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Identification of mustelids using mitochondrial DNA and non-invasive sampling

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One of the most fundamental issues in conservation biology is the determination of species distribution and richness. Many species, including forest carnivores, are elusive and secretive, making it difficult or impossible to make even basic conservation decisions on listing, delisting, or threat status. Non-invasive sampling of hairs left on hair snares can substantially increase our detection of such species. In an extension of a nationwide project using hair snares across 12 states to identify lynx (*Lynx canadensis*) over three years (McKelvey et al. 1999; Mills et al. 2000; McDaniel et al. 2000), we developed DNA protocols to identify other forest carnivores. With genetic protocols already in place for felids, canids, and ursids (Mills et al. 2000; Paxinos et al. 1997; Pilgrim et al., unpublished) we felt the need to target additional mid-sized forest carnivores. We concentrated our efforts on mustelid identification protocols for fisher (*Martes pennanti*) and wolverine (*Gulo gulo*) because of their sensitive status (USDA Forest Service and the Bureau of Land Management classify them as sensitive species) but also due to the lack of knowledge about present distributions. In addition, we were able to develop protocols for marten (*Martes americana*), mink (*Mustela vison*), and striped skunk (*Mephitis mephitis*). Our methods were designed to be cost and time efficient and work well on low-quality DNA samples.

Initially we used known cytochrome b (cyt b) sequences from wolverine, fisher, marten, mink, and striped skunk (GenBank accession AB051245, AF057131, AF154964, AB026109, X94927) to investigate potential diagnostic restriction sites. Using the program Sequecher version 3.1 (Genecodes Corp., Ann Arbor MI) we generated restriction maps showing potential restriction sites with a corresponding enzyme. We tested these restriction sites using known tissue samples from a wide geographic range: 27 wolverines (6 Montana, 5 Idaho, 1 Oregon, 5 Wyoming, 2 Alaska, 2 BC, 1 Ontario, 2 Yukon, 1 Manitoba, 2 Alberta), 80 fisher (18 Montana, 5 Oregon, 7 Idaho, 5 Minnesota, 3 Wyoming, 4 Wisconsin, 38 California), 25 marten (11 Montana, 4 Minnesota, 4 Alaska, 1 Idaho, 5 Wyoming), 24 mink (3 Oregon, 6 Washington; from fur farms, 2 Washington, 12 New York, 1 Russia), and six striped skunk (2 Montana, 3 Wisconsin, 1 Minnesota). Geographic range validation was used to ensure the protocol correctly identified the mustelid species for many locations. We also tested known hair samples from wolverine (8) and fisher (11) to ensure the protocol worked on hair samples. Several other carnivore species were included during protocol development to test our ability to distinguish the five targeted species: long-tailed weasels (4; *Mustela frenata*), least weasel (1; *Mustela nivalis*), badger (13; *Taxidea taxis*), river otter (13; *Lutra canadensis*) and raccoon (8; *Procyon lotor*).

Genomic DNA was extracted from tissues using standard Qiagen protocols for tissue (Dneasy tissue kit). Genomic DNA was extracted from hair samples using the Qiagen tissue protocol with slight modifications following the approach of Mills et al. (2000). A target of 5–10 hairs (preferably with follicles) were

