Mastozoología Neotropical, 13(2):239-244, Mendoza, 2006

ISSN 0327-9383
Versión on-line ISSN 1666-0536
www.cricyt.edu.ar/mn.htm

IDENTIFICATION OF ANDEAN FELID FECES USING PCR-RFLP

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Key words. Andean cat. Feces. Geoffroy's cat. Molecular scatology. Pampas cat. Puma.

Scat analysis is a useful method to determine distribution, abundance and diet of animals (Putman, 1984; Kohn and Wayne, 1997; Wasser et al., 2004). These methods are particularly relevant for monitoring elusive and secretive carnivores for which feces are often the only available materials (Foran et al., 1997; Riddle et al., 2003). As feces of similar-sized carnivore species can resemble each other in morphology and composition, distinguishing among them can be problematic (Davidson et al., 2002; Prugh and Ritland, 2005). Development of methods for identification of the species from which feces originated is crucial for scat collection-based monitoring programs.

DNA methods on scat use intestinal cells of the animal that are incorporated into feces. Molecular scatology has been demonstrated to be an efficient method to identify carnivore species in a large number of publications, including felids (e.g. Farrel et al., 2000; Ernest et al., 2000; Palomares et al., 2002; Wan et al., 2003; Zuercher et al., 2003). However, no protocol specific to Andean felids has been published. Several projects focussing on Andean cat (*Oreailurus jacobita*), one of the rarest felids of the world and the most threatened of South America, are in progress (Villalba et al., 2004). Therefore the development of such a method is highly relevant.

The aim of this work was to develop a PCR-based method for distinguishing between scats of Andean felids, namely Andean cat (*Oreailurus jacobita*), pampas cat (*Lynchailurus colocolo*) – the most common felid in the Andes–, Geoffroy's cat (*Oncifelis geoffroyi*), puma (*Puma concolor*), as well as other sympatric carnivores such as culpeo fox (*Pseudalopex culpaeus*), domestic cat (*Felis catus*), and domestic dog (*Canis familiaris*). A portion of the mitochondrial genome was PCR-amplified and RFLP patterns were used to distinguish felid species from one another and from other carnivore species.

The sequences of mitochondrial 16S ribosomal DNA from Andean felids and other carnivores (domestic cat and dog, canids, ursids, herpestids) were aligned using the CLUSTALW program. Restriction sites were identified as those providing a diagnostic pattern for felid species (**Fig. 1**). Because fecal DNA is often of low quality, we selected a segment as short as possible that included restriction sites. Two primers (5'-AATTGACCTTCCCGTGAAGAGG-3' and 5'-CCTAGGGTAACTTGTTCCGTTG-3') were designed to amplify an expected segment of 257bp for Andean cat and Geoffroy's cat, 258bp for other felids and 257-263bp for other carnivores.

Samples for Andean cat, pampas cat, puma, and culpeo fox from Peru and Argentina; Geoffroy's cat from Argentina; and domestic cat, and domestic dog from various sources were obtained (skin, feces, tissues, see **Fig. 2**). DNA from feces was isolated with DNeasy

Recibido 16 setiembre 2005. Aceptación final 18 febrero 2006.

