High-resolution melting of the cytochrome B gene in fecal DNA: A powerful approach for fox species identification of the Lycalopex genus in Chile

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Abstract
Easy, economic, precise species authentication is currently necessary in many areas of research and diagnosis in molecular biology applied to conservation studies of endangered species. Here, we present a new method for the identification of three fox species of the Lycalopex genus in Chile. We developed an assay based on high-resolution melt analysis of the mitochondrial cytochrome B gene, allowing a simple, low cost, fast, and accurate species determination. To validate the assay applicability for noninvasive samples, we collected fecal samples in the Atacama Desert, finding unexpectedly one species outside of its known distribution range. We conclude that the assay has a potential to become a valuable tool for a standardized genetic monitoring of the Lycalopex species in Chile.

KEYWORDS
barcode, HRM, Lycalopex, PCR

1 | INTRODUCTION
Six species of foxes of the genus Lycalopex Burmeister, 1854 are found in South America, distributed from Ecuador to southern Patagonia (Ochoa, 2011; Zunino, Vaccaro, Canevari, & Gardner, 1995). Lycalopex is represented by three species in Chile: Lycalopex culpaeus (culpeo or Andean fox), the largest in size and distributed throughout Chile, except on the Island of Chiloé (southern Chile); Lycalopex griseus (gray fox) distributed from the Antofagasta to the Magallanes regions, including the Island of Chiloé; and Lycalopex...
without the use of sequencing or hybridization procedures (Reed & Aten, Roos, & Dunnen, 2009), so it is a useful technique for obtaining species-specific genotypes. Due to the increased demand for rapid, economic, easy, high-throughput genotyping analyses, there has been a considerable focus on HRM, which can detect sequence variants based on the analysis of fecal DNA by the mitochondrial cytochrome B gene using HRM techniques.

2 | METHODS

Two hundred Lycalopex fecal samples were obtained in the Atacama Desert, northern Chile, between Lat. 26°31′29.07″ and 26°34′59.86″S, Long. 70°30′57.68″ and 70°20′45.27″W during September 2014 and February 2015. The search and collection of the feces were carried out along transects defined according to the geomorphological characteristics of the stream and then from the prospective terrain to the study area to identify potential habitats such as trails, caves, and bushes used by foxes. Twenty-seven sampling stations were selected in scrub, rocky, and sandy environments, which were georeferenced and where camera traps were installed to constantly monitor places and target species. With this information, a map where each sampling point was plotted. For the collection of samples in the field, the feces were distinguished from those of other carnivores by their characteristic shape, excluding the white or dry appearance, to ensure to collect feces from foxes.

2.1 | Sample collection

2.1.1 | References samples

Fresh frozen skin tissues from each of the taxonomically determined Chile-inhabiting species L. culpaeus, L. fulvipes, and L. griseus were used to obtain reference DNA for the PCR end-time and Real-Time reactions with HRM.

2.1.2 | Unknown samples from feces

DNA was extracted from 200 fecal samples, with the Isolate Fecal DNA kit (Bioline), following the manufacturer’s instructions. As input material we used an amount of 150 mg taken from initial sample of the external contour of the feces. The DNA concentration was estimated by standard fluorometric methods, using the Qubit 2.0 (Thermo Fisher Scientific) following the manufacturer’s instructions. The integrity of the extracted DNA was checked by agarose gel electrophoresis in 0.8% agarose gel. The concentration of the DNA samples was normalized to 25 ng/µl to dilute potentially present PCR-inhibitors and stored at −20°C for further use.

2.2 | End point PCR standardization of the cytochrome B gene

DNA amplification using end point PCR was performed using the Applied Biosystems™ SimpliAmp™, 0.2 µM forward
primer, 0.2 µM reverse primer, and 1 µl of 25 ng/µl DNA. The primer pair used for the cytochrome B gene was: LC CYTB-F: 5’TTCAGCACACCATCAATTTCGCG 3’ and LC CYTB-R: 5’GGGCGGTTGGCATGATGTAACG 3’. (Quinga, 2012), using an initial denaturing step at 96°C for 4 min followed by 35 cycles of 96°C for 30 s, 66°C for 30 s, and 72°C for 30 s. The amplicons were electrophoresed in agarose gel 2% TAE 1X and stained with GelRed (Biotium). These primers amplify a region of the mitochondrial cytochrome B gene. For general specificity validation, we performed a primer BLAST search (Ye et al., 2012) confirming that the primers are specific for Lycalopex as indicated in Quinga (2012). Amplification tests were performed by end point PCR to standardize the reaction conditions. It is optimized according to the size of the amplicon.

