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METHODS PAPER

A novel real-time TaqMan™ PCR assay for simultaneous detection of Neotropical fox species using noninvasive samples based on *cytochrome c oxidase subunit II*

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Abstract Strategies to evaluate and monitor elusive mammal species require the development of genetic techniques and their application to unambiguous biological material for ecological and genetic studies. In order to assess *cytochrome c oxidase subunit II* gene inter- and intraspecific variations, we compared sequences from different Neotropical canids and domestic dogs. We developed a primer pair to amplify a 154-bp fragment of this gene and a species-specific multiplex TaqMan™ assay for accurate identification of two native fox species occurring in sympatry in South America, the crab-eating fox (*Cerdocyon thous*) and the pampas fox (*Lycalopex gymnocercus*). The assays can also distinguish domestic dogs (*Canis lupus familiaris*) from both wild foxes. The use of different fluorescent reporter dyes for species identification in a multiplex probe PCR-RT assay reduces labor and costs. The methodology presented in this study demonstrates an efficient approach to enable high-performance analysis and represents a reliable cost-effective tool for molecular ecology research to monitor the wild canid populations by noninvasive genetic sampling. This standardized assay will allow large-scale high-throughput analyses in a routine and reliable way.

Keywords mtDNA · *COII* gene · Molecular ecology · Canid · Biodiversity monitoring · Uruguay

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Introduction

In the Neotropical Region (Procheş and Ramdhani 2012; Morrone 2014), two wild fox species are sympatric: the crab-eating fox (*Cerdocyon thous*) and the pampas fox (*Lycalopex gymnocercus*) (Fig. 1). In addition to their overlapping distribution range, they have a similar body size and share eating habits (Vieira and Port 2007). The crab-eating fox is the widest distributed canid of South America, ranging from Uruguay and northern Argentina to Paraguay, Colombia, Venezuela, and throughout Brazil, except for the Amazon basin lowlands (Berta 1982). Even though the species is listed as Least Concern for the IUCN (International Union for Conservation of Nature) (Courtenay and Maffei 2010), it is on Appendix II of CITES as a vulnerable species with regard to exploitation and trade activities (Nowak 1999). The pampas fox has a typical southern geographical distribution, occurring in eastern Bolivia, western and central Paraguay, Uruguay, north and central Argentina, and southeastern Brazil. It has also been assigned to the Least Concern category of the IUCN (Lucherini and Luengos Vidal 2008) and is included on Appendix II of CITES. The pampas fox seems to occupy only open areas including grasslands and dirt roads. Meanwhile, the crab-eating fox is found in more diverse habitats including the interior of forests as well as the edge of forests and grasslands, but more frequently in woodland areas (Lucherini et al. 2004). In addition, in natural areas, it is possible to find domestic dog (*Canis lupus familiaris*). The Uruguayan Zoonosis Commission estimates more than 146,000 dogs inhabit rural areas (Lete J., personal communication) so it is common sampling areas with dogs present. The high number of dogs present in such areas is affecting wild fauna because they predate small- and medium-sized animals such as southern tamandua (*Tamandua tetradactyla*). Transmission of diseases such as rabies and canine distemper

