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Molecular identification of mesocarnivores through feces in the Sierra del Abra Tanchipa Biosphere Reserve, San Luis Potosi, Mexico

Octavio César Rosas-Rosas[1], Canuto Muñoz-García[2], Miguel Paul Conde-Hinojosa[3], Luis Antonio Tarango-Arámbula[1], Reyna Isabel Rojas-Martínez[4], Héctor Eduardo Benítez-Alemán[5], and Cesar Cortez-Romero[1,5*]

[1]Posgrado en Innovación en Manejo de Recursos Naturales, Colegio de Postgraduados, Campus San Luis Potosí, Iturbide No. 73, Centro, Salinas de Hidalgo, San Luis Potosí, México. C.P. 78620. E-mail: octaviocrr@colpos.mx (OCR-R); ltarango@colpos.mx (LAT-A); ccortez@colpos.mx (CC-R)
[2]Facultad de Medicina Veterinaria y Zootecnia. Universidad Autónoma de Guerrero. Pungarabato, Guerrero, México C.P. 40610. E-mail: cmunoz@uagro.mx (CM-G)
[3]Instituto de Ciencias Agropecuarias. Universidad Autónoma del Estado de Hidalgo. Avenida Universidad Km. 1 s/n Exhacienda Aquetzalpa, Tulancingo, Hidalgo, México. C.P. 43600. E-mail: miguel_conde@uaeh.edu.mx (MPC-H)
[4]Programa de Fitopatología, Colegio de Postgraduados, Campus Montecillo, km. 36.5 Carretera México-Texcoco, Montecillo, Texcoco, Estado de México, México. C.P. 56264. E-mail: rojas@colpos.mx (RIR-M)
[5]Posgrado en Recursos Genéticos y Productividad, Programa de Ganadería, Colegio de Postgraduados, Campus Montecillo, km. 36.5 Carretera México-Texcoco, Montecillo, Texcoco, México. C.P. 56264. E-mail: benitez.hector@colpos.mx (HEB-A)
*Corresponding author: ccortez@colpos.mx

Molecular identification of sympatric carnivore species through feces is not a simple task, especially for those of similar body size and similar droppings. This fact can be a disadvantage for several types of field research, including distribution, demography, and feeding habits. The objective of this study was to identify mesocarnivore species from feces collected in the Sierra del Abra Tanchipa Biosphere Reserve, San Luis Potosí, northeastern Mexico, using the PCR-RFLP technique. We collected 125 fecal samples from roads and latrines during February-May 2018, November 2018, and February 2019. DNA was extracted and a fragment of the cytochrome *b* gene was amplified with two pairs of primers: one specific for five Neotropical feline species and the other for the universal carnivore. To include control samples, DNA was extracted and amplified from hair follicles of the following species: captive ocelot (*Leopardus pardalis*), bobcat (*Lynx rufus*), jaguarundi (*Puma yagouaroundi*), margay (*Leopardus wiedii*), gray fox (*Urocyon cinereoargenteus*), domestic species, dogs (*Canis lupus familiaris*) and cats (*Felis catus*), and one sample from tissue of puma (*Puma concolor*). The PCR products from the control samples were sequenced to identify the restriction enzymes *Hpa II*, *MluC I*, *Hae II*, and *Mbo I,* which were used for the PCR-RFLP. Identification of restriction fragments was performed by banding patterns for each species on 2 % agarose gels. A total of 108 samples were identified (86.4 % of the total collected): 82 (75.3 %) *L. pardalis*, 11 (10 %) *U. cinereoargenteus*, 6 (5.5 %) *Puma concolor*, 6 (5.5 %) *P. yagouaroundi*, and 3 (2.7 %) *L. wiedii.*

Keywords: Feces, felids, molecular tools, restriction enzymes

La identificación molecular de carnívoros simpátricos a partir de heces no es una tarea sencilla, especialmente para aquellas con tamaño corporal y excrementos similares. Esto puede ser una desventaja para investigaciones de campo, distribución, demografía y de hábitos alimentarios. El objetivo de este estudio fue identificar especies de mesocarnívoros a partir de heces recolectadas en la Reserva de la Biosfera Sierra del Abra Tanchipa, San Luis Potosí, noreste de México, mediante la técnica de PCR-RFLP. Se recolectaron 125 muestras fecales de caminos y letrinas, durante febrero-mayo de 2018, noviembre de 2018 y febrero de 2019. Se extrajo ADN y se amplificó parte del gen citocromo b del ADN mitochondrial con dos pares de primers: uno específico para cinco especies de felinos neotropicales y el otro universal para carnívoros. De las muestras testigo se extrajo y se amplificó ADN de folículos pilosos de ocelote (*Leopardus pardalis*), gato montés (*Lynx rufus*), jaguarundi (*Puma yagouaroundi*), tigrillo (*Leopardus wiedii*) y zorro gris (*Urocyon cinereoargenteus*) en cautiverio; de especies domésticas como perros (*Canis lupus familiaris*) y gatos (*Felis catus*), así como de una muestra de tejido de puma (*Puma concolor*). Los productos de PCR de las muestras testigo se secuenciaron para identificar las enzimas de restricción *Hpa II*, *MluC I*, *Hae II* y *Mbo I*, las cuales fueron utilizadas en la técnica PCR-RFLP. La identificación de fragmentos de restricción se realizó mediante patrones de bandas en geles de agarosa al 2 % para cada especie. Se identificó un total de 108 muestras (86.4 % del total recolectadas): 82 (75.3 %) *L. pardalis*, 11 (10.0 %) *U. cinereoargenteus*, 6 (5.5 %) *P. concolor*, 6 (5.5 %) *P. yagouaroundi* y 3 (2.7 %) *L. wiedii*.

Palabras clave: Enzimas de restricción, félidos, heces, herramientas moleculares

Predators are highly specialized animals that have developed traits to capture and subdue their prey. Mammals of the Order Carnivora are characterized by having teeth capable of cutting and slicing, as they have sharp carnassial teeth and highly developed canines (Kitchener 1991; Ceballos and Oliva 2005). This order encompasses 245 recent terrestrial species, grouped into 107 genera and 13 families (Wilson *et al.* 1995; Bellani 2020). We found in Mexico six of the 37 world species (Ceballos and Oliva 2005), of which the Felidae family is the most morphologically specialized (Bellani 2020). In general, felids are top predators within ecosystems, since

they keep the ecosystem in balance by controlling the population size of primary prey, influencing the diversity and dynamics of the plant community and, consequently, its diversity (Wang 2002; López-Pérez *et al.* 2021).

Medium-sized carnivores (<20 kg), known as "mesocarnivores," are more numerous than large carnivores, both in species richness and abundance, and in their behavior and ecology, given their small size and ability to thrive in diverse habitats. However, these species are assumed to have a lower ecological impact and, consequently, mesocarnivores have received little or no attention (Emmons 1988; Roemer *et al.* 2009). Among the mesocarnivores of felids are: ocelot (*Leopardus pardalis* Linné 1758), margay (*Leopardus wiedii* Schinz 1821), and jaguarundi (*Puma yagouaroundi* Geoffroy Saint-Hilaire 1803). These medium-sized felids have a neotropical distribution and inhabit a wide variety of habitats (de Oliveira 1998; Bianchi *et al.* 2011; Peña-Mondragón and del Val 2022). On the other hand, some canids have a wide distribution across the American continent, including coyote (*Canis latrans* Say 1823) and gray fox (*Urocyon cinereoargenteus* Schreber 1775), which are considered sympatric species that occupy diverse habitats within the same region. Coexistence of these species makes it difficult to identify their feces in the field by shape and color (Alberts *et al.* 2017) and, consequently, makes it difficult to describe their diets and to determine whether they may share certain prey preferences.

