



Non-invasive genetic sampling reveals a habitat use extension of *Chrysocyon brachyurus* and *Leopardus guttulus* inside a protected area of Southeastern Brazil

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Abstract: Anthropogenic activities have modified landscapes leading to environmental damages and to a threatened biodiversity. As a result, protected areas have become the last refuge for many species. Protected areas surrounded by a highly modified landscape may accumulate species, especially large mammals, which may alter their habitual habitat use. Here we used non-invasive DNA analysis, molecular species identification, and landscape analyses to assess the habitat use of carnivore species in an isolated protected area, Itatiaia National Park (PNI). Two species were by far the most sampled within PNI, *Chrysocyon brachyurus* and *Leopardus guttulus*. The spatial distribution of each species was assessed to the following landscape variables: altitude; land use/land cover; slope and Euclidean distances from water and from urban buildings. The habitat use of *C. brachyurus* and *L. guttulus* was related to altitude and land use/land cover. We tested whether there were differences in the environmental indicators considering both species, which showed that higher altitudes, forest and/or grassland formation were indeed associated with them. We highlighted the unprecedented presence of both species at altitudes up to 2,631 meters. Our results suggest a habitat use extension for both species inside the PNI that could be a consequence of the highly modified landscape where PNI is inserted. Therefore, the results can be helpful for better understanding the species dynamics and their conservation in the face of landscape changes. Further, this study may be of help for management and conservation policies of this emblematic protected area.

Keywords: Conservation; non-invasive DNA; landscape; land use/land cover; carnivores.

Amostragem genética não-invasiva revela uma extensão do uso de habitat por *Chrysocyon brachyurus* e *Leopardus guttulus* em uma área protegida do Sudeste do Brasil

Resumo: As atividades antropogênicas modificaram as paisagens levando a danos ambientais e a uma biodiversidade ameaçada. Como resultado, as áreas protegidas se tornaram o último refúgio para muitas espécies. Áreas protegidas cercadas por uma paisagem altamente modificada podem acumular espécies, especialmente grandes mamíferos, o que pode alterar o uso de seu habitat habitual. Neste trabalho, usamos análise de DNA não-invasivo, identificação molecular de espécies e análises de paisagem para avaliar o uso de habitat de carnívoros em uma área protegida isolada, o Parque Nacional de Itatiaia (PNI). Duas espécies foram de longe as mais amostradas dentro do PNI, *Chrysocyon brachyurus* e *Leopardus guttulus*. A distribuição espacial de cada espécie foi avaliada com as seguintes variáveis paisagísticas: altitude; uso e cobertura do solo; declividade e distâncias euclidianas da água e de construções urbanas. O uso de *C. brachyurus* e *L. guttulus* no habitat estava relacionado à altitude e ao uso e cobertura do solo. Testamos se existiam diferenças nos indicadores ambientais considerando ambas as espécies, o que mostrou que altitudes mais elevadas, formação de florestas e/ou campestres estavam de fato associadas a elas. Destacamos a presença incomum de ambas as espécies em altitudes de até 2.631 metros. Nossos resultados sugerem uma extensão do uso do habitat conhecido para ambas as espécies como possível consequência da paisagem

altamente modificada onde o PNI está inserido. Portanto, os resultados podem ser úteis para entender melhor a dinâmica das espécies e sua conservação diante das mudanças na paisagem. Além disso, este estudo pode ser útil para as políticas de manejo e conservação desta emblemática área protegida.

Palavras-chave: Conservação; DNA não-invasivo; paisagem; uso e cobertura do solo; carnívoros.

Introduction

Anthropogenic activities are the main source of environmental changes once they have intensified the process of landscape fragmentation and enhanced ecological damage (Cerqueira et al. 2003). These land use modifications represent the greatest immediate threat to biodiversity, and it can lead to variations in the ecosystem functioning, including biodiversity loss (Cardinale et al. 2006).

