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PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

IDENTIFICAÇÃO DE ESPÉCIES DE CARNÍVOROS BRASILEIROS (MAMMALIA: CARNIVORA) A PARTIR DE AMOSTRAS DE FEZES UTILIZANDO SEQÜÊNCIAS DE DNA E MICROSCOPIA ÓPTICA DE PÊLOS

Vanessa Godoy Graeff

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PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA

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Orientador: Dr. Eduardo Eizirik

DISSERTAÇÃO DE MESTRADO

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“In the end, our society will be defined not only by what we create, but by what we refuse to destroy.”

John C. Sawhill (1936-2000)
President, The Nature Conservancy, 1990-2000

Dedico àqueles que um dia sofrerão as conseqüências da insensatez humana.

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SUMÁRIO

RESUMO

A habilidade para detectar e analisar indícios de animais na natureza, parte integral da pesquisa e manejo da vida silvestre, se torna fundamental quando a espécie em estudo é um carnívoro. Para as espécies desse grupo, geralmente raras e/ou difíceis de capturar, a análise das fezes é um dos melhores métodos não-invasivos para a identificação, caracterização e monitoramento das populações. Contudo, identificações de espécies a partir de amostras fecais podem ser subjetivas quando baseadas em critérios tradicionais. Neste estudo, comparamos a eficiência de dois métodos de identificação de espécies de carnívoros: análise do DNA fecal e microscopia óptica de pêlos-guarda encontrados em fezes. Ambos foram aplicados a 102 amostras de fezes coletadas em uma área de Mata Atlântica no Rio Grande do Sul. Através da análise das seqüências de mtDNA obtidas a partir de 70 fezes foi possível identificar 75,7% destas amostras como pertencentes a 3 espécies de felinos, 21,3% a uma espécie de canídeo silvestre e 3% ao cão doméstico. Pêlos-guarda foram encontrados em 56% das fezes coletadas. Através da análise microscópica desses pêlos identificou-se 55,6% das amostras em nível de espécie (três espécies de felídeos) e 44% em nível de família (Canidae ou Felidae). Foram analisadas comparativamente 44 amostras sobrepostas pelos dois métodos. Desacordos na identificação entre os métodos ocorreram em apenas três amostras. No total, 77 amostras de fezes foram identificadas em nível de espécie por pelo menos um dos métodos, permitindo uma caracterização da dieta destes carnívoros. A identificação baseada em microscopia de pêlos requer poucos gastos ou tecnologia, sendo simples e rápida para análises em campo. Contudo, algumas características morfológicas dos pêlos-guarda podem influenciar o poder de identificação por microscopia, em alguns casos podendo levar a uma interpretação errônea ou incompleta dos padrões observados. A identificação baseada na análise do DNA fecal pode ter alto custo e ser tecnicamente difícil, mas as informações que podem ser obtidas através deste método superam em quantidade e qualidade outros métodos. Assim, considerando as vantagens e desvantagens dos métodos analisados, as diferenças entre eles permitem que sejam usados de forma a se complementarem mutuamente em diversos estudos, propiciando maior acurácia na identificação de espécies a partir de fezes.

APRESENTAÇÃO

O presente trabalho, intitulado “Identificação de espécies de carnívoros brasileiros (Mammalia: Carnivora) a partir de amostras de fezes utilizando seqüências de DNA e microscopia óptica de pêlos” foi desenvolvido como parte dos requisitos necessários para a obtenção do título de Mestre junto ao Programa de Pós-Graduação em Zoologia da Pontifícia Universidade Católica do Rio Grande do Sul.

Este trabalho teve como principais objetivos caracterizar e comparar duas técnicas de identificação de espécies de carnívoros a partir de amostras de fezes, avaliando sua adequação e eficiência para estudos ecológicos, e aplicando as amostras identificadas em um estudo da comunidade de carnívoros do CPCN Pró-Mata, RS. Com base na relação entre os resultados obtidos com informações já disponíveis sobre estas técnicas, procurou-se contribuir para a consolidação de metodologias confiáveis para a identificação de espécies de carnívoros brasileiros, auxiliando no desenvolvimento de estratégias adequadas para sua conservação.

Esta dissertação é apresentada no formato de um artigo científico a ser submetido ao periódico *Journal of Zoology*.

PREFACE

The present study, entitled “DNA sequencing *versus* hair microscopy: a comparison of two methods for the identification of non-invasive samples from Atlantic Forest sympatric carnivores” has been developed as part of the requirements for the M.Sc. degree at the Graduate Program in Zoology of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS).

The main goals of this project were to characterize and compare two techniques for the identification of carnivore species based on faecal samples, evaluating their suitability and efficiency for ecological studies involving this group, and applying the identified samples in an investigation of the carnivore community occurring at the Pro-Mata Research Center, Brazil. We also aimed to relate the results with available information about these techniques, contributing to the elaboration of reliable protocols for the identification of Brazilian carnivores, which are required for the development of adequate conservation strategies for these taxa.

This thesis is written as a scientific article to be submitted to the Journal of Zoology.

DNA sequencing *versus* hair microscopy: a comparison of two methods for the identification of non-invasive samples from Atlantic Forest sympatric carnivores

VANESSA G. GRAEFF, PAULO B. CHAVES, CRISTIANO M. MARIN and EDUARDO EIZIRIK

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DNA sequencing *versus* hair microscopy: a comparison of two methods for the identification of non-invasive samples from Atlantic Forest sympatric carnivores

VANESSA G. GRAEFF[1], PAULO B. CHAVES[1], CRISTIANO M. MARIN[1], EDUARDO EIZIRIK[1, 2]

[1] Faculdade de Biociências, PUCRS, Porto Alegre, Brazil.

[2] Instituto Pró-Carnívoros, Brazil.

Corresponding author:

Dr. Eduardo Eizirik

Faculdade de Biociências - PUCRS

Av. Ipiranga 6681, CEP 90619-900 Porto Alegre, RS, Brazil

Telephone number: 55 (51) 3320.3500 Ext. 4727

Fax number: 55 (51) 3320.3612

Email: eduardo.eizirik@pucrs.br

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ABSTRACT

Non-invasive sampling methods provide a means for studying species such as mammalian carnivores, which are difficult to survey using traditional techniques. The collection of carnivore faeces, a common non-invasive method, has been widely used in wildlife ecology studies. However, species identification based on fecal samples can be inaccurate or biased, potentially compromising the quality of all downstream data. We compared the effectiveness of two methods for carnivore species identification: faecal DNA analysis and hair microscopy of guard hairs found in scats. We collected 102 carnivore scats in a protected area in the Brazilian Atlantic Forest, and submitted these samples to parallel DNA- and hair-based analyses. The target mtDNA segment was successfully amplified from 98% of the samples, and reliable sequence-based identification could be obtained for 70 scats. This analysis identified all samples at species level, 75.7% of which belonged to three species of wild felids, 21.3% to a wild canid and 3% to a domestic dog. Guard hairs were found in 57 scats, and microscopic analysis of these samples identified *ca.* 56% of them at species level (three felids) and 44% at family level (Canidae or Felidae). The comparative analysis used a total of 44 samples that were identified by both methods. Identification disagreement between them occurred in only three scat samples. Overall, 77 samples were identified at species level by at least one of the methods, allowing a dietary investigation of the included carnivores, and a more detailed comparison of the food habits of *Leopardus tigrinus* and *Puma yagouaroundi*. Identification by hair microscopy is a method that has proved to be simple, quick and inexpensive. However, some morphological features of guard hairs might influence the power of hair-based identification, possibly leading to incomplete or erroneous interpretation in some cases. On the other hand, routine DNA-based identification of faeces might be costly and technically demanding, but our results support the expectation that it performs better (both in terms of quality and quantity of information) than the other method. Overall, it is likely that both methods can be applied in a complementary fashion in several sampling situations, with their joint assets providing higher accuracy for carnivore faecal identification.