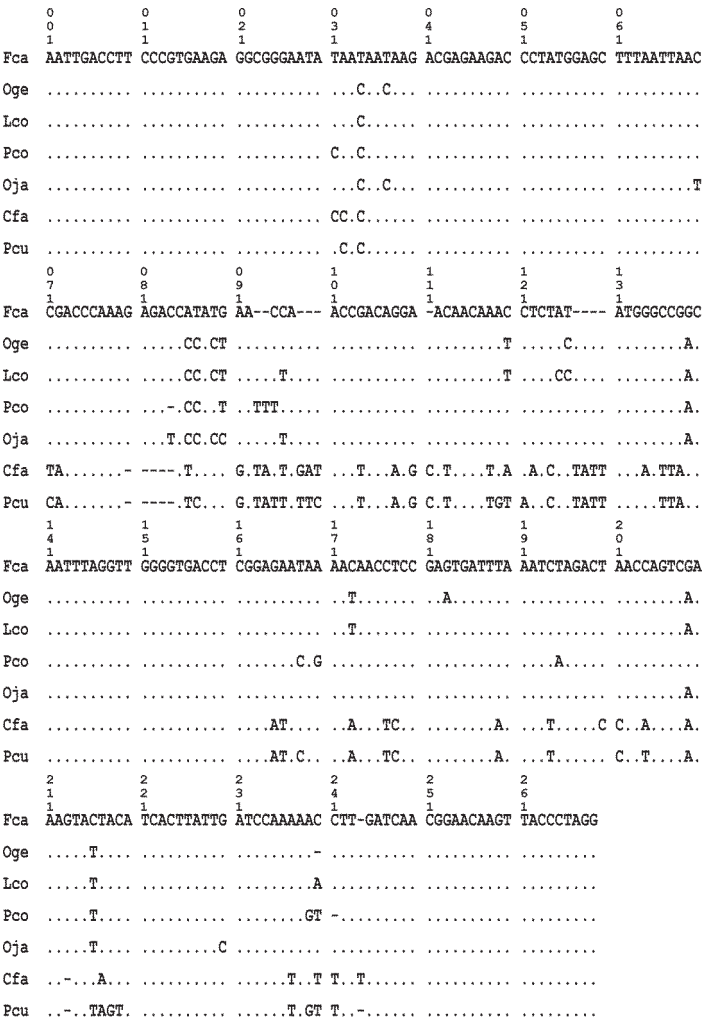

Fig. 1. Aligned sequences of the 16S rRNA gene segment from Andean carnivores. The first position of the diagnostic restriction sites is indicated : *Dra*I (TTTAAA) 187; *Hae*III (GGCC) 134; *Nla*III (CATG) 126; *Nco*I (CCATGG) 125; *Ase*I (ATTAAT) 65, 88; *Mbo*I (GATC) 82 and *Hpa*II (CCGG) 136. Lco: pampas cat, Oja: Andean cat, Pco: puma, Oge: Geoffroy's cat, Fca: domestic cat, Cfa: domestic dog, Pcu: culpeo fox

Tissue Kits (QIAGEN, Valencia, USA) according to Pires and Fernandes (2003). DNA from other samples was isolated by standard method of Proteinase K digestion, phenol-chloroform extraction and precipitation with ethanol (Sambrook et al., 1989).

Fifty-ul PCR reactions contained a final concentration of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP and 0.8 pM of each primer, 0.8 mg/ml of BSA, 0.8 unit of Taq DNA polymerase and approximately 50 ng of template DNA. The following program was performed: an initial denaturing step at 92°C for 2 min, 45 cycles of 92°C for 15 sec, 52°C for 15 sec, and 68°C for 30 sec, and a final extension step at 68°C for 5 min. Negative controls were used in the amplification reactions to monitor contamination.

Following amplification, PCR products were digested in seven reactions with *Dra*I, *Hae*III,

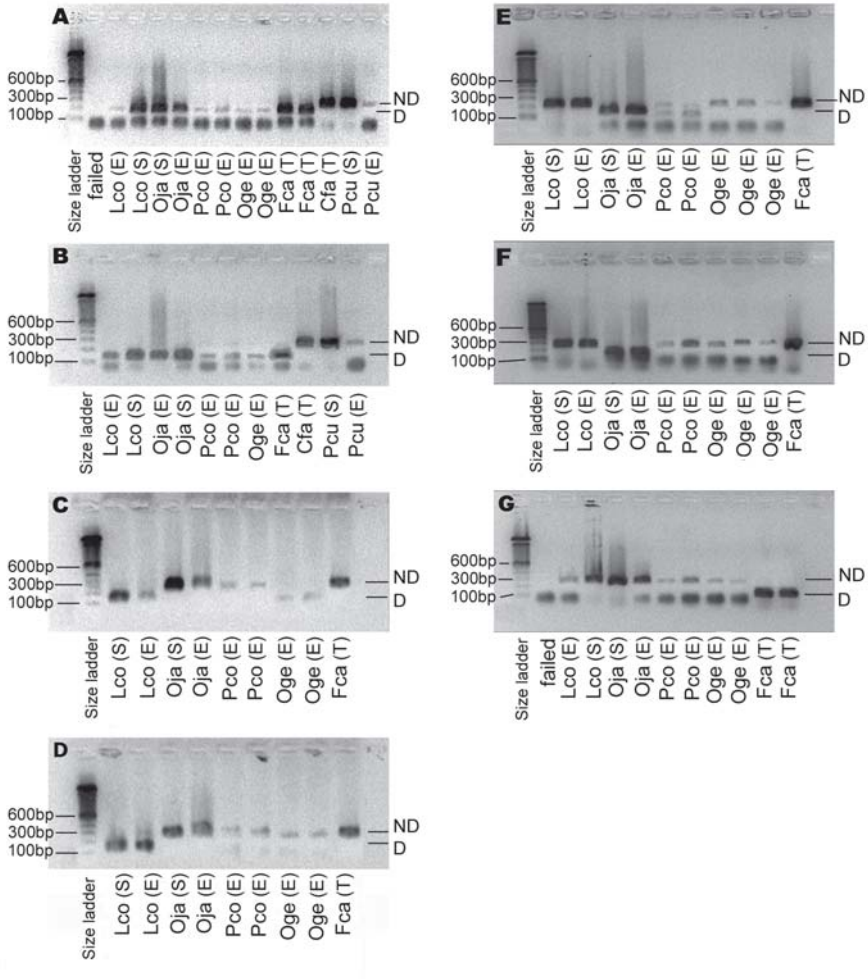

Fig. 2. Fragment profiles following digestion of the 16S rRNA gene segment. The origin of the extracted DNA (skin, tissue or scat samples) is indicated for each sample. S: skin, E: excrement, T: tissue. A: *Dra*I, B: *Hae*III, C: *Nla*III, D: *Nco*I, E: *Ase*I, F: *Mbo*I, G: *Hpa*II. Lco: pampas cat, Oja: Andean cat, Pco: puma, Oge: Geoffroy's cat, Fca: domestic cat, Cfa: domestic dog, Pcu: culpeo fox. ND: undigested fragment, D: digested fragment.

