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Tools and Technology

Molecular Techniques for Identifying Intraguild Predators of Fishers and Other North American Small Carnivores

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ABSTRACT Identifying predators of threatened and endangered species is important for understanding and reducing the impacts of predation. Visible evidence collected from a carcass alone is often insufficient to accurately identify predator species. The DNA from the predator left on the carcass allows for a definitive identification of predator species associated with the carcass, but DNA can be difficult to isolate independently from the prey. We developed field collection and molecular protocols for amplifying canid and felid predator DNA from saliva on fisher (*Martes pennanti*) carcasses without amplifying fisher DNA itself. We tested the protocol on fisher carcasses suspected of having been killed by a bobcat (*Lynx rufus*), mountain lion (*Puma concolor*), coyote (*Canis latrans*), and domestic dog. We successfully amplified and sequenced DNA from these 4 predator species, confirming predation by them on fishers. We confirmed that these protocols could also identify other felid and canid predators of several other small North American carnivores.

KEY WORDS fisher, forensic, intraguild predation, *Martes pennanti*, polymerase chain reaction, predator.

Determining predators of threatened or endangered species is essential to conservation efforts (Ratz et al. 1999, Ernest et al. 2002, Benson et al. 2010). Identification of predator species based solely on physical evidence visible at kill sites is difficult, even for seasoned field ecologists acutely familiar with their focal species (Larivière 1999, Rosas-Rosas et al. 2008). Poor carcass or environmental conditions and similarities in attack pattern by different species can hinder accurate identification of predators (Williams and Johnston 2004). For example, bite wound size and position are often used to identify predator species (Yom-Tov et al. 1995, Lyver 2000), but environmental conditions or autolytic state of carcasses often promote morphological changes and loss of skin structure, resulting in alteration of wound size, spacing, and shape, and ambiguity and inaccuracy in identifying predators (L. Munson, University of California Davis School of Veterinary Medicine, personal communication).

Technological advances have added to the tools available for identification of predators. For example, remote cameras have been used to identify nest predators (Williams and Wood 2002). Scats have been used to identify predators both from prey remains in scats (Neale et al. 1998, Sundqvist et al. 2008) and from predator DNA from scats left at a carcass (Ernest et al. 2002). However, scat-based methods are not ideal for distinguishing scavenging from predation. The DNA swabbed from wounds confirmed to be antemortem because of associated hemorrhage provides a direct means of determining predator species and has been used, for example, to identify individual predators of livestock (Blejwas et al. 2006). The considerable phylogenetic divergence between carnivores and most prey species reflected in mitochondrial DNA facilitates specific amplification of carnivore DNA, avoiding contamination by the prey DNA. This approach is more difficult for the identification of predators in the same taxonomic order as prey (i.e., Carnivora), and therefore, has not yet been used to study intraguild predation.

The recent designation of the fisher (*Martes pennanti*) as a candidate for listing under the U.S. Endangered Species Act has stimulated a flurry of research on this species in California, providing opportunities to investigate cases of suspected intraguild predation in northern California and in the southern Sierra Nevada mountains. We developed field collection and genetic protocols for the collection and analysis of forensic data and samples from carcasses of fishers

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to assist in the determination of species responsible for intraguild predation.

STUDY AREA

We investigated fisher carcasses from 2 California fisher research projects, one in the southern Sierra Nevada Mountains (Kings River Fisher Project) and one in northwestern California (Hoopa Valley Indian Reservation Fisher Project). Elevations within the Kings River Fisher Project ranged from 1,100 m to 2,282 m and dominant forest types included montane hardwood conifer, mixed conifer, and pine (*pinus* spp.) with small patches of montane chapparal, barren rock, and wet meadows. The Hoopa Valley Indian Reservation Fisher Project was located about 50 km northeast of Eureka, California, where elevations ranged from 98 m to 1,170 m. The dominant vegetation types were Douglas-fir (*Pseudotsuga menziesii*) and montane hardwood conifer, and meadows occur sparsely throughout the project area.

