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PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

FACULDADE DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA

CÓDIGO DE BARRA DE DNA DE MAMÍFEROS NEOTROPICAIS, E SUA APLICAÇÃO EM ESTUDOS ECOLÓGICOS DE CARNÍVOROS

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2010

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PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
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DISSERTAÇÃO DE MESTRADO
PORTO ALEGRE – ERASIL
2010

AGRADECIMENTOS

Ao Duda, meu orientador e amigo por todo apoio, paciência e confiança desde a época da graduação, obrigado por tudo.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior pela bolsa de mestrado.

Aos colegas do Genoma por toda a amizade, discussões, conselhos e risadas. Em especial à Marina O. Favarini, Tatiane Trigo, Flávia P. Tirelli, Mirian T.N.T. Jerep.

Aos amigos de jornada com quem dividi felicidades e angústias Talita Pereira, Fernando Dalmas, Andréa Thomaz e Fernanda Pedone

À Cladinara Roberts Sarturi pelo apoio, companheirismo, amizade, ensinamentos e até pelos puxões de orelha nas horas certas.

Ao pesquisadores que colaboraram para execução deste trabalho, em especial, Pedro C. Estrela, Lívia Müller e o prof. Thales R.O. de Freitas.

Aos meus amigos de longa data pela compreensão e compaheirismo: Guilherme D. Rodrigues, Karen Rosa, Renata Perez e Alice Tagliani.

À minha mãe que por toda a compreensão, carinho e amizade e que apesar de não entender bem o que eu faço sempre esteve presente e me apoiou incondicionalmente.

SUMÁRIO

Resumo

Desde 2003 o uso de um marcador molecular para identificação de espécies surgiu como uma solução plausível para uma grande variedade de pesquisas da biodiversidade. Na literatura, o número que trabalhos que conseguiu observar padrões de diversidade desconhecidos aumenta cada vez mais. O presente estudo propõe a utilização de uma abordagem molecular para a identificação de espécies de roedores encontradas no trato digestivo de quatro espécies de gatos selvagens neotropicais (*Leopardus tigrinus*, *L. geoffroyi*, *L. wiedii* e *Puma yagouaroundi*), e realizar uma comparação entre os hábitos alimentares de *L. tigrinus* e *L. geoffroyi*. Fomos capazes de identificar 59% das nossas amostras em nível de espécie, e separar os itens não identificados em agrupamentos, através de uma análise de distância, que tornou possível evidenciar a diversidade de espécies amostradas, mesmo sem uma identificação taxonômica precisa. Os táxons identificados foram *Cavia magna*, *Mus musculus*, *Sooretamys angouya*, *Oligoryzomys nigripes*, *Rattus rattus*, *Oxymycterus quaestor*, *Oxymycterus* sp., *Akodon paranaensis* e *Akodon azarae*. Além disso, cinco outros agrupamentos foram identificados, não correspondendo a qualquer das espécies amostradas. Estes grupos provavelmente correspondem a táxons de nível específico, possivelmente não descritos ou não reconhecidos como ocorrentes na região investigada. Este resultado salienta o potencial da aplicação desta técnica ao conteúdo da dieta de predadores para inventariar a diversidade de suas presas, inclusive com a possibilidade de descoberta de formas ainda desconhecidas. A análise ecológica comparativa de *L. tigrinus* e *L. geoffroyi* evidenciou um forte padrão de especialização da dieta para ambas as espécies e uma fraca sobreposição de nicho entre elas. Tendo em vista que a identificação detalhada de presas destas espécies tem se mostrado historicamente muito difícil, com base em métodos tradicionais, os resultados aqui obtidos ilustram a utilidade deste método para que se possa efetuar uma comparação aprofundada da dieta destes predadores, viabilizando uma melhor compreensão de sua interface ecológica em áreas de simpatria no sul do Brasil.