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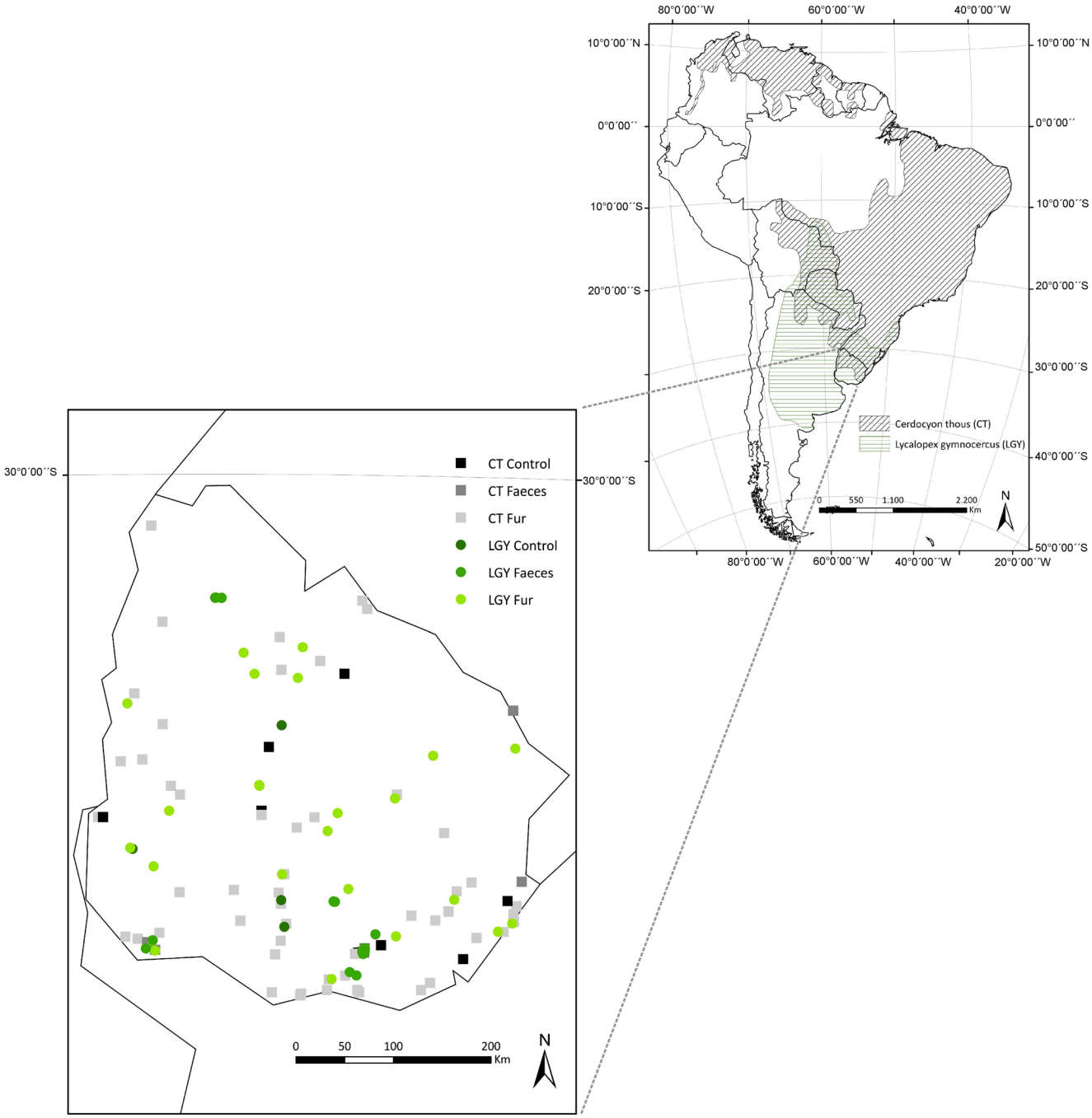

Fig. 1 Map of Uruguay showing the results in different sample types (control, fur, or feces) and fox species (*Cerdocyon thous*: *CT*; *Lycalopex gymnocercus*: *LGY*). *Top right*: fox species geographic distribution and sympatric area

virus may also affect wildlife (Hughes and Macdonald 2013). In Uruguay, studies on conflicts between domestic dogs and wildlife are needed to suggest accurate management activities.

Samples obtained from noninvasive methods, such as feces or shed hairs, are recognized as a valuable source of DNA in different molecular approaches for assessing genetic structure, demography, and life history of mammals (Kohn and Wayne 1997; Beja-Pereira et al. 2009; Hausknecht et al. 2010; Ebert et al. 2012). Hence, the identification at individual or species level obtained by genetic analysis from noninvasive samples allows to monitor the presence and distribution of elusive species and to estimate their abundance applying capture-

recapture methods (Petit and Valiere 2006; Mondol et al. 2009; O'Meara et al. 2014). The first key step in fecal and other noninvasive biological sample analysis is accurately assigning species; this provides needed information to resolve continuing further molecular analyses.