The study of wildlife, particularly of elusive species such as carnivores, represents a significant methodological challenge. Conventional methods of direct capture and handling can be costly, logistically complex, and potentially harmful to the individuals studied (Paez *et al.* 2021). In response to these limitations, non-invasive techniques have become increasingly relevant in wildlife research and population monitoring. Among the most widely used tools are camera traps, track transects, hair snares, and fecal collection (Torres-Romero *et al.* 2018). Comparative studies have shown that camera traps are significantly more efficient than live-capture traps and comparable in effectiveness to other non-invasive methods, but only provide information on the ecological aspects of felines; collected data do not distinguish between species or sex (Wearn and Glover-Kapfer 2019), being particularly valuable for detecting nocturnal species and populations at low density. Hair snares, for example, have demonstrated their potential in tropical ecosystems of Mexico for sampling felid populations, including records of rarely detected species such as the jaguarundi and the margay (Garcia-Alaniz *et al.* 2009). Taken together, these minimally invasive approaches (including camera trapping and genetic identification) detect a similar number of individuals compared to conventional trapping, with additional advantages in animal welfare and cost reduction (Paez *et al.* 2021).

Identification of signs, including tracks, burrows, hair, and feces, is an indirect, non-invasive technique that allows researchers to study the biological and ecological aspects of a species in a given ecosystem (Torres-Romero *et al.* 2018). Differentiating feces of Neotropical felids is complicated by their great morphological similarity (Alberts *et al.* 2010; Aranda-Sánchez 2012). Morphological characteristics such as diameter, length, and disjoint segments of feces may guide field identification; however, in many cases morphometric patterns cannot be a reliable technique to distinguish among different sympatric species (Akrim *et al.* 2018). Furthermore, in habitats with similarly sized sympatric carnivores, misidentification of fecal samples in the field is frequent and can lead to significant bias in research results (Laguardia *et al.* 2015), with direct implications for management and conservation decisions.

To address this problem, molecular scatology techniques have been developed to identify species from DNA present in intestinal epithelial cells adhered to the outer surface of feces (Reed *et al.* 1997). These techniques can be used to establish species distributions, model habitat requirements, analyze diet, estimate abundance and population density, and are important for population and conservation analyses (Rodgers and Janečka 2013). Molecular scatology consists of a genetic analysis of fecal samples to extract genetic material (DNA) from an individual without physical contact, thus determining the species, sex, and population size, among other parameters (Nagata *et al.* 2005; Alberts *et al.* 2010; Bach *et al.* 2022). Since DNA obtained from fecal samples is often degraded by environmental conditions, the use of multiple molecular markers is critical for obtaining more reliable identifications (Rodríguez-Castro *et al.* 2020). Among the most frequently used mitochondrial markers for the identification of Neotropical species are regions of the cytochrome *b* gene and, more recently, primers for a region of the ATP6 gene under a DNA mini-barcoding approach (Alberts *et al.* 2017).

Molecular studies offer a more accurate method, such as PCR-RFLP, because they use specific primers (Mukherjee *et al.* 2010). In this way, PCR-RFLP (where a PCR with common primers is followed by restriction digestions targeting species-specific motifs). PCR-RFLP is a technique that differentiates organisms by analyzing patterns derived from the cleavage of a previously amplified DNA strand with restriction enzymes or endonucleases. If two organisms differ in the distance between sites cut by a particular restriction enzyme, the lengths of the fragments will differ when the DNA is digested with the endonuclease (Bidlack *et al.* 2007). The similarities or differences in the patterns generated in an agarose gel can be used to differentiate sequences obtained from sympatric species. Several studies have evaluated and applied this approach in carnivore communities worldwide. In Europe, reliable non-invasive methods based on nested PCR of a mitochondrial D-loop region, followed by restriction enzyme digestion, were developed to differentiate morphologically similar sympatric mustelid species (Gómez-Moliner *et al.* 2004). Researchers in India developed primers for felids and canids targeting the 16S rRNA region of the mitochondrial

genome. They also selected restriction enzymes that differentially cut this region for various species, generating diagnostic banding patterns on agarose gel (Mukherjee *et al.* 2010). For Andean felids, Cossíos and Angers (2006) demonstrated the utility of PCR-RFLP for discriminating feces of sympatric species, while for Neotropical felids with broader distributions, Roques *et al.* (2011) developed the RCP-PCR protocol — a single-tube multiplex PCR system that generates species-specific banding patterns on agarose gel — achieving high identification success rates for puma, jaguar, jaguarundi, and ocelot/margay feces. These precedents confirm the viability and versatility of PCR-RFLP as a non-invasive monitoring tool for carnivores across different biogeographic contexts.

Therefore, due to the great diversity of carnivores distributed in the Sierra del Abra Tanchipa Biosphere Reserve, San Luis Potosí, Mexico, a specific protocol is required to accurately identify its feces. The objective of this research was to identify mesocarnivore species from fecal samples collected using the PCR-RFLP technique.

Materials and methods

Study Area. The Biosphere Reserve Sierra del Abra Tanchipa (RBSAT) is a natural protected area (NPA) located in the municipalities of Ciudad Valles and Tamuín, San Luis Potosí, and Antiguo Morelos, El Mante, Tamaulipas, which encompasses about 21,464.44 hectares (22° 24'-22° 03' N, 90° 59'-98° 53' W). The vegetation types include tropical deciduous forest, medium subdeciduous forest, low subevergreen forest, tropical oak forest, palm forest, and grassland (de Nova-Vázquez *et al.* 2018).

Collection and preservation of samples. Feces and Hair follicles. We collected fecal samples every two weeks from February to May 2018, November 2018, and February 2019. We conducted field surveys along transects on the main roads in the western section of the RBSAT. We placed feces collected in acid-free paper bags and labeled them, including location, date, collecting time, and habitat type; samples were stored at room temperature.

To validate the extracted DNA from fecal samples, we used a set of genetic material known as "witness samples." For this purpose, we used hair follicles from captive ocelot, bobcat, jaguarundi, margay, gray fox, and puma fur. Chapultepec Zoo, Ciudad de México, donated follicle samples. We also used follicles from local dogs and house cats. We stored the samples in 70% ethyl alcohol.