Abstract

Since 2003 the use of a standardized molecular marker for species identification has emerged as a practical solution for a great variety of biodiversity surveys and other applications. In the, literature the list of examples of hidden diversity uncovered through this technique increases continuously. The present study proposes the use of a molecular approach for species identification of dietary contents of four species of Neotropical wild cats (*Leopardus tigrinus*, *L. geoffroyi*, *L. wiedii* and *Puma yagouaroundi*), and includes a more detailed comparison of the food habits of *L. tigrinus* and *L. geoffroyi*. We were able to identify 59% of our samples at species level, and to classify the unidentified ones into well-supported clusters, which revealed the likely diversity of species sampled in the area, even without proper taxonomic identification. The identified taxa were *Cavia magna*, *Mus musculus*, *Sooretamys angouya*, *Oligoryzomys nigripes, Rattus rattus, Oxymycterus quaestor, Oxymycterus* sp*., Akodon paranaensis* and *Akodon azarae*. Five other clusters were identified which did not match any of the reference species in our database. These groups likely correspond to species-level taxa that are either undescribed or not known to occur in the sampled region. This result highlights the potential for application of this technique to the dietary content of predators to inventory the diversity of their prey, including the possibility of discovering currently unknown taxa. The comparative ecological analysis of *L. geoffroyi* and *L. tigrinus* showed a strong pattern of dietary specialization for both species and a weak niche overlap between them. Considering that the detailed identification of prey items from these species has been historically difficult on the basis of traditional methods, the results obtained here illustrate the usefulness of this approach to perform a more in-depth comparision of the diet of thse felids, making it possible to gain a better understanding of their ecological interface in areas of sympatry in southern Brazil.

APRESENTAÇÃO

O presente trabalho intitulado “Código de Barra de DNA de mamíferos neotropicais, e sua aplicação em estudos ecológicos de carnívoros” foi desenvolvido como parte dos requisitos necessários para 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 (i) avaliar o potencial do segmento ‘barcode’ do gene mitocondrial *COI* para efetuar uma identificação confiável em nível de espécie de pequenos mamíferos, especialmente roedores; e (ii) testar a eficácia deste marcador para a aplicação em estudos ecológicos de carnívoros, com foco na identificação de suas presas, especialmente no caso da ingestão de roedores.

Esta dissertação é apresentada no formato de artigo científico sobre a identificação de itens alimentares de quatro espécies de felinos neotropicais e análises ecológicas subsequentes, a ser submetido ao periódico *Proceedings of the Royal Society B*.

Cats find mice: Hidden diversity of rodents revealed by DNA barcoding of prey items of wild cats in southern Brazil

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Summary

Since 2003 the use of a standardized molecular marker for species identification has emerged as a practical solution for a great variety of biodiversity surveys and other applications. In the, literature the list of examples of hidden diversity uncovered through this technique increases continuously. The present study proposes the use of a molecular approach for species identification of dietary contents of four species of Neotropical wild cats (*Leopardus tigrinus*, *L. geoffroyi*, *L. wiedii* and *Puma yagouaroundi*), and includes a more detailed comparison of the food habits of *L. tigrinus* and *L. geoffroyi*. We were able to identify 59% of our samples at species level, and to classify the unidentified ones into well-supported clusters, which revealed the likely diversity of species sampled in the area, even without proper taxonomic identification. The identified taxa were *Cavia magna*, *Mus musculus*, *Sooretamys angouya*, *Oligoryzomys nigripes, Rattus rattus, Oxymycterus quaestor, Oxymycterus* sp*., Akodon paranaensis* and *Akodon azarae*. Five other clusters were identified which did not match any of the reference species in our database. These groups likely correspond to species-level taxa that are either undescribed or not known to occur in the sampled region. This result highlights the potential for application of this technique to the dietary content of predators to inventory the diversity of their prey, including the possibility of discovering currently unknown taxa. The comparative ecological analysis of *L. geoffroyi* and *L. tigrinus* showed a strong pattern of dietary specialization for both species and a weak niche overlap between them. Considering that the detailed identification of prey items from these species has been historically difficult on the basis of traditional methods, the results obtained here illustrate the usefulness of this approach to perform a more in-depth comparision of the diet of thse felids, making it possible to gain a better understanding of their ecological interface in areas of sympatry in southern Brazil.

Keywords: *Leopardus tigrinus*, *Leopardus geoffroyi*, *Leopardus wiedii*, *Puma yagouroundi*, Neotropical, DNA barcodes.