METHODS

Field Protocol

Four fisher carcasses were recovered $\leq$3 days after detection of a very high frequency mortality signal by field crews between spring 2007 and winter 2012. Because predation was suspected as the cause of death, we photographed all visible bite wounds and location of the carcass, and recorded if and where carcasses were cached. We measured distance between canine tooth punctures. We collected any non-fisher hairs on or near the carcass. One fisher was suspected of having been killed by a bobcat (*Lynx rufus*) because of putative bobcat hairs found on the carcass; another was suspected of having been killed by a coyote (*Canis latrans*) because of a recent coyote sighting in the area; the third fisher was suspected of having been killed by a mountain lion (*Puma concolor*) because fresh mountain lion scat was found near the carcass; and the last fisher was suspected of having been killed by a domestic dog due to dog tracks near the carcass. In an effort to obtain DNA from predator saliva (including shed epithelial cells), we collected 2 types of forensic samples: 1) synthetic-tipped swabs rubbed aggressively within bite wounds, and 2) matted fisher fur clipped (to avoid hair roots) within 1 cm of any bite wounds. Swabs were stored dry in 1.5 ml or 2.0 ml air-tight plastic vials and frozen at -20°C until further analysis.

Molecular Analyses

To extract DNA from the bite wound swabs and matted fur samples, 250–400 μL of 1× phosphate-buffered saline solution was first added to each vial containing the swab or fur sample, and the vial was gently vortexed for 60–90 s. We then used a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) to extract DNA from 200 μl of this solution according to the manufacturer's protocol for blood samples. To extract DNA from predator hairs left on the carcass, hair follicles were digested overnight following manufacturer's instructions for tissue extraction in the DNeasy Blood and Tissue Kit.

We chose primer pairs developed by the Forensics Unit of the University of California Davis Veterinary Genetics Laboratory that were family-specific for Felidae (Felid HV1A, Felid HV1B2) and Canidae (Canid HV1A, Canid HV1C) that would amplify variable regions of the mitochondrial genome in Hypervariable region I of the D loop, allowing us to produce sequences to differentiate species within each family (Table 1). Alignments of the primers with sequences of these 4 predator species indicated they were family-specific (Fig. 1). These D-loop fragments ranged from 200 base pairs to 300 base pairs (bp) for felids and from 300 bp to 400 bp for canids. We conducted polymerase chain reactions (PCR) in 25 μl reactions, which included 3 μl of DNA template, 1 U *Taq* polymerase (Titanium Taq; Clontech, Mountainview, CA), 6 μl of 5× reaction buffer (with MgCl), 1.2 mM of total DNTPs, and primers (i.e., felid or canid) at 0.7 μM concentration. Reactions were conducted with an initial denaturation step of 1 minute at 95°C, followed by 36 cycles of 20 s of denaturation at 95°C, 30 s of annealing at 55°C (felid) or 51°C (canid), and 40 s of extension at 72°C, and lastly, a final extension of 10 min at 72°C.

We electrophoresed the PCR products on a 1.0% agarose gel with GelStar (Lonza Group Limited, Basel, Switzerland) as a nucleic acid stain and visualized them using a Dark Reader non-ultraviolet transilluminator (Clare Chemical Research, Inc., Dolores, CO). We excised 1–2 of the strongest gel bands from each carcass in the range of 200–300 bp for felids and 300–400 bp for canids and gel-extracted them using the Qiagen Gel Extraction kit (QIAGEN) according to the manufacturer's instructions. We sequenced the 5′–3′ DNA strand PCR products using Big Dye Terminator cycle-sequencing kit (Applied Biosystems, Foster City, CA) on an ABI 3730 DNA Analyzer (Applied Biosystems). Sequences were aligned using RidomTraceEdit (Ridom GmbH, Würzberg, Germany) and cross-referenced on GenBank using the basic local alignment search tool to determine closest match to published species sequences.