DNA extraction. Feces. We extracted DNA from 125 field-collected fecal samples using the QIAamp DNA Stool Mini Kit from QIAGEN (QIAGEN®, Mexico) using a protocol modified by Chaves *et al.* (2010) and divided them into two days. On the first day, we cut fine fragments of the cortex of each sample (an average of 200 mg), using a new scalpel, forceps, and a disposable Petri dish. We placed the samples in a 2 mL Eppendorf tube, added 1.5 mL of ASL buffer and mixing gently. Some samples had a large amount of hair and insoluble material, which had to be dissolved in 4 mL of ASL buffer. A 1.5 mL aliquot was taken after the sample was dissolved in a 2.0 mL Eppendorf tube, and then the sample was incubated at 65 °C for 12 h. On the second day, the sample was centrifuged at 6000 x *g* for three min. Subsequently, 1.5 mL of the supernatant was transferred to a new 2.0 mL Eppendorf tube, and an Inhibitex (buffer) tablet was added until it was completely dissolved. After a 1-minute incubation at room temperature, the mixture was centrifuged at 6000 x *g* for 12 min. Subsequently, 25 µL of proteinase K and 600 µL of the supernatant from the pellet precipitation performed earlier were added to the Eppendorf tubes containing the sample, and the mixture was carefully mixed to obtain a homogeneous solution. Next, we added 600 µL of buffer AL to the same tube and incubated at 70 °C for 15 min. After the incubation time, we added 600 µL of 100 % ethanol and stirred for 15 s to form a homogeneous mixture. We transferred 600 µL of the above mixture to a QIAamp spin column with a 2 mL collection tube and centrifuged at 6000 x *g* for one minute. We repeated these steps until the entire sample was filtered. Subsequently, we transferred the column to a clean collection tube, then added 500 µL of buffer AW1 and centrifuged at 6000 x *g* for one minute; the filtrate was discarded. Again, we added 500 µL of buffer AW2, centrifuged at 6000 x g for two min, and discarded the filtrate. Then, we transferred the filtrate column to a 1.5 mL Eppendorf tube and performed two washes within the column with 60 µL of buffer AE directly on the membrane. It was left to incubate for one hour at room temperature for each wash, where we obtained a final volume of 120 µL with DNA in the Eppendorf tube, identified and stored at -20 °C.

For this research, we used two pairs of oligonucleotides; the first pair was an oligonucleotide specific for five species of Neotropical felids (MxCtF 5´-CCATCCAACATCTCAGCATGATG-3´ and MxCtR 5´-GAGGCTCCGTTGGCATGTAT-3´), which was developed by Garcia-Alaniz (2009), where fragments of the cytochrome *b* gene with 165 bp, considering fragments between 148 bp and 486 bp from mitochondrial DNA. These fragments are reported in GenBank with accession numbers FJ490206 (165 bp) puma (*Puma concolor*), FJ490207 (165 bp) ocelot (*Leopardus pardalis*), FJ490208 (165 bp) jaguarundi (*Puma yagouaroundi*) and FJ490209 (165 bp) margay (*Leopardus wiedii*).

The second pair of oligonucleotides, from universal carnivores, amplified a fragment of the mitochondrial DNA cytochrome *b* gene (HCarn200; 5´-ATTCAGCCRTARTTAACGTC–3´) (Bidlack *et al.* 2007), used in conjunction with another (CanidL1; 5´-AATGACCAACATTCGAAA-3´) (Paxinos *et al.* 1997). These two oligonucleotides amplified fragments of 234 bp, corresponding to the 148 bp and 486 bp fragments from mitochondrial DNA. These oligonucleotides amplified fragments of the aforementioned species, and we also amplified for coyote (*Canis latrans*), gray fox (*Urocyon cinereoargenteus*) and bobcat (*Lynx rufus*), as well as for domestic dog (*Canis lupus familiaris*) and domestic cat (*Felis catus*).

Hair follicles. We extracted DNA from all control samples

and directly amplified using the Phire Animal Tissue Direct PCR Kit (Termo Fisher Scientific™, Mexico). Samples were dried at room temperature and cut with a clean scalpel to no more than 5 mm in length from the start of the follicle. We placed ten follicles in 1.5 mL Eppendorf tubes, where 25 µL of 2x Phire Animal Tissue PCR Buffer (Termo Fisher Scientific™, Mexico), 25 µM of each oligo, and finally, we added 1 µL of Phire Hot Start DNA Polymerase (Termo Fisher Scientific™, Mexico).

DNA Amplification. Feces samples were amplified by PCR, in a 25 µL final volume reaction containing 100 ng μL^{-1} of DNA, 1X TAE reaction buffer (Tris Acetate-EDTA, Sigma Aldrich®, Mexico), 2.5 mM $MgCl_2$, 0.2 mM (200µM) of dNTP (Promega®, USA), 20 µM of each primer, and 1.5 units per reaction of Taq polymerase (Sigma Aldrich®, Mexico). We used an initial incubation in a thermocycler (Tc-512 brand; Techne®) at 94 °C for three minutes, followed by 40 cycles at 94 °C for one min, 54 °C for one min and 72 °C for two minutes, and a final extension at 72 °C for five minutes. In each test, we added a positive and a negative control. For follicles, we placed the DNA samples in a thermal cycler (Tc-512 brand; Techne®), with an initial denaturation temperature at 98 °C for five minutes, followed by 40 cycles at 98 °C for five seconds, 54 °C for five seconds, and 72 °C for 20 s and a final extension at 72 °C for 1 min.

Although the DNA extracted from the fecal samples had low DNA integrity, the concentration in many samples was greater than 100 ng μL^{-1}; those with concentrations below 50 ng µL-1 were discarded from the study. In some samples with a sweep, PCR amplification could be performed with the specific primer (fragment size of 165 bp) for felids developed by Garcia-Alaniz (2009), from which 91 % of the samples were amplified (Supplementary data SD1). The samples that did not amplify with the previous primer were subjected to amplification with the universal carnivore primer (fragment size of 234 bp) reported by Bidlack *et al.* (2007) which allowed the amplification of the remaining 9.0 % (Supplementary data SD2).

PCR-RFLP technique. We sequenced amplicons from control samples and imported into NebCutter software v. 2.0 (https://academic.oup.com/nar/article/31/13/3688/2904148) to determine the type of restriction enzymes used to obtain restriction fragments, generate positive controls, and identify fecal samples. The enzymatic reactions had a final volume of 15 µL, containing 1.5 µL of 10x buffer, 1 µL of enzyme (5U), 2 µL (20 ng of DNA) of PCR product, and Milli-Q water (Merck®, Mexico) for the remainder of the reaction volume. We added positive and negative controls to each reaction.

Agarose gels. We run by electrophoresis DNA samples, PCR products, and PCR-RFLP in a vertical chamber (Model MVG-216-33; C.B.S Scientific CO®, USA) in agarose gels 1 % in TAE running buffer (Sigma Aldrich®, Mexico) at 247 volts for 90 min, with a molecular weight marker (GeneRuler 1 kb DNA Ladder; Thermo Scientific®, USA). We stained the gel with ethidium bromide, then added it to the agarose gel. Three µL of the amplicon and 3 µL of Green GoTaq® Flexi buffer (Promega©, Wisconsin, USA) were deposited in each well. We viewed the gel on a photodocumenter (Quantum®) with Vision Capt® software.

Results

DNA extraction and amplification from feces. The PCR enabled amplification of DNA from 108 of 125 samples collected (60 from roadsides and 65 from a latrine).-

DNA extraction and amplification from hair follicles. We performed DNA amplification from follicles as control samples using the Phire Animal Tissue Direct PCR Kit with both pairs of primers, and we observed amplification for all samples. With the universal carnivore primer used by Bidlack *et al.* (2007), we also observed that the feline follicle samples amplified (234 bp), but the band in the agarose gel was fainter, unlike the bands from the canid follicles sampled (Figure 1).