1. Introduction

In the last few years, the DNA barcoding approach has emerged as a large-scale initiative proposing the use of standardized molecular markers for species-level identification of living organisms (Borisenko *et al.* 2009). In taxonomy, DNA barcoding can play an important role in species surveys and the discovery of hidden diversity (Hebert *et al.* 2004). The presence of unidentified clusters in a biodiversity inventory could flag the occurrence of undescribed species or, at least, of taxa that require better sampling (Papadopoulou *et al.* 2008). The use of a standard molecular marker can accelerate the discovery of new species, aiding traditional taxonomy in the description and directed investigation of previously unknown life forms (Hebert *et al.* 2003). It has become widely apparent that this technique can help improve the analysis in other research areas that depend on taxonomy, such as forensics, ecology and medicine (Teletchea *et al.* 2005).

Ecology is one of the research fields in which this technique can be most useful. Valentini *et al.* (2008) reviewed the subject and listed several approaches that could benefit from more precise answers provided by DNA barcoding; most of them were related to non-invasive sampling, such as the identification of predators using scats (e.g. Napolitano *et al.* 2008, Haag *et al.* 2009). Another potential field of application pertains to the study of dietary contents of various species, whose precise identification would benefit not only trophic analyses but could also allow improved ecological surveys with respect to the prey item groups. There are several reported cases of insect groups that had been previously described as generalists and, after a DNA-based analysis, were shown to possess complex dietary relationships (see Bickford *et al.* [2006] for examples).

Since the proposition of the DNA barcoding approach (Hebert *et al.* 2003), some studies have employed this method to identify dietary contents of vertebrates as well. Deagle *et al.* (2007) analyzed the diet of Macaroni Penguins through the molecular identification of prey obtained from fecal material; the same approach was employed by other authors in other taxonomic groups, such as bats (Clare *et al.* 2009) and seals (Deagle *et al.* 2009), and also in the identification of plant content in the diet of rodents (Soininen *et al.* 2009).

Dietary studies of carnivore species are relatively common, as they can be performed on the basis of faecal sampling (e.g. Wang 2002, Manfredi *et al.* 2004) or stomach content analysis (e.g. Novaro *et al.* 2000). Several studies have been performed on the food habits of Neotropical carnivores, most of which used fecal material (see Abreu *et al.* 2007; Martins *et al.* 2008; Tófoli *et al.* 2009). A problem that frequently emerges in these studies is the difficulty of identifying prey items at species level, due to the fragmentary or degraded nature of the materials recovered from these sources. For example, Abreu *et al.* (2007) studied the feeding habits of *L. pardalis* and observed a high level of predation on small prey (<1kg), but from these items they could not identify 25% of the sampled mass.

Felids play an important role in most Neotropical mammalian communities. With ten extant species, they occupy a wide variety of habitats from dense forest formations to open fields, from sea-level to high altitudes (Nowak *et al.*1999). The group includes top predators (*e.g. Panthera onca* and *Puma concolor*), meso-predators (*e.g. Leopardus pardalis*, *Puma yagouaroundi*) and also small representatives (*e.g. L. tigrinus*, *L. guigna*), whose trophic interactions are still poorly understood. It is likely that trophic niche overlap and inter-specific competition play relevant roles in the dynamics of these communities, as has

already been observed in some cases (Martins *et al.* 2008; Tófoli *et al.* 2009) Likewise, predation pressure by felids may be an important factor regulating the composition and dynamics of mammal communities in the Neotropics. Testing these ecological hypotheses would require a better understanding of felid dietary composition, especially in the case of smaller wild cats, whose food habits have not yet been characterized in detail.

Within this guild, *P. yagouaroundi* has a broad distribution occupying different habitat types, including the Amazon and Atlantic forest, as well as open areas such as the Cerrado and Pampas. In the literature it is described as preying on a wide variety of groups, depending the area of occurrence (Emmons, 1997). However, very few studies have focused on its dietary contents, so that our understanding of its food habits is still quite preliminary. The *Leopardus* comprises seven species occupying a great variety of habitats and presenting different ecological features. One member of this genus is *L. wiedii*, which seems to be quite unique due to its likely arboreal habits as inferred and by morphological adaptations and some field observations (Sunquist and Sunquist 2002). As result of these adaptations and habits, *L. wiedii* is expected to prey more extensively on birds and arboreal mammals than other small Neotropical felids (Emmons, 1997), a hypothesis that has not yet been thoroughly tested.