In this scenario, protected natural areas are one of the most important mechanisms to slow down biodiversity loss. (Godet and Devictor 2018; Wintle et al. 2019). They are also essential to watershed protection and carbon storage, as well as to cultural services that are more challenging to quantify (DeFries et al. 2007). Nevertheless, even protected areas may face several threats such as deforestation and fragmentation, invasion of alien species, wildfires, logging, and hunting (Ervin 2003; Carey et al. 2000). Protected areas surrounded by a highly modified landscape may suffer with the potential negative consequences associated with neighbor land use changes (Hansen and DeFries 2007). A most common consequence of these landscape modifications to animals, mainly large mammals, is altering their range distribution and habitat use (Ripple et al. 2017).

Nonetheless, understanding the habitat use and its conservation becomes a challenge when focusing on large mammals, due to recurring problems in observing or capturing most of these species. The use of non-invasive samples left in the environment by animals as scats, hair, urine, saliva, nails, and feathers, may decrease the expenditure of capturing these animals and the time spent in the field (Duque et al. 2018). Furthermore, the increased interest in molecular techniques among ecologists has been improving our knowledge of the biology and ecology of these large mammals. As an example, the use of non-invasive samples as DNA sources (Deyoung and Honeycutt 2005; Ruell and Crooks 2007) for species identification or for assessing social organization system, demographic history, and population dynamics (Marker et al. 2007; Miotto et al. 2014; Riley et al. 2006; Saranholi et al. 2017; Souza et al. 2017).

Among mammals, the carnivores have been increasingly suffering from habitat loss and landscape modifications (Noss et al. 1996; Ripple et al. 2014). However, because of their important ecological function of regulating other populations through predation (Santos et al. 2004; Ripple et al. 2014), their persistence in the ecosystem is critical.

In this study, we employed non-invasive-derived DNA from carnivores' scats to understand how these species use available habitats inside an important protected mountain area inserted in a highly modified landscape in Southeastern Brazil. We focused in *Chrysocyon brachyurus* and *Leopardus guttulus*, which are by far the two carnivore species most sampled in the studied area. Regarding habitat use, we expected that *C. brachyurus* would be associated with grassland and *L. guttulus* with forest formations. In addition, *C. brachyurus* inhabits the Brazilian savannas in altitudes up to 2000 m (Queirolo et al. 2011; Paula & DeMatteo, 2015; Bereta et al. 2017) and *L. guttulus* appears in areas of dense vegetation, such as Atlantic Forest from the coastal plain

to fields with altitudes no higher than 1300 m (Oliveira and Cassaro 1999; Goulart et al. 2009), although it can reach up to 2000 m of altitude (Oliveira et al. 2016; Sartor et al. 2021). Hence, we also expected to find both species up to a maximum altitude of 2000 meters.

Material and Methods

The sample collection was made in previously existing trails in the emblematic Itatiaia National Park (PNI), the first national park created in Brazil in 1937. The area is classified by the Brazilian legislation as an integral protection conservation unit (IPCU), where only indirect use of natural resources is allowed. PNI has a total area of 28,084 hectares (ICMBio 2012), ranging altitude from 549 to 2791 meters and is located between the states of Rio de Janeiro and Minas Gerais (Fig. 1a) - boundaries from the Brazilian Institute of Geography and Statistics, Southeastern Brazil. The conservation area is integrally inserted in the Atlantic Forest biome, and is considered a priority area for the conservation of biodiversity of extreme relevance due to the high concentration of endemic and endangered species (Bencke et al. 2006). Also, it is a priority area for future mammal censuses considering the absence of data, especially about medium and large mammals (Galetti et al. 2009).

Despite being a protected area, the PNI is circumvented by municipalities historically recognized for agricultural activities, which present a series of conflicts concerning PNI (Aximoff and Rodrigues 2011; Richter 2004). Between the alarming activities, we can highlight the use of fire for cleaning and maintenance of pastures, disorderly tourism, hunting, and illegal extraction (de Medeiros 2002; Aximoff and Rodrigues 2011; Dib et al. 2020). The unregulated land tenure also causes irregular parceling of the soil and may even allow people living in the PNI, culminating in the generation of waste, effluents, and others (ICMBio 2012).

