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TECHNICAL NOTE

Molecular species identification for multiple carnivores

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Abstract Species identification is crucial for carnivore conservation and ecological studies. We present a simple molecular genetic test that amplifies DNA of 16 wild carnivore species from three continents. The test is based on co-amplification of two mitochondrial DNA fragments and scoring of the resulting species-specific size patterns. We evaluated the performance of this method using 332 known tissue, blood, hair and fecal samples from 23 carnivore and 11 potential prey species. Results demonstrate that this test can distinguish many Caniform species but not members of Felidae. The test can be performed with a single PCR and capillary sequencer run for cost-effective processing of large sample numbers typical of non-invasive genetic projects.

Keywords Carnivores · Fragment analysis · Mitochondrial DNA control region · Non-invasive genetic samples · Species identification

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Species identification (ID) is crucial in many wildlife conservation and management applications. Given the conservation interest and ecological relevance of the order Carnivora, several molecular species ID tests have been developed for this taxonomic group including sequencing of diagnostic DNA markers (Farrell et al. 2000; Chaves et al. 2012), mitochondrial DNA (mtDNA)-RFLP (Paxinos et al. 1997; Mills et al. 2000), amplification with mtDNA species-specific primers (Palomares et al. 2002; Dalén et al. 2004; Fernandes et al. 2008), amplification of nuclear markers (Pilot et al. 2007; Oliveira et al. 2010), and real-time PCR (Berry and Sarre 2007; Matejusová et al. 2013). Recent approaches have moved toward more efficient methods to detect entire carnivore communities (Oliveira et al. 2010) and the use of standardized loci for the order (Chaves et al. 2012).

We report the development of a rapid, cost-effective molecular species ID test for 16 wild carnivores found in North America, South America and Europe. The method consists of co-amplification and fragment analysis of two short segments of the mtDNA control region using a single dye-labeled forward primer SIDL 5′-TCTATTTAAACTA TTCCCTGG-3′ (Murphy et al. 2000), and two different reverse primers H16145 5′-GGGCACGCCATTAATCGA CG-3′ (corrected sequence from Murphy et al. 2000) and H3R 5′-CCTGAAGTAGGAACCAGATG-3′ (Dalén et al. 2004).

We validated the method on 274 known hair (n = 60), fecal (n = 115), and blood/tissue samples (n = 99) of 21 wild carnivores from five families and the domestic dog (*Canis familiaris*) and cat *(Felis catus)* (Table 1). We analyzed 58 samples from 11 mammalian prey to evaluate the possibility of misidentification of carnivore fecal samples due to amplification of prey (Table 1) and tested human DNA to evaluate potential misidentification from human contamination. Samples were preserved in 95 %

Table 1 The number and locality of samples analyzed for each carnivore and prey species