Two other species from this genus that show relevant questions regarding their natural history and trophic biology are *L. tigrinus* and *L. geoffroyi*. In Rio Grande do Sul state, southern Brazil, these species have a mostly allopatric distribution, with a narrow overlap (*ca.* 100km wide) at the center of the state. This contact zone is likely caused by the encounter between two different vegetational formations that dominate the northern and southern part of the state, namely the Atlantic Forest and the Pampas grasslands,

respectively (Eizirik *et al.* 2006). Within this contact zone, we have identified an ongoing process of hybridization between these species (Trigo *et al.* 2008), highlighting the interest in unraveling their evolutionary history and ecological interactions in this area of range overlap.

The diets of *L. tigrinus* and *L. geoffroyi* have been investigated in a few previous studies, based on both faecal samples and stomach content analysis (*e.g.* Wang 2002, Manfredi *et al.* 2004, Novaro *et al.* 2000). Prey items in these studies were identified using morphological features of hairs, bones, teeth, feathers, etc. and in many cases could not be classified at species level. Moreover, the number of studies performed so far (in many cases incorporating small sample sizes and limited geographic coverage) is insufficient to allow an adequate comparison of the diets of these species. We have recently attempted to contribute to the understanding of this issue by analyzing stomach contents collected from road-killed individuals of these two cat species (Trigo *et al. in prep*.). Even though that study used morphological features to identify prey items, the use of stomach contents instead of faeces allowed a more precise identification of several items, as their remains were more intact than we usually observe in scats. In that study we found a strong trophic niche separation between these felids when the items were categorized at species level. This analysis showed the importance of a strict categorization of the dietary items, because this is the only way to unveil some hidden patterns in the ecological relationships between these sympatric predators. In spite of the interesting results observed in that study, several prey items could not be identified at species level, due to the difficulty in diagnosing closely related species that are morphologically very similar. This was especially the case of small

rodents, raising the question of whether species-level identification in this group could be accomplished with DNA-based methods.

In the present study, we aimed to evaluate the power of the DNA barcoding approach to perform trophic analyses of Neotropical wild cats. More specifically, we focused on the species-level identification of rodents found in the stomach content of the felids *Leopardus tigrinus*, *L. geoffroyi*, *L. wiedii* and *Puma yagouaroundi*, which is the first attempt to investigate their diets using a molecular approach. We focused particularly on the niche partitioning between *L. tigrinus* and *L. geoffroyi*, two species presenting intriguing patterns of spatial segregation and ongoing hybridization in southern Brazil. Additionally, we used our data set to investigate patterns of species distribution for the rodents identified as prey items, an approach that revealed likely instances of hidden diversity in this very speciose group which is still poorly characterized in this region of South America.

2. Material and Methods

(a) *Sampling*

Sampling was divided into two fronts: the first step was the generation of a sequence database for rodent species that occur in the same areas as the investigated wild cats (Table 1). We preferentially selected samples that had been previously identified by experts and deposited in museums, or those that had already been used in other studies. In addition, we complemented the database with sequences downloaded from BOLD (Ratnasinghan & Hebert 2007) and GenBank that would represent rodent species that occur in the same regions. The second step was the gathering of rodent samples removed from the stomach of

road-killed wild cats (Table 2), collected in different localities in Rio Grande do Sul and Santa Catarina states (Figure 1). After the opening of the stomach, the samples were separated by morphotypes, preferentially selecting recognizable rodent parts such as the legs, tail or head, to assess the correct number of specimen in each stomach. Each sample was then washed in ethanol and a tissue sample was taken for the rest of the procedure. We analyzed the dietary content of 10 *L. tigrinus,* 6 *L. geoffroyi*, 2 *L. wiedii* and 2 *Puma yagouaroundi* individuals, making up a total sample size of 20 stomach contents.