The traveled trails totaling 352.20 km covered the entire extension of the PNI (Fig. 1a), varying between 773 m and 2766 m in altitude, with 46% in Forest Formation, 49% in Grassland Formation, 4% in Mosaic of Agriculture and pasture, and 1% in Rocky outcrops.

The trails were covered once by foot in non-periodic visits and the sample collection was carried out between July 2017 and April 2018. This was done preferably in the dry season when finding fresh scats is easier (Miotto et al. 2012) and considering that the presence of carnivores is not strongly affected by seasonality (Nagy-Reis et al. 2019; Trolle et al. 2006; de Almeida Jácomo et al. 2004).

Scats morphologically identified in the field as belonging to medium and large carnivores were collected along the trails in rocks and open places up to 3 m maximum around the trails since carnivores can use these places to mark their territory (Kleiman 2011; Lyra-Jorge et al. 2008). All scat samples collected were stored in 95% alcohol at -20 °C for molecular species identification, and the sampling geographic coordinates were recorded using a GPS device (Table S1).

Total DNA was extracted from fecal using QIAamp Fast DNA Stool Mini Kit (Qiagen) following the manufacturer's recommendations. We used the scat surface to minimize contamination from non-target DNA (Ball et al. 2007); additionally, two carnivore-specific fragments of mitochondrial DNA from the 12S (148 bp, Rodríguez-Castro et al. 2018), and ATP6 (179 bp, Haag et al. 2009; Chaves et al. 2012) genes were amplified by PCR for molecular identification. The use of taxon-specific primers avoided amplification of non-target species, such as the prey on which carnivores feed, facilitating the DNA amplification of the species of interest. PCRs were performed on the Applied Biosystems ProFlex PCR System thermocycler (Life Technologies) following the conditions proposed for suitable amplification of mitochondrial regions of carnivore scats using the Car12Ss2 rRNA primers (Rodríguez-Castro et al. 2018), and ATP6-DF3 (Haag et al. 2009), and ATP6-DR1 (Chaves et al. 2012).

PCR products were verified in electrophoresis using 1.5% agarose gel with GelRed (Biotium), purified using ExoSAP-IT enzyme (Affymetrix), and sequenced in ABI3730XL automatic sequencer (Applied Biosystem). Sequences obtained were manually edited and aligned using the CLUSTAL method (Thompson et al. 1994) with the Geneious software (Kearse et al. 2012). We compared each obtained sequence with the deposited sequences in the National Center for Biotechnology Information (NCBI), using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). Likewise, the sequence was contrasted with sequences previously obtained by the Laboratory of Molecular Biodiversity and Conservation (Department of Genetics and Evolution/Federal University of São Carlos), using the neighbor-joining distance-based method (Saitou and Nei 1987). Only sequences that showed the identity similarity value above 98% with the reference sequences were considered.

The landscape variables land use/land cover, Euclidean distance (ED) from water bodies, ED from the nearest urban buildings and altitude which have already been identified in other studies as influencing the occurrence of carnivores (Queirolo et al. 2011; Bereta et al. 2017; Cruz et al. 2019) were analyzed. The fine-scaled measure of climate variables was not available and was not included in the analyses. We extracted the variables from each fecal sample point and added random points obtained through the Create Random Points tool in QGIS. All points were used to test whether each species occurrence had a specific pattern, given the possible habitats within PNI. Land use/land cover were obtained from the MAPBIOMAS PROJECT 3.0 platform (<http://mapbiomas.org>). The annual series used (2017) was obtained with Landsat images using Google Earth Engine with 30 m resolution and accuracy validated by the platform owner (Fig. 1b). Based on information from the drainage network, the distance between the samples and the nearest water body was calculated (Fig. 1f), aiming to establish the geographical range of water bodies by Euclidean distance using QGIS. The urban infrastructure locations were obtained from the PNI's Management Plan, Map 2.1, booklet 2, where they were named as Villages, Hamlets and Neighborhoods. These locations were completed using Google Earth Pro. The ED calculated between urban buildings and the species location is shown in Fig. 1e.

