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Development and testing of an optimized method for DNA-based identification of jaguar (*Panthera onca*) and puma (*Puma concolor*) faecal samples for use in ecological and genetic studies

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Abstract The elusive nature and endangered status of most carnivore species imply that efficient approaches for their non-invasive sampling are required to allow for genetic and ecological studies. Faecal samples are a major potential source of information, and reliable approaches are needed to foster their application in this field, particularly in areas where few studies have been conducted. A major obstacle to the reliable use of faecal samples is their uncertain species-level identification in the field, an issue that can be addressed with DNA-based assays. In this study we describe a sequence-based approach that efficiently distinguishes jaguar versus puma scats, and that presents several desirable properties: (1) considerably high amplification and sequencing rates; (2) multiple diagnostic sites reliably differentiating the two focal species; (3) high information content that allows for future application in other carnivores; (4) no evidence of amplification of prey DNA; and (5) no evidence of amplification of a nuclear mitochondrial DNA insertion known to occur in the jaguar. We demonstrate the reliability and usefulness of this approach by evaluating 55 field-collected samples from four locations in the highly fragmented Atlantic Forest biome of Brazil and Argentina, and document the presence of one or both of these endangered felids in each of these areas.

Keywords Faecal DNA · Mitochondrial DNA · *Panthera onca* · *Puma concolor* · Species identification

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Introduction

The jaguar (*Panthera onca*) and puma (*Puma concolor*) are the only large felids currently present in the Neotropics. Both species are now threatened by habitat loss and fragmentation, along with direct persecution by ranchers due to conflict over livestock depredation (Nowell and Jackson 1996). Resilience to human disturbance varies between them, with the jaguar being considerably more sensitive to anthropogenic threats (Polisar 2002; Novack 2003; Silveira 2004). To maintain viable populations of these felids, urgent conservation efforts are needed in several areas, and

a first step towards this goal is the establishment of reliable methods to assess the presence and abundance of each of these species in different areas, so as to allow the adequate design of ecological, behavioral and genetic studies.

A major impediment to the development of such efforts is the difficulty and cost of obtaining direct information on these animals throughout their geographic range, given their low density and elusive behavior (Johnson et al. 2001). As a consequence, intensive studies such as those based on capture and radio-telemetry data are expensive and restricted to some focal areas, which in most cases still lack long-term monitoring of populations. In recent years, technical and analytical advances in approaches such as camera trapping have allowed a substantial increase in the number of studies investigating the presence of these species. In spite of the relevance of this approach, it still lacks the ability to provide biological samples of the identified individuals, or to provide information on important ecological and behavioral aspects such as diet, hormonal levels or interactions with pathogens. Biological samples are also critical for the development of genetic studies, which have the potential to illuminate issues such as loss of allelic diversity in fragmented populations, evolutionary history of demographic units, social interactions among individuals and patterns of territoriality and dispersal. In this context, recent advances in molecular biology have permitted the use of noninvasive samples (e.g. scats, hairs) as a reliable source of DNA, allowing genetic studies of free-ranging animals to be performed without having to capture or even observe them (Taberlet et al. 1999).

Analyses based on faecal DNA are now widespread, and have been applied to a broad array of taxa to address a variety of questions (Reed et al. 1997; Wasser et al. 1997; Kohn et al. 1999; Sloane et al. 2000; Parsons 2001; Palomares et al. 2002; Adams et al. 2003; Ernest et al. 2003; Wan et al. 2003; Pilgrim et al. 2005; Bergl and Vigilant 2007). Once scats are collected in the field, a first step for their use in genetic or ecological studies is their identification at species level. Morphology-based criteria have been shown to often be unreliable (Farrell et al. 2000; Davison et al. 2002; Reed et al. 2004), leading to a growing concern regarding the development of rigorous approaches for species-level assignment of field-collected scats. This is particularly relevant when the focal species for a field study occurs in sympatry with related taxa, whose scat size, morphology and scent may be quite similar. In such cases, molecular methods based on DNA sequences, nuclear VNTRs, PCR-RFLP or haplotype-specific Polymerase chain reaction (PCR) have been shown to successfully identify carnivore species, leading to a reliable alternative to traditional means of identification (Farrell et al. 2000; Davison et al. 2002; Palomares et al. 2002; Bhagavatula and Singh 2006; Lucentini et al. 2007; Pilot et al. 2007).