(b) *Sequence generation*

DNA extraction for all samples was conducted using a standard phenol/choloroform protocol (Sambrook *et al.* 1989). A 658bp of the *COXI* gene was amplified via the polymerase chain reaction (PCR) using two primer cocktails, composed by three primers each (forward: VF1_t1, VF1d_t1, VF1i_t1; and reverse: VR1_t1, VR1d_t1, VR1i_t1) (Ivanova *et al.* 2007). Alternatively, we used a second pair of primers amplifying a sub segment spanning 170 bp in the case of samples that presented its DNA in advanced stage of degradation (Chaves 2008). PCR experiments were performed in 20 μL final volume containing 2.0mM $MgCl_2$, 0.2mM dNTPs, 0.5U of Taq Platinum, and 0.2μmM each of the forward and reverse primers. Thermo cycling profiles consisted of a touchdown PCR as described in Tchaicka *et al.* (2007), with the annealing temperature decreasing from 60˚C to 51˚C in 10 cycles, followed by 30-35 cycles in which it was kept constant at 50˚C.

PCR products were analyzed on a Gel-Red stained 1% agarose gel and then purified using the enzymes exonuclease I and Shrimp alkaline phosphatase. Purified PCR products were sequenced using DYEnamic ET kit (Amersham) and then analyzed in a MegaBACE 1000

automated sequencer. Sequence electropherograms were verified using FinchTV (Geospiza) and the consensus of both strands was assembled using Phred/Phrap/Consed (Ewing *et al.* 1998; Ewing & Green 1998, Gordon *et al.* 1998). After that, all sequences were aligned using the CLUSTALW algorithm implemented in MEGA 4.1 (Tamura *et al.* 2007), and all the sequences were reviewed and edited by hand.

(c) *Data analysis*

We identified each stomach content sample through a distance-based phylogenetic analysis using the Neighbor-joining (Saitou *et al.* 1987) algorithm and the Kimura-2-parameter (Kimura, 1980) model, with 1000 bootstrap pseudoreplicates used to assess nodal support. To assign a prey sample to a given species, it had to cluster with a reference sample with booststrap support > 75%. Beyond that, each sequence was compared through a BLAST search (Altschul *et al.* 1990) on the NCBI and BOLD websites, aiming to check for any unsampled taxa in our database, especially in the case of rodent species not expected to occur in our sampling area. Items that could not be assigned to any reference species were treated as unidentified clusters

(d) *Ecological Analysis*

We analyzed the prey data using two estimators: the frequency of occurrence (FO) of each item and its relative frequency (RF). The frequency of occurrence is the proportion of samples in which a particular item has been found, and is obtained by dividing the number of samples that contained that food item by the total number of samples. The relative frequency is the proportion of occurrence of the item with respect to the total number of

identified items for that species. This frequency is calculated by dividing the number of times a particular item has been recorded by the total number of items (including multiple occurrences per item). The relative frequency was used for most downstream analyses, as it represents more accurately the proportion of intake for each item.

The food niche breadth was calculated for each felid species using the total number of items identified in the stomach content. Levin's index (Krebs, 1989) was used as an exploratory measurement of niche breadth, given as: BA (B−1)/(*n*−1), where BA is the standardized Levin's index for the number of items (*n*) and $B=1/\sum p_i^2$, where *pi* is the frequency of the item in all analyzed species. The trophic niche overlap between *L. tigrinus* and *L. geoffroyi* was also calculated using Pianka's index (Pianka 1973), and the distinction between the two diets was assessed with chi-square tests.

3. Results and Discussion

(a) *Stomach content identification and predator/prey relationships*

Our data set allowed the identification of seven different rodent species and one additional rodent genus among the prey items of the sampled felid stomach contents (Figure 2). The identified species were *Cavia magna*, *Mus musculus*, *Sooretamys angouya*, *Oligoryzomys nigripes, Rattus rattus, Oxymycterus quaestor, Oxymycterus* sp*., Akodon paranaensis* and *Akodon* azarae. Additionally, we found 10 other items that could not be confidently assigned to any reference taxon. Our phylogenetic analysis indicated that these 10 samples represented five different clusters, whose divergence from the remaining taxa

(*e.g.* cluster 1) and/or consistent clustering with respect to each other (*e.g.* cluster 3) suggest that they constitute additional rodent species not represented in our reference data base.