Keywords: faeces, scats, mammals, mitochondrial DNA, DNA barcodes, optical microscopy, fur, dietary

INTRODUCTION

Mammalian carnivores are often difficult to study because many are elusive, rare, wide ranging or frequently occupy inaccessible areas. The inability to collect sufficient amounts of reliable data on carnivore distribution, numbers, population structure, and habitat requirements is a severe impediment to the development of effective conservation efforts on behalf of these species, and the lack of reliable information often leads to controversies (Johnson *et al.*, 2001).

Given these difficulties in detecting carnivore species, it is critical to develop new tools to improve the collection of field data. In this regard, the ability to detect and analyze animal sign in the wild, an integral part of wildlife research and management, becomes essential when the study focuses on one or more carnivore species. For this group, faecal analysis is one of the best methods to identify, characterize and monitor populations (Foran, Crooks & Minta, 1997). Faecal samples can provide vital information on a species' distribution and abundance, as well as individual spatio-temporal movement, diet and diseases (Kohn & Wayne, 1997). However, unambiguous species identification from scats is problematic. Classifications based on traditional criteria such as size and morphology can be quite subjective (Davison *et al.*, 2002) and can be confounded by a number of factors, including large intra-specific variability and the occurrence of sympatric species with similar scats (Foran *et al.*, 1997; Farrell, Roman & Sunquist, 2000; Prugh & Ritland, 2005). In addition, in some cases the identification is performed based on the habitat where the sample was found and/or on the food items identified, which would lead to circular and possibly erroneous inferences.

Considering the widespread difficulties in identifying carnivore species based on their scats, more reliable methods are required, especially those that can be standardized and cross-compared among studies and geographic regions. Two different approaches have been receiving increased attention in this regard, namely hair microscopy and molecular genetic techniques, both of which have an interesting potential for aiding in carnivore studies relying upon non-invasive sampling (*e.g.* scats). Scientists working in conservation biology and in ethology are particularly interested in non-invasive sampling techniques, as they allow the investigation of various problems without having to capture, disturb, or even observe the animal (O'Brien, 1996; Taberlet, Luikart & Geffen, 2001; Johnson *et al.*, 2001).

The analysis of hair characteristics in the context of field studies (which may be termed ecological trichology) has been applied in several studies of carnivore food habits, in which both predator and prey may be identified using macroscopic features or characters visualized under light

microscopy (Teerink, 1991; Quadros & Monteiro-Filho, 2006a) or scanning electron microscopy (Chernova, 2002, 2003). There is a growing trend for employing light microscopy to characterize the cuticular and/or medullar morphology of guard hairs (a.k.a. overhairs), allowing the development of reference collections (Teerink, 1991) and keys for the identification of predators and their prey (*e.g.* Gamberg & Atkinson, 1988; Inagaki & Tsukahara, 1993; Cowell *et al.*, 2001; Chernova, 2002; Chernova, 2003; González-Esteban, Villate & Irizar, 2006). In the case of predator identification, the approach is based on the fact that overhairs may be ingested during self-grooming, and can be found later in the animal's faeces. A challenge in this case is to isolate the predator's hairs amidst a much larger universe of prey remains. In addition, it is critical to develop comprehensive reference collections and identification keys allowing for a broad standardization of this approach, as well as to evaluate the reliability, consistency and discriminatory power of diagnostic morphological characters observed in the hairs of different species. In the case of Brazilian mammals, an important contribution in the direction of a standardized approach for the use of this technique was provided by Quadros (2002) and Quadros & Monteiro-Filho (2006a, 2006b), who developed a reference collection and identification key for multiple species, proposed an integrated nomenclatural scheme for hair micro-structural features, and assessed the performance of the method in the analysis of carnivore scats collected in one study site.

With the development of the Polymerase Chain Reaction (PCR) (Saiki *et al*., 1985), it has become possible to use hair, feathers or faeces from free ranging animals as a source of DNA for various types of analyses (Höss *et al*., 1992; Wayne, 1996; Kohn & Wayne, 1997; Johnson *et al.*, 2001; Taberlet *et al.*, 2001; Frankham, Ballou & Briscoe, 2004; Prugh *et al*., 2005). Analysis of scat DNA (often referred to as molecular scatology) is a rather recent approach that uses epithelial cells sloughed off the colon wall and deposited in scats as a reliable source of DNA (Albaugh *et al.*, 1992) to determine species origin and several other types of information. By isolating DNA from scats and using molecular assays that can be compared against reference samples, it is possible to accurately determine species identity, especially with the use of direct DNA sequencing (Paxinos *et al.*, 1997; Wasser *et al*., 1997; Hansen & Jacobsen, 1999; Farrell *et al*., 2000; Palomares *et al.*, 2002; Miotto *et al.*, 2007).

The reliability of the various methods employed for species-level identification of carnivore scats has seldom been assessed in a rigorous fashion, especially by directly comparing different approaches. Few examples are available of such comparisons, and most have focused on morphological *versus* molecular methods (e.g. Davison *et al.*, 2002; Prugh & Ritland, 2005). While it seems quite clear that a simple assessment of faecal size, shape, and odor will often be error-prone, and is unlikely to provide

the basis for a standardized approach across multiple study sites and carnivore assemblages, little is known as to the relative performance of scat DNA *versus* guard hair microscopy. Both methods seem to have their own assets. The hair-microscopy approach is currently less expensive, requires simpler laboratory conditions, and may have sufficient power to discriminate all carnivore species, or at least all those expected to occur in a given geographic region. In contrast, the DNA-based method is likely to have more discriminatory power (as more characters can be surveyed by choosing one or more genomic fragments exhibiting high variability), and should be fully amenable to digital standardization leading to worldwide sequence data bases of all carnivore species. Given the promise of both approaches, it is relevant to address the following questions:

(i) what is the success rate of each one in the species-level identification of carnivore scats?

(ii) when applied to the same samples, are both methods congruent in carnivore species assignment?

(iii) in the case of discrepancies, which method is more likely to be accurate?

In this study we attempted to address these questions by investigating the species-level identification of carnivores occurring in a protected area in the Brazilian Atlantic Forest, employing both hair microscopy and DNA sequencing to the same standardized set of field-collected faecal samples. We compared the performance of both methods, assessed their congruence, and used the identified scat samples to investigate the food habits of this little-studied carnivore community.