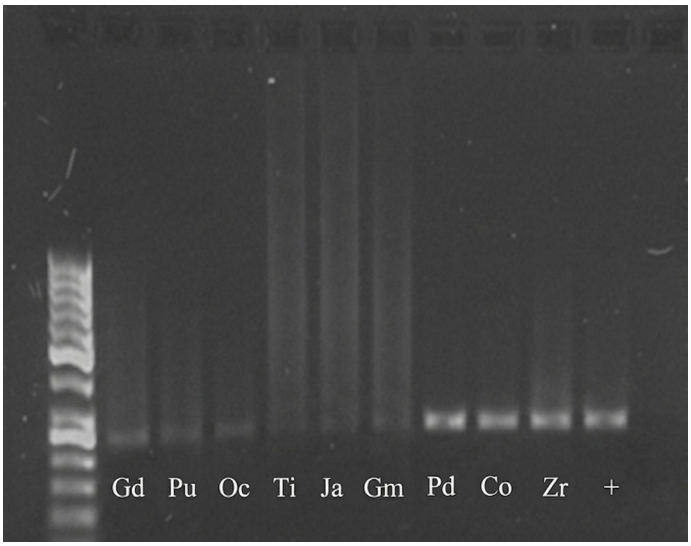

Figure 1. Amplification of samples from follicles using the universal carnivore primer. 50 bp molecular marker (Mm). Gd: Domestic cat, Pu: Puma, Oc: Ocelot. Ti: Margay, Ja: Jaguarundi, Gm: Bobcat. Pd: Domestic dog, Co: Coyote, Zr: Gray fox, + Positive control (Pd), - Negative control.

In the case of the specific primer developed by Garcia-Alaniz (2009), a faint band was observed for the domestic cat and the bobcat. In wild canids, we did not observe amplification. For the ocelot, margay, puma, and jaguarundi control samples, the bands were completely labeled (165 bp, Figure 2). Amplifications of control samples from nine carnivore species for the mitochondrial DNA cytochrome *b* gene are shown in Table 1.

Identification of restriction enzymes. We sequenced PCR-amplified control samples to identify the restriction enzymes used in the PCR-RFLP. The sequences obtained were subjected to BLAST (Basic Local Alignment Search Tool) analysis to verify similarities with other reported cytochrome *b* gene sequences in GENBANK. Similarities ranged from 88% to 98%.

After verification of the sequences of the cytochrome *b* gene fragments for different mesocarnivore species,

Table 1. Fragment of the cytochrome *b* gene sequences obtained from DNA amplification of follicles from nine carnivore species.

Species	Sequence	bp	GenBank accession number
Puma concolor (Puma)	GAGGGGATGATCTCCTCCCAACATTTCAGCATGATGAAACTTTGGCTCCCTACTAGGGGTCTGCCTAATCCTA-CAAATCCTAACCGGCCTCTTCCTGGCCATACACTATACATCAGACACAATGACTGCCTTTTCATCAGTCACTCA-CATCCTGTCGGACGTCAAACTACG	165	FJ490206
Leopardus pardalis (Ocelot)	TGGGTCCTTATTAGCAGTTTGCCTACTTTTACAGATTCTCACAGGCCTCTTTCTAGCCATACACTATACATCAGA-TACAGCAACCGCCTTTTCCATCATTTACCCAGATCTGCCGCGACGTCAACTATGGCTAAATCATCCGATACAT-ACATGCCAACGGAGCCTCA	165	FJ490207
Puma yagouaroundi (Jaguarundi)	GGTTCCTTAATAGGAGTCTGCCTAATCCTACAGATTCTGACCGGCCTATTCCTAGCCATACACTACACATCAGA-CACAACAACCGCCTTCTCATCAGTCACCCACATCTGCCGCGACGTTAACTAGGGCTGAATCATCAGATATATA-CATGCCAACGGGGCTTCCA	165	FJ490208
Leopardus wiedii (Margay)	GGCTCCTTATTAGGAGTTTGCCTAATCCTACAAATTCTCACTGGCCTTTTCCTAGCTATACACTACACATCAGA-CACCACAACCGCTTTCTCATCAGTTACCCACATCTGCCGCGACGTCAACTATGGCTGAATTATCCGATACCTA-CATGCCAACGGAGCCTCC	165	FJ490209
Felis catus (Domestic cat)	AATCACACCCCGTTATCAACATTATTACTCACTCATCCATCGATCTACCTAGCCCATCTTACATCTCAGCATGAT-GAAACTTCGGCTCCCTTCTAGGAGTCTGCCTAACCTTACAAATCCTCACCGGCCTCTTTTTGGCCATACACTA-CACATCAGACACAATAACCGCCTTTTCATCAGTTACCCACATCTGTCGCGACGTTAACTACGGCTGAATAATC-CGATATTTACAC	402	AJ300702
Lynx rufus (Bobcat)	AAAGTCACACCGTATTACTAAAGTATACAACCGATAATTCATCGATTCACCCGCCCCATCAACATCTCAGCAT-GATGAAACTTCGGCTCCCTGCTAGGAGTCTGCCTAATCCTACAGATCCTCACCGGCCTCTTCCTAGCCATACAC-TACACATCAGACACGCTAACCGCCTTTTCATCAGTCACCCATATCTGCCGCGACGTTAACTATGGCTGAATAATC-CGATAC	226	DQ471842
Canis familiaris (Domestic dog)	AAAACCCACCCACTAGCCACAATTGTTAATAACTCATTCATTGACCTCCCAGCGCCGTCTAACATCTCTGCTT-GATGGAACTTCGGATCCTTACTAGGAGTATGCTTGATTCTACAGATTCTAACAGGTTTATTCTTAGCTATGCAC-TATACATCGGACACAGCCACAGCTTTTTCATCAGTCACCCACATCTGCCGAGACGTTAACTACGACTGAATTATC-CGCA	277	DQ236096
Canis latrans (Coyote)	CACCCACTATGCAAAGTTGTCAATAACTCATTCATTGACCTCCCAGCGCCATCTAACATCTCTGCTTGATG-GAATTTCGGATCCTTACTAGGAGTATGCCTGATTCTACAGATTCTAACAGGTTTATTTTTAGCTATACACTATA-CATCGGACACAGCCACAGCTTTTTCATCAGTCACCCACATCTGTCGAGACGTTAACTACGGCTGAATTATCCGC-TACATACA	253	KT946976
Urocyon cinereoargenteus (Gray fox)	AAAACCCACCCGCTCGGTAAAATCGTCAACAGCTCGTTCATCGACCTACCTGCACCATCTAACATTTCTGCAT-GATGGAACTTCGGGTCCCTGTTAGGAATCTGCCTTATTCTACAGATTATAACAGGCTTATTCTTGGCCATACAC-TATACATCAGATACCGCTACAGCCTTTTCATCCGTTACCCATATCTGTCGAGACGTTAACTACGGCTGAATAATC-CGCTATAT	226	DQ471838