In the specific case of jaguar and puma, these felids are sympatric over almost all the range of the former species. It is thus unlikely that field studies focusing on jaguars will be carried out in areas devoid of pumas, so that both species will probably be sampled in most scat collection efforts. This issue is compounded by the established knowledge that it is difficult to distinguish jaguar and puma scats on the basis of their morphological features (Emmons 1987; Farrell et al. 2000; Chame 2003), rendering the problem of species-level identification a critical impediment for reliable field studies on these felids. It is thus very important to devote attention to the development of DNA-based assays that reliably discriminate these species, and that also present other desirable features such as high PCR success rate and no co-amplification of prey DNA.

Although several assays based on mitochondrial DNA (mtDNA) data have so far been applied to carnivore scat identification (Farrell et al. 2000; Novack et al. 2005; Bhagavatula and Singh 2006; Weckel et al. 2006; Lucentini et al. 2007; Miotto et al. 2007), very few studies have included Neotropical species, and puma and jaguar in particular. Farrell et al. (2000) developed primers targeting the mtDNA *cytochrome b gene* (*cyt b*) to identify carnivore scats in the context of an ecological study in Venezuela investigating four species (*P. concolor*, *P. onca*, *Leopardus pardalis* and *Cerdocyon thous*). In that study, 20 of 34 scats (59%) were successfully amplified and sequenced, allowing species-level identification. More recently, Miotto et al. (2007) used those same primers in a study to determine the presence of pumas and their estimated minimum population in two protected areas in Brazil, and also achieved 60% success rate in amplification and sequencing. Higher amplification success rates (83% and 85%, respectively) were observed with *cyt b* markers in the studies of Adams et al. (2003) and Onorato et al. (2006) in different areas of the United States. However, both of these papers reported a certain amount of prey DNA amplification using these primers (13 and 8%, respectively), which suggests that additional marker development is desirable to maximize the efficiency of assays capable of identifying carnivore species.

In the case of the jaguar, a complicating factor for the development of mtDNA-based assays is the presence in all five *Panthera* species of a large nuclear insertion (*numt*) containing most of the mitochondrial genome. A detailed study investigating this *Panthera numt* suggested that it encompasses a long segment spanning eight protein coding genes (including *cyt b*), two rRNA genes, 17 tRNA genes, and the control region (Kim et al. 2006). Since the amplification (or coamplification) of a *numt* is a known complication that can hamper or confound genetic analyses (Zhang and Hewitt 1996; Kim et al. 2006), including DNA-based identification, it is important to develop markers that

target mtDNA regions that are not contained in this translocation.

In this paper we describe a DNA-based assay for the identification of jaguar and puma faecal samples that bears the following assets: (1) high specificity, as sequence-based identification leads to multiple diagnostic characters between the two species; (2) high sensitivity, as the amplified fragment is short and leads to high amplification rates; (3) avoidance of *numt* amplification by targeting a mtDNA region not included in this translocation; and (4) no detected amplification of prey DNA. In addition to accomplishing successful discrimination between jaguar and puma scats, the method proposed here has a potential for much broader application in carnivores, as its sequence-based diagnosis allows for multiple sympatric species to be reliably identified.

Materials and methods

In order to develop an effective molecular approach for reliable identification of *P. onca* scats, especially with respect to distinguishing it from and *P. concolor*, we used 52 jaguar reference samples that spanned the geographic distribution of this species (Table 1), including blood ($n = 15$), tissue ($n = 1$), hair ($n = 2$) and faeces ($n = 34$) collected from captive animals. For comparison, we analyzed nine reference samples of pumas (eight blood samples and one tissue), representing multiple geographic regions where it is sympatric with the jaguar. Additional analyses included a marker test using a faecal sample of domestic cat (*Felis catus*) collected in Argentina, paired samples of blood and scat collected from four captive jaguars (see Table 1), and comparisons with multiple sequences available in GenBank or generated in our laboratory for other purposes.