The geographical relief was obtained from a Digital Elevation Model (DEM), which reproduces the spatial representation of a terrain's surface from points that describe its three-dimensional elevation (Montgomery 2003). Planialtimetric data were acquired using ALOS Palsar satellite

images with 30 m resolution. The primary attributes of relief features (Klingebiel et al. 1987) obtained from the DEM and considered in this study were the altitude and slope (Figures 1c, d). Those values were taken from the pixel of each sampling point using QGIS (pixel resolution 30 m).

We tested whether species occurrence was related to environmental variables (land use/land cover, altitude, slope, ED from water bodies and urban buildings), comparing the sample fecal points to a null random sampling points for each species, using R (R Development Core Team 2020). For the land use/land cover, the unique categorical variable, we also compared species across vegetation types using the "lsmmeans" package (Lenth et al. 2016). Since *C. brachyurus* and *L. guttulus* shown similar presence with regard to the environmental variables, we compared species occurrence in a single generalized mixed model, one species relative to the other. We used sets of generalized mixed models with binomial distribution, and the PCA-axis-built with ecological variables as fixed-factors, in all possible arrangements for PCA following Lindstrom and Bates (1990).

Generalized mixed models were fitted using the 'lme4' package (Bates et al. 2015). In all cases, we selected the best model relating species occurrence to these variables, using the lowest Akaike Information Criterion (AIC; Akaike 1974) values and significant differences. The significance of each explanatory variable was tested using the 'Anova' function in the 'car' package (Fox and Weisberg 2019).

Results

From a total of 244 scats, we were able to identify 175 (71.7%) using non-invasive-derived DNA for molecular species identification. The remaining 81 samples were removed from the analyses because they presented degradation in the genetic material likely due to prolonged exposure to environmental conditions or they did not belong to carnivores.

The scat samples were identified as following: 94 (57.6%) as belonging to *C. brachyurus* (maned wolf), 52 (31.9%) *L. guttulus* (southern tiger cat), three (1.84%) *Cerdocyon thous* (crab-eating fox) and 14 (8.58%) of *Canis familiaris* (domestic dog). Only the two most sampled species ($n > 50$), *C. brachyurus* and *L. guttulus*, were analyzed hereafter. *C. brachyurus* scats were mainly found in grassland formation (69 scats, 73%), following by forest formation (25 scats, 27%). Otherwise *L. guttulus* scats were mostly found in forest formation (35 scats, 67%), following by grassland formation (16 scats, 31%) and mosaic of agriculture and pasture (1, 2%), Table S1.

Seven different types of land use and land cover were determined within the PNI, mostly natural elements of the landscape, such as Forest formation (79.3%), Grassland formation (15.55%), Rock Outcrop (0.79%). Nevertheless, other types, such as the Mosaic of Agriculture and pasture (3.3%), Pasture (0.83%), Non-vegetated areas (0.04%) and Forest plantation (0.01%), were generated by the human presence (Fig. 1b).

Using DEM analysis, we found altitudes ranging from 549 to 2790 m (Pico das Agulhas Negras – Fig. 1c). According to the classification proposed by Lepsch (1983), the relief of the PNI can be characterized as strongly wavy to steep, with slope above 15% on most of its land (Fig. 1d). The ED results for each sample to water bodies ranged from 1 to 750 m (Fig. 1f), while for urban buildings ranged from 1 to 6650 m (Fig. 1e).