MATERIALS AND METHODS

Study area

We conducted this study at the Pro-Mata Research Center (henceforth Pro-Mata RC), a private biological reserve located in Rio Grande do Sul state, southernmost Brazil (29º28'51.96"S, 50º10'28.01"W) (Figure 1). The climate is mesothermic, with an average annual temperature of 14.5°C, mild winters and hot summers (Bertoletti & Teixeira, 1995), corresponding to Köppen-Geiger's *Cfa* zone (Peel, Finlayson & McMahon, 2007). The study area comprises approximately 2,994 ha ranging from 600 to 900 m a.s.l., including plateau areas surrounded by steep slopes leading to adjacent valleys. The plateau vegetation is composed of grasslands and natural patches of Araucaria forest (mixed ombrophilous forest, dominated by the Neotropical pine *Araucaria angustifolia*), while the slopes are covered with dense ombrophilous forest (Bertoletti & Teixeira, 1995). These two forest landscapes are important components of the Atlantic Forest biome (Guedes *et al.*, 2005), harboring a diverse array of species, including many endemics. The Atlantic Forest original range covered more than 1.5 million

km^2, stretching along a wide latitudinal range and representing the second largest rainforest of the Neotropics (Galindo-Leal *et al.*, 2005). Today it represents a critical example of the alarming situation of most tropical forests in the world, with only 7% of the original area left. It has been recognized as one of the 25 worldwide biodiversity hotspots, but its current system of protected areas is still insufficient to guarantee the conservation of its biological legacy (Tabarelli *et al.*, 2005). In spite of its relevance and critical conservation status, many aspects of this biome are still poorly known, including the composition and ecological dynamics of the mammalian community in most of the remaining fragments.

Faecal sampling

We collected scats opportunistically along roads and trails between September 2006 and July 2007. A portion of approximately 5 cm in length of each scat was collected for DNA analysis, and was then preserved in silica desiccant or ethanol 96% and stored at -20°C prior to extraction. The remaining portion of each scat was stored in a zip lock bag at -20°C until it was processed. We recorded the GPS location (UTM system) of each scat sample, and estimated its age category (old or recent) at the time of collection, based upon appearance, exposure of the deposition site, and weather conditions.

DNA-based identification of carnivore species

Genomic DNA was extracted from all faecal samples by scraping the surface of each scat followed by the use of the QIAamp Stool DNA Mini Kit (Qiagen Inc.), according to the manufacturer's instructions. DNA extractions and subsequent handling of the extracted DNA were carried out in a separate laboratory area, in a UV-sterilized laminar flow hood, dedicated to the molecular analysis of non-invasive samples.

A short segment (*ca.* 170 bp) of the mitochondrial *ATP6* gene was amplified by PCR using primer ATP6-DF3 (5'-AACGAAAATCTATTCGCCTCT-3') in combination with either ATP6-DR1 (5'-CCAGTATTTGTTTTGATGTTAGTTG-3') or ATP6-DR2 (5'-TGGATGGACAGTATTTGTTTTGAT-3'). Each 20 µl PCR reaction contained 1-9 µl of DNA, 1x PCR Buffer (Invitrogen), 2.5 mM $MgCl_2$, 100 µM of dNTPs, 5 µM of each primer and 1.0 unit of *Taq* DNA Polymerase (Invitrogen). The PCR began with 10 cycles (*Touchdown*) of 94°C for 45s, 60-50°C for 45s, and 72°C for 90s; this was followed by 30 cycles of 94°C for 45s, 50°C for 45s, 72°C for 90s and a final extension of 72°C for 3min. PCR products were stained with *GelRed* (Biotium), visualized on a 1% agarose gel, sequenced using the *DYEnamic ET Dye Terminator Sequencing Kit* (Amersham Biosciences) and analyzed in a MegaBACE 1000

automated sequencer (Amersham Biosciences). Sequences were visually checked using CHROMAS 2.0 (www.technelysium.com.au/ chromas.html), manually corrected using MEGA 3.1 (Kumar, Tamura & Nei, 2004) and checked against a reference sequence data base of Neotropical carnivores developed in our laboratory (Chaves *et al.*, in preparation). Species identification was conducted using simple phylogenetic analyses (*e.g.* the Neighbor-Joining or UPGMA clustering methods based on a matrix of either the absolute number of differences or a p-distance) performed with MEGA, since there was virtually no within-species variation in the mtDNA segment used here. This procedure was therefore simply a test of species-level monophyly, using 1,000 nonparametric bootstrap replicates as a measure of nodal support.

Species identification using hair microscopy

Upon arrival in the laboratory, the portion of each scat that had been stored in a zip lock bag was placed for 5 days in a glass flask containing a solution composed of 91% ethanol (70% concentrated), 5% formaldehyde (10% concentrated), 4% acetic acid, and drops of liquid detergent for conservation and sterilization (Mantovani, 2001). The scats were subsequently washed in flowing water through a fine (<1mm) nylon sieve. Hairs contained in the faecal sample (including those originating from the predator itself [in a lower amount] as well as from mammalian prey) were separated from the total organic matter and were dried at 40°C for 3 days to avoid fungal growth.

Carnivore identification using hair microscopy was performed totally independently from the DNA-based analysis described above, using a blind-test approach (*i.e.* results from the two methods were not cross-compared until all identifications were concluded). Guard hairs found in the samples were prepared for observation with optical microscopy following the technique developed by Quadros & Monteiro-Filho (2006b). Cuticular impressions were obtained pressing hairs against a thin nail varnish layer let dry for 15 to 20 minutes on glass slides. In order to observe the medullar pattern, hairs were submitted to a discoloration process with commercial peroxide for 80 minutes. After this process, the hairs were enclosed in synthetic balsam on glass slides. Microscopic analyses of the hairs and species identification were conducted based on the patterns of hair cuticle and medulla described by Quadros (2002). In that study, she described the hair micro-structural features of 11 Brazilian carnivores, including several (but not all) species expected to occur in our study area. In order to verify our ability to detect the diagnostic characters identified by Quadros (2002), as well to complement her list of reference species, we assembled a collection containing multiple hairs of several Neotropical

carnivores gathered from two Brazilian museums (Museu de Ciências e Tecnologia – PUCRS [MCT-PUCRS] and Museu de Ciências Naturais - FZB/RS [MCN-FZB]). The number of samples varied per institution and totalled 25 specimens belonging to 15 species and five carnivoran families (3 *Cerdocyon thous*, 1 *Conepatus chinga*, 1 *Conepatus semistriatus*, 1 *Eira barbara*, 1 *Galictis cuja*, 2 *Leopardus colocolo*, 2 *Leopardus geoffroyi*, 2 *Leopardus pardalis*, 1 *Leopardus tigrinus*, 2 *Leopardus wiedii*, 2 *Lycalopex gymnocercus*, 1 *Nasua nasua*, 2 *Procyon cancrivorus*, 1 *Puma concolor* and 3 *Puma yagouaroundi*). Five of these species (*C. chinga*, *C. semistriatus*, *L. gymnocercus*, *L. colocolo* and *L. geoffroyi*) had not been included in the reference collection assembled by Quadros (2002), so that no standard trichological pattern was available for them. To allow for the possibility of detection of non-native carnivores, we also generated reference cuticle and medullar slides for domestic dogs (*Canis familiaris*), using multiple hairs collected from individuals roaming around the Pró-Mata RC headquarters (*i.e.* within the study site).

Dietary analysis

Prey remains found in the faecal samples (such as bones, teeth, claws, hairs, seeds, arthropod fragments, and feathers), were analyzed to investigate the food habits of the carnivore community of the Pró-Mata RC. The identification was based on macroscopic and microscopic features of these items in comparison with the reference collections of MCT-PUCRS and MCN-FZB. Due to the high fragmentation of the mammalian prey remains, it was not possible to use cranial characters for the species identification of small mammals, and the analysis was thus concentrated on dental features, more specifically on the first molar. For other mammalian groups, characters such as the shape and size of teeth, claws and bones were included.