Species name	Common name	Locality	Samples		Amplification success	
			Type	N	Fragment 1	Fragment 2
Predator						
Canidae						
Canis familiaris	Dog		Hair	5	5	5
Canis latrans	Coyote	AL, AZ, ID, NF, VA, UT	Feces	30	30	30
		CA, ME, NC, TX	Blood/tissue	20	20	20
Canis lupus	Gray wolf	ID, IT	Feces	10	10	10
		AK	Tissue	5	5	5
Canis rufus	Red wolf	NC	Blood	5	5	5
Lycalopex culpaeus	Andean fox	EC	Feces	5	5	5
Urocyon cinereoargenteus	Gray fox	NC	Feces	5	5	5
		UT	Hair	5	5	5
		NC	Tissue	3	3	3
Vulpes macrotis	Kit fox	UT	Hair	5	0	5
			Feces	5	0	5
Vulpes velox	Swift fox	CO, OK	Blood/tissue	7	0	7
Vulpes vulpes	Red Fox	NC, NF, WY	Feces	15	0	15
		NC	Tissue	1	1	1
Felidae						
Felis catus	Domestic cat	ID	Hair	5	0	0
Lynx canadensis	Canada lynx	NF	Feces	5	0	0
		MT	Tissue	5	0	0
Lynx lynx	European lynx	CH	Feces	5	0	0
Lynx rufus	Bobcat	NC	Feces	5	0	0
		AZ	Hair	5	0	0
		AZ	Blood/tissue	3	0	0
Puma concolor	Mountain lion	AZ	Hair	5	0	0
		ID	Tissue	5	0	0
Mustelidae						
Gulo gulo	Wolverine	MT, WY	Blood/tissue	5	5	0
Lontra canadensis	River otter	ID	Tissue	3	3	3
Martes americana	American marten	AK, BC, MT	Blood/tissue	25	25	25
Martes pennanti	Fisher	ID, MT, WI	Blood/tissue	8	8	8
Procyonidae						
Procyon lotor	Raccoon	NC	Feces	5	0	0
Ursidae						
Tremarctos ornatus	Andean bear	EC	Feces	5	5	5
Ursus americanus	Black bear	AL, NF, VA	Feces	15	15	15
		AK, AZ, ID	Hair	15	15	15
		NC	Blood	4	4	4
Ursus arctos	Brown bear	AK, IT	Hair	10	10	10
		ID	Feces	5	5	5
Ursus maritimus	Polar bear	NU	Hair	5	5	5
Prey						
Antilocapridae						
Antilocapra americana	Pronghorn	MT	Tissue	5	0	5
Bovidae						
Ovis canadensis	Bighorn sheep	ID	Blood	5	0	0

Table 1 continued

Species name	Common name	Locality	Samples		Amplification success	
			Type	N	Fragment 1	Fragment 2
Castoridae						
Castor canadensis	Beaver	WA	Hair	5	0	0
Cervidae						
Alces alces	Moose	MI, NF	Tissue	8	0	8
Cervus canadensis	Elk	ID	Tissue	5	0	5
Odocoileus virginianus	White-tailed deer	ID	Tissue	4	0	4
Rangifer tarandus	Caribou	NF	Hair	5	0	5
Equidae						
Equus caballus	Horse	NC	Feces	5	0	5
Leporidae						
Lepus americanus	Snowshoe hare	NF	Tissue	3	0	3
Lepus californicus	Black-tailed jackrabbit	ID	Tissue	5	0	0
Sylvilagus nuttallii	Mountain cottontail	ID	Tissue	5	0	0
		ID, OK	Blood/tissue	3	0	0

Fragment 1 is the fragment produced by primers SIDL and H16145 and Fragment 2 is the fragment produced by primers SIDL and H3R

AL Alabama, *AK* Alaska, *AZ* Arizona, *BC* British Columbia, *CA* California, *CH* Switzerland, *CO* Colorado, *EC* Ecuador, *ID* Idaho, *IT* Italy, *ME* Maine, *MI* Michigan, *MT* Montana, *OK* Oklahoma, *NC* North Carolina, *NF* Newfoundland, *NU* Nunavut, *SE US* South East United States, *UT* Utah, *VA* Virginia, *WI* Wisconsin, *WY* Wyoming

ethanol (tissue, feces), lysis buffer (blood, tissue), DETs buffer (feces), and silica desiccant (hair), and extracted using a Qiagen DNeasy Blood and Tissue Kit (tissue, blood, hair) and QIAmp DNA stool kit (feces) (Qiagen Inc., Valencia, CA). The 7 µl PCR contained 1× Qiagen Multiplex PCR Master Mix, 0.5× Qiagen Q Solution (Qiagen Inc.), 0.29 µM SIDL, 0.2 µM H16145, 0.1 µM H3R, 1 µl DNA extract. Thermocycling conditions included 15 min denaturation at 94 °C, 35 (hair, feces) or 30 (blood, tissue) cycles of 30 s at 94 °C, 90 s at 46 °C, 60 s at 72 °C and 30 min elongation at 60 °C. DNA extraction and PCR set up for hair and feces were performed in a laboratory dedicated to low quantity DNA samples. Negative controls were used in each extraction and PCR to monitor contamination. PCR products were loaded on an ABI3130xl DNA sequencer and fragments scored with Genemapper 3.7 software (Applied Biosystems) using size bins for each species.