Tests of Protocol Specificity

To verify that the primers would not amplify DNA from non-target carnivore species (especially fishers) and to determine other North American felids and canids detectable by the corresponding primers, we conducted PCR on DNA extracted from blood from several other North American carnivores. Test species included coyote, domestic dog, gray wolf (*Canis lupus*), red fox (*Vulpes vulpes*), gray fox (*Urocyon*

Table 1. Primers used to identify intraguild predator species of fishers (*Martes pennanti*) using DNA from bite wounds on carcasses collected between spring 2007 and winter 2012 at the Hoopa Valley Indian Reservation Fisher Project, Hoopa, CA and Kings River Fisher Project in the Sierra Nevadas, CA.

Primer	Sequence (5′-3′)
Felid HV1A	CCACTATCAGCACCCAAAGC
Felid HV1B2	TTATGTGTGATCATGGGCTGA
Canid HV1A	CCCTGACACCCCTACATTCA
Canid HV1C	TTATGTGTGATCATGGGCTGA

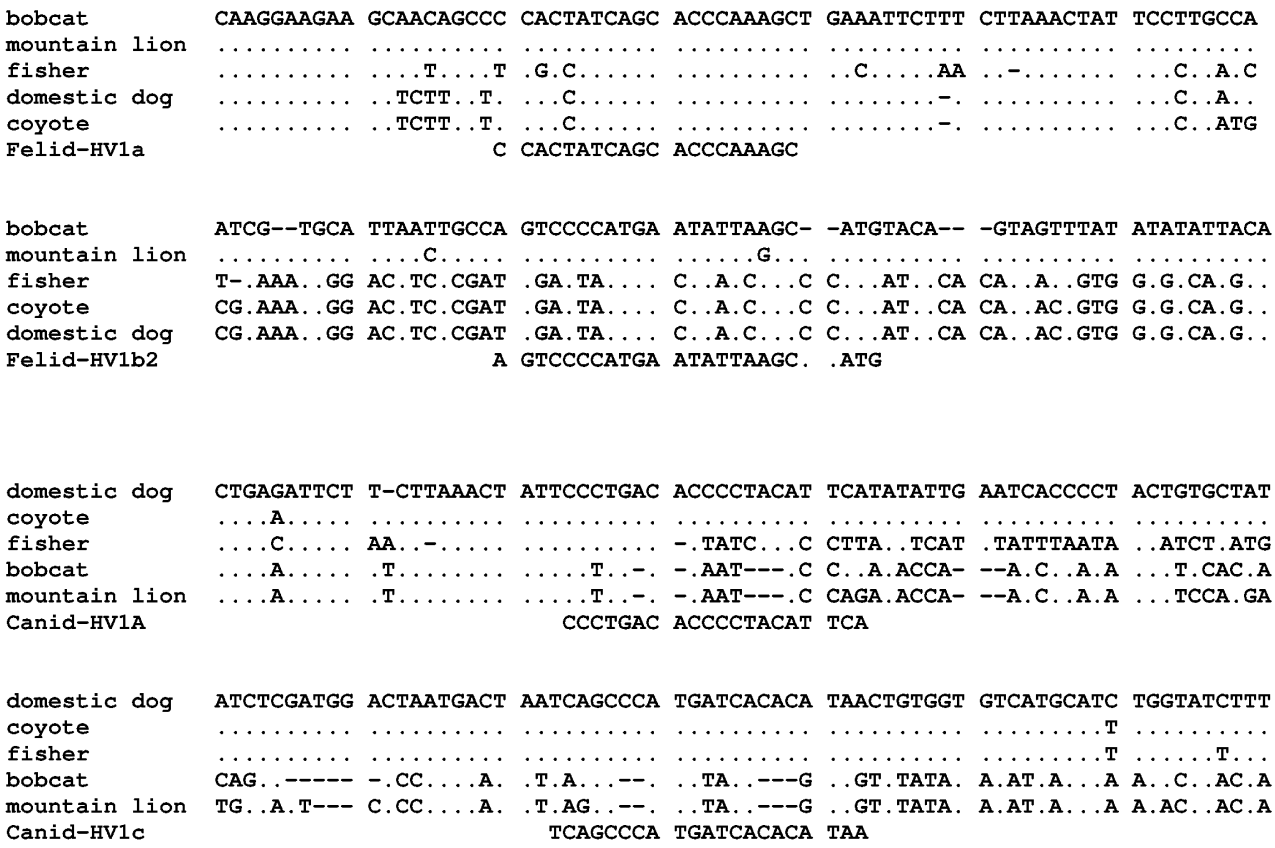

Figure 1. Alignments of 4 predator species' partial sequences (bobcat, *Lynx rufus;* mountain lion, *Puma concolor;* domestic dog; and coyote, *Canis latrans*) from the D-loop region of the mitochondrial genome with the 4 primers used to identify predators of fisher (*Martes pennanti*) carcasses collected between spring 2007 and winter 2012 at the Hoopa Valley Indian Reservation Fisher Project, Hoopa, CA and Kings River Fisher Project in the Sierra Nevadas, CA. Periods indicate the same nucleotide as the reference sequence at the top of each alignment, and dashes represent deletions.

cinereoargenteus), bobcat, mountain lion, Canada lynx (*Lynx canadensis*), fisher, marten (*Martes americana*), black bear (*Ursus americanus*), raccoon (*Procyon lotor*), ringtail (*Bassariscus astutus*), striped skunk (*Mephitis mephitis*), and western spotted skunk (*Spilogale gracilis*). The PCR reactions were conducted on 2 DNA samples from each of these species according to the methods outlined above.

RESULTS

We sampled wounds on each carcass from injuries confirmed to have occurred before death (antemortem) by the presence of subcutaneous hemorrhaging, which was verified through gross necropsy and histology. For the fisher suspected to have been killed by a bobcat, a non-fisher hair, 5 swabs, and 1 matted fur sample from wounds on the fisher yielded DNA fragments of approximately 200 bp that were amplified using the felid primers but not the canid primers (Table 2). The non-fisher hair sample and one swab sample sequenced matched a bobcat sequence with 100% homology (GenBank no. GQ979707.3). For the fisher suspected to have been killed by a mountain lion, 4 swabs and 1 matted fur sample from wounds on the fisher yielded DNA fragments of approximately 260 bp that were amplified using the felid primers but not the canid primers (Table 2). Both a swab and matted fur sample were sequenced and matched a mountain lion sequence with 98% homology (GenBank no. JN999997.1). For the fisher suspected to have been killed by a coyote, 3 swabs and 1 matted fur sample from wounds on the fisher yielded DNA fragments of approximately 380 bp that were amplified using the canid primers but not felid primers (Table 2). The matted fur sample was sequenced and it most closely matched a coyote sequence, with 97% homology (GenBank no. FJ213925.2). For the fisher suspected to have been killed by a dog, 2 swabs from wounds on the fisher yielded DNA fragments of approximately 400 bp that were amplified using the canid primers but not felid primers (Table 2). Both sequences most closely matched a dog sequence with 100% homology (GenBank no. HE687017.1).

In tests of known carnivore DNA samples, the felid primers amplified an approximately 200-bp DNA fragment from bobcat and 260-bp DNA fragments from mountain lion and Canada lynx, but did not amplify DNA from any of the other species tested. The canid primers amplified an approximately 380-bp DNA fragment from coyote and 400-bp DNA fragments from dog and wolf, but only weakly amplified red fox and gray fox. The canid primers also amplified an approximately 270-bp DNA fragment from ringtail (GenBank no. KC427988) and weakly amplified an approximately 270-bp DNA fragment from one striped skunk DNA

Table 2. Number of swab, hair, and matted fur samples taken from 4 fisher (*Martes pennanti*) carcasses suspected of being killed by bobcat (*Lynx rufus*), mountain lion (*Puma concolor*), coyote (*Canis latrans*), and domestic dog between Spring 2007 and Winter 2012 at the Hoopa Valley Indian Reservation Fisher Project, Hoopa, CA and Kings River Fisher Project in the Sierra Nevadas, CA; and number of samples with successful predator DNA amplification through polymerase chain reaction. NA indicates that no samples of this type were tested through polymerase chain reaction and, therefore, no results were obtained.