The importance of each type of prey was analyzed based on its absolute frequency (the percentage of scats in which the item was found) as well as its relative frequency (the percentage of occurrence of the item relative to the total number of items). We calculated the food niche breadth for each of the identified carnivore species using the standardized Levins index (Krebs, 1998), given as: $B_A=(B-1)/(n-1)$ where B is Levins's index ($B= 1/\sum p_i^2$), where p_i is the relative frequency of each item) and n is the number of dietary items. Values approaching 1.0 indicate that the resources are used in similar frequencies, while a diet concentrated on some of the items will generate B_A values close to 0.

We also calculated the dietary niche overlap between the two species for which the sample size was largest (the felids *Leopardus tigrinus* and *Puma yagouaroundi*), using Pianka's (1973) index:

$O_{jk}=\Sigma p_{ij}\ p_{ik}/(\Sigma p^2_{ij}\ \Sigma p^2_{ik})^{½}$, where p_i is the proportion of item i in the diet of the species j and k, respectively (Pianka, 1973). This index ranges from 0 (no overlap) to 1 (complete overlap). To evaluate if the estimated niche overlap could have arisen by chance, we tested for statistical significance of the calculated value by comparing it to a null distribution generated by randomizing the original data matrix (1,000 iterations) using the program EcoSim (Gotelli & Entsminger, 2007).

RESULTS

In the course of 11 field trips we traversed a total of 115 km of dirt roads and forest trails, averaging 8 hours or 5 km per day, with a mean of 9 scat samples found per survey (Figure 1). All samples selected for collection and analysis were categorized as "recent" if they were very fresh (*i.e.* deposited in the previous 1-2 days) or "old" if they were drier and bearing initial signs of fungal growth (*i.e.* likely deposited in the previous 3-7 days). All faeces that appeared to be much older than this latter standard (*i.e.* extremely dry, exhibiting severe fungal growth, or seemingly devoid of fresh organic matter) were not collected, and neither were those that had been severely rain-washed. Overall, a total of 102 faeces were collected, most of which (49%) were found during the Spring season (September-November - Figure 2).

DNA extraction was performed for all 102 collected scats, and for 98% of them (N=100) the target mtDNA segment could be successfully amplified (this included "old" scats having experienced modest rain-wash and/or showing some fungal cover). High quality DNA sequences could be obtained from 73 of these samples, in every case allowing clear species-level identification (Figure 3 and Table 1). Of these 73 samples, two were identified as having been produced by a domestic dog (*Canis familiaris*), 15 by a crab-eating fox (*Cerdocyon thous*) and 56 by wild felids (eight *Puma concolor*, 17 *Leopardus tigrinus* and 31 *Puma yagouaroundi*).

Overhairs were found in 57 of the 102 scats (56%) collected in the field. Microscopic analysis of these hairs identified 32 samples at species level (four felids, see below), 24 only at family level (either Canidae or Felidae), and one was left as unidentified as it presented unusual morphological features (this sample was subsequently found to originate from a domestic dog on the basis of the DNA evidence). Microscopic analyses of medullar and cuticular patterns of the hairs collected in museum collections showed that each of the investigated species has a unique pattern that can provide diagnostic characters, with the exception of the foxes *Lycalopex gymnocercus* and *C. thous*. These two canid species appear to have essentially identical patterns, precluding their discrimination with the hair

micro-structural characters surveyed in this study. This is a relevant limitation, as both canids are known to occur at the study site, and their species-level discrimination is an important requirement for detailed ecological studies in the area. The domestic dog hairs analyzed here were quite variable, and could not be conclusively distinguished from the two wild canids mentioned above. Therefore the identification of any canid sample could only be confidently achieved at the family level using the hair microscopy approach (Table 1). As for the felids, there was a single sample identified as *Leopardus wiedii*, which was subsequently found to have a discrepant identification based on the DNA method (see below) and was thus excluded from all downstream analyses (e.g. dietary investigation). The remaining samples identified at species level using the trichological approach were all assigned to *P. concolor*, *L. tigrinus* or *P. yagouaroundi* (Table 1). Figures 4 and 5 show the micro-structural patterns of medulla and cuticle of the four carnivore species identified in this study (considering the DNA-based identification of *C. thous* for some of the analyzed samples).

Forty-four samples could be identified using both methods, which allowed an assessment of their congruence, and a comparison of their performance. There were only three cases of clear identification discrepancy between the two methods, one of them involving the felids *Leopardus tigrinus* (DNA) *vs. L. wiedii* (hair), the second consisting of *L. tigrinus* (DNA) *vs. P. concolor* (hair), and the third being *P. yagouaroundi* (DNA) *vs.* Canidae (hair). These three samples showing evidence of identification disagreement were eliminated from all subsequent analyses (*e.g.* dietary investigation). For the remaining 41 samples, both methods agreed in the carnivore identification at the family level. However, 20 of these samples (eight canids and 12 felids) could only be identified at species level using the DNA-based approach. The two domestic dog samples contained guard hairs, which in one case were inconclusive and in the second led to family-level identification as a canid.

Given the observation that the two methods were largely congruent, and that few instances of discrepancy were observed, we pooled the samples identified by both methods to perform a dietary analysis of the carnivore species surveyed in this study. A total of 77 samples (excluding the domestic dog scats) could be identified at species level by at least one of the methods, allowing for a comparison of the food habits of four carnivore species (Table 2).

The most common food item found in the scats samples of *C. thous* was insects, followed by rodents (see Table 2). *L. tigrinus* exhibited the most restricted diet, based very strongly on small mammals (*ca.* 90% of the total items), particularly rodents. *P. concolor* exhibited a varied diet composed predominantly of vertebrates (including mostly mammals but also a bird and an amphibian),

and *P. yagouaroundi* also presented a rather generalist feeding ecology, with over 50% of the food items consisting of mammals, but other components including various fruits and insects.

The estimated food niche breadth (B) varied among species, with *L. tigrinus* found to have the lowest index (3.7) and *P. concolor* the highest (10.3). The calculated value for *C. thous* was also quite high (9.3), and an intermediate value was observed for *P. yagouaroundi* (6.8). The standardized breadth index (B_A) also indicated a broad dietary niche for *P. concolor* (1.04) and *C. thous* (0.93), with lower values estimated for *L. tigrinus* (0.68) and *P. yagouaroundi* (0.44).

Finally, we performed a more detailed analysis focusing on the two species that presented the narrowest dietary niche (*i.e.* identified as those with the most specialized food habits), the small cats *Leopardus tigrinus* and *Puma yagouaroundi*. These were also the two species with the largest sample size (combined n=52), allowing for a more reliable comparison than any other pair of surveyed species. An overall assessment indicated that their diet is similar (Pianka's overlap index= 0.938), with both species relying heavily on small mammals (Table 2). However, this overlap was found not to be homogeneous throughout the year, and a seasonal pattern of variation emerged upon further scrutiny (Figure 6). The overlap index was lowest in the Fall (0.433) and greatest in the Winter (0.988). Overall, it could be observed that *L. tigrinus* seems to be more specialized in small mammals, particularly rodents (a pattern which is maintained throughout the year), while *P. yagouaroundi* seasonally complements this staple resource with insects and fruits.

