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Definition of mtDNA Markers for Studies in Pampas Deer (Ozotoceros bezoarticus) based On Fecal Samples

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***Abstract*—**Pampas deer, *Ozotoceros bezoarticus* (Linnaeus, 1758), is found within a wide geographic distribution in Latin America with five subspecies recognized (*O. b. bezoarticus*, *O. b. celer*, *O. b. uruguayensis*, *O. b. arerunguaensis* and *O. b. leucogaster*). The limited access to biological material and the lack of genetic structure information, however, hinder the conservation strategies for the species and the management of free-living populations. Therefore, we selected informative regions and developed primers for amplification of small sequences of mitochondrial DNA (Cytochrome-b, Dloop, ND5 and COI) to provide tools for studies using non-invasive genetic sampling. We evaluated the polymorphisms and phylogenetic signal of each region and successfully amplified it in fecal DNA samples of pampas deer from Pantanal. As results we described a set of primers that allow amplified DNA present in any fecal material to be applied in different approaches of conservation genetics. The methodological approach described here will favor genetic analyses of wild populations and highlights the relevance of non-invasive sampling to assist the decision-making and conservation strategies for this species.

Keywords— Odocoileini, fecal DNA, non-invasive genetic sampling, minibarcoding, conservation.

I. INTRODUCTION

Neotropical deer species represent a large part of the Cervidae family's diversity. They are under a high risk of extinction and a lack of data [1]. One emblematic example of the group is pampas deer (*Ozotoceros bezoarticus* – Linnaeus, 1758), a medium-sized species (20-40kg) that have branched antlers with up to three tips and occupy the open grasslands of the South American continent [2]. Although the species is globally classified as Near Threatened by the IUCN, populational decline and its habitat loss and fragmentation are of great concern [3]. It is estimated to have a 98% historical reduction in its original distribution [4]. The main threats are the conversion of its natural environment in agriculture, hunting, and the transmission of diseases through contact with exotic and domestic ungulates [1][3].

There are five subspecies of pampas deer: *O. b. bezoarticus* (Linnaeus 1758), occurring in the Brazilian Cerrado; *O. b. leucogaster* (Goldfuss, 1817) in the Pantanal and Chaco of Brazil, Bolivia, Paraguay, and Argentina; *O. b. celer* [5] in the Argentine Pampa; and finally, *O. b. arerunguaensis* and *O. b. uruguayensis* [6], represented respectively and exclusively by the populations of El Tapado and Sierra de Los Ajos in Uruguay.

The conservation status of each subspecies is distinct and depends on regional factors. The *O. b. leocogaster* subspecies has the best conservation status due to its large population [2][7]. Nevertheless, it is considered vulnerable in the Brazilian list of threatened fauna due to decline projections [8]. In contrast, the three southern subspecies (*O. b. celer*; *O. b. arerunguaensis*; *O. b. uruguayensis*) are considered to be at high risk of extinction, as they are restricted to one or a few populations with a low number of individuals [2]. Finally, the *O. b. bezoarticus* subspecies, previously considered to have deficient data, is now listed as vulnerable due to the intense environmental impact in the Brazilian Cerrado during the last two decades [8]. Estimates point to more than a 50% loss of the biome's original cover and an intense fragmentation [9] [10] with only 3% of its distribution within protected areas of strict conservation management [11].

Given the extensive distribution of pampas deer across the continent, its high genetic diversity and geographic isolation, it is evident that knowledge about its genetic structure and phylogeographic relationships is limited. Understanding these aspects will be essential for planning management actions aimed at the long-term conservation of the species in a scenario of small and disconnected populations. Thus, we evaluated the polymorphism of small mtDNA regions for use in phylogenetic studies to

enable genetic analysis from non-invasive sampling. We proposed the development of primers for amplification of these regions and tested the amplification efficiency in fecal DNA samples collected in nature.

The methodological approach described here will favor genetic analyses of wild populations and highlights the relevance of non-invasive sampling to assist the decision-making and conservation strategies for this species.

II. RELATED WORK

Genetic divergences between Brazilian populations of pampas deer were investigated through the analysis of mitochondrial DNA sequences from the Emas National Park and the Pantanal biome [12]. Those haplotypes are not geographically exclusive, thus, these populations may not be geographically isolated and the genetic divergence and extinction risk may not be as pronounced as previously imagined.

Additionally, [13] investigated the genetic structure and diversity of different subspecies using RAPD (Random amplified polymorphic DNA) markers, which revealed a low genetic variation that could differentiate populations. The variation found was attributed as an individual feature. These results contradict [12] but are justified by the type of molecular marker used in each study [13].