2.3 | High-resolution melting (HRM) analysis

Real-Time PCR was performed using the Eco Real-Time PCR system (Illumina) in order to determine characteristic Tm of the different Lycalopex species to be distinguished. The reaction mixture for real-time PCR and HRM analysis consisted of a total volume of 15 µl. This contained 7.5 µl of 2× Kapa SYBR qPCR Mix (Kapa Biosystems Roche), (Cowan & Elkins, 2018; Solano et al., 2019) 0.2 µM forward primer, 0.2 µM reverse primer, and 1 µl of 25 ng/µl DNA. SYBR fluorescence dye was used to monitor the accumulation of the amplified product during PCR and the HRM process to derive the Tm value. The same primer pair and PCR program as for end point PCR was used (Quinga, 2012).

Fluorescence data were measured at the end of each extension step during the PCR cycles. Subsequently, the PCR amplicons were denatured for HRM at 95°C for 15 s, and then annealed at 50°C for 15 s to form a random DNA duplex. The RT-PCR HRM protocol collected fluorescence data at 0.1°C temperature increments. For the HRM analyses, the fluorescence data were collected in 0.1°C increments between 78 and 82°C. No cross-species tests were used, since the primers were specific for the genus Lycalopex.

We used the EcoStudy Software v 5.0 to plot a normalized curve of decreasing fluorescence with increasing temperature. The negative derivative of the fluorescence (F) over temperature (T) (dF/dT) curve gives the Tm. Three control DNA samples were obtained from fresh tissues of taxonomically identified Lycalopex species. To generate normalized melting curves and difference melting curves, pre- and postmelt normalization regions were set to define the main temperature boundaries of the normalized and difference plots in these controls, which were set as the species references. The control samples were provided by government agencies.

2.4 | Sequence analysis

PCR products were directly sequenced in two directions for each product using a BigDye Terminator V3.1 Cycle Kit in an automated Genetic Analyzer 3500 xl (Applied Biosystems, HITACHI), with software Data Collection v3. Sequences were aligned and proofread using the software MEGA 5 and submitted to GenBank.

3 | RESULTS

3.1 | End point PCR standardization of the cytochrome B gene

Of the 200 DNA samples extracted, 150 were successfully amplified. These 150 samples were used for the HRM analysis. The samples analyzed were amplified approximately to a 200-bp product, as previously reported for the cytochrome B gene (Yacoub et al., 2013) (Quinga, 2012). (data not shown).

3.2 | Species determination of reference and unknown samples using HRM analysis of cytochrome B gene

Melting curves (Figure 1a,b) were generated by plotting the fluorescence against the temperature. Fluorescence declines as the DNA melts. DNA melting is visualized through the use of a saturating duplex-dependent DNA intercalating dye. As the DNA melts, the dye is released; unbound dye does not fluoresce. The normalized curve (Figure 1c) was generated by taking the negative derivative of fluorescence with respect to temperature and plotting these values against temperature (−dF/dT vs. T). When L. fulvipes is selected as reference (Figure 1d) the obtained difference graph can clearly be distinguished by variation in peak position, positive peak for Lycalopex culpaeus, and negative peak for L. griseus, shape change, and Tm.

PCR of fresh tissue (reference samples) and fecal samples was successfully amplified; in both cases, it was possible to obtain amplifiable DNA for the region of interest. This is very important, since the conservation status of feces is often bad, especially in extreme environments like the Atacama Desert. In some cases of fecal samples, the amplification is not appreciated, precisely because of the presence of inhibitors elements in the DNA samples. Despite this difference in the amplification products, the temperatures and shapes of the melting curves for each amplicon are maintained.

The DNA fusion profile was analyzed to investigate whether there is a polymorphism in the cytochrome B gene of different fecal samples of the Lycalopex genus. This variation was clearly detectable in the derived and normalized melting curves (Figure 1a–d).

The obtained HRM curves could be categorized into three different characteristic peaks in the range between 80.4°C and 80.9°C. (L. culpaeus 80.9°C, L. fulvipes 80.6°C, L. griseus 80.4°C), this values did not vary within a species, hence, there were no deviations in Tm detectable. All profiles of Lycalopex fecal samples produced only one peak. The HRM melting curves were different for each species of the Lycalopex genus. The samples were genetically distinguishable and therefore corresponded to the different genotype.