DISCUSSION

The success rate of mtDNA amplification from our faecal samples was quite high, above the levels reported in several previous studies (*e.g.* Davison *et al.*, 2002; Prugh & Ritland, 2005; Prugh *et al.*, 2005; Smith *et al.*, 2006; Miotto *et al.*, 2007). DNA sequences could be obtained from most of these amplified fragments, but only those considered to be high quality (n=70) were used in the present study. This implies that further effort on repeated attempts at amplification and sequencing of these fragments would yield even higher success rates at obtaining reliable DNA sequences from these samples. The success rate observed in this study did not seem to be affected by the scat age category, weather conditions or deposition site, contrary to reports in previous studies identifying such factors as influential for amplification success. It is possible that the field-based triage which excluded the oldest and most decomposed scats was sufficient to lead to collection of only faecal samples still containing suitable DNA for analysis.

The number of scat samples identified with the hair microscopy approach was lower than that achieved with the DNA-based method. So far, few papers have reported the success rate of employing microscopic analysis of hairs found in faeces to identify carnivore species, which limits the comparative assessment of our results relative to other studies. Wachter, Jauernig & Breitenmoser (2006), studying the diet of *Acinonyx jubatus*, found that 33% of the scats contained hairs ingested by auto-grooming. In another study, Garla, Setz & Gobbi (2001) found only 17.8% of the faecal samples containing hairs ingested by *Panthera onca* during its grooming. These lower results might explain the preference for microscopic analysis from hairs collected in fur traps (Lynch, Brown & Rochford, 2006; Depue & Ben-David, 2007) or shelters (Cowell *et al.*, 2001), as opposed to obtaining them from scats, when such approaches are adequate to address the ecological problem at hand.

Therefore, the ability to isolate the predator's own hairs from among a much larger amount of prey remains (usually composed mostly of fur), seems to be a substantial limitation of this approach. In addition, the grooming behavior may differ among carnivore species or even for a single species across seasons, possibly leading to fluctuations in the number of predator hairs found in scats. Another obstacle to the complete success of carnivore identification using hair microscopy can be the quality of the guard hair itself. In some cases, a missing portion of the shaft may bias the species-level identification, if the standardized cuticle pattern occurs only in that specific region of the hair. Moreover, we observed that some hairs exhibited differences along the shaft, such as the dimension and/or shape of scales, possibly misleading the identification based on cuticular patterns. In this study, all the samples exhibiting such problems were identified only at the family level. On a positive note, there were several cases of complete congruence between the hair- and DNA-based identifications (*i.e.* for the three felids identified in the area), indicating that this method does have a good potential for accurate identification in the case of species that consistently exhibit diagnostic characters.

It is relevant to attempt to understand the causes of the observed cases of identification discrepancy between the two methods. Although hairs are known to be very resistant to various extrinsic disturbances, it cannot be ruled out that some forces might occasionally alter their cuticular pattern. One of the three samples analyzed here that showed a discrepancy between the two identification methods might have been caused by such a process. The DNA-based method identified this sample as belonging to *L. tigrinus*, but the hair-based analysis positively identified the *L. wiedii* cuticle pattern. The cuticle patterns of these species differ only in the shape but not in the size of the scales. *Leopardus wiedii* has a narrow petal pattern, while *L. tigrinus* has a narrow diamond petal

pattern (Teerink, 1991). Therefore, it is possible that the cuticle suffered mechanical friction which could have made the scale edges prominent, misleading the hair-based identification in this case. Further investigation using controlled comparisons would be important to verify if this situation can indeed occur, and how relevant it is in the context of field-based identifications.

The two other cases of identification discrepancy could not be easily attributed to any natural cause. The most likely explanations would involve cross-contamination among samples, which could have occurred either with the spurious inclusion of extrinsic hairs in a sample while it was being processed, or via undetected contamination in the DNA assays (no contamination was observed in the negative controls). The rigorous monitoring of the error rate of both approaches should be an important component of all studies employing carnivore faecal analysis, but this is difficult to accomplish when only a single method is applied. It may thus be interesting to consider employing both methods for at least a sub-sample of the analyzed faeces as a means of independently assessing the identification error rate.

A peculiar case of predator identification involved a sample for which there was no discrepancy between the two methods. The sample was identified by the DNA-based analysis as having been produced by a *Cerdocyon thous*, and as a canid by the hair microscopy approach. However, the amount of *C. thous* (*i.e.* canid) hairs present in the faecal sample was much larger than is usually found for the predator as ingested by self-grooming; this suggests that *C. thous* was more likely to have been a prey than the predator in this case, which would not have been distinguished by the DNA assay. In addition, the food item analysis revealed the presence of three molar teeth belonging to a young coati (*Nasua nasua*). A plausible albeit unlikely inference here is that the sample belongs to a young *N. nasua* that might have fed on a fox carcass and also swallowed its own teeth. This sample was excluded from the dietary analysis due to this uncertain provenance, but the case illustrates the complexity of performing a reliable identification of the predator when the prey is also a carnivore.

Our dietary analysis results are consistent with some aspects previously reported on the feeding ecology of the investigated felid species (Oliveira, 1994; Wang, 2002; Moreno, Kays & Samudio, 2006). Surprisingly, despite the knowledge that *P. yagouaroundi* is a generalist that includes fruits and insects in its diet, in this study the dietary analysis shows the large importance of these items in the diet of this species during the Fall season. We expected to find this kind of pattern in the diet of *C. thous*, a species that is known to be more of a feeding generalist. Although numerous studies demonstrate the importance of fruits and rodents in this species' diet (Motta-Junior, Lombardi &

Talamoni, 1994; Juarez & Marinho-Filho, 2002; Gatti *et al.*, 2006), we found a greater presence of insects, some rodents and an insignificant proportion of fruits in its diet.

Another unexpected result was the presence of several intact ticks (Arachnida: Ixodida) in the samples. Several studies investigating the feeding ecology of carnivores have not detected any evidence of ticks in the scats (e.g. Azevedo *et al.*, 2006; Gatti, 2006; Moreno *et al.*, 2006; Vieira & Port, 2006; Walker *et al.* 2007). According to Rosalino *et al*. (2007), two possible origins for the collected ticks can be hypothesized: they could be the result of self-grooming behavior, which is common among several mammals (Seyfarth, 1977; Patenaude & Bovet, 1984; Wilkinson, 1986; Schino, 1988; Mooring, Blumstein & Stoner, 2004) including carnivores (Eckstein & Hart, 2000) or they could have been ingested as prey ecto-parasites. Assuming that ticks ingested with prey might be found more intact in scats than those groomed (since carnivores usually ingest partly intact portions of the prey through large bites - Bicknevicius & Valkenburgh, 1996), the unaltered appearance of most of the identified ticks suggest that they originate in the prey, and not in the predator itself. All the scat samples containing ticks also contained rodents, which may be an interesting indication that these mammals might be the source of these ecto-parasites, and that the latter are perhaps particularly abundant in the area.