The presence of a substantial number of unidentified clusters in the analysis (representing 41% of the total number of items) reflects the state of knowledge about this group, especially in the Neotropical region. Brito (2004) reviewed this issue and addressed the importance of improving the available information on these taxa, since this could be used in conservation and management decisions. Another fact that may have contributed to the lack of identification of these clusters is the sampling strategies usually employed to capture small wild rodents. The most common trap techniques used in ecological surveys are focused on the ground, mainly on trails, and cats can forage in a wide variety of different places, such as water bodies and tree branches, not contemplated by this sampling technique. It is therefore plausible that the wild cats are foraging on rodent species that have not yet been sampled (at least in these regions) using the standard survey techniques.

In our study, *L. geoffroyi* had the greatest number of unidentified items in proportion to identified ones; these items possibly represent three species that do not have a barcode in any of the data bases used in this study. Sousa & Bager (2007) analyzed the diet of the Geoffroyi's cat through morphological identification in both stomach and scats contents. When we compared the items identified in their study with the rodents sampled in our database, only *Myocastor coypus* was not present, even being considered relatively common in the region, and it might be part of the unidentified clusters. These data indicate that there is a need for further studies in the region to assess the diversity of rodents in order to solve those questions.

In general, the diet of *L. tigrinus* is constituted of small mammals and birds (Gardner, 1971; Ximenez 1982, Mondolfi, 1986; Guix, 1997; Wang, 2002). Their habitat is strongly related to forest formations, such as evergreen and deciduous forest and brush, also including lowlands in some regions and cloud forest and high elevation thickets in others (Nowell & Jackson 1996). Their distribution in southern Brazil is linked to the Atlantic Forest (Oliveira 1994), the same occurring with *Sooretamys angouya* (Weksler *et al.* 2006), which was found in this study be its most frequent prey item (27%). The other prey items identified for this felid were *Rattus rattus* and *Mus musculus*, two invasive species that are already known to exclude competitive native species in different areas of the globe (Harris & Macdonald, 2007; Stokes *et al.*, 2009). This finding is very interesting not only in the sense of indicating the effect of these invasive species on the current composition of Neotropical rodent biotas, but also because it documents the dietary flexibility of *L. tigrinus* as it shifts its diet to take advantage of newly available exotic species. As the interplay between dietary flexibility (and thus adaptability to varying prey availability) and niche specialization is still unclear for these cats, DNA barcoding associated with more extensive sampling may emerge as a powerful approach to unravel these interactions.

We observed high bootstrap support for the node uniting clusters 3 and 4 (each likely containing unidentified taxa) and reference samples of *Oligoryzomys fulvescens* and *O. vegetus* (fig.1). This result is intriguing, as our clusters 3 and 4 contain unidentified rodents from southern Brazil, including prey items from cats sampled in the extreme south of the Brazilian Pampas (see Fig. 2 and Table 2). In contrast, the reference sequences from *Oligoryzomys fulvescens* and *O. vegetus* that were generated in a mammalian survey in Suriname (Borisenko *et al.* 2007), and the distribution of these species is thought to be

mostly restricted to the Amazon region. It is therefore likely that the prey items from southern Brazil represent distinct species (perhaps as of yet undescribed) that are closely related to these Amazonian taxa. Further sampling in these areas (including targeted surveys of rodent species) will be required to further clarify their identity.

One of the identified items for the margay (*L. wiedii*) was *Oxymycterus quaestor*, which is very interesting since this species has its known occurrence restricted to Paraná state (Oliveira & Bonvicino, 2006) and this stomach content was collected in Rio Grande do Sul, > 700 km south of the former state. The identification was performed on the basis of a high bootstrap value, which is further supported by the fact that we had a good taxonomic coverage for this genus. One important consideration in this regard is that the taxonomy of *Oxymycterus* is still not very clear (Gonçalves & Oliveira, 2004), so that additional investigations are required to fully characterize its extant diversity. Although margays are known to forage in trees, several authors have observed more generalist habits regarding their feeding ecology (Mondolfi, 1986; Konecny, 1989; Oliveira, 1998; Wang, 2002) and *O. quaestor* is described as foraging in forest borders and open areas (Bonvicino *et al.* 2005), not necessarily exploiting arboreal habitats within closed environments. This finding illustrates the view that margays may not necessarily target arboreal species or preferentially forages above ground, in spite of their perceived morphological adaptations for arboreal life. In this sense, it becomes even less clear what factors drive the ecological segregation between margays and other small sympatric felids, highlighting the need for in-depth investigation of this topic.