In addition to samples originated from known specimens, we analyzed 55 scats collected by field researchers in different areas, and identified as "large felid" based on morphological features such as diameter, size and shape, as well as associated tracks. "Large felid" scats are usually interpreted as originating from either jaguar or puma, and discriminating between the two is a known and recurrent challenge in most cases. All 55 samples were collected from areas in the Atlantic Forest biome where both species are thought to occur, and where field projects addressing ecological aspects of one or both of them are currently being carried out. The field sites were: Reserva Natural Vale, Espírito Santo State, Brazil (RV; $n = 9$); Parque Estadual do Rio Doce, Minas Gerais State, Brazil (RD; $n = 1$); Parque Estadual das Várzeas do Rio Ivinhema, Mato Grosso do Sul State, Brazil (PI; $n = 3$), and several forested locations in the Misiones Province, Argentina (MP; $n = 42$).

Blood samples were preserved in a salt-saturated solution (100 mM Tris, 100 mM EDTA, 2% SDS) and tissues and hairs were kept in ethanol 96%. Approximately 6 g of faeces were collected and stored in a 15 ml vial containing silica gel at a 4 g silica/g faeces ratio (Wasser et al. 1997). All samples were stored at −20°C prior to DNA extraction. Genomic DNA was extracted from blood and tissue samples using a standard Proteinase-K digestion and phenol-chloroform-isoamyl alcohol protocol (Sambrook et al. 1989). Extractions from the hair samples were performed with the Puregene DNA Purification Kit (GENTRA), and those of scat samples used the QIAamp DNA Stool Mini Kit (QIAGEN), following the manufacturers' instructions. Scat DNA extractions were carried out in a separate laboratory area, in a UV-sterilized laminar flow hood dedicated to the analysis of DNA from noninvasive samples. Each batch of extractions ($n = 10$) included one negative extraction control to monitor the occurrence of contamination with extrinsic DNA.

In order to avoid amplification of the *Panthera numt* and to aim for high amplification success and sequence variability, we targeted a short segment of the mtDNA *ATP synthase subunit 6* (*ATP6*) gene, including its overlapping portion with the *ATP8* gene. Previous studies indicated that this segment was quite variable in carnivores (Trigo et al. 2008; E. Eizirik et al., unpublished), and that it lay outside of the *numt* (Kim et al. 2006). We employed the reverse primer ATP6-DR1 (5′-CCAGTATTTGTTTTGATGTTAG TTG-3′), originally reported by Trigo et al. (2008), and designed two new forward primers: ATP6-DF2 (5′-ATGA ACGAAAATCTATTCGC-3′) and ATP6-DF3 (5′-AACG AAAATCTATTCGCCTCT-3′). These forward primers anneal at very similar positions (PCR product size in combination with ATP6-DR1 is 175 bp for DF2 and 172 bp for DF3), and were designed to maximize the probability of successful amplification in carnivores, given a preliminary alignment including sequences drawn from GenBank that represented the major lineages of this mammalian order (i.e. Feliformia, Arctoidea, and Cynoidea). On the basis of initial tests, both forward primers appeared to perform well in carnivores (not shown), and primer ATP6-DF2 was employed throughout this study.