In this study, the number of identified carnivore species was smaller than that expected given the known carnivore community occurring in the study area. We found tracks of *Procyon cancrivorus* and *Eira barbara* in the area, and *Nasua nasua* was detected as a food item (Table 2), but none of these species was identified among the carnivores producing the faecal samples analyzed here. In addition, the fox *Lycalopex gymnocercus* has been directly observed in the area several years ago, and a camera-trapping study has documented the presence of *N. nasua*, *P. cancrivorus*, *Leopardus pardalis* and *L. wiedii* (Cerveira, 2005). Our results may be influenced by the fact that in some cases only large carnivores defecate along trails or territorial boundaries (Macdonald, 1980). Additional hypotheses are that these species avoid trails due to their movement behavior (*N. nasua*), potential dog attacks or habitat preferences (*P. cancrivorus*), or use of different territorial marking sites (*e.g.* on trees, such as perhaps *L. wiedii* and as is known to occur in *L. geoffroyi* [Johnson & Franklin, 1991]). In the case of *E. barbara* and *L. pardalis*, it is possible that their densities are low in the area, so that scat deposition along the trails by these species may be sporadic. In the case of the fox *L. gymnocercus*, the absence of detected scat samples may be due to presence of the likely competitor *C. thous*, which is known to be more of a habitat generalist (Juarez & Marinho-Filho, 2002; Vieira & Port, 2006), as well as the observed reduction of grassland habitat in the area (Pillar, 2003; Behling et al., 2004; Oliveira & Pillar,

2004). As *L. gymnocercus* seems to be more restricted to open habitats (García & Kittlein, 2005; Vieira & Port, 2006), it may be currently absent or occur at lower densities in the surveyed areas (see Figure 1).

The identification of carnivore scats using hair microscopy was found to be simple, quick and inexpensive, in some cases requiring only one hair per sample. However, according to González-Esteban *et al*. (2006) and the results reported here, this method requires guard hairs that are well-formed and complete for a successful application. In addition, there are two types of guard hairs, the primary and secondary hairs (Teerink, 1991). The latter type displays a somewhat homogeneous hair cuticle pattern throughout the hair shaft in all mammalian groups (Meyer, Schnapper & Hülmann, 2002) which could become a problem when species identification of hairs found in scats is aimed. Moreover, according to Noback (1951), the structure may differ significantly at different developmental stages of the same individual hair. These morphological features might influence the identification power of hair microscopy because the guard hairs found in the scats could be of any type or age, and the researcher might not be able to distinguish them, thus proceeding with the downstream analyses based on an incorrect interpretation of the patterns observed.

Regarding the molecular analysis, routine DNA identification from faeces might be costly and technically demanding, as DNA is known to be considerably degraded in this type of material, making it advisable for these studies to use only relatively fresh samples (which is not the case when employing the hair-based method). However, faecal samples represent an easy way of non-invasive sampling, potentially making a large number of individuals available for analysis. The mitochondrial DNA is extremely useful for these studies, and as the results presented here demonstrate that it can be successfully applied in relatively old and lightly rain-washed samples.

The possibility of reliably identifying carnivore species employing non-invasive methods opens up new research avenues in the area of carnivore field ecology, and more rigorous and efficient approaches should be constantly sought and compared. Our results indicate that the DNA-based method applied here was superior to the hair microscopy approach, although the latter did identify samples that the former could not, and there was substantial congruence between them in several cases. It will therefore be interesting to further investigate the strengths and limitations of both approaches in additional ecological settings, spanning a broader range of field conditions and sampling different carnivore species, so as to identify cases in which the use of one or both is particularly recommended. The combination of the resolving power of the DNA-based approach with the low cost

and simple requirements of the trichological method may lead to a future strategy that integrates both tools, allowing for greater reliability and effectiveness of carnivore identifications in the field.

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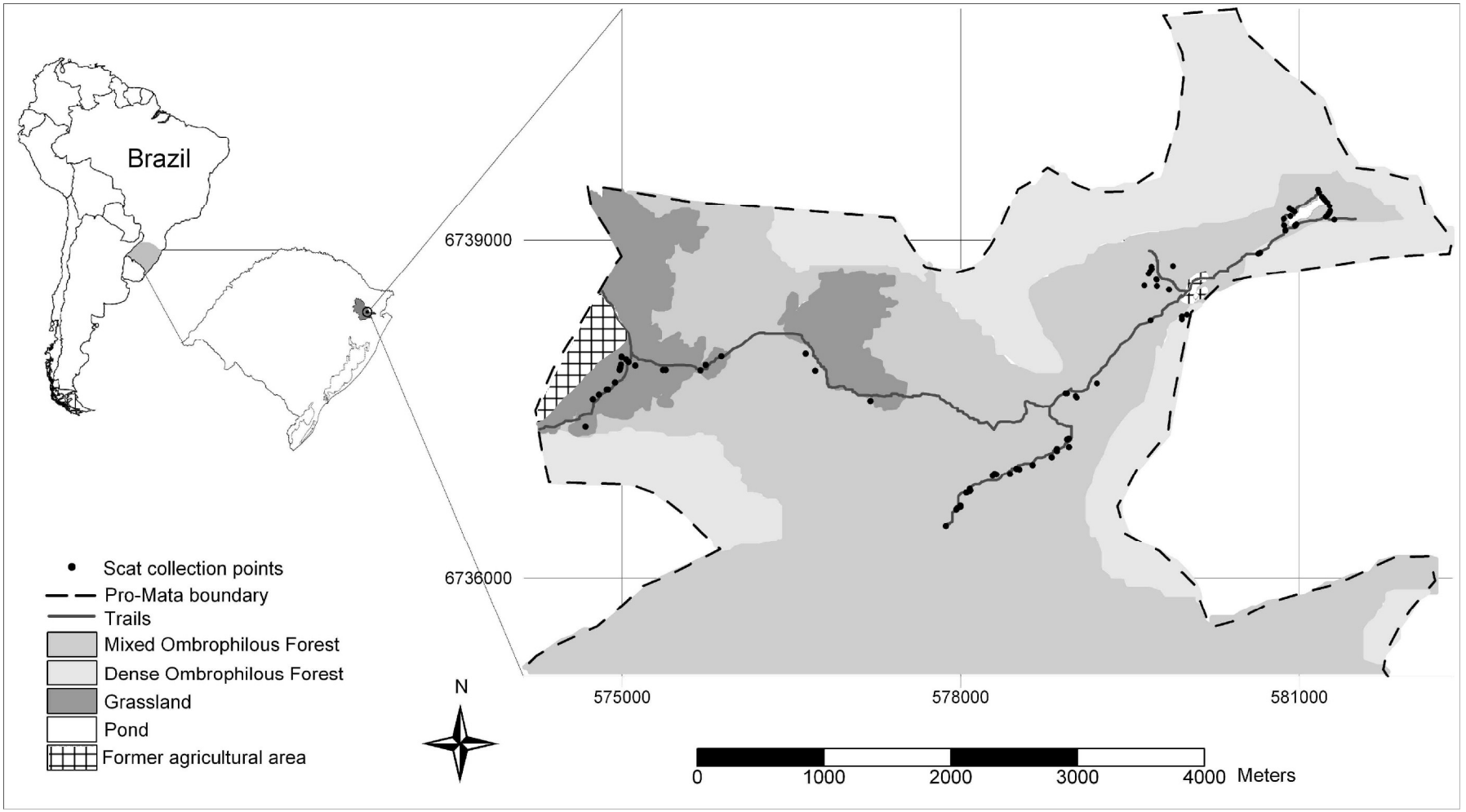

Figure 1. Map of the study area showing the different vegetation landscapes occurring at the Pro-Mata RC, São Francisco de Paula Municipality, Rio Grande do Sul State, Brazil, with a depiction of scat collection points.