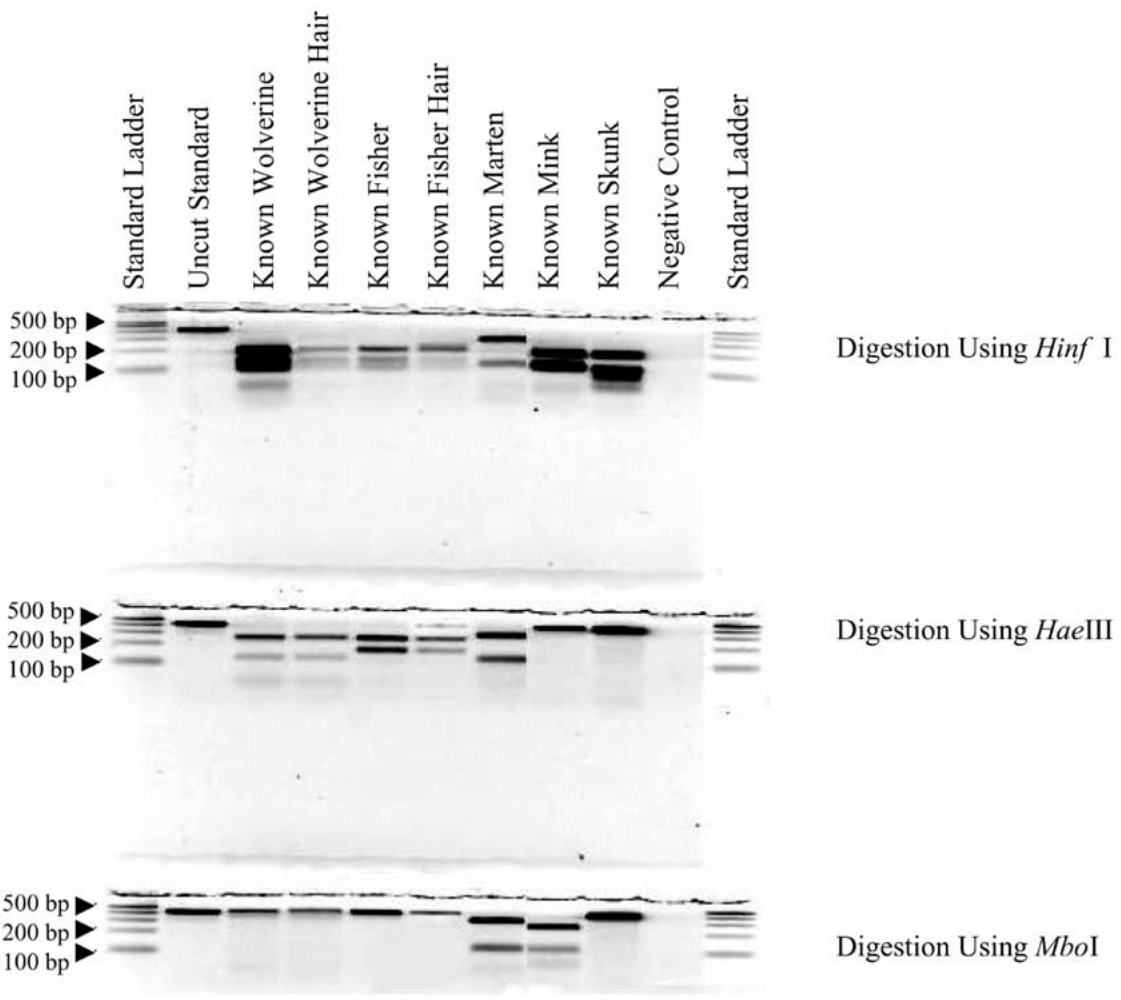

Figure 1. 2.5% agarose gel showing the differences among species in the cytochrome b region of mitochondrial DNA. The first panel shows PCR products digested with *Hinf*I, the middle with *Hae*III, and the bottom with *Mbo*I. Restriction products are shown for the five target species (wolverine, fisher, marten, mink and striped skunk), and unless otherwise noted are from DNA extractions from tissue. The "uncut standard" contains PCR product of a wolverine not subjected to the restriction digests. The first and last lanes are a 1 Kb standard ladder.

Table 1. Diagnostic restriction enzyme patterns for five mustelid species following amplification of a 442 bp region of cytochrome b. A sample that does not show the characteristic pattern for all three enzymes is designated as "other species." Restriction products are in bp.

Restriction enzyme	Fisher	Wolverine	Marten	Mink	Striped skunk
*Hinf*I	212, 132, 98	212, 132, 98	329, 113	212, 117, 113	212, 107, 77, 46
*Hae*III	259, 183	259, 140, 43	302, 140	No cut	No cut
*Mbo*I	No cut	No cut	313, 129	256, 129, 57	No cut

used in the extractions. To minimize contamination, hair extractions took place in a laboratory designated solely for this purpose.

Mitochondrial DNA (mtDNA) was amplified using the polymerase chain reaction (PCR) and primers (CanidL1 and H15149) for the cytochrome b region (Kocher et al. 1989; Paxinos et al. 1997). The reaction volume (50 μl) contained 50–100 ng DNA, 1× reaction buffer (Perkin-Elmer), 2.5 mM $MgCl_2$, 200 μM each dNTP, 1 μM each primer, 1 U *Taq* polymerase (Perkin-Elmer). The PCR program was 94 °C/5 min, [94 °C/1 min, 50 °C/1 min, 72 °C/1 min 30s] × 34 cycles, 72 °C/5 min. PCR products (442 bp) were run in a 2% agarose gel containing ethidium bromide (1.5 μl) and 1x TAE buffer (Ausubel et al. 1989).

Following DNA amplification, PCR products were digested in three restriction reactions with *Hinf*I, *Hae*III, and *Mbo*I, respectively (Figure 1, Table 1). The reaction volume (10 μl) contained 5.0 μl PCR product, 0.6 μl restriction enzyme, 1.0 μl digest buffer, 3.4 μl distilled water. Products were digested for a minimum of 4 hours at 37 °C and run in a 2.5% agarose gel with ethidium bromide (1.5 μl) and 1× TAE buffer (Ausubel et al. 1989).

One benefit of our protocol is that it relies on the high copy number of mtDNA for improved species resolution with low quantity, low quality samples. Also, the use of multiple restriction sites reduces the chance of misidentification. In addition to the geographic range tests discussed previously, we also have conducted "blind tests" of our protocol based on our target species (12 wolverine, 12 fisher, nine marten, six mink, six striped skunk) and 19 others (badger, raccoon, bobcat (*Lynx rufus*), moose (*Alces alces*), elk (*Cervus elaphus*), California red-backed vole (*Clethrionomys californicus*), snowshoe hare (*Lepus americanus*), grizzly bear (*Ursus arctos*), black bear (*Ursus americanus*), porcupine (*Erethizon dorsatum*), wolf (*Canis lupus*), and human (*Homo sapien*). All 64 samples were identified correctly as one of the target species, or as 'other species'.

Initially our protocol was implemented on hair samples collected from the nationwide lynx survey. However, mustelid hair samples are not expected to be found in large numbers for the survey because mustelids are unlikely to respond to the lynx pad and bait configuration. To date the nationwide lynx survey has only yielded two mustelid hair samples, a wolverine detected in the Lewis and Clark National Forest, Montana, and a fisher in northwestern Maine. In addition our DNA protocol has identified fishers successfully from a study using hair snares designed for fishers (W. Zielinski and R. Schlexer unpublished) and we are currently developing a wolverine specific snare. Our protocol can be used to gain insights into these secretive and elusive forest carnivores, especially the wolverine and fisher, using non-invasive sampling and low quality/low quantity samples.

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