Similar to other Neotropical deer species, there is a sampling deficiency for pampas deer where only those two populations were sampled in Brazil. This limitation results from the difficulty to detect, capture, and collect samples in the wild, which hinders the collection of robust data for elusive deer [14]. This is a common scenario in rare or evasive species that has been reverted in recent decades due to non-invasive genetic sampling techniques and the use of dogs trained to detect feces [15][16][17].

The search for feces in nature and the analysis of fecal DNA has already allowed extensive genetic sampling and distribution studies of forest deer species in Brazil [18] [19][20][21]. Despite the advantages of using DNA from feces, some characteristics limit its use for genetic studies, such as the presence of inhibitors, which prevent the amplification of genetic material in PCR, and the degradation of DNA, which hinders the amplification of large fragments [17][22].

III. METHODOLOGY

Study Design, Samples and Sequences

Five regions of mitochondrial DNA were analyzed from sequences deposited in GenBank/NCBI and from good quality biological samples (tissue or blood). We evaluated two regions of the Cytochrome-B gene (CytB; a total of 868bp), a fragment of the NADH dehydrogenase subunit 5 gene (ND5; 562bp), part of the Cytochrome Oxidase I gene (COI; 543bp) and finally, a fragment of the control region (Dloop; 389bp). An internal fragment of smaller size was selected (~150- 350bp) for each region to compose a fecal DNA study design. We analyzed polymorphism and phylogenetic signals to assess whether the sequences obtained from fecal material confer congruent evolutionary relationships with those of large fragments (GenBank/ tissue and blood).

All 5 original regions were amplified and sequenced for a set of *Ozotoceros bezoarticus* tissue and blood samples (n=20) from the *Tissue and Cell Banking from the Deer Research and Conservation Center* (NUPECCE), São Paulo State University (UNESP), Jaboticabal city, Brazil. They come from populations previously studied at the Emas National Park and Pantanal, in Brazil, and Uruguay, samples identification in Table 1.

As for the GenBank/NCBI sequences, 10 fragments from Duarte et al (2008) [23] were used for the CytB gene (DQ789190.2-DQ789199.2) and 45 sequences produced by González *et al.,* (1998) [12] for the Dloop region (GI2393960-GI2394005). Finally, all aligments were added with a sequence of a pampas deer mitogenome (JN632681.2) deposited by Hassanin *et al.* [24].

Tissue Samples Sequencing and Alignment

We extracted DNA from tissue and blood samples using the phenol-chloroform purification protocol [25] and the primers described for CytB [26] [27], Dloop [27], ND5 [28] and COI [29]. PCR reactions were standardized for all regions in a final volume of 30ul (27ul of mix and 3ul of DNA with approximately 50ng/ul), containing 1x buffer and 1U of Taq DNA polymerase (both *PlatinumTM, Thermo Fisher Scientific*), 1.8 mM MgCl 2, 3.6 mM dNTP, 1 mg/ml BSA, and 0.5 pM of each primer (Table 1). Reactions were performed in a *Bio-Rad C1000 Touch™ Thermal Cycler* under the following conditions: initial step of 94°C for 2 min followed by 36 cycles of (1) 94°C for 1 min, (2) 55°C for 30 seconds, (3) 72°C for 30 seconds, and at the end, a 10-min extension at 72°C. The PCR products were then analyzed by 2% agarose gel electrophoresis and purified with the *GE Healthcare GFX™ PCR DNA and Gel Purification Kit.* Subsequently, they were submitted to an ABI 3730XL sequencer at the Center for Biological Resources and Genomic Biology at UNESP. The produced sequences had their electropherograms visually revised in the BioEdit 7.2.6 program [30] and were aligned for each of the five regions on the Mafft online server [31]. All sequences produced were submitted to GenBank (MZ493187 to MZ493229 and MZ502281 to MZ502290).

Fecal DNA Design and Primer Selection

The amplification of fecal DNA used three sets of primers described in the literature and included the design of primers for the CytB and ND5 genes. The primers design was performed based on GenBank sequences and selected on the PrimerQuest Tools platform. Parameters were defined for PCR in General (General PCR – Primers only), annealing temperature (primer Tm(C^o), minimum at 52^o C, optimal at 60^o C, maximum 64^o C), Primer GC (%) 40 to 60, primer size 17 to 22 (nt), and amplicon from 100 to

350 bp, optimum at 250 bp. The suggested regions were manually and individually located in the aligned array of GenBank fragments. Those primers located in conserved regions without polymorphisms, indels or gaps for all sequences were selected, and the presence of internal polymorphic regions to the amplicon was also observed (Table 1).