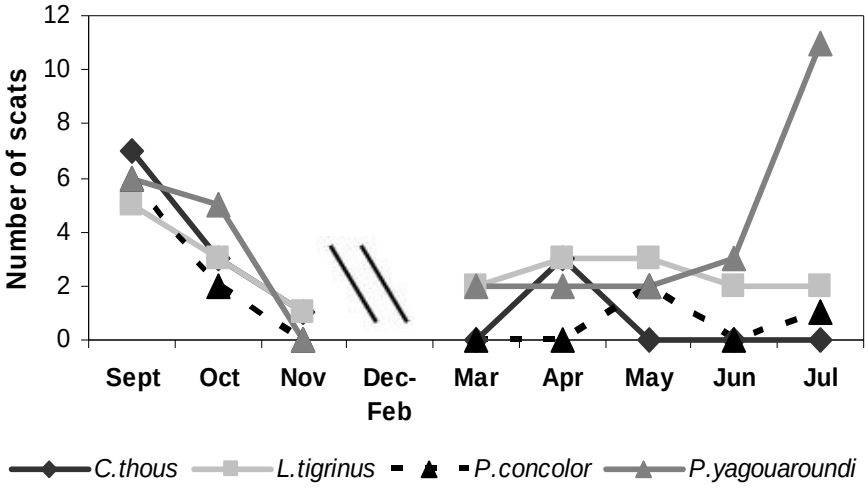

Figure 2. Total number of scats collected at Pro-Mata RC, São Francisco de Paula Municipality, Rio Grande do Sul State, each month over one year of field work. Values presented here excluded December-February due to the limited sample size.

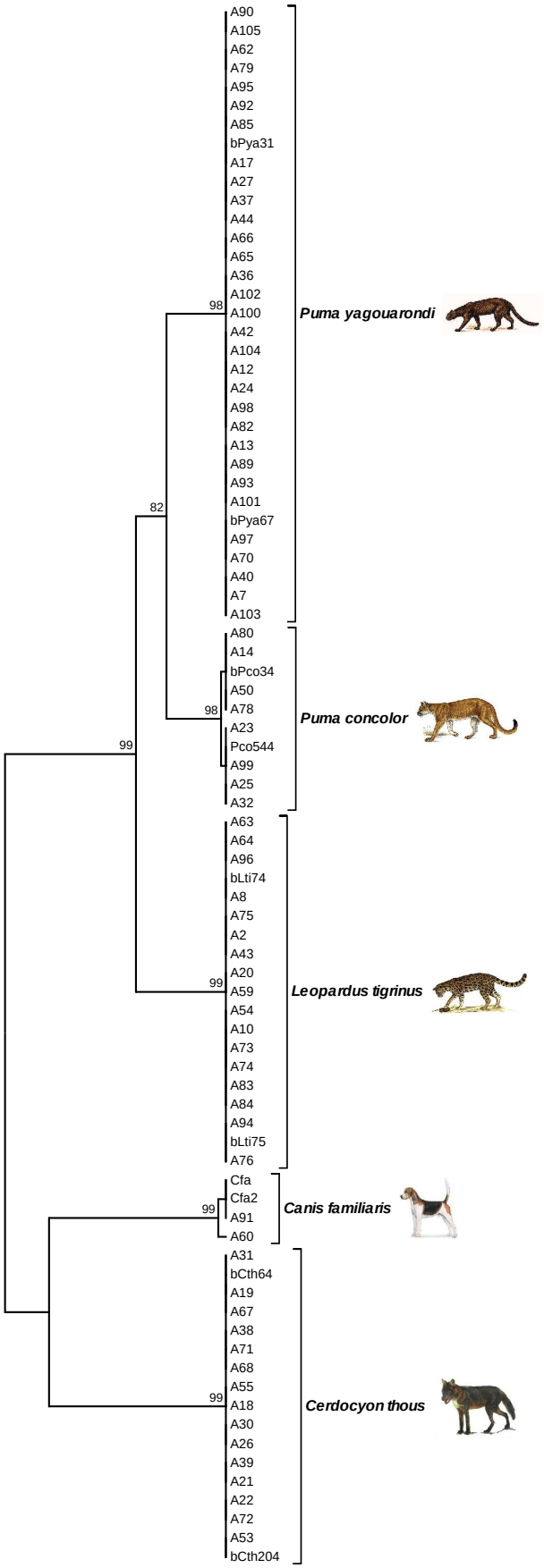

Figure 3. Phylogenetic tree of the mtDNA *ATP6* gene segment inferred with the UPGMA method. Sequences labeled A2-A105 are derived from faecal samples collected in the Pro-Mata RC. Sequences derived from voucher specimens are also included (bPya- *P. yagouaroundi*; bPco- *P. concolor*; bLti- *L. tigrinus*; Cfa- *C. familiaris*; bCth- *C. thous*). Numbers above branches are bootstrap values based on 1,000 replications. Carnivore pictures were modified from Biblioteca Virtual Luis Ángel Arango (2007) and Iwokrama International Centre for Rain Forest Conservation and Development (1999).

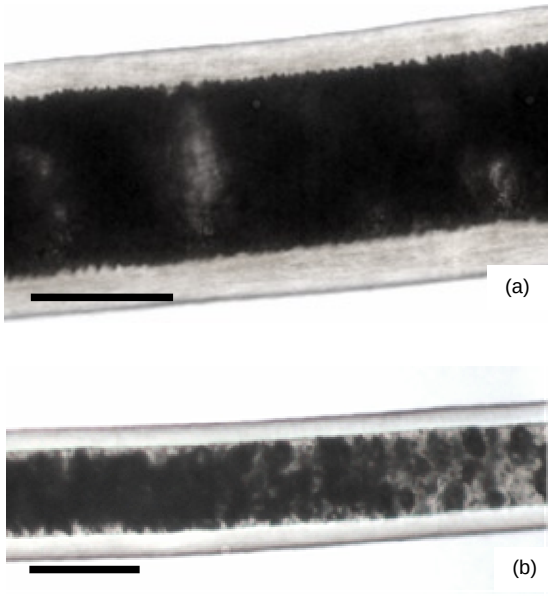

Figure 4. Medullar pattern of reference carnivore guard hairs (shield region) enclosed in synthetic balsam: (a) Felidae: reversed cloisonné with fringed margins; (b) Canidae: anisocellic. The hair pattern nomenclature shown in (a) is based on Teerink (1991), while that shown in (b) is based on Quadros & Monteiro- Filho, (2006a). Scale bars = 70 µm.