More precise, fast, and inexpensive methods for identification of species are thus sought by molecular ecology and conservation genetics researchers. The amplification of small fragments (ca. 150-bp) from mitochondrial DNA (mtDNA) by polymerase chain reaction (PCR) is a powerful tool for species identification (Chaves et al. 2012; O'Meara et al. 2014; Rodriguez-Castro et al. 2017). Additionally, different TaqMan™ probes in multiplex assays is a well-documented technique to determine DNA sequence variation (Holland et al. 1991; Schoske et al. 2003; Ali et al. 2014). This methodological approach in combination with real-time PCR (PCR-RT) assays is the technique of choice for rapid, accurate, and sensitive analysis (Walker 2002) and even for low template DNA concentrations (O'Neill et al. 2013).

In this study, we report the development of a novel TaqMan™ probe PCR-RT assay based on the sequence of a fragment of the *cytochrome c oxidase subunit II* (*COII*) gene (mtDNA), which can be used to discriminate sympatric Neotropical Canidae species and dogs.

Materials and methods

Skin, muscle, and liver tissues from crab-eating fox (*C. thous*) ($n = 13$), pampas fox (*L. gymnocercus*) ($n = 8$), and ambiguous or non-accurate species determination canids ($n = 112$) were obtained from roadkill animals in Uruguay, collected between 1997 and 2016. Blood samples from domestic dogs ($n = 10$) were obtained from breeders. Additionally, fecal samples with a preliminary indication on canid species (ambiguous or non-accurate) were collected during surveys in Uruguay ($n = 112$) between 2009 and 2016. All tissue and fecal samples were stored in 95% ethanol at 4 °C just after collection and until DNA extraction. DNA was isolated from tissue samples following González et al. (2015) protocol. To isolate DNA from feces, we used QIAamp DNA Stool Mini Kit® (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. This protocol for DNA isolation was carried out in a separate room with several safeguards against contamination including laminar flow chambers, UV lamps, and dedicated equipment and reagents which are used neither for tissue samples nor post-PCR applications. In addition, extraction negative controls were included. DNA was eluted in 100 μL H_2O for all sample types and stored at 4 °C. Quantification of DNA from tissue and fur samples was carried out in a NanoDropTM ND-1000 UV-vis Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE).