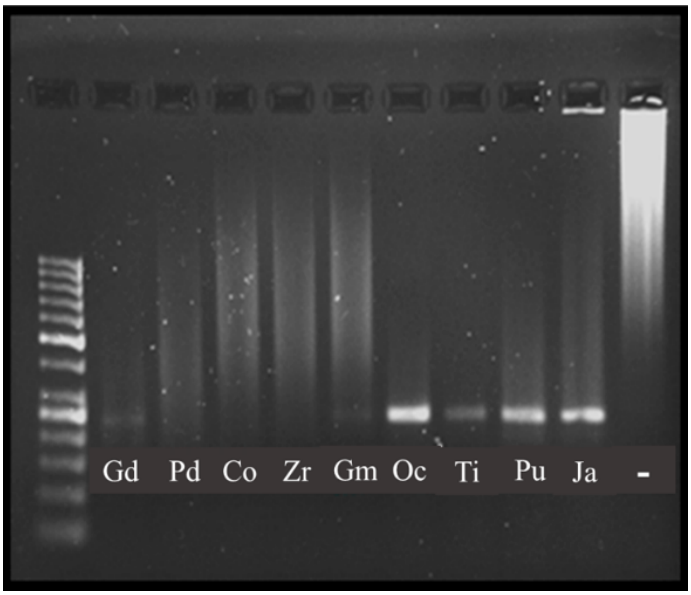

Figure 2. Amplification of follicle samples using the feline-specific primer. 50 bp marker, Gd: Domestic cat, Pu: Puma, Oc: Ocelot. Ti: Margay, Ja: Jaguarundi, Gm: Bobcat. Pd: Domestic dog, Co: Coyote, Zr: Gray fox, (-) Negative control.

we performed PCR-RFLP method. Then we searched for restriction enzymes (endonucleases) using the online NebCutter v. 2.0 program (New England, Biolabs), which cut at different points. We used these sequences to generate banding patterns to differentiate each species. In the first case, we obtained the sequences for ocelot, margay, jaguarundi, and puma. We identified the *Hpa II* enzyme, which had no effect on the ocelot sequences. We cut the puma sequence at base 85, which generated two fragments of similar size, in the same way, *Hpa II* cut the sequence obtained from jaguarundi at base 42, which generated two restriction fragments: the first with a length of 42 bp and the second with a length of 125 bp (Figure 3a). For the sequences obtained from ocelot and jaguarundi, we identified *Hae III* enzyme to be used in conjunction with the *Mbo I* enzyme, which cut the ocelot sequence at base 44, generating two restriction fragments. The first one with a length of 44 bp and the second one of 121 bp. This allowed to differentiate the three fragments generated by this same pair of enzymes for the ocelot sequence, two of 61 bp and one of 46 bp (Figure 3b).

Validation of restriction enzymes. This procedure was first verified using PCR products obtained from follicular samples, then tested with PCR products from fecal samples, and amplified with the species-specific primer for Neotropical felids for identification. We observed a band in the agarose gel, since both fragments in the use of the *Hpa II* enzyme for puma are similar in size (85 bp and 87 bp) (Figure 4a). For the same enzyme in the jaguarundi, we also observed two bands, each of a different size: the lower band is 42 bp and appears faint, while the upper band is 125 bp (Figure 4a). When restriction enzymes *Hae III* and *Mbo I* were used together to select the sequences of the ocelot and

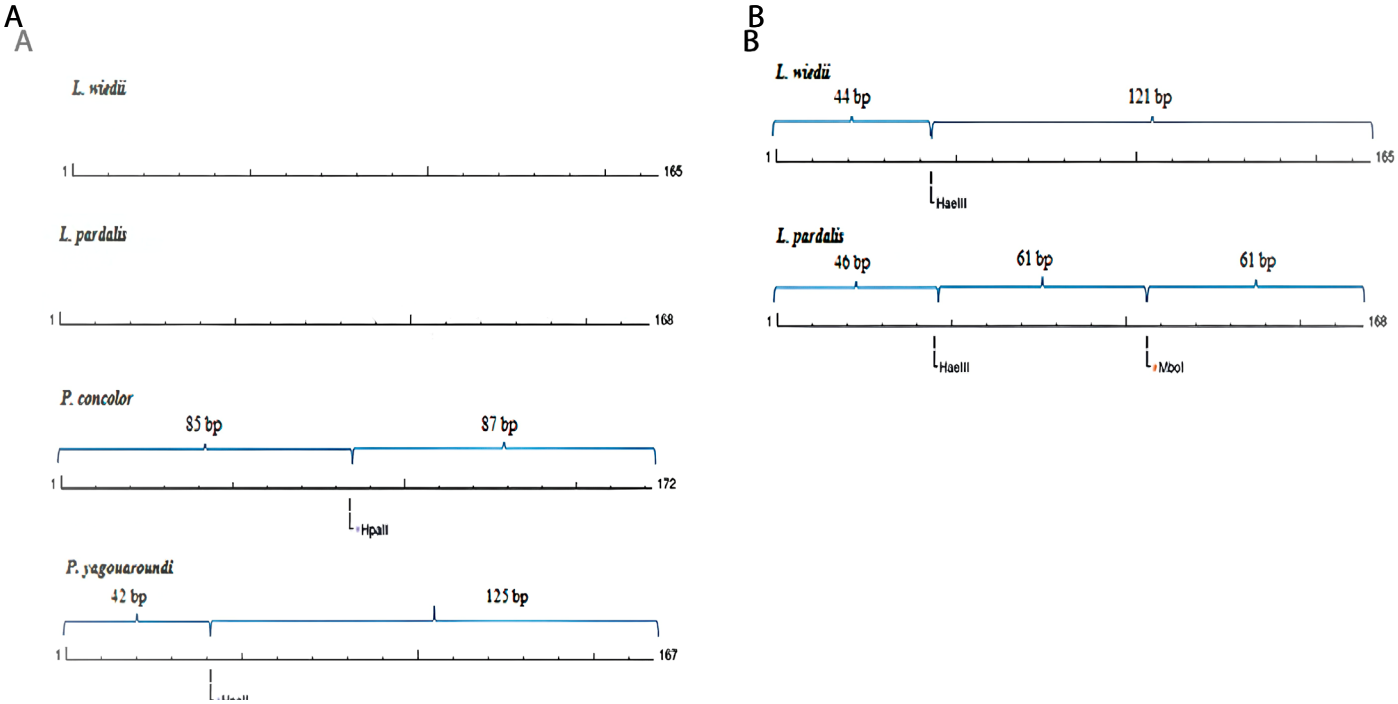

Figure 3. Cutting patterns created by the restriction enzymes *Hpa II*, *Hae II*/*Mbo I* for four feline species: (3a) fragments for margay, ocelot, puma, and jaguarondi, and (3b) fragments for margay and ocelot.

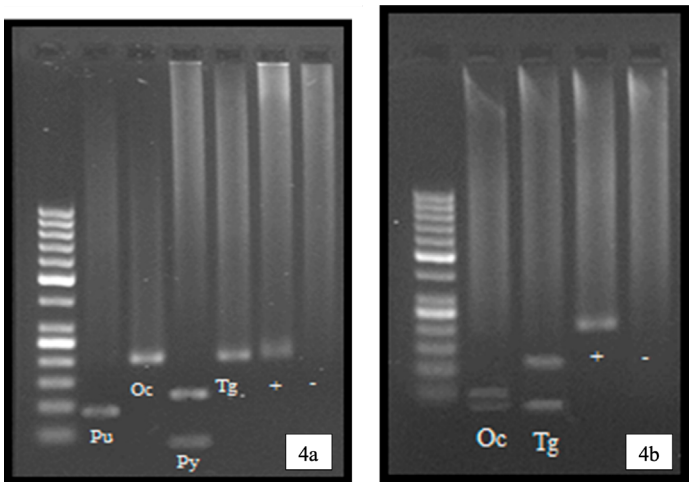

Figure 4. Amplification of feline fecal samples. 50 bp marker. (a) Pu: Puma, Oc: Ocelot, Py: Jaguarundi, Tg: Margay. (b) Oc: Ocelot, Tg: Margay. Positive control (+), Negative control (-).

the margay, two bands were observed in the agarose gel for the ocelot, generated by three fragments: two of 61 bp, which means that only one band is observed, and another smaller fragment of 46 bp. We used this same pair for the margay sequence, where only the *Hae III* enzyme acted, and generated two easily visible bands in the agarose gel: one of 44 bp and a larger one of 121 bp (Figure 4b).