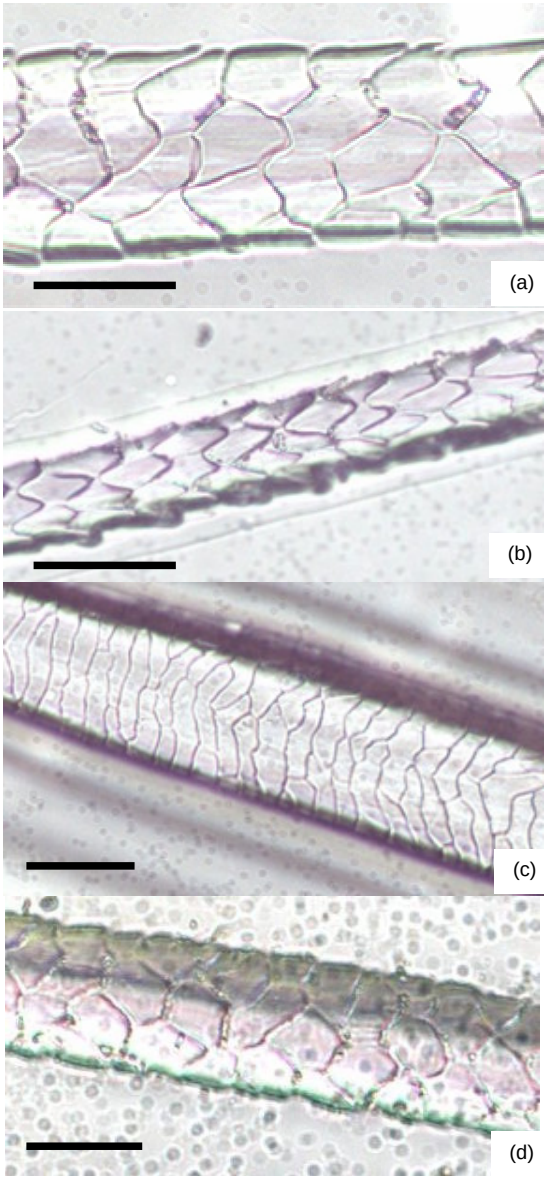

Figure 5. Cuticular pattern of reference carnivore guard hairs (shaft region), printed on nail varnish: (a) medium diamond petal pattern of *Cerdocyon thous*; (b) narrow diamond petal pattern of *Leopardus tigrinus*; (c) regular wave shape of *Puma concolor*; (d) large diamond petal pattern of *Puma yagouaroundi*. Hair pattern nomenclature is based on Teerink (1991). Scale bars = 70 µm.

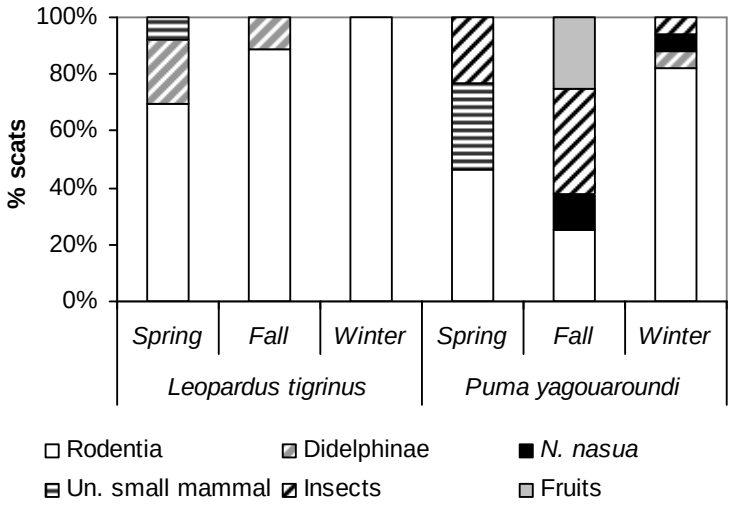

Figure 6. Relative frequency of food items found in 52 faecal samples of two small Neotropical cats (*L. tigrinus*, N=21; and *P. yagouaroundi*, N=31) in Pro-Mata RC, Rio Grande do Sul state, southern Brazil. Values presented here are derived from all samples grouped per season (except for the Summer, which was excluded from the analysis due to the small available sample size). Ticks and unidentified vegetable matter were excluded from this analysis, as they were not considered to be nutritional items.

Table 1. Success rate of two methods of species identification (light microscopy of guard hairs and mtDNA sequencing) applied to 102 carnivore faecal samples collected at Pro-Mata RC, southern Brazil. Three samples whose identification was discrepant between the two methods were excluded from this comparison (see text for details).

	Identification method	
Family- or Species-level identification	**Hair** (*n*= 54)	**DNA** (*n*= 70)
Canidae	18.5%	24.3%
Canis familiaris	--	3.0%
Cerdocyon thous	--	21.3%
Felidae	81.5%	75.7%
Leopardus tigrinus	18.5%	20.0%
Puma concolor	16.7%	11.4%
Puma yagouaroundi	20.4%	44.3%

Table 2. Food items found in 77 faecal samples from carnivore species at Pro-Mata RC, São Francisco de Paula Municipality, Rio Grande do Sul state, southern Brazil.

	Cerdocyon thous		*Leopardus tigrinus*		*Puma concolor*		*Puma yagouaroundi*	
	% scats	% items	% scats	% items	% scats	% items	% scats	% items
Food items	(*n*=14)	(*n*=31)	(*n*=21)	(*n*=30)	(*n*=11)	(*n*=17)	(*n*=31)	(*n*=52)
Mammals								
RODENTIA								
Sigmodontinae								
Un. small size sp. [a]	35.7	16.1	57.1	40.0	36.4	23.5	58.1	34.6
Un. medium size sp. [b]	7.1	3.2	43.0	30.0			16.1	9.6
Echimyinae								
Phyllomys sp.	7.1	3.2						
Erethyzontinae								
Sphiggurus villosus					9.1	5.9		
Un. small size rodent [a]	21.4	9.7	4.8	3.3	9.1	5.9	6.4	3.8
DIDELPHIMORPHIA								
Didelphinae			19.0	13.3			3.2	1.9
PROCYONIDAE								
Nasua nasua					18.2	11.8	6.4	3.8
Un. small size mammal [c]	28.6	13.0	4.8	3.3	9.1	5.9	9.7	5.7
Un. medium size mammal [d]					9.1	5.9		
Birds					9.1	5.9		
Amphibians					9.1	5.9		
Insects								
Coleoptera	42.9	19.3			9.1	5.9	12.9	7.7
Orthoptera	21.4	9.7					6.4	3.8
Hymenoptera	7.1	3.2			9.1	5.9		
Un. larvae	14.3	6.4					3.2	1.9
Arachnids								
Ixodida	7.1	3.2	9.5	6.7	9.1	5.9	6.4	3.8
Fruits								
Arecaceae								
Syagarus romanzoffiana							3.2	1.9
Ebenaceae								
Diospyros sp.	7.1	3.2					6.4	3.8
Myrtaceae								
Feijoa sellowiana							6.4	3.8
Rosaceae								
Rubus sp.							3.2	1.9
Un. fruit							3.2	1.9
Vegetable matter	21.4	9.7	4.8	3.3	18.2	11.8	16.1	9.6

* % scats, percentage of total of scats in which each item was found; % items, percentage of occurrence of each item in relation to the total number of items. Un., unidentified.

[a] Small size rodent, mean weight $\leq$ 30 g. Sigmodontinae prey in this range likely belongs to the genus *Oligoryzomys* (25g) or *Akodon* (30g).

[b] Medium size rodent, mean weight > 30 g. Sigmodontinae prey in this range likely belongs to the genus *Holochilus* (100g).

[c] Small size mammal, mean weight < 500 g. Mammals in this size range likely belongs to the groups Rodentia or Didelphimorphia.

[d] Medium size mammal, mean weight $\geq$ 500 g. The appearance of fragments suggests that these prey items may belong to the genus *Dasypus.*