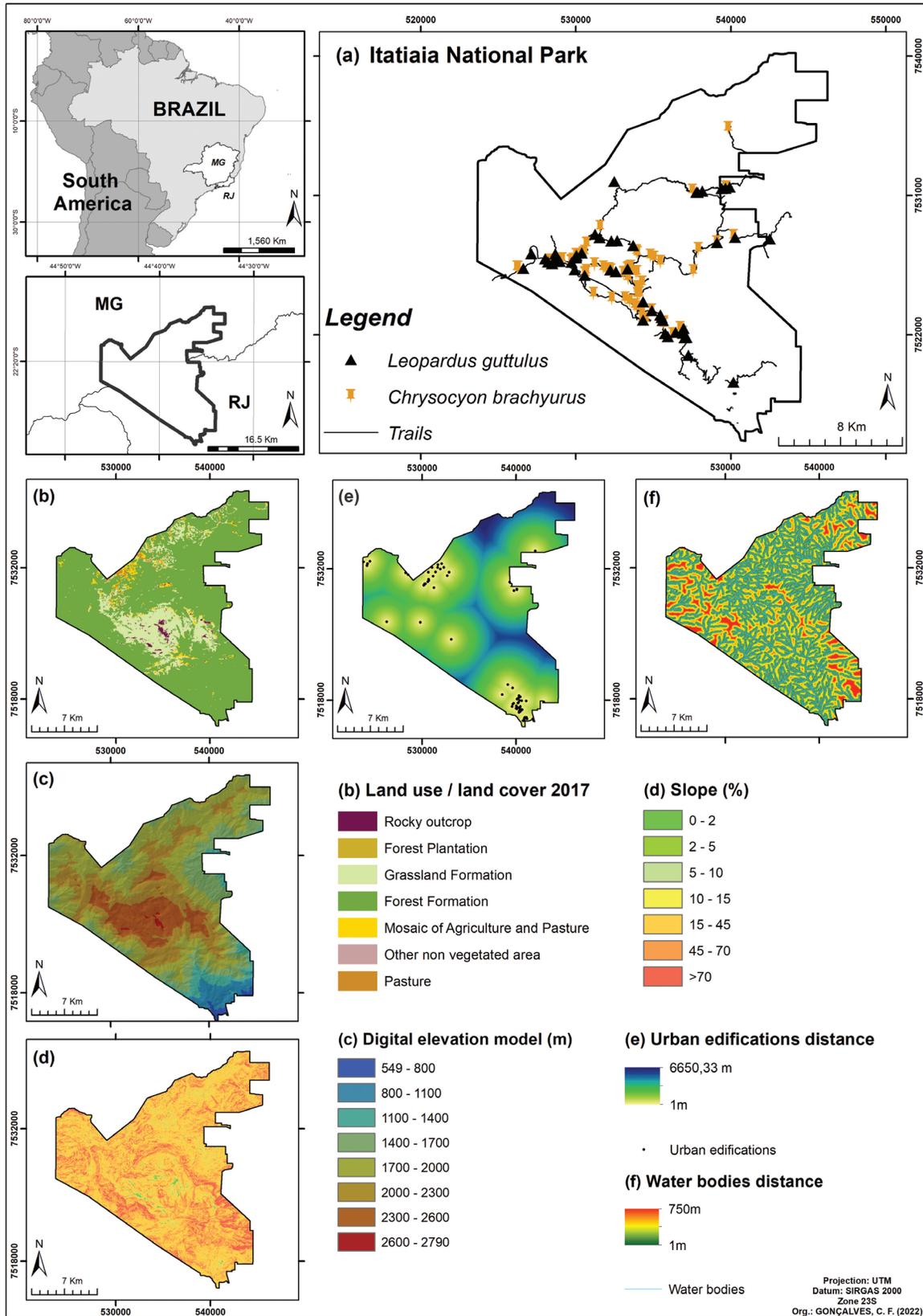


Figure 1. (a) Location of Itatiaia National Park (PNI), trails and samples location; (b) Land use/land cover; (c) digital elevation model; (d) slope; (e) Distance from urban buildings and their locations (f) Distance from water bodies.

Table 1. Coefficients of generalized mixed models using a random sampling showing correlation between presence of species (*C. brachyurus* and *L. guttulus*) and environmental variables (altitude, forest formation, grassland formation and mosaic of agriculture and pasture).