Predator species	No. swabs collected and tested	No. swabs yielding predator DNA	No. matted fur samples collected and tested	No. matted fur samples yielding predator DNA	No. non-fisher hairs tested	No. non-fisher hairs yielding predator DNA
Bobcat	5	5[a]	3	1	1	1[a]
Mountain lion	4	4[a]	1	1[a]	0	NA
Coyote	4	3	1	1[a]	0	NA
Domestic dog	2	2[b]	0	NA	0	NA

[a] One DNA sample of this type was successfully sequenced and the predator species was confirmed when cross-referenced on GenBank.
[b] Two DNA samples of this type were successfully sequenced and the predator species was confirmed when cross-referenced on GenBank.

sample, although this product was not sequenceable. Neither primer set amplified DNA from fisher, American marten, raccoon, bear, or western spotted skunk.

DISCUSSION

We developed a protocol for genetic analysis of fisher carcasses that enabled determination of fisher predators from both Felidae and Canidae. Our results show that DNA left on the carcass from saliva of the predator can be amplified through PCR and differentiated from DNA of the fisher. Previous predation forensics work focused on wildlife predators of livestock and was successful in identifying individual coyotes (Williams et al. 2003, Blejwas et al. 2006) and dogs or wolves (Sundqvist et al. 2008) responsible for killing sheep. Our research builds on this foundation by extending the approach to intraguild predators of smaller carnivores. The greater similarity in mitochondrial sequences among species within the Carnivora order than between orders (e.g., Carnivora vs. Ruminantia) required us to choose different primers for the 2 primary carnivore families most likely to prey on fishers to achieve the necessary specificity. Nevertheless, this protocol was successful in differentially amplifying felid and canid DNA from saliva left on the 4 fisher carcasses.

Additionally, tests of this protocol on other carnivore species demonstrated its potential utility for identification of other felid and canid predators, including Canada lynx and wolves. These species have been suspected of killing fishers and other small carnivores across North America (Apps 1999, White et al. 2002). However, poor success of the canid primers in amplifying DNA from the 2 fox species suggests they would not be as useful in identifying fox predators of smaller carnivores. Although we found no documentation of intraguild predation from gray foxes, red foxes have killed smaller carnivores such as American marten (Thompson 1994) and European pine marten (*Martes martes;* Lindström et al. 1995).

Lack of amplification of DNA from American marten, raccoon, and western spotted skunk using both primer pairs suggests that they would be effective in differentiating predator DNA from carcasses of these species in addition to fisher. Correspondingly, bobcats and coyotes have been suspected of killing American martens (Bull and Heater 2001), coyotes of killing raccoons (Kamler and Gipson 2004), and dogs of killing eastern spotted skunks (*Spilogale putorius;* Crabb 1948). Our protocol could, therefore, be used to assess predation by canids and felids on these small carnivore species. Unfortunately, our canid primers amplified ringtail and weakly amplified striped skunk DNA, which indicated the need for further optimization before using our approach on these species.

MANAGEMENT IMPLICATIONS

Given the potential significance of intraguild predation in the population dynamics and life history of small to mid-sized carnivores (Palomares and Caro 1999), methods to identify predator species are essential. Without definitive knowledge of predator species, quantitative estimates of impacts due to specific predators cannot be addressed appropriately through conservation and management programs. The protocol described here provides an accurate and effective way to identify felid and canid predators of fishers and other small carnivores in North America, such that field researchers and wildlife managers can correctly estimate the predator-specific impacts of predation on small and mid-sized carnivore populations and take appropriate management and conservation actions.

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