A PCR product was observed for 16 of the 22 wild predators plus the dog (Table 1). Primers SIDL and H16145 produced a 115–220 bp fragment (Fragment 1) in 13 predators, and primers SIDL and H3R produced a 315–401 bp fragment (Fragment 2) in 15 predators (Table 2). Ten species (American marten, fisher, black bear, Andean bear, river otter, gray fox, kit fox, swift fox, red fox, and Andean fox) exhibited a species-specific fragment size pattern (Table 2). Gray wolf (*C. lupus*), wolverine (*Gulo gulo*), and dog samples produced the same fragment or fragments (Table 2) and could not be distinguished. Also, coyotes (*C. latrans*) and red wolves (*C. rufus*), plus brown (*Ursus arctos*) and polar (*U. maritimus*) bears produced the same fragment size pattern respectively (Table 2). However, these primers will still be useful in areas where their ranges do not overlap. No amplification was observed for any felid species or for raccoons (*Procyon lotor*).

To further investigate these patterns, we performed in silico PCR using the program ecoPCR (Ficetola et al. 2010) and mammalian sequences from EMBL nucleotide library (release 117) and examined mismatches using Primer-BLAST (NCBI). These analyses revealed that ≥2 base pair mismatches within the last 4 bases on the 3′ end of a primer generally prevented PCR amplification. The horse was the only prey species that produced a PCR product in the predator species size range, but the 375 bp fragment did not overlap with predator fragments. Six other prey species also produced fragments, however, the size of the fragments was longer than observed for the predator species and unlikely to amplify in low quality samples (Table 2). Human DNA amplified at 573 base pairs.

This species ID method provides multiple benefits. First, 16 wild predator species of wide distribution and conservation interest can be identified with a single PCR and capillary sequencer run, allowing simple, rapid, and inexpensive processing of large sample numbers (ca. USD$1.5 in supplies after DNA extraction). This will be particularly useful for species identification of hair, feces, saliva or degraded tissue samples in areas where canids, ursids and

Table 2 Size ranges in base pairs for Fragment 1 and Fragment 2 for each species

Species name	Common name	Fragment 1	Fragment 2
Predator			
Canidae			
Canis familiaris	Dog	123–128	365–368
Canis latrans	Coyote	115–120	360–364
Canis lupus	Gray wolf	123–128	365–368
Canis rufus	Red wolf	115–120	360–364
Lycalopex culpaeus	Andean fox	111–113	354–356
Urocyon cinereoargenteus	Gray fox	123–128	369–371
Vulpes macrotis	Kit fox	–	336–337
Vulpes velox	Swift fox	–	334–335
Vulpes vulpes	Red fox	–	343–347
Mustelidae			
Gulo gulo	Wolverine	125	–
Lontra canadensis	River otter	220	318
Martes pennanti	Fisher	121–122	315–316
Martes americana	American marten	125–127	317–322
Ursidae			
Ursus americanus	Black bear	158–165	396–401
Ursus arctos	Brown bear	143–153	380–387
Ursus maritimus	Polar bear	143–153	380–387
Tremarctos ornatus	Andean bear	156–158	390–393
Prey			
Antilocapridae			
Antilocapra americana	Pronghorn		465–466
Cervidae			
Alces alces	Moose		500–502
Cervus canadensis	Elk		570–574
Odocoileus virginianus	White-tailed deer		583
Rangifer tarandus	Caribou		499–500
Equidae			
Equus caballus	Horse		375
Leporidae			
Lepus americanus	Snowshoe hare		503

mustelids are sympatric. Moreover, additional primers could be added to the multiplex and co-amplified for target species presently not detected (i.e. felids). Other benefits include the amplification of relatively short, high copy number mtDNA fragments favoring the analysis of degraded DNA, and the co-amplification of two markers producing distinct PCR products in most species.

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