Variable	Estimate	Std. error	t-value	P (> t)
<i>C. brachyurus</i>				
Intercept	-1.775 ⁺⁰⁰	2.893 ⁻⁰¹	-6.134	<0.001
Altitude	6.959 ⁻⁰⁴	8.797 ⁻⁰⁵	7.911	<0.001
Grassland Formation	7.295 ⁻⁰¹	1.873 ⁻⁰¹	3.895	<0.001
Forest Formation	1.025 ⁺⁰⁰	2.076 ⁻⁰¹	4.936	<0.001
Mosaic agriculture and pasture	1.959 ⁻⁰¹	2.994 ⁻⁰¹	0.654	0.5136
<i>L. guttulus</i>				
Intercept	-1.734 ⁺⁰⁰	2.964 ⁻⁰¹	-5.849	<0.001
Altitude	6.798 ⁻⁰⁴	9.126 ⁻⁰⁵	7.450	<0.001
Grassland Formation	7.229 ⁻⁰¹	1.882 ⁻⁰¹	3.841	<0.001
Forest Formation	9.995 ⁻⁰¹	2.111 ⁻⁰¹	4.735	<0.001
Mosaic agriculture and pasture	1.914 ⁻⁰¹	3.008 ⁻⁰¹	0.636	0.5254

The top-ranked models of generalized mixed models performed to evaluate the relationship between presence of species (*C. brachyurus* and *L. guttulus*) and environmental variables are shown in Table S2, and the model selected included altitude and land use/land cover, considering the lowest AIC with significant P values. Because we did not obtain fecal samples in rocky outcrop, forest plantation, other non-vegetated area and pasture, these variables were not included in the GLMM analyses. The generalized mixed models using random sample ($P < 0.001$, Table 1) and both species (Table S3), performed to assess the association of species occurrence to environmental variables according to availability within the PNI, showed that altitude, forest and grassland formation were relevant for their habitat use.

Altitude, forest and grassland positively affected the presence of both *C. brachyurus* and *L. guttulus* ($P < 0.001$, Table 1). Since the habitat use was similar between the two species, we have included both in a single model only with observational data to test whether there were any differences between them, and the results were similar (Table S3). Of note, among the numerical variables analyzed, altitude was the only variable that differed between species (Figure S1).

Discussion

Our findings suggest a habitat use extension of *C. brachyurus* inhabiting grassland and forest in the studied area. In contrast, *L. guttulus* was mostly found in the forest formation (67%) as expected, corroborating its habitat use already reported (Oliveira and Cassaro, 1999; Goulart et al. 2009; Sartor et al. 2021), although scats of this species were also obtained in grassland formation (31%), suggesting an important association with this habitat in PNI. It is well known that *C. brachyurus* is affected throughout its original range by habitat loss

and modification due to urbanization, agricultural and livestock raising (Paula et al. 2013; Queirolo et al. 2011, 2014; Vynne, 2014). These human-promoted habitat modifications can be leading to habitat use expansion in this species (Queirolo et al. 2011). Thus, even though the original range of *C. brachyurus* did not include Atlantic Forest, it has been already reported inside this domain (Rodrigues and Oliveira 2006; Lourenzutti and Almeida 2006) and had its first record for PNI in 1954 (Ávila-Pires & Gouvea, 1977).

In the present study, *C. brachyurus* was observed in the grassland formation, as frequently reported across its distribution area (Dietz 1984; Rodden et al. 2004; Lyra-Jorge et al. 2010; Coelho et al. 2018), but an expressive amount of scats (27%) was sampled in the forest, contrasting with other studies which showed that this animal avoids these areas (e.g. Coelho et al. 2008). It is important to notice that the proportion of grassland formation in PNI is low (16%), but it concentrates 73% of all records obtained for *C. brachyurus*, demonstrating a strong association of this canid with this vegetation. However, it is suggested that PNI represents an important refuge for the local population of *C. brachyurus* that once was putatively expelled from the highly modified areas nearby. Limited by the geographical and vegetational characteristics of PNI, *C. brachyurus* seems to be expanding its habitat use to the forest.