According to literature, *P. yagouaroundi* feeds on small mammals, mainly rodents, birds and reptiles (Manzani & Monteiro, 1989 Oliveira 1998, Tófoli 2009). Found in many

habitats, from rainforests to fields, savanna, and dense thicket in scrub, they can live in secondary vegetation near villages (Emmons, 1997). Among the identified items in its diet, *Akodon paranaensis* has a distribution that includes the northern portion of Rio Grande do Sul state, with a strong relationship to forest formations. The specimen where this item was found was collected in the southeastern portion of the state (Figure 1), again indicating that further investigations are necessary to better define this rodent's distribution.

(b) *Ecological Analysis of L. tigrinus and L. geoffroyi*

The number of analyzed items for each species and its frequency of occurrence and relative frequency are shown in Table 3 and also Figures 3 and 4. It is possible to observe in the frequency figures a clearly separation in the dietary contents of each species. The most common item in the diet of *L. tigrinus* was *Sooretamys angouya*, while for *L. geoffroyi* the most frequent item was the unidentified species grouped into cluster 4 (figs 2 and table 3), an item that was also present in the diet of *L. tigrinus*.

The estimated niche breadth was low for both *L. tigrinus* and *L. geoffroyi*, which presented values of 0.36 and 0.12, respectively. These values indicate a high degree of specialization, mainly in the case of *L. geoffroyi*. When compared to previous studies, in all cases where the identification of dietary items allowed a fine-scale characterization, the dietary specialization became quite clear (Johnson and Franklin, 1991; Manfredi *et al.* 2004), suggesting that overlap observed in some cases may be due to coarse-level prey identification. In the case of *L. tigrinus*, we obtained a value similar to that reported by Wang (2002), who also assessed the niche breadth, and obtained a value of 0.44 for Levin's index, indicating a certain level of specialization. The same occurred for *L. geoffroyi*, but no other

study presented such a strong level of specialization for this species (Manfredi *et al.* 2004). This difference again may be due to a finer level of identification for prey items in this study relative to previous ones, made possible by the DNA barcoding technique. In a traditional study, these items possibly would have their identification performed only at family o subfamily level, which would likely decrease the observed level of specialization, since all items are part of rodent subfamily Sigmodontinae.

For the niche overlap analysis, Pianka's index was 0.34, indicating a small degree of overlap between the two species. This was caused by the sharing of only one unidentified item (cluster 4). Although the chi-square test between two cat species showed no significant values, figs. 3 and 4 illustrate this pattern, indicating a clear pattern of trophic niche segregation between these species.

4. Conclusions

(a) *Taxonomy and Molecular identification*

There are 165 species of rodents described as occurring in Brazil, and 7.3% of them suffer some type of threat (Costa *et al.* 2000). However, this number is highly underestimated due to the lack of detailed knowledge about this group in almost every biome, especially the Atlantic Forest and the Amazon (Voss & Emmons, 1996; Britto, 2004). It has been pointed out that, for small mammals, a major factor exacerbating their threat of extinction is the lack of scientific knowledge on systematics, distribution and natural history (Costa *et a.l* 2000). The coordinated work among museum collections and the performance

of field expeditions focused on traditional taxonomy allied to the gathering of tissue samples for barcoding scanning could help solve these questions.

(b) *Ecological perspectives*

Understanding the trophic relationships of carnivores is an important goal, not only to improve our knowledge about these species' biology, but also to understand the community as a whole. The possibility of improving our knowledge on the dietary composition of a species, while also supporting the identification and characterization of new taxa among the prey items, could be one of the greatest advantages of the barcoding method when compared to the traditional methodology in ecological studies. Although further sampling will be required to better characterize these felids' diets and identify all items at species level, at the present study demonstrated the potential of the DNA barcoding technique in this area, and may serve as a basis for future efforts addressing similar topics.