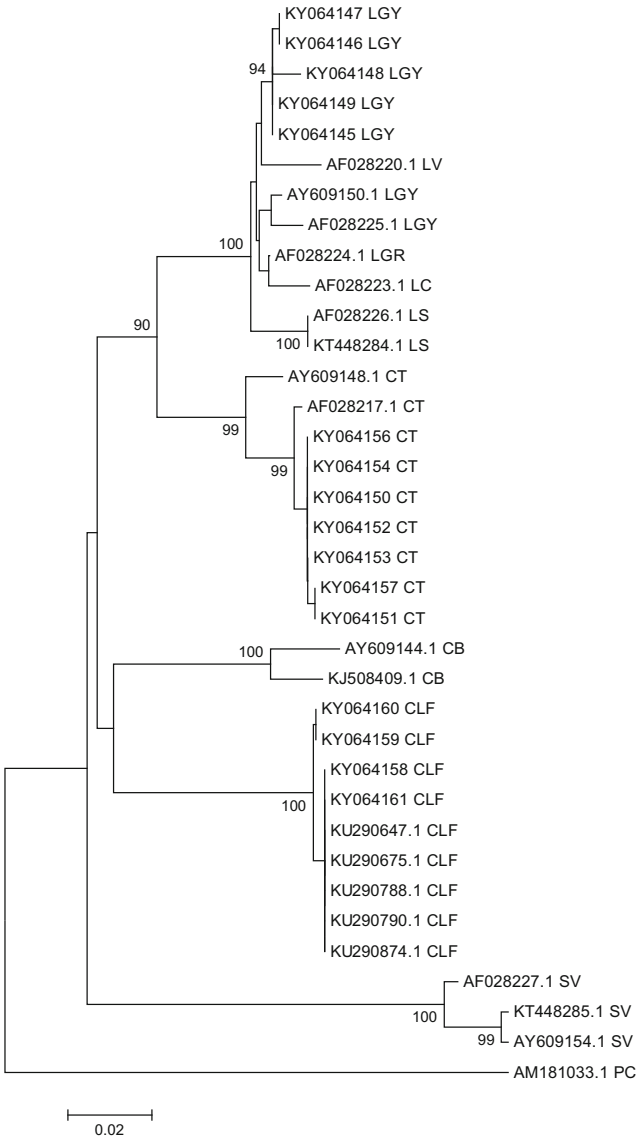

Fig. 2 Gene tree derived from *COII* gene based on 608-bp data sets containing sequences from 35 canids and *Phoca caspica* (*PC*) as external outgroup. Neighbor-Joining method (*NJ*) with bootstrap replicates (1000). Numbers above the branches indicate nodes supported in 90% of bootstrap replicates. *LGY*: *L. gymnocercus*; *LV*: *L. vetulus*; *LGR*: *L. griseus*; *LC*: *L. culpaeus*; *LS*: *L. sechurae*; *CT*: *C. thous*; *CB*: *C. brachyurus*; CLF: *C. l. familiaris*; *SV*: *S. venaticus.* GenBank accession number are shown; those starting with *KY* correspond to sequences obtained from this study

In order to assess *COII* inter- and intraspecific variation, we compared sequences from different Neotropical canids and domestic dogs deposited in GenBank (ncbi.nlm.nih.gov), using ClustalW tool in Mega6 (Tamura et al. 2013). The alignments were compared using *Canis lupus familiaris* ($n = 101$), *C. thous* ($n = 2$: AF028217, AY609148), *L. gymnocercus* ($n = 2$: AY609150, AF028225), *Lycalopex vetulus* ($n = 1$: AF028220.1), *Lycalopex culpaeus* ($n = 1$: AF028223.1), *Lycalopex griseus* ($n = 1$: AF028224.1), *Lycalopex sechurae* ($n = 2$: AF028226.1, KT448284.1), *Speothos venaticus* ($n = 3$:

Table 1 TaqMan™ probe developed. Probe IDs correspond with CLF: *C. lupus familiaris*, CT: *C. thous*, LGY: *L. gymnocercus*

TaqMan™ MGM probe ID	Probe sequence (5′–3′)	Reporter	Quencher	Tm (°C)	Length (bp)
CLF FOX *COX2* PROBE	C CAC AGC TTT ATA CCC ATT GTT CTT GAA A	CY5	BHQ2	66.2	29
CT FOX *COX2* PROBE	AC AGC TTT ATA CCT ATT GTC CTC GAA A	HEX	BHQ1	63.7	27
LGY FOX *COX2* PROBE	AC AGC TTC ATA CCC ATC GTT CTT GAA A	FAM	BHQ1	65.3	27

AF028227.1, KT448285.1, AY609154.1) and *Chrysocyon brachyurus* (*n* = 2: AY609144.1, KJ508409.1).

Tissue samples from different canid species-identified individuals were amplified using COII-F (5′-GTAA AACATTACATGACTTTGTC-3′) and COII-R (5′-AGAG GTTAAAACTCCCAGTCTT-3′) (Bardeleben et al. 2005a). Each PCR contained 0.2 U Taq DNA Polymerase (Invitrogen), 1X PCR Buffer, 0.2 μM $MgCl_2$, 0.4 μM each