In the case of the samples amplified with the non-specific primer, we identified the enzyme *MluC I*. Additionally, we observed two restriction sites at bases 20 and 215 for the domestic dog, which generated three restriction fragments; the first was 20 bp in size, the second 195 bp, and the third 11 bp. We similarly observed in the coyote sequence two restriction sites at bases 72 and 209, which generated three fragments of 72 bp, 137 bp, and 18 bp, respectively (Figure 5a). The sequences of the gray fox, bobcat, and domestic cat were not cut by this enzyme, but they were cut by the enzymes *Hae III* and *Mbo I* when used together, which identified restriction zones at base 139 in the gray fox sequence, which generated two fragments: the first of 139 bp and another of 91 bp by the simple action of the *Hae III* enzyme. In the bobcat sequence, we found two restriction sites at the height of bases 116 and 128, which generated three fragments of 116 bp, 12 bp and 100 bp. The domestic cat sequence had three restriction sites at the height of bases 41, 127 and 138; therefore, we obtained four restriction fragments of 41 bp, 86 bp, 11 bp, and 95 bp (Figure 5b).

The follicle PCR products as control samples, and the PCR products of the DNA extracted from the fecal samples for identification, both amplified with the nonspecific primer, were subjected to the action of the aforementioned restriction enzymes, their products were placed on an agarose gel and separated by electrophoresis. The *MluC I* enzyme generated two visible bands in the domestic dog sample; one of approximately 195 bp and the second of 20 bp. For the coyote sample, three bands were easily observable: the first of 137 bp, followed by one of 72 bp, and the last, barely visible, of 18 bp. No action of this enzyme was observed in the gray fox, bobcat, and domestic cat samples (Figure 6a), which were subjected to the action of *Hae III* and *Mbo I* together to differentiate these three species. We observed in the gray fox the presence of two bands, one of 139 bp and the other of 91 bp. To differentiate the wildcat sample, is the only band that could be observed, generated by two restriction fragments, which have a size of 116 and

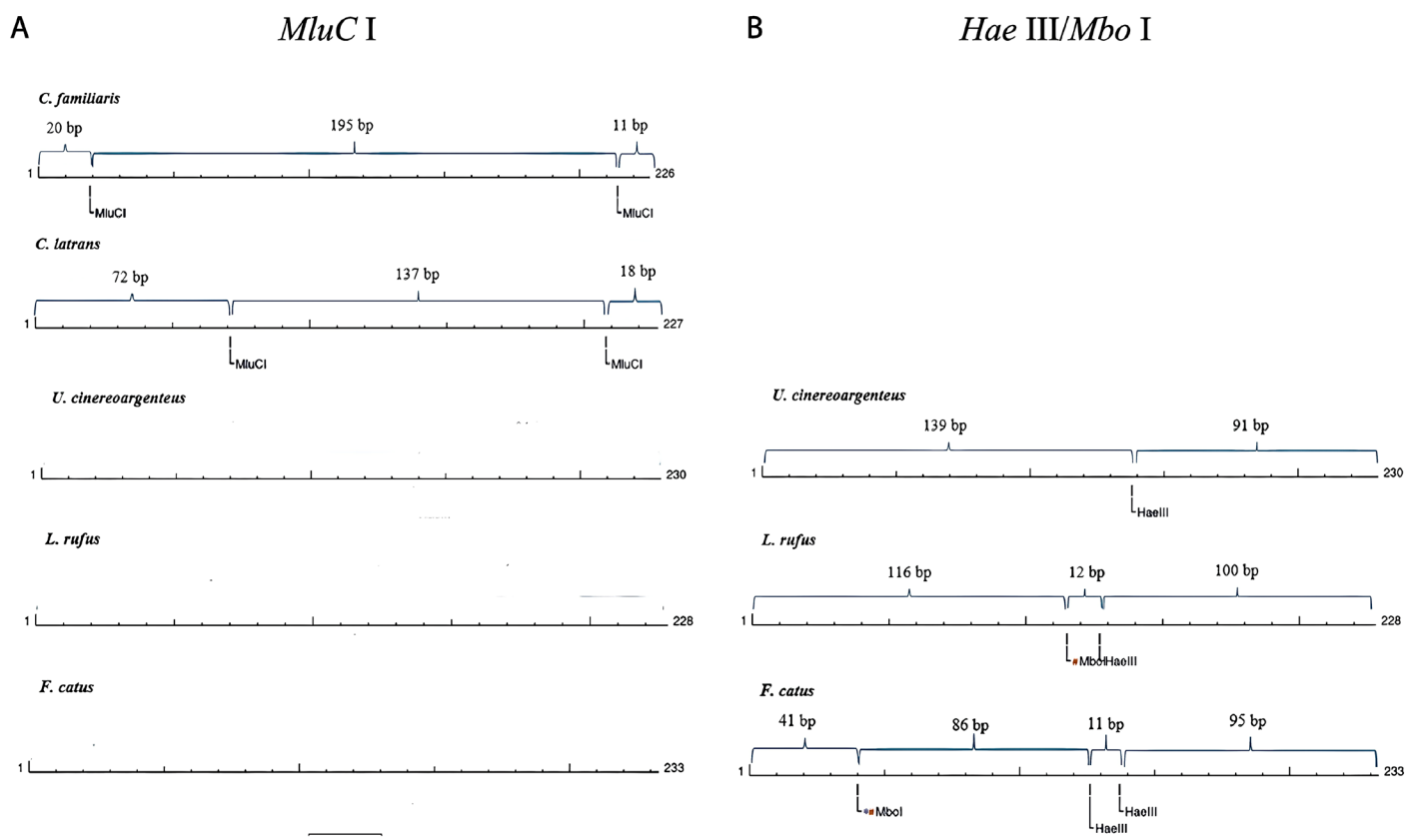

Figure 5. Cutting patterns created by the restriction enzymes *MluC* I and *Hae II*/*Mbo I* for sequences generated by the nonspecific primer; (5a) fragments for domestic dog, coyote, gray fox, bobcat, and domestic cat, and (5b) gray fox, bobcat, and domestic cat.

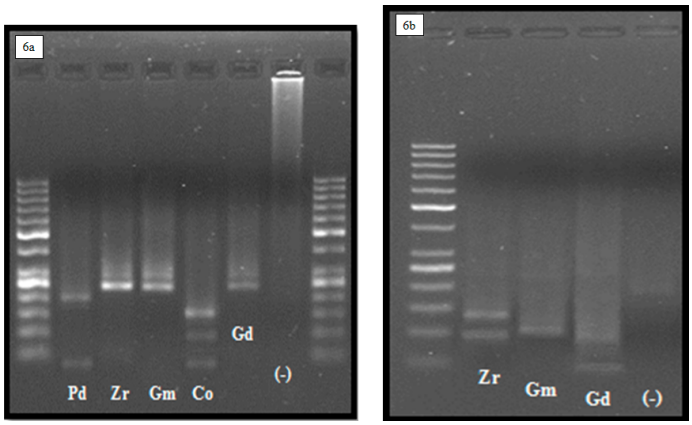

Figure 6. Amplification of feline follicle samples. 50 bp marker. (a) Pd: Domestic dog, Zr: Gray fox, Gm: Bobcat, Co: Coyote, and Gd: Domestic cat. (b) Zr: Gray fox, Gm: Bobcat, Gd: Domestic cat, (-) Negative control.