Table 1. Sequences of primers for small mtDNA amplification of pampas deer (*Ozotoceros bezoarticus)* fecal DNA, amplicon size (pb) and reference.

Gene	Primers		
	Primer Sequence	***Amplicon size (bp)***	***Reference***
CytB-A	F 5′CATCCGACACAATAACAGCA3′ R 5′TCCTACGAATGCTGTGGCTA3′	224	Gonzalez *et al.,* (2009) and Gonzalez *et al.,* (2018)
DL	F 5′GCGGCATGGTAATTAAGCTC3′ R5′GCTAGATTGCCAGCTAAAGGG3′	159	Gonzalez *et al.,* (2012) and Gonzalez *et al.,* (2018)
COI	F 5′GTGCTCCAGATATAGCATTTCC3′ R 5′GCTAGATTGCCAGCTAAAGGG3′	159	Gonzalez *et al.,* (2018)
CytB-B	F 5′CCATCAGACGCAGACAAA3′ R 5′GCGTTGTTTAGATGTGTGAAG3′	306	present study
ND5	F 5′TGTCACATGGTCCATCATAG3′ R 5′GCAGAGCTGCTGTGTTT3′	224	present study

Polymorphism, Saturation and Phylogenetic Analysis

These analyzes were applied against two matrices, one made with the largest fragments (1973pb) and the other with the basic sequences of fecal DNA (741pb), both from concatenated gene fragments (CytB + COI + ND5).

The level of polymorphisms, variable sites and parsimoniously informative sites (PIS; those with mutations present in at least two individuals), were observed with MEGA X [32]. The occurrence of saturation signal in each alignment was also evaluated by observing the correlation plot between transversions/transitions as a function of genetic distance (GTR) generated by the DAMBE 7.2 program [33]. Finally, Bayesian Inference (BI) phylogenetic analyses were performed using Mrbayes 3 [34], implemented by the online platform CIPRES Science Gateway [35]. For this, evolutionary models for each alignment were estimated using the program jModelTest 2.1.4 [36], with the best model selected by the lowest Bayesian Information Criterion (BIC) number. Phylogenetic analysis of BI was performed in two runs with four Markov Chain Monte Carlo chains with 10 million generations with a 25% burn-in. All analyses had an average standard deviation of split frequencies < 0.01.

Fecal DNA Amplification Success

The efficiency of primers designed to amplify faecal DNA was tested on DNA samples obtained from feces collected in nature. We used twenty fecal samples from free-living pampas deer collected during 2012 in the Pantanal. This is a heterogeneous set that included samples from different exposure times to weather conditions and were formed with the species' natural diet. The samples had their DNA extracted through a protocol based on DNA capture by the combination of silica and guanidine thiocyanate, adapted from [37]. The primers tested in this study were amplified with reactions normalized to a final volume of 30 µl containing 3 µl of 1x buffer (10 mM Tris-HCl, pH 8.4, 50 mM KCl); 0.6 mM dNTP; 2.5 mM $MgCl_2$; 2.1 mg/ml BSA; 0.7 pM of each primer; 1.5 U of Taq polymerase and 5 ul of DNA from the fecal sample. The reactions were performed in a Bio-Rad C1000 Touch™ Thermal Cycler in a touchdown protocol that totaled 45 cycles under the following conditions: initial step of 94°C for 2 min; followed by 5 cycles of 94°C-50 sec/ 58°C-50 sec/72°C-50 sec; 6 cycles of 94°C-50 sec/ 57°C-45 sec/ 72°C-50 sec; 8 cycles of 94°C-50 sec/ 56°C-40 sec/ 72°C-50 sec; 13 cycles of 94°C-50 sec/55°C-30 sec/72°C-50 sec; 13 cycles of 94°C-50 sec/ 54°C-30 sec/ 72°C-50 sec, and finally a 10-min extension at 72°C. The PCR products were then analyzed by 2% agarose gel electrophoresis and their amplification efficiency and specificity were observed. To confirm that the amplifications were target genes, a sample of each fragment was sequenced and confirmed by searching for BLAST similarity in GenBank.

IV. RESULTS AND DISCUSSION

The innovative and practical merit this work lies in the description of new primers, that allow the amplification of DNA present in any fecal material from *Ozotoceros bezoarticus*, with the achievement of adequate quality sequences to be applied in phylogenetic analyses and a tool for conservation genetics.