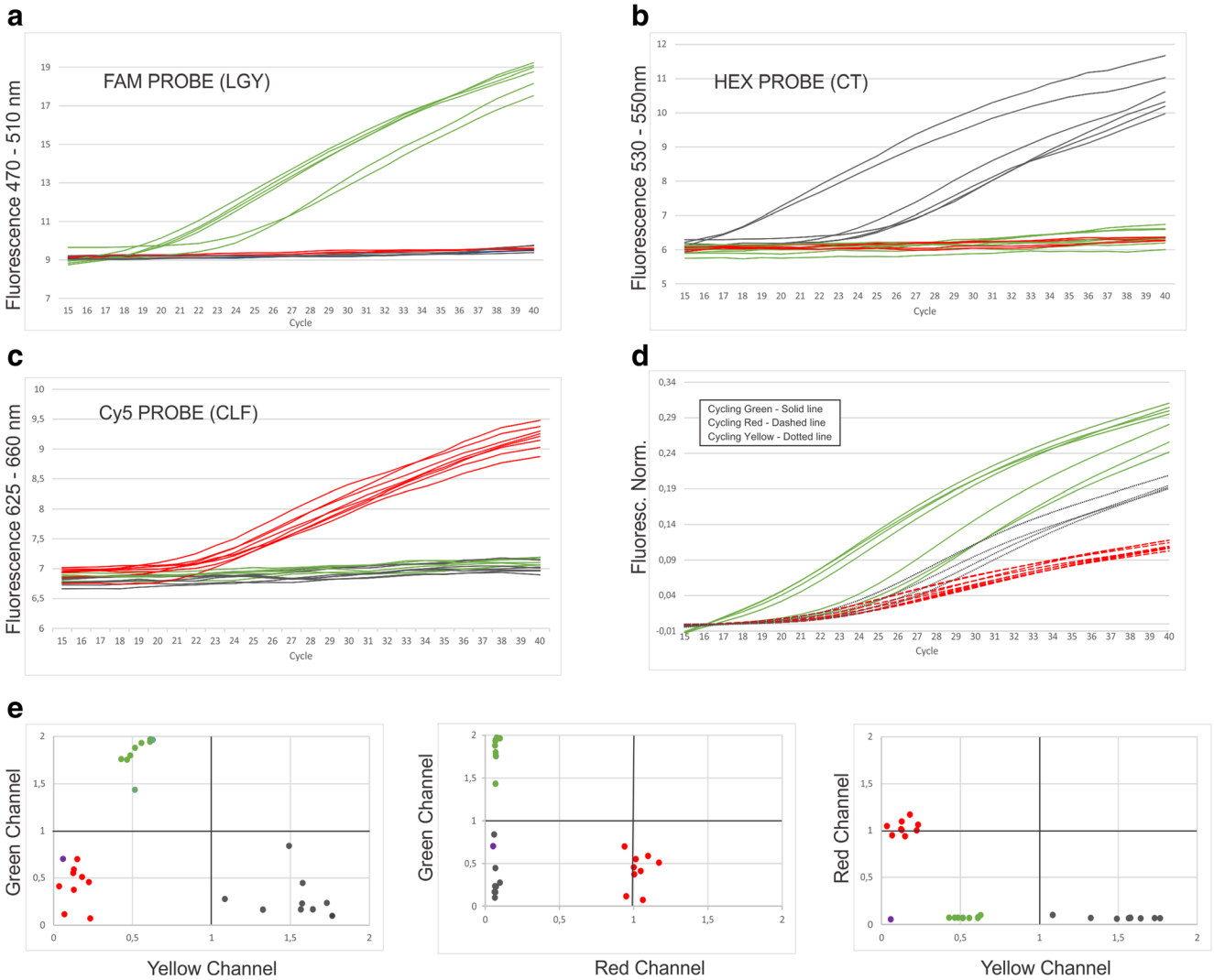

Fig. 3 Real-time TaqMan™ method for species identification. Probe hybridization of specimens. **a** Fam probe. **b** Hex probe. **c** Cy5 probe. **d** Allelic discrimination of haplotypes by auto analysis software 6.0 program of Rotor-Gene after amplification. **e** Cluster plots. The *x*-axis and *y*-axis represents the signal intensities to each channel (*green*, *yellow*, and *red*). Every *dot* symbolizes the signal for each probe from one individual. *Colors* represent the assigned species: *green*: LGY; *black*: CT; *red*: CLF; purple: negative controls