100 bp, because they are so similar in size, only one band is visible. In the domestic cat sample, we observed two bands: the first, formed by two restriction fragments of 95 and 86 bp, and the second band, barely visible, by a 41 bp fragment (Figure 6b).

Identification of mesocarnivores from fecal samples. Finally, we identified 108 samples (86.4 %) from a total of 125 feces collected in the field (60 on roads and 65 in a latrine), 82 (76.3 %) correspond to *L. pardalis*, 11 (10 %) to *U. cinereoargenteus*, 6 (5.5 %) to *P. concolor*, 6 (5.5 %) *P. yagouaroundi,* and 3 (2.7 %) to *Leopardus wiedii* (Figure 7).

Out of the 60 feces collected on roads, only 51 (85 %) were identified, corresponding to 35 (68 %) of ocelot, 3 (6%) of margay, 5 (10 %) of jaguarundi and 8 (16 %) of gray fox and, of 65 collected in a latrine, 57 (87 %) were identified, belonging to ocelot 47 (82.5 %), 6 (10.5 %) of puma, one (1.7 %) of jaguarundi, 3 (5.3 %) of gray fox (Figure 8).

Discussion

This study demonstrates the efficacy of PCR-RFLP for identifying mesocarnivores from fecal samples in the Sierra del Abra Tanchipa Biosphere Reserve (RBSAT), achieving successful identification in 108 of 125 collected samples (86.4%). The predominant species was the ocelot (*Leopardus pardalis*), representing 82 samples (75.3 %), followed by gray fox (*Urocyon cinereoargenteus*) with 11 samples (10 %), and puma (*Puma concolor*), jaguarundi (*Puma yagouaroundi*), and margay (*Leopardus wiedii*) each with 6, 6, and 3 samples (5.5 %, 5.5 %, and 2.7 %, respectively). Ocelot scats were most abundant on roads (68 % of 51 identified) and especially in latrines (82.5 % of 57 identified), reflecting their behavioral preference for trails in foraging and latrine use for territorial marking and intraspecific communication in Neotropical habitats, as documented by Emmons (1988), Kitchener (1991) and King *et al.* (2017).

These results confirm the sympatric presence of these mesocarnivores in RBSAT tropical deciduous and subdeciduous forests, aligning with prior distribution records for ocelots in the region amid ongoing habitat fragmentation from agriculture and human activities. The high ocelot prevalence suggests sustained

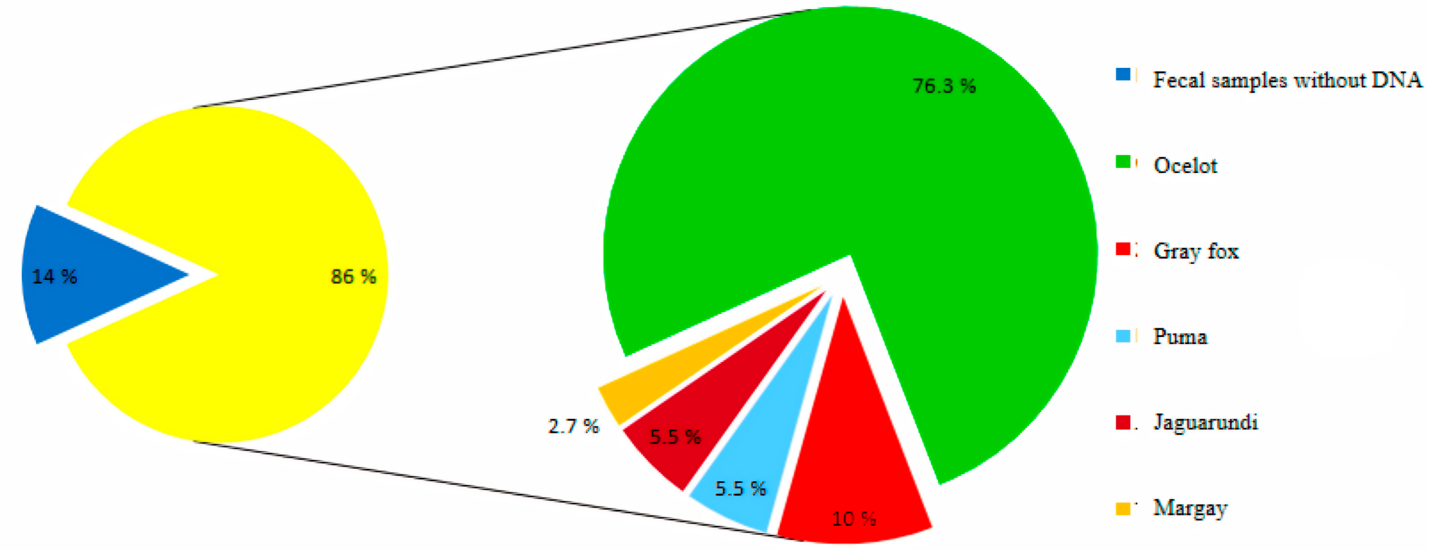

Figure 7. Percentage of samples of mesocarnivore species identified by PCR-RFL.