Polymerase chain reactions were performed in a final volume of 20 µl, containing 1× PCR buffer (Invitrogen), 2.0–2.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each primer, 0.5 unit of regular *Taq* DNA polymerase (Invitrogen) or Platinum *Taq* DNA polymerase (Invitrogen) and 1–6 µl of empirically diluted template DNA. The reaction profile was as follows: 10 cycles (Touchdown) of 94°C for 45 s, 60–51°C for 45 s, 72°C for 1.5 min, followed by 30 cycles of 94°C for 45 s, 50°C for 45 s, 72°C for 1.5 min, and a final extension at 72°C for 3 min. Products were visualized on a 1% agarose gel stained with GelRed (Biotium), purified with >PEG8000, sequenced using the DYEnamic ET Dye

Table 1 Samples utilized as reference in the present study

ID	Sample	Geographic origin	Institution/Contact
Panthera onca			
bPon-01	Blood	Paraná state, Brazil	Proj. Carnívoros-Ibama—P. G. Crawshaw Jr.
bPon-03	Blood	Mato Grosso do Sul state, Brazil	Proj. Porto Primavera—P. G. Crawshaw Jr.
bPon-13	Blood	Amazonas state, Brazil	CIGS, Manaus
bPon-15[a], bPon-27[a], bPon-32[a]	Blood and faeces	Mato Grosso do Sul state, Brazil	CENAP/ICMBio; I. Pró-Carnívoros; Ilha Solteira Zoo; V. Queirós
bPon-16, bPon-35	Blood	Mato Grosso do Sul state, Brazil	CENAP/ICMBio; Pró-Carnívoros
bPon-18[a]	Blood and faeces	São Paulo state, Brazil	CENAP/ICMBio; I. Pró-Carnívoros; Ilha Solteira Zoo; V. Queirós
bPon-24, bPon-51	Blood	São Paulo state, Brazil	Inst. Pesquisas Ecológicas (IPE)/L. Cullen and A. Nava
bPon-34	Muscle	French Guiana	Benoit de Thoisy
bPon-55[a], bPon-56[a], bPon-102[a], bPon-103[a], bPon-104[a]	Faeces	Captivity, Brazil	Sapucaia do Sul Zoo/R. von Hohendorff
bPon-59[a]	Faeces	Paraná state, Brazil	CASIB/W. de Moraes
bPon-66	Blood	Mato Grosso do Sul state, Brazil	Embrapa-Pantanal/G Mourão
bPon-81[a], bPon-82[a]	Faeces	Captivity, Brazil	Americana Zoo/M. Falcade; Limeira Zoo/A. C. A. Sorg
bPon-83[a]	Faeces	Amazonas state, Brazil	Limeira Zoo/A. C. A. Sorg
bPon-90[a], bPon-91[a], bPon-93[a], bPon-94[a]	Faeces	Captivity, Brazil	Parque Municipal "Danilo Galafassi"/L. E. S. Delgado
bPon-95[a], bPon-96[a], bPon-97[a]	Faeces	Captivity, Brazil	CEBUS/C. D. P. Coelho and L. C. Silva
bPon-98[a], bPon-99[a]	Faeces	Captivity, Brazil	Goiania Zoo/R. F. de Carvalho and D. Nogueira
bPon-100[a]	Faeces	Captivity, Brazil	Campinas Zoo/E. F. Santos
bPon-101[a]	Faeces	Acre state, Brazil	Campinas Zoo/E. F. Santos
bPon-105[a], bPon-112[a]	Faeces	Captivity, Brazil	Guarulhos Zoo/C. E. Bolochio
bPon-107[a]	Faeces	Acre state, Brazil	Parque Ambiental Chico Mendes/J. O. Guimarães
bPon-108[a]	Faeces	Rondonia state, Brazil	Parque Ambiental Chico Mendes/J. O. Guimarães
bPon-114[a], bPon-115[a]	Faeces	Captivity, Brazil	Parque Cyro Gevaerd/M. R. Achutti
bPon-116[a]	Faeces	Amazonas state, Brazil	Curitiba Zoo/M. L. Javorouski
bPon-117[a]	Faeces	Santa Catarina state, Brazil	Curitiba Zoo/M. L. Javorouski
bPon-120[a]	Faeces	Amazonas state, Brazil	Pomerode Zoo (Fund. Hermann Weege)/C. H. Maas
bPon-123[a]	Faeces	Mato Grosso do Sul state, Brazil	Pomerode Zoo (Fund. Hermann Weege)/C. H. Maas
bPon-126 (Pon-31)	Blood	San Luis Potosí, Mexico	Leon Zoo
bPon-127 (Pon-50)	Blood	Chaco, Paraguay	Itaipu, Paraguay
bPon-128 (Pon-54)	Blood	Amazonas, Venezuela	Las Delicias
P31-1	Hair	Misiones Province, Argentina	Proyecto Yaguareté, CeIBA/Carlos De Angelo
P3-2	Hair	Misiones Province, Argentina	Proyecto Yaguareté, CeIBA./Carlos De Angelo
Puma concolor			
bPco-72 (Pco-356)	Blood	Texas, USA	Laboratory of Genomic Diversity (LGD), USA
bPco-73 (Pco-541)	Blood	Panama	Laboratory of Genomic Diversity (LGD), USA
bPco-74 (Pco-556)	Blood	Guatemala	Laboratory of Genomic Diversity (LGD), USA
bPco-75 (Pco-560)	Blood	Argentina	Laboratory of Genomic Diversity (LGD), USA
bPco-76 (Pco-700)	Blood	Paraiba state, Brazil	Laboratory of Genomic Diversity (LGD), USA
bPco-77 (Pco-704)	Blood	Venezuela	Laboratory of Genomic Diversity (LGD), USA
bPco-78 (Pco-707)	Blood	Bolivia	Laboratory of Genomic Diversity (LGD), USA

