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TECHNICAL NOTE

A novel primer set for mammal species identification from feces samples

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Abstract We described a technique for detecting mammal species, based on the analysis of a control region fragment of mitochondrial DNA by establishing taxonomic identity from non-invasive samples. We detected a polymorphic fragment that varies in sequence and length within different mammalian species but maintains its identity among individuals of the same species. We amplified a single fragment in all the mammalian species tested from tissue samples and identified feces samples at species level. The use of a unique set of primers to assess the presence of different mammal species with non-invasive sampling allowed us to differentiate sequences from more than one species per environmental sample. Thus, it constitutes a powerful molecular tool for inventory and description of the mammal diversity distribution in natural areas.

Keywords Noninvasive sampling · Species determination · Mammalia monitoring · Wildlife forensics

The combination of noninvasive genetic sampling and novel tools for molecular species identification enables monitoring the geographical occupancy of species (Waits and Paetkau 2005; Schwartz et al. 2007). Feces contain DNA that can be amplified by PCR analysis generating species-specific sequences to unambiguously identify samples (Kohn and Wayne 1997; Taberlet and Gordon 1999; González and Duarte 2007). We described a molecular ecology technique for detecting mammal species, based on the analysis of a fragment of the control region (CR) of mitochondrial DNA, establishing taxonomic identity from noninvasive samples, such as feces.

Two types of samples were used: tissue, (N = 33) with precise taxonomic identification and feces (N = 34), with taxonomic order level determination. Tissue samples belonged to the Conservation Genetics-IIBCE tissue and DNA Bank, whereas feces were obtained in different surveys in the locality of Centurion, department of Cerro Largo (32° 6′30.52″S; 53°44′ 44.39″W). Muscle and skin DNA was isolated following the Medrano et al. (1990) protocol. DNA extraction from feces was performed with DNeasy kit Mericon Food, following the manufacturer's protocol.

Primers (MAMCODEF 5′ATGGGCCCGGAGCGAGAAGA/MAMCODER 5′AGAATNTCAGCTTTGGGWG) were designed over a 55 GenBank sequences database for both flanking ends of a variable region amplified with primers Thr-L15926 5′CAATTCCCCGGTCTTGTAAACC/DL-H16340 5′CCTGAAGTAGGAACCAGATG (Vila et al. 1999). For the fecal material, a nested PCR was carried out to increase the efficiency of the reaction and restrict the amplified products to mammals' species only. It consists of a first step that amplifies a fragment with primers Thr-L15926/DL-H16340 and a second reaction with previously described primers. The first PCR contained 0.2 U Taq DNA Polymerase (Invitrogen), 1 × PCR Buffer, 0.2 μM MgCl2, 0.2 μM each primer, template (≈90 ng DNA) and H_2O to a final volume of 15 μL; the second, 0.7 μM each primer and as a template 1 μL of the product of the first reaction. Amplification was performed with an initial step of 5 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at annealing temperature (AT)

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and 45 s at 72 °C, and a final step of 20 min at 72 °C. The AT was 51 °C and 59.5 °C for the first and second PCR. To confirm product amplification an electrophoresis analysis in agarose 2 % was performed for 120 m. In feces, when various bands were observed for the same sample, purification was performed cutting the different products from the agarose gel. Sequences were analyzed on an ABI3130 DNA analyzer (Applied Biosystems) and aligned by eyed using MEGA5 (Tamura et al. 2011). Finally, all sequences were blasted using default settings in the National Center for Biotechnology Information (NCBI) GenBank.

We amplified a single fragment in all the mammalian species tested from tissue samples and identify 64.7 % of the feces samples at species level (Table 1). A high proportion of the samples (44.1 %) presented more than a single band product in the electrophoresis gel analysis (Table 2 see +). The products showed size variability depending on the species isolated, corresponding to mammals' species in all cases and resulting in different informative sequences (Table 2).

Overall, 8 species were identified through the scat assessment. Two PCR products were inconclusive, either due to excessive sequence variation or to poor taxon coverage in the database (Table 2 see *). One of the feces (ANI56) was macroscopically identified as a carnivore, however the sequence corresponded to *Lepus europaeus*. It represents a dietary item, since the identification of dietary components is one of the potentials of this technique (Symondson 2002; Sheppard and Harwood 2005). Furthermore, it was possible to identify two species, *Cuniculus paca* and *Lycalopex gymnocercus* (grey fox) from another rodent fecal sample. Most likely the sample had been marked with urine by the fox, remaining in the sample and allowing us to detect both species. The same applies to another macroscopically rodent-like sample which sequence corresponded to *Canis lupus familiaris*. Several studies have described the possibility of extracting DNA from environmental samples of urine (Hausknecht et al. 2007). These cases highlight the potential of, not only detecting the individual to which the sample belongs, but also capturing other individuals present in a mixed sample.

The novelty of this technique compared to standard DNA barcoding, the markers described in Nowak et al. (2014) or detection of species or groups (Bozarth et al. 2010) is that by using our primer set we can capture and discriminate different DNAs present in a sample and harness mammal detection in an area. Unlike Pun et al. (2009) we managed noninvasive samples of non-model mammals and increased the amplification success of the CR by implementing a nested PCR. We highlight the importance for considering potential limitations on the publicly availability CR reference database of Neotropical mammals. Nevertheless, it constitutes a powerful novelty approach among already existing systems for monitoring mammals in natural areas.

Table 1 Species identification in scat and tissue samples

Mammalia orders	Scat			Tissue		
	i	sp.	%	i	sp.	%
Artiodactyla	8	2		5	5	
Carnivora	11	4		21	9	
Pilosa	0	0		1	1	
Rodentia	2	2		6	5	
Total	21	8	64.7	33	20	100

Identified samples (i), species detected (sp.) and PCR efficiency (%)

Table 2 Species identification from 34 mammal scats

Sample ID.	Species	SQL (pb)
S1MF1	*Leopardus geoffroyi*$^{+}$	392
S1MF4	*Leopardus pardalis or Leopardus wiedii*$^{*+}$	465
S1MF6	*Leopardus geoffroyi*$^{+}$	490
S1MF7	*Cerdocyon thous*$^{+}$	259
S2MF1	*Cerdocyon thous*$^{+}$	218
S2MF2	*Mazama guazoubira*$^{+}$	398
S2MF3	*Mazama guazoubira*	172
S2MF5	*Cerdocyon thous*	259
S3M1	*Canis lupus familiaris*$^{+}$	107
S3MF1	*Mazama guazoubira*	225
S3PF1	*Chaetophractus vellerosus**	281
S4M1	*Mazama guazoubira*	392
S4MF1	*Mazama guazoubira*	134
S4P1	*Cerdocyon thous*	273
S4PF2	*Bos taurus*$^{+}$	341
S5M1	*Mazama guazoubira*	66
S5M2	*Mazama guazoubira*$^{+}$	383
S5M3	*Cuniculus paca/Lycalopex gymnocercus*$^{+}$	377/272
ANI51	*Cerdocyon thous*	296
ANI53	*Canis lupus familiaris*	309
ANI56	*Lepus europaeus*	462
ANI63	*Cerdocyon thous*	236
ANI64	*Cerdocyon thous*	245
ANI66	*Hydrochoeris hydrochaeris*	401

Shown are sample identification numbers (ID.), species names. SQL values show the maximum continuous readable sequence length in the sequence trace file

* Unsuccessful species identification via BLAST search

$^{+}$ Represent samples with more than one band product

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