Mitochondrial genome position (bp)	7651	7654	7660	7663	7666	7669
Fox probes position (bp)	5	8	14	17	20	23
Transitions C/T in third codon position	AGT/C	TTT/C	CCT/C	ATT/C	GTT/C	CTT/C
Aminoacid	Ser (S)	Phe (F)	Pro (P)	Ile (I)	Val (V)	Leu (L)
Canis lupus familiaris	C	T	C	T	C	C
Cerdocyon thous	C	T	T	C	T	T
Lycalopex sp. Except *Lycalopex sechurae*	C	C	C	T	C	C
Lycalopex sechurae	T	T	C	C	T	T
Speothos venaticus	C	T	T	T	T	C
Chrysocyon brachyurus	C	T	T	T	T	T

Fig. 4 *COII* 18-bp sequences showing the specie-specific polymorphism and the synonymous substitutions observed in the *COII* binding probe region

primer, template (≈ 90 ng DNA), and H_2O to a final volume of 15 μL. The cycling conditions began with an initial step of 10 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C, 90 s at 72 °C, and a final step of 20 min at 72 °C using sterile filtered pipette tips. PCR products were purified and sequenced with an ABI3130 (Applied Biosystems) at Macrogen Korea sequencing service. Sequences were aligned using ClustalW in Mega 6 (Tamura et al. 2013) including a sample of *Phoca caspica* (PC) as external outgroup. The sequence similarity among species were evaluated using the neighbor-joining (NJ) algorithm (Saitou and Nei 1987) as executed in MEGA 6 with the Kimura 2-parameter (K2P) model (Kimura 1980) of nucleotide substitution. The degree of information support for clades was assessed after bootstrap resampling of 1000 pseudo-replicates (Felsenstein 1985).

We developed a primer pair for the amplification of a short fragment of mtDNA *COII* from *Cerdocyon*, *Lycalopex*, and *Canis* species. Three TaqMan™ probes were designed using intraspecies conserved sequences to detect *C. thous*, *L. gymnocercus*, and *Canis l. familiaris*. Primer Express 3.0 software from Applied Biosystems (Life Technologies) was used. The TaqMan™ fluoregenic probes were labeled with reporter and quencher dyes with different absorption spectra (Johansson 2006).

Novel primers and probes were validated by PCR-RT assays using DNA isolated from tissue of known-species canids ($n = 24$). Each PCR contained 1X SensiFastprobe™ PCR master mix (Bioline), 2 mM MgCl, 0.4 μM each primer, 0.15 μM each probe, template (≈ 60 ng DNA), and H_2O to a final volume of 20 μL. The analysis was conducted using the following cycling conditions on a Rotor-Gene 6000 (Corbett Research) thermocycler: initial step of 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 60 s at 62 °C. Fluorescence acquisition was determined during the extension step on the green channel (470 nm excitation, 510 nm detection), on the yellow channel (530 nm excitation, 550 nm detection), and on the red channel (625 nm excitation, 660 nm detection) to detect the binding of the probe to the target sequences of *L. gymnocercus*, *C. thous*, or *C. lupus familiaris*, respectively. Positive and negative controls were included in all PCR reactions. The acquisition curves analyses were performed using Rotor-Gene™ 6000 software v.1.7 and the algorithm provided. Additionally, the efficiency of the TaqMan™ probes developed for species identification were assessed using DNA obtained from noninvasive samples like feces ($n = 112$). In these cases were conducted at least two replicates for each PCR with 1 and 2 uL DNA volume. From seven roadkilled foxes, we collected tissue and feces present in the rectum. We conducted real-time PCR TaqMan™ probe on both types of samples collected from the same animal. Furthermore, in four feces samples, we performed not only the probe experiment but also sequenced the COII probe region.

Results

We obtained 17 sequences from *COII* mtDNA [*C. thous* ($n = 8$), *L. gymnocercus* ($n = 5$), *and C. l. familiaris* ($n = 4$)]. The fragments generated were 608-bp long. For the *COII* analysis, the alignment was constructed with sequences available from GenBank and those obtained in this work, summing 35 sequences (608-bp) that included eight Neotropical canid species and domestic dogs. Except *L. gymnocercus*, all species represented by more than one sequence formed monophyletic groups (≥99% BS) (Fig. 2). Conserved flanking region sequences for PCR primer design were identified. We also identified sequence variation that could be used to distinguish canid species present in Uruguay: *C. l. familiaris*, *C. thous*, and *L. gymnocercus* as well as *S. venaticus*, *C. brachyurus*, and *L. sechurae*.