Table 1 continued

ID	Sample	Geographic origin	Institution/Contact
bPco-79 (Pco-7)	Blood	Oregon, USA	Laboratory of Genomic Diversity (LGD), USA
bPco-014	Tissue	São Paulo state, Brazil	F. Olmos
Felis catus			
F3-120[a]	Faeces	Buenos Aires, Argentina	Proyecto Yaguareté, CeIBA/Carlos De Angelo

[a] Faeces collected in captivity

Terminator Sequencing Kit (GE Healthcare), and analyzed in a MegaBACE 1000 automated sequencer (GE Healthcare). To assess the performance of a fast, straightforward and lower-cost identification strategy, PCR products were routinely sequenced for only one strand (using the forward primer ATP6-DF2), and only bases that could be reliably scored were kept in the data set. To verify sequence accuracy and to ascertain the validity of only using a single strand for identification purposes, we sequenced the reverse strand for one jaguar and one puma sample (bPon-127 and bPco-014, respectively), in both cases confirming the original result. Sequences were visually checked and manually corrected using CHROMAS 2.0 (http://www.technelysium.com.au/chromas.html). All haplotypes identified here have been deposited in GenBank (Accession numbers FJ596283-FJ596287).

DNA sequences were aligned with the CLUSTALW algorithm implemented in MEGA 3.1 (Kumar et al. 2004). MEGA was also used to identify identical haplotypes, to assess the presence and consistency of diagnostic sites between the two species, and to perform phylogenetic analyses. In most cases the identification of samples could be performed with a direct, character-based approach, as field-collected scats often contained identical haplotypes to those observed in reference samples. To test whether different haplotypes observed in each species could be reliably grouped, so as to provide an easy and consistent identification tool even in cases where haplotypes were not identical, we performed several phylogenetic analyses. These included maximum parsimony and distance-based approaches using the Neighbor-Joining algorithm (Saitou and Nei 1987) with various types of genetic distances. The reliability of inferred nodes was assessed using 1,000 bootstrap replications. In addition to the data generated here, phylogenetic analyses also included sequences from other felid species available in GenBank, especially domestic cat (U20753) and cheetah (*Acinonyx jubatus;* AY463959).

Results and discussion

The use of PCR primers ATP6-DF2 and ATP6-DR1 led to excellent amplification success (100%) using blood, tissue or hair as DNA sources. High quality DNA sequences could thus be obtained from all reference samples. PCR products were 175 bp long in both species, yielding a 130 bp long analyzable segment after removal of primer sequences. When employing only forward sequences for streamlined and lower-cost sample identification, 96 sites could be reliably used after additional removal of bases that did not present high quality scores with this DNA strand alone. However, if the reverse strand was also used, all 130 sites could be reliably scored. Even considering only the 96 sites scored with the forward strand, species-level identification was found to be highly accurate, with a minimum of 15 diagnostic sites identified between any jaguar and puma sequence (Table 2).