The proportion of variable sites, PIC values and the selected evolutionary models, for each fragment and internal region analyzed is described in Table 2. The CytB+COI+ND5 concatenated matrix for the fecal design was 38% of the original size, with 54% of the polymorphic sites and 63% of the PISs in the largest alignment. Additionally, the proportions of transversions/transitions showed that most alignments did not show any sign of saturation.

Table 2. Polymorphism and evolution model comparison between fecal DNA fragment design and the original full-length sequence. The proportion of fragment sequence length, variables sites, and PISs are demonstrated in percentages (%) in relation to the full-length (FL) sequence data.

Aligment	*Amplicon/ size*	*Variables Sites (bp)*	*PICs (bp)*	*BIC Model*
CytB	FL (868bp) Fecal1 (184pb) Fecal2 (267pb)	109 5 (5%) 61 (56%)	75 3 (4%) 47 (63%)	TrN+1 TrN TrN+1
COI	FL(543pb) Fecal (103pb)	13 1 (8%)	2 0 (0%)	TrN+G HKY+1
ND5	FL(562pb) Fecal(187pb)	33 11 (33%)	17 8 (47%)	TrN+1 HKY+1
DL	FL(389pb) Fecal(117pb)	97 28 (29%)	48 16 (33%)	GTR+G HKY+G
CytB + COI + ND5	FL(1973pb) Fecal(741pb)	127 69 (54%)	82 52 (63%)	TrN+G TrN+G

However, the Dloop region showed a high level of saturation with a high proportion of transversions in relation to the increase in genetic distance. The plots of transitions and transversions proportion in relation to the increase in genetic distance can be found in Figure 1.

The phylogenetic trees obtained with the concatenated regions CytB-COI-ND5 showed support above 0.9 (Figure 1). The phylogenetics results obtained separately for each region can be observed in detail in Figure 1. The results between the original regions and the fecal design were similar in the average support value and were congruent in topology. The phylogenetic hypotheses obtained did not demonstrate a monophyletic relationship between any of the Pampas deer subspecies with the sample set we used. On the contrary, clades composed of samples from different locations were recovered.

Fecal DNA Amplification Success

The efficiency of primers was confirmed by the quality of the target DNA amplifications present in the fecal material. Therefore, the designed primers amplified the DNA sequences of the *Ozotoceros bezoartica*s target fecal material with specificity and quality.

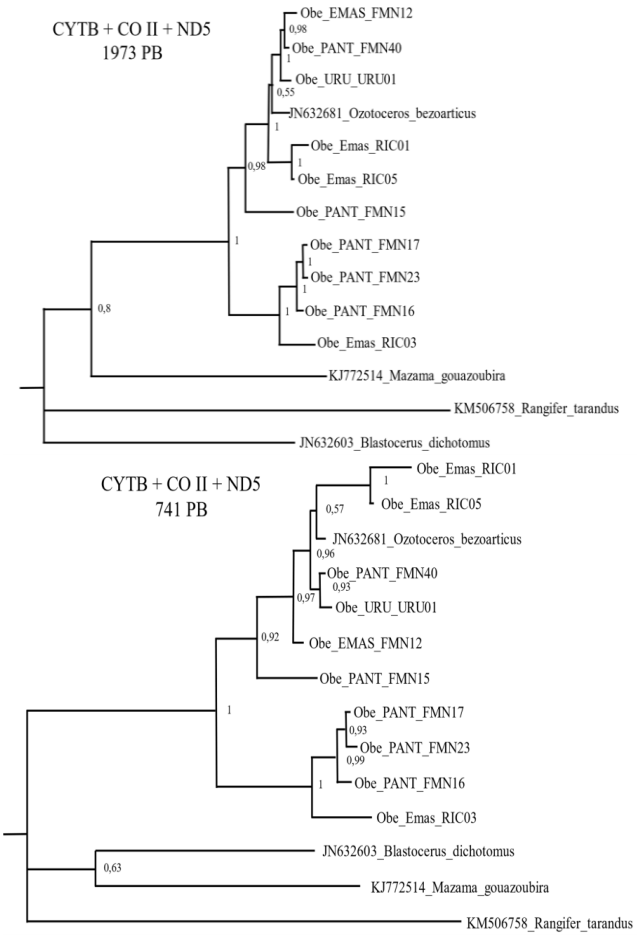

Figure 1 - Phylogenetic hypothesis comparison between the original full length (1973 bp) sequence and fecal DNA fragment design (741 bp) from CytB+COI+ND5 concatenated alignment, obtained through Bayesian Inference analysis for the pampas deer (*Ozotoceros bezoarticus*). EMAS= Emas National Park (Cerrado) samples; PANT= Pantanal samples, URU= Uruguay sample.