The primer pair designed (FOX154F: CGC TAT YCC AGG ACG ACT A and FOX154R: GGC TGA TCA GGT TTC AAA G) successfully amplified a 154-bp fragment of *COII* mtDNA and the binding site for TaqMan™ probes designed (Table 1) for *C. thous*, *L. gymnocercus*, *and C. l. familiaris* were also unequivocally validated by assaying all control samples, i.e., samples of crab-eating foxes ($n = 14$), pampas foxes ($n = 10$), and dogs ($n = 4$).

From 112 roadkilled samples, 95 were correctly identified and it represents that 84.8% of the experiments were successful. In 71.4% of cases, the result were obtained in the first experiment, while in 13.4%, it was achieved in the second one. The 15.2% of PCR did not amplify in these kind of samples. From roadkilled samples, 64 were crab-eating foxes (*C. thous*) and 31 were pampas foxes (*L. gymnocercus*). Only in one case we detected a misidentification in a sample that was primary identified as *L. gymnocercus* and resulting in *C.thous* from two independent PCRs. In addition, from 112 feces samples we identified 26 as crab-eating foxes (*C. thous*) and 40 as pampas foxes (*L. gymnocercus*), i.e., 58.9% of the feces samples could be used for identification at the species level (Fig. 3). Feces showed 48.2% of success on the first PCR amplifications and only 10.7% of samples were identified after replicates. The accuracy of this technique with noninvasive samples like feces was confirmed from the same and stable pattern of Taqman™ probes with fur and rectal samples from the same individuals in seven evaluated cases. Finally, the sequenced *COII* fragment from feces samples showed concordance with the probe experiments.

Discussion

COII gene (partial region of 608-bp) was suitable for Neotropical fox species identification with high bootstrap values for each clade, except for *L. gymnocercus*, which is not represented as a monophyletic clade (Fig. 2). This pattern in *L. gymnocercus* was observed by Tchaicka et al. (2016) with mtDNA control region and these authors suggested an inter-species hybridization process or a secondary admixture. Nevertheless, the *COII* analyzed region (608-bp) shows homoplasy and the tree performed with NJ method based in this region seem not to reflect the phylogenetic relation of the species (Pamilo and Nei 1988; Wayne et al. 1997; Bardeleben et al. 2005b; Bardeleben et al. 2005a; Galimberti et al. 2015).

For noninvasive DNA sampling, mtDNA sequences tend to result in better amplification success (70–90%) against nuclear markers (Broquet et al. 2007). The novel primer pair designed in this study could be used for PCR amplification of a 154-bp *COII* gene fragment in the Neotropical canid species and the domestic dog. The length of the PCR product was suitable for amplification of low-quality and low-quantity DNA, given that amplification success of fecal DNA from carnivore species was 71.9%.

The TaqMan™ probe binding region (18-bp) has synonymous substitutions with a polymorphism level that allows distinguishing Neotropical canid species in Uruguay. Additionally, this 18-bp region has potential for being useful to identify several other Neotropical canid species (Fig. 4). Nevertheless, the *L. gymnocercus* TaqMan™ probe binds to all the species of the genus (*L. culpaeus*, *L. griseus*, *L. vetulus*) except for *L. sechurae*. The polymorphism for *Lycalopex fulvipes* was not analyzed because there were no sequences available in GenBank.

The use of different fluorescent reporter dyes for species identification in a multiplex probe PCR-RT assay reduces labor and costs. The methodology presented in this study demonstrates an efficient approach to facilitate high-throughput analysis and critical evaluation of *C. thous*, *L. gymnocercus*, and dog samples in a single analysis. The Scatter Analysis Graph with the software provided by Rotor-Gene Q (V.2.3.1) allows an easy taxonomic determination (Fig. 3e). This standardized assay will allow large-scale high-throughput analyses in a routine and reliable way.

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