*Nla*III, *Nco*I, *Ase*I, *Hpa*II and *Mbo*I. Restriction enzyme incubations were run in 7 ul volume consisting of 0.7 ul of the appropriate 10x buffer, 2-4 U of restriction enzyme and 5 ul of the PCR product. Incubations were performed for at least 4h at 37°C and fragments were visualized in a 1.5% agarose gel.

The expected fragment profile (**Table 1**) was observed for all combinations of species and enzymes (**Fig. 2**). The enzymes *Dra*I and *Hae*III discriminate felids from other carnivores, digesting only felid species (**Fig. 2A** and **B**). *Dra*I may digest herpestids but these species are not expected to be found in South America. The other restriction enzymes discriminate felid species from one another. The enzyme *Nla*III provides restriction fragments only for pampas cat and Geoffroy's cat (**Fig. 2C**). Both species can then be discriminated with *Nco*I that digests only pampas cat (**Fig. 2D**). The

Table 1

Expected fragments size in bp of the 16S gene-amplified segment following digestion with diagnostic restriction enzymes. Fragments less than 25bp are not shown. ND indicates undigested fragments. For carnivore species different than felids, only *Dra*I and *Hae*III fragment patterns are shown.

	Identification of felids		Identification of Andean felid species					References and Genebank accession numbers
	***Dra*I**	***Hae*III**	***Nla*III**	***Nco*I**	***Ase*I**	***Mbo*I**	***Hpa*II**	
FELIDAE								
Geoffreoy's cat*	179, 78	132, 125		134, 123	ND	ND	NDND	AF006427; AF241818
Pampas cat*	179, 79	133, 125	135, 123	139, 119	ND	ND	ND	AF006411; Johnson et al. (1998)
Puma*	180, 78	132, 126	ND	ND	170, 88	ND	ND	AF288630-4; AF241851-3
Andean cat*	179, 78	132, 125	ND	ND	191, 66	151, 81	ND	Johnson et al. (1998)
Domestic cat*	179, 79	133, 125	ND	ND	ND	ND	136, 126	AF288633; AF288629
CANIDAE								
Culpeo fox*	ND	ND	-	-	-	-	-	DQ402045 (This study)
Domestic dog*	ND	ND	-	-	-	-	-	AF064567-8; AF006453
Dhole	ND	ND	-	-	-	-	-	AY289971-2
Wolf	ND	ND	-	-	-	-	-	AY289946; AY289960
Golden jackal	ND	ND	-	-	-	-	-	AY289970
HERPESTIDAE								
Indian mongoose	178, 79	ND	-	-	-	-	-	AY873843
URSIDAE								
Asiatic black bear	ND	ND	-	-	-	-	-	AY504956-7
Brown bear	ND	ND	-	-	-	-	-	NC 003427; AF303110
American black bear	ND	ND	-	-	-	-	-	NC 003426; AF303109

* species that can be found in Andean environments

*Ase*I digestion pattern discriminates Andean cat and puma from other felids and both species can be differentiated according to the fragment sizes that differ by 20 bp (**Fig. 2E**). *Mbo*I can be used to identify unambiguously Andean cat (**Fig. 2F**). Finally, digestion with *Hpa*II provided fragments only for domestic cat (**Fig. 2G**). The combination of enzymes allowed identification of each felid species on the basis of positive results (digested segment) making identification unambiguous in agarose gel.

PCR-RFLP on this segment of the mitochondrial 16S ribosomal DNA appears to be a suitable technique for differentiation between Andean felids. The method was designed to be performed with minimal laboratory equipment, to be cost and time efficient. It allows the identification of species from low quality DNA from scat samples, as well as from hair, museum skins and carcasses. Hairs and other undigested rests from preys present on fecal samples may however interfere, producing a 'hybrid' pattern (Paxinos et al., 1997). Special attention must be dedicated to sample preparation and extraction in order to reduce the risk of identifying the DNA of the prey (Wasser et al., 1997). One limitation of this technique is the identification of putative hybrids among species such as pampas cat and Geoffroy's cat (Johnson et al., 1999). Because of the strictly maternal inheritance of the mtDNA, nuclear markers must be developed to identify such hybrids.

Unambiguous identification of the Andean felids using feces and other non-invasive sampling provides a useful tool for estimation of felids diversity and for conservation purposes. In addition, identified samples can be further analyzed, opening up the possibility of studying other ecological and evolutionary aspects of these species.

We are grateful to the Wildlife Conservation Network, who supported this work, and to S Walker, P Perovic, M Lucherini, A Madrid, D Ascencios and JL Condori for providing samples.

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