The amplification success in wild pampas deer fecal samples was 80% (n=16/20) for the CytB region (frag2) and 75% (n=15/20) for the ND5 region. Using the primers described in the literature, the success was 80% (n=16/20) for the COI, 75% (n=15/20) for CytB (frag1), and 70% (n=14/20) in the amplifications of Dloop region.

Discussion

The Pampas deer is a widely distributed species that presents a significant sampling deficiency and a lack of genetic studies on its populations and subspecies' diversity, phylogenetic relationships, and their respective spatial patterns. In addition to several other important populations that are known in the Cerrado and were never sampled [8], isolated populations were identified in the far north of Brazil, to the east, on Ilha de Marajó, at the mouth of the Amazon River [38] and to the west, in the Campos Amazônicos regions [39], as well as relictual populations in the grasslands of southern Brazil [40] [41]. In this scenario, non-invasive genetic sampling represents an essential tool for obtaining biological material for more robust analyses of the species, with the inclusion of more populations.

The results and methodology described here indicates the effectiveness of using small mtDNA sequences obtained from fecal DNA. The use of mitochondrial genes has already been applied in several works when analyzing the phylogenetic and phylogeographic relationships in deer, with the CytB gene being the most used region of choice [42] [43] [44] [45] [46]. In the same sense, amplifications of ribosomal DNA sequences are used for the most diverse organisms to verify similarity or evolutionary relationships between species or strain [47] [48] [49]. The ND5 gene had also been used to compare the sequences of gray brocket [28], water deer [50], and white-tailed deer [51]. Additionally, the COI gene is widely known to be the proposed marker in the barcoding of life initiative for species identification [52]. The COI and CytB fragments (frag 1) were originally selected due to the observation of interspecies polymorphism and no intra-specific polymorphism [53] [54] which justify the results observed here. The Dloop region, in turn, considered to be highly polymorphic [12] and a comparison of the polymorphism distribution in the mitogenome of white-tailed deer subspecies showed Dloop represents the most varied region of mtDNA [51]. However, the saturation signal observed for Dloop in the present study for the species raises an alert for its application in phylogenetic studies. Saturation of transitions results in a homoplastic phylogenetic signal and consequently bias the reconstruction of robust hypotheses [55][56]. The application of the framework proposed here in future studies should focus on the CytB and ND5 genes, use more regions of these genes, and analyze the same sample set for all regions in a concatenated way to guarantee greater support in the phylogenetic analysis.

We can discern that the phylogenetic relationships between the populations of Emas National Park and the Pantanal biome are not geographically exclusive, therefore a polyphyletic relationship for these subspecies. However, it is essential to carry out more robust future studies with larger population samples. Geographically relation and mtDNA phylogenetic structure analyzes of the genus Cervus, using complete mitochondrial genomes, reveal different lineages between Western lineage and Eastern, accumulate of deleterious substitutions in their mitochondrial protein-coding genes, probably due to drift and convergent changes in the composition of some mitochondrial genes between lineage; approach that seems necessary for Pampas deer, given these results and of a recent evolutionary deer history [57].

The successful amplification of the studied regions from fecal samples collected in nature with the described primers here was possible and applicable, though the efficiency for identification of individual animals by fecal DNA is correlated by storage method and sample condition [58]. The observed success is consistent with those found in previous studies that ranged between 78-94% in amplifying mtDNA from forest deer feces [19][20][53], and also with other work on fecal DNA in hot climate localities involving duikers (92%) [59], leporids (83-90%) [60] pumas (60%) [61][62], and jaguars (87%) [63].

The primers developed in this genetic work are important tools for future genetic studies, especially for the blastocerin subtribe, in particular the species *O. bezoarticus* and its intraespecifics varieties [12][6]. Due to the small size of the mitochondrial DNA fragments amplified by these primers, their use in forensic samples such as animal hair, feces and bones allow non-invasive approaches [18][19][20][64][65]. The use of small mtDNA fragments has enabled the identification of neotropical deer species [66] as well as works on population ecology and identification of endangered species [19][20][21], population estimate [67] and niche modeling [68].

V. CONCLUSION AND FUTURE SCOPE

The primers used in this work, to DNA amplifications fecal material, proved to be safe int terms of quality, specificity and in obtaining short sequences to be used in phylogenetic analysis or in other applications in conservation genetics to pampas deer. This opening up possibilities to understand the diversity and genetic structure this group, but it also represents a new tool for application in other elusive species with geographic sampling gaps. This type of genetic information is essential to support management actions that involve translocations, reintroductions or "*ex-situ*" conservation strategies.

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