Two different haplotypes, differing by a single nucleotide, were observed in the *P. onca* samples. Among pumas, two variable sites were identified, leading to three different haplotypes (Table 2). In addition to the character-based analysis that clearly diagnosed pumas versus jaguars, phylogenetic analyses also demonstrated that the two species could be easily differentiated using this mtDNA segment (Fig. 1).

PCR amplification of this segment from faecal samples also led to promising results. Thirty-four out of 39 fresh scat samples collected from captive jaguars were successfully amplified and sequenced, corresponding to an 87% success rate. Field-collected scats yielded variable success rates, likely due to heterogeneity in environmental conditions as well as sample age (when collected) and storage time. A success rate of 78% (seven out of nine samples) was obtained with scats from the RV location (see Materials and methods), in contrast to only 50% (21 out of 42 scats) for the samples from Misiones (MP). We examined the underlying causes of this lower success rate with the MP samples by partitioning these scats into different categories of field-assigned quality ("freshness") and storage time. If only samples categorized as "fresh" were considered, the success rate became 89% (8/9 scats) for scats from MP. Within the group categorized in the field as "intermediate" in freshness, a 59% success rate (10/17) was obtained for samples that had been stored for <1 year prior to extraction, and only 22% (2/9) for those stored for ≥1 year. Finally, the group of samples categorized as "low

Table 2 Mitochondrial DNA *ATP6* haplotypes identified from jaguar (PonH1, PonH2) and puma (PcoH1, PcoH2, PcoH3) samples

Haplotype ID	Variable sites	Known samples	Field samples[b]
	112344556667889		
	369067025013692171		
PonH1	TCACACTCGGTCTTACGT	bPon01, 03, 13, 15[a], 16, 18[a], 24, 27[a], 32[a], 34, 35, 51, 55, 56, 59, 66, 82, 83, 90, 91, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 107, 108, 112, 114, 115, 116, 117, 120, 123, 126, 127, 128; P3-2, 31-1	RV04, 06, 07, 08, 09, 19, 31; PI32, 40; MP5-9, 5-10, 5-12, 4-120, 23-18, 43-200
PonH2	..........T.....	bPon81	
PcoH1	CTCTGACAAACTCCGGAC	bPco14, 72, 73, 74, 75, 76, 77, 78, 79	RD01; MP18-31, 38-55, 56-26, 57-1, 2-635, 2-636, 2-711, 2-659
PcoH2	C.NTGACAAACTCCGGAC		MP48-2, 61-3, 12-101
PcoH3	C.CT.ACAAACTCCGGAC		MP4-13

Only variable sites are shown. Site numbers (vertical notation) refer to the aligned position in our 96 bp data set. Known samples (see Table1) and field-collected scats bearing each haplotype are also indicated

[a] Individuals for which paired blood and faecal samples were analyzed, in every case leading to identical results

[b] Field samples consist of scats collected in the following locations: Reserva Natural Vale, Espírito Santo state, Brazil (RV); Parque Estadual do Rio Doce, Minas Gerais state, Brazil (RD); Parque Estadual das Várzeas do Rio Ivinhema, Mato Grosso do Sul state, Brazil (PI); and Misiones Province, Argentina (MP)

