Phylogeographic localization of mtDNA haplotypes

Extensive DNA sequence information on ursid mitochondrial genes is now available, due to the work of many researchers (e.g. Taberlet and Bouvet 1994; Paetkau and Strobeck 1996; Talbot and Shields 1996*a,b*; Waits et al. 1998, 1999; Matsuhashi et al. 1999). This information is useful for identifying inter and intra-species differences as demonstrated by Shields and Kocher (1991), Avise (1994), Kohn et al. (1995), Taberlet et al. (1995), and Wooding and Ward (1997). A comparison of DNA sequences of black to grizzly bear showed that grizzly bears have 2 transitions (T to C [cytosine] and A to G [guanine]) upstream of the insertion–deletion region that result in the loss of a *MseI* site (Taberlet and Bouvet 1994, Paetkau and Strobeck 1996). Although this restriction site loss was apparently conserved in all the grizzly bears we examined (n = 16), it appears to be retained in clades of grizzly bears we did not examine (Clades I–III, Talbot and Shields 1996*a*). This being the case, PCR–RFLPs may be of further use to delineate grizzly bear mtDNA contact zones in North America, particularly between Clades III and IV, as demonstrated by Taberlet et al. (1995) in brown bear lineages in Sweden.

Wooding and Ward (1997) used a PCR–RFLP method with the restriction enzymes *AflII* (5'CTTAAG) and *MboI* (5'GATC) to distinguish between 2 highly divergent mtDNA clades of black bears (Clades A and B) roughly separated by the Continental Divide. Black bear Clade B was detected at 2 sampling localities: Mendocino County, California, and the Yaak River in British Columbia. Additional sites in Alberta and Montana showed varying degrees of introgression with Clade A. The restriction enzyme *DraI* (5'TTTAAA) also differentiates between

Clades A and B. All 9 of the black bear samples we tested from 3 separate geographic locations in Washington state were from Clade B, with no detectable mitochondrial DNA introgression with Clade A.

MANAGEMENT IMPLICATIONS

We have developed a rapid, reliable, and non-invasive genetic method for discriminating grizzly from black bears in the absence of visual confirmation and expensive DNA fluorescent sequencers. These results demonstrate the usefulness of PCR–based RFLPs to assign species to unknown samples for forensic, management, or conservation purposes. In addition, RFLPs used in conjunction with sequence information can give informative lineage classifications and offer ways to quickly assess potential contact zones. This procedure offers a powerful management tool to aid recovery efforts of endangered species in general.

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