To confirm the identity of the specimens, the three species under study were sequenced and aligned with sequences retrieved from NCBI. Our results were also confirmed by sequence analysis of cytochrome B amplicons, which revealed that all three species of foxes were present in this analysis (L. culpaeus, L. griseus and L. fulvipes).
FIGURE 1  (a) Melting curves derived from reference samples; (b) Melting curves derived from fecal DNA plus reference samples; (c) Melting curves normalized from reference samples; (d) Difference graph. Lycalopex fulvipes is used as a reference; (e) Electropherogram of sequences of cytochrome B gene in: (e) Lycalopex culpaeus; (f) L. fulvipes and (g) Lycalopex griseus
DISCUSSION

Significant improvements have been reported based on fecal DNA analysis for both extraction and quantification of nucleic acids as part of genotyping and species identification (Kanthaswamy, Premasuthan, Ng, Satkoski, & Goyal, 2012). Molecular analysis of feces is a noninvasive method with a high degree of integrity and quality when using specific commercial kits for these complex matrices (Deshpande, Villarreal, & Mills, 2016; Ramón-Laca, Soriano, Gleeson, & Godoy, 2015; Tende, Hansson, Ottoisson, & Bensch, 2014). The main challenge of this study was to obtain DNA of good quality from fecal samples collected in the Atacama Desert. The DNA samples obtained from these matrices were used to obtain information about the presence and distribution of Lycalopex species in the Atacama Desert, which is of great importance for promoting and orienting conservation measures.

Feces contain epithelial cells from the intestine, mucus, and hairs. DNA can be extracted from this material and amplified by PCR. Although there are previous studies that have used mtDNA from feces (Chaves, Graeff, Lion, Oliveira, & Ezirik, 2012; Quinga, 2012; Ray & Sunquist, 2001; Taberlet, Waits, & Luikart, 1999), there are no reports of the use of mtDNA to analyze the presence and distribution of Lycalopex species in Chile and South America. We developed an assay based on HRM analysis of the mitochondrial cytochrome B gene, used for identifying genetic variations in nucleic acid sequences. We also describe the HRM method for genotyping Lycalopex species, which shows the potential of the mitochondrial cytochrome B gene DNA barcode coupled with the HRM method to provide a fast and very accurate method of taxonomic identification and species identification.

We were able to identify all the three species of Lycalopex foxes inhabiting Chile using the developed assay; this is the first report of the presence of L. fulvipes foxes in northern Chile. The Chilean species of this genus are in different conservation categories. There are no reports on hybridization between species of the Lycalopex genus, only observations and stories of local people in the field, which refer to the possible hybridization without viability of the offspring. The melting curves obtained from amplified fecal DNA samples are grouped or positioned with respect to the curves obtained from the control reference samples of the taxonomically determined species (three species of the Lycalopex genus that live in Chile). We can also add that our evidence indicates that an approximate proportion of 60% was obtained for L. griseus and approximately 40% for L. culpeus. (Medel & Jaksic, 1988).

Surprisingly, we could detect one L. fulvipes sample, which was not expected to be present in our study area. This is the first report of the presence of foxes of L. fulvipes in northern Chile. Therefore, it is important to determine their presence and distribution in different ecosystems throughout the country. Although the presence of L. fulvipes may be less frequent in these latitudes, the deepening of monitoring of Lycalopex species in northern Chile is very important because due to possible unknown distribution ranges of the Lycalopex species. A possible cause for migrations into new habitats could be a response to climate change or events such as forest fires, increasingly common in southern Chile and specifically in the ecosystems where L. fulvipes lives.

There are no previous reports of the use of mtDNA coupled to HRM analysis to establish the presence and distribution of Lycalopex species in Chile and South America. This work detects the bases for further studies of the distribution of these species in Chile, to understand their movement flows in different geographical areas and their adaptation to diverse environments and ecosystems.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHORS CONTRIBUTIONS

Leonardo Anabalón: developed the experimental design techniques and elaborated the manuscript; Francisco Encina: developed the experimental design and elaborated the manuscript; Pamela Sanchez: designed the sample and involved in field activities; Jaime Solano: involved in technical development and laboratory work; Felipe Benavente: involved in analysis of sequences; Flavio Olivares: involved in field activity and sampling; Carlos Oberti: involved in handwritten elaboration; Basilio Guíñez: provided the taxonomy control samples; Rolando Vega: elaborated the manuscript and involved in analysis of sequences.

DATA AVAILABILITY

DNA sequences: Genbank accession AF028151.1.

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