Our findings of differential occurrence in relation to altitude also have some novelties. Although *L. guttulus* occurred preferably in lower altitudes, our data revealed its presence at altitudes ranging from 1436 to 2544 m, representing the highest altitudinal limit observed for the species (Oliveira et al. 2016; Goulart et al. 2009). In a recent modelling study, the presence of *L. guttulus* reached altitudes of about 1000 m, declining to a minimum value at 2000 m (Sartor et al. 2021). It is suggested that our findings enlarge the altitudinal range for the species and the presence of forested areas at these altitudes could be facilitating *L. guttulus* to expand its height scope in PNI. However, further studies to evaluate the occurrence of this species in different altitudes are still needed. Similarly, our results on the *C. brachyurus* occurrence also showed higher altitudes than it has ever been reported. While this species preferably appears to inhabit altitudes up to 2000 m (Queirolo et al. 2011; Paula & DeMatteo, 2015; Bereta et al. 2017), we found this species in higher altitudes ranging from 1574 to 2631 m. Together, these results represent the highest altitude reported for both species this far.

In sum, our findings suggest a potential habitat use extension in *C. brachyurus* and *L. guttulus* inhabiting an emblematic protected area in southeastern Brazil. Similar results were already reported for both species living in different studied areas (Rodrigues and Oliveira 2006; Lourenzutti and Almeida 2006; Queirolo et al. 2011; Sartor et al. 2021). We also point out that PNI represents an important refuge for the species that suffer the consequences of the increasingly landscape modifications in this geographical region. The use of molecular species identification through non-invasive sampling was critical for assessing presence and ecological information of both carnivores studied. The integration of molecular species identification and landscape analyses allowed to infer how these species may be responding to anthropic disturbances. Even further, it is gathering useful information for a better understanding of the species dynamics, conservation plans of studied species in a changing scenario, and management and conservation policies of this important protected area.

Supplementary Material

The following online material is available for this article:

Table S1 – Geographic coordinates and landscape variables for each data.

Table S2 – Top-ranked models of generalized mixed models between presence of species (*C. brachyurus* and *L. guttulus*) and environmental variables (ED from water bodies, ED from urban buildings, altitude, slope, land use/land cover).

Table S3 – Coefficients of generalized mixed models showing correlation between presence of species (*C. brachyurus* and *L. guttulus*) and environmental variables (altitude, land use/land cover).

Figure S1 – Boxplot showing the environmental variables describing the occurrence of the studied species. Grey boxplot indicates significant difference after the GLMM tests ($P < 0.001$).

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Author contributions

Camila F. Gonçalves: substantial contribution in the concept and design of the study; data collection; analysis and interpretation; manuscript preparation and critical revision, adding intellectual content.

Karen Giselle Rodriguez-Castro: substantial contribution to data analysis and interpretation; manuscript preparation and critical revision, adding intellectual content.

Lais Verdan Dib and Alyne da Silva Barbosa: substantial contribution to data collection.

Luiz Eduardo Moschini: substantial contribution in the analysis and interpretation.

Pedro M. Galetti Jr: substantial contribution in the concept and design of the study; analysis and interpretation; manuscript preparation and critical revision, adding intellectual content.

Conflicts of Interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

Ethics

All research was conducted based on the protocols approved by Ethics Committee on Animal Experimentation (CEUA, Instituto Biomédico, Universidade Federal Fluminense), and SISBIO-ICMBio (Authorization System and Biodiversity Information-Chico Mendes Institute for Biodiversity Conservation, Ministry of Environment, Federal Government, Brazil, number 57635-1), and through the National Management System for Genetic Heritage, and Associated Traditional Knowledge (SisGen), under registration code AFB4EA3.

Data Availability

The dataset generated in this study from AT6 region are available in the Dryad repository under DOI number <https://doi.org/10.5061/dryad.djh9w0vxx>, for 12S and COI in GenBank-NCBI under accession numbers MN509185-MN509198, MN608174-MN608176 (Dib et al. 2020). And all the dataset are available in the Supplementary Information in the Figshare under DOI number <https://doi.org/10.6084/m9.figshare.20217044>.

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