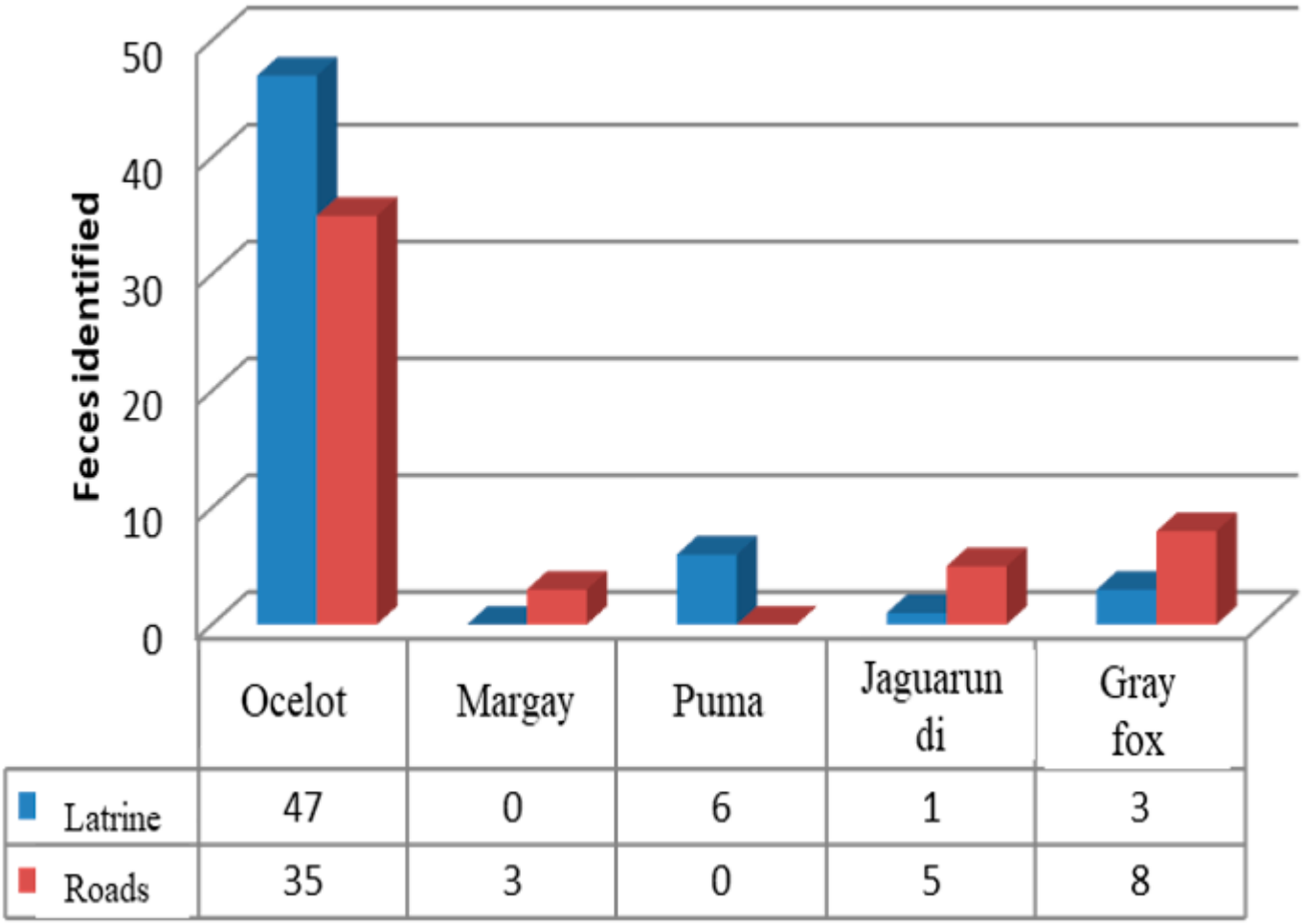

Figure 8. Comparison of feces identified in latrines and roads.

populations capable of regulating prey dynamics, though mesocarnivores generally receive less conservation attention than larger carnivores due to their adaptability and perceived lesser ecological impact (Roemer *et al.* 2009). Detection of puma and jaguarundi primarily in latrines highlights potential spatial overlap in scent-marking sites, offering opportunities for noninvasive monitoring of multi-species interactions, diet, and abundance in this protected area (Moreno and Giacalone 2006; Cacelin-Castillo *et al.* 2020).

DNA extraction from feces yielded fragmented but sufficient material (>100 ng μL^{-1} in most cases), enabled by a modified QIAamp protocol that enhanced lysis despite environmental degradation (Morin *et al.* 2001; Chaves *et al.* 2010). Amplification succeeded in 91 samples (73 %) with felid-specific primers (165 bp; Garcia-Alaniz 2009) and nine more with universal primers (234 bp; Bidlack *et al.* 2007), minimizing bias and capturing canids. Follicle controls validated enzyme specificity: *Hpa II* differentiated puma and jaguarundi; *Hae III*/*Mbo I* distinguished ocelot and margay; *MluC I* separated canids, with *Hae III*/*Mbo I* resolving gray fox, bobcat, and domestic cat patterns (Wilson *et al.* 1995).

PCR-RFLP provided high-resolution identification (86.4 % success) for morphologically cryptic scats, surpassing traditional methods prone to errors among similar-sized sympatric species (e.g., ocelot vs. margay; gray fox vs. domestic dog) (Rosas-Rosas *et al.* 2003; Haag *et al.* 2009; Aranda-Sánchez 2012). This technique's dual-primer strategy—specific for felids, universal for others—followed by targeted restriction digests (*Hpa II*, *MluC I*) generated diagnostic banding patterns without sequencing, proving cost-effective and field-applicable (Zuercher *et al.* 2003; Mukherjee *et al.* 2010). Comparable approaches achieved 86 % accuracy across seven carnivores (Zuercher *et al.* 2003) or Andean felids via 16S rRNA (Cossíos and Angers 2006), while hair-morphology keys reached only 52-70 % reliability (Ruell and Crooks 2007).

In contrast, metabarcoding offers broader taxonomic coverage but requires advanced sequencing infrastructure and risks contamination, whereas PCR-RFLP agarose gel visualization is suitable for resource-limited settings such as RBSAT studies on distribution, demographics, and feeding ecology (Mills *et al.* 2000; Cossíos and Angers 2006). Despite challenges such as low fecal DNA quality, strategic collection (roads/latrines), and controls mitigated failures, supporting scalable noninvasive scatology for mesocarnivore research in biodiverse, fragmented ecosystems (Davison *et al.* 2002). Future enhancements could integrate detection by trained dogs to boost sample recovery 5-15-fold (Long *et al.* 2007).

Conclusions and recommendations

Through identification and analysis of feces, we determined that methods based on molecular scatology, for identifying the origin of fecal samples, are successful methods since up to 90 % of the total samples can be identified when they are in good condition and properly preserved for analysis, in addition to reducing the risk of errors due to identifications based on associated track and sign. Indeed, it is the most accurate technique for the identification of feces collected in the field, therefore for describing food habits of sympatric carnivores. Currently, molecular techniques have decreased in cost, making them more accessible. The availability of kits and technologies reduces the time required to obtain and analyze results. The PCR-RFLP technique for identifying carnivore feces had an 86 % success rate. This could be because samples were selected based on lower levels of disintegration caused by environmental factors such as rain, high temperatures, and sunlight. In addition, the sampling was conducted during seasons with little rainfall. It is recommended that the PCR-RFLP technique for identifying fecal samples be performed on samples from the same sampling site, as discrepancies in banding patterns may occur among species from different populations, caused by variations in polymorphisms in the target sequences. BLAST testing of the obtained sequences is also recommended to avoid errors in fragment amplification. Increasing the number of samples by searching with the help of trained dogs could help achieve greater representativeness of some other species that are more difficult to identify and sample at a simple view. Our results support the identification of individuals through fecal samples in the field, where various species of mesocarnivores are present.

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Declaration of Artificial Intelligence use

The authors declare that they did not use any artificial intelligence tools in writing this article.

Autor contributions

Octavio C. Rosas-Rosas: conceptualization, study design and fieldwork planning, data collection, analysis and interpretation of the results, and preparation of the first draft of the manuscript. Canuto Muñoz-García: conceptualization, study design, and fieldwork planning; reviewed and provided critical revisions to the manuscript. Miguel Paul Conde-Hinojosa: conceptualization, reviewed, and provided critical revisions to the manuscript. Luis Antonio Tarango-Arámbula: conceptualization, study design and fieldwork planning, funding acquisition, reviewed and provided critical revisions to the manuscript. Reyna Isabel Rojas-Martínez: sample processing in the molecular biology laboratory and provided critical revisions to the manuscript. Héctor E. Benítez-Alemán: conceptualization, study design and fieldwork planning, sample processing in the molecular biology laboratory. Cesar Cortez-Romero: conceptualization, study design and fieldwork planning, funding acquisition, reviewed and provided critical revisions to the manuscript.

Supplementary data

SD1. Amplification of DNA extracted from fecal samples using the feline-specific primer (fragment size of 165 bp).

SD2. Amplification of DNA extracted from stool samples using the nonspecific primer (fragment size of 234 bp).

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