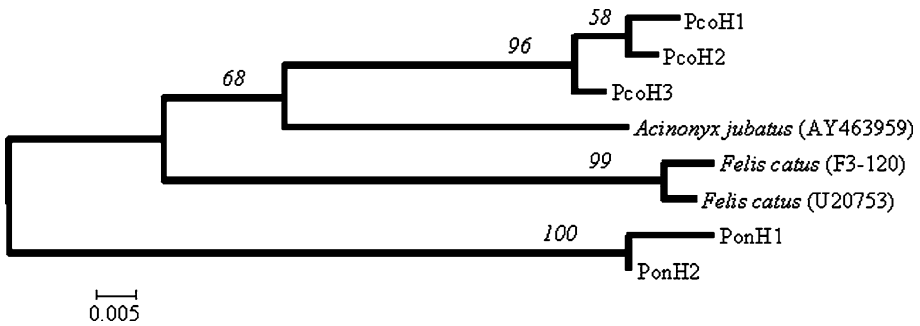

Fig. 1 Phylogenetic tree depicting the evolutionary relationships among mtDNA haplotypes sampled in jaguars (PonH), pumas (PcoH) and other carnivores (see text and Table 2). The tree is based on 96 bp of the mtDNA *ATP6* gene, and was constructed using the Neighbor-joining algorithm on the basis of a p-distance matrix. *Numbers* above branches represent bootstrap support values generated with 1,000 replicates

quality" in the field did exhibit the lowest success rate (1/7 scats, i.e. 14%), indicating that this initial assessment at the collection stage did predict to some extent the success rate of PCR and sequencing. Scats from other locations presented satisfactory success rates (1/1 for RD; 2/3 for PI), though their smaller sample size precludes a more detailed assessment of local variables.

Throughout all analyses of this mtDNA segment from scat samples, no evidence of prey DNA amplification was observed. If affirmed by further sampling and analyses of other carnivore communities, this result would indicate that the markers proposed here might have advantages in terms of identification performance relative to others that have been published previously (e.g. see Adams et al. 2003 and Onorato et al. 2006). In addition, we also saw no evidence of *numt* amplification or co-amplification (e.g. double peaks, high sequence background) in the jaguar samples, supporting the prediction that this segment excludes this nuclear insertion. This would represent an additional advantage of employing this marker relative to the *cyt b* gene, targeted by other studies (e.g. Farrell et al. 2000).

Since very little variation was observed within each species (see Fig. 1), most field-collected scats bore identical haplotypes to those observed in reference samples (Table 2), leading to straightforward identification. In the few cases where a unique haplotype was identified in scat samples (e.g. PcoH2 and PcoH3, see Table 2), the presence of multiple diagnostic sites made species-level identification conclusive with either a character-based or a phylogenetic approach (see Fig. 1). Employing this assay, we where thus able to confidently detect the presence of one or both species in all surveyed field locations (see

Table 2). For MP, which presented the largest available sample size, 12 scats were identified as originating from pumas and six from jaguars, allowing the use of these samples in downstream analyses addressing fragment occupation, diet and population genetics. Interestingly, all seven scats that could be sequenced from the RV site were identified as jaguars, providing the first genetic samples of *P. onca* collected from a Coastal Atlantic Forest location. Further sampling from this area will be extremely important to allow for an initial survey of the genetic diversity and evolutionary distinctiveness of this isolated and critically endangered remnant population.

Finally, in addition to its diagnostic power for pumas and jaguars, the informative content of this mtDNA segment holds promise to provide reliable identification for other carnivore species. For example, as a control in this study, we amplified and sequenced DNA from a domestic cat faecal sample, which was easily grouped with the reference sequence for this species available in GenBank (see Fig. 1). In the context of the inherent uncertainty of field-based scat identification, especially in ecosystems harboring multiple sympatric species, the usefulness of a sequence-based assay is noteworthy. One graphic example was observed among the samples investigated in this study. Of the 21 sequenced samples from the MP location, all of which had been identified in the field as originating from a "large felid", 18 were identified as jaguar or puma (see above and Table 2). Three others were found to bear considerably different *ATP6* haplotypes, and were subsequently identified as originating from ocelots (*L. pardalis*) by comparison with a multi-species data base (P. B. Chaves et al. unpublished data). The finding that ocelot samples were identified as belonging to a "large felid" by experienced field researchers highlights the urgent issue of procuring accurate and standardized faecal identification methods, so as to provide a reliable basis for the development of non-invasive ecological and genetic investigations on jaguars and pumas, and on carnivore species in general.

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