Acknowledgments

The authors thank Thales R.O. de Freitas, Livia Muller, Yuri Leite, Ana Carolina Loss, Victor Valiati, Diego Jung, Jamile Queiroz, Felipe Peters, Leonardo Machado, Alexandre Christoff, Eduardo Reginato, Ivana Regina Silveira, Carla S. Fontana, Flávia Pereira Tirelli, Marina Ochoa Favarini, Anelisie da Silva Santos, Alexsandra Schneider, and Cladinara Roberts Sarturi for help with sample collection and experimental procedures. We also thank Capes and CNPQ for financial support.

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Figures

Figure 1 – Map of collected samples.

Distribution map for the collected road-killed wild cats.

Figure 2 – Neighbor-joining tree from the identified items

Neighbor-joining tree based on the Kimura-2-parameter model with 1000 bootstrap pseudoreplicates employed to assess nodal support. Each cluster identified with a letter represents successfully identified items based on comparison with reference samples, while clusters identified with numbers include samples without any reliable correspondence to reference species present in our database. Only clusters with more than 75% of bootstrap support were considered.

Figure 3 - Frequency of occurrence for *L. tigrinus* and *L. geoffroyi*

Proportion of samples in which a particular item has been found for both species.

Figure 4 – Relative frequency for *L. tigrinus* and *L. geoffroyi*

Proportion of occurrence of a given item with respect to the total number of identified items for that species.

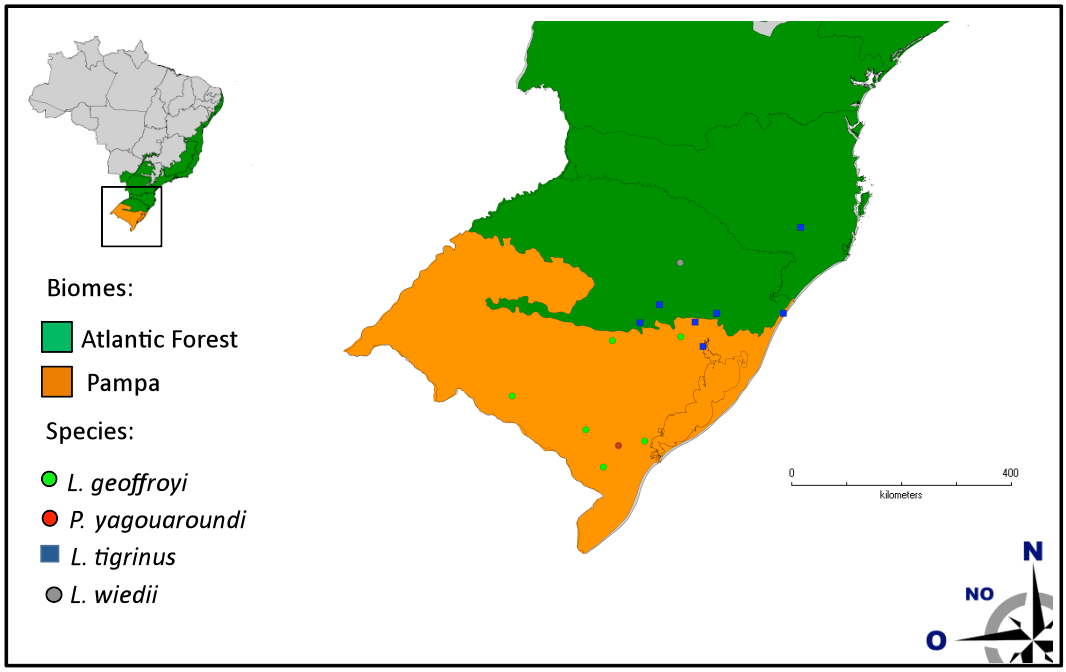
Biomes:
Atlantic Forest
Pampa
Species:
L. geoffroyi
P. yagouaroundi
L. tigrinus
L. wiedii
0
400
kilometers
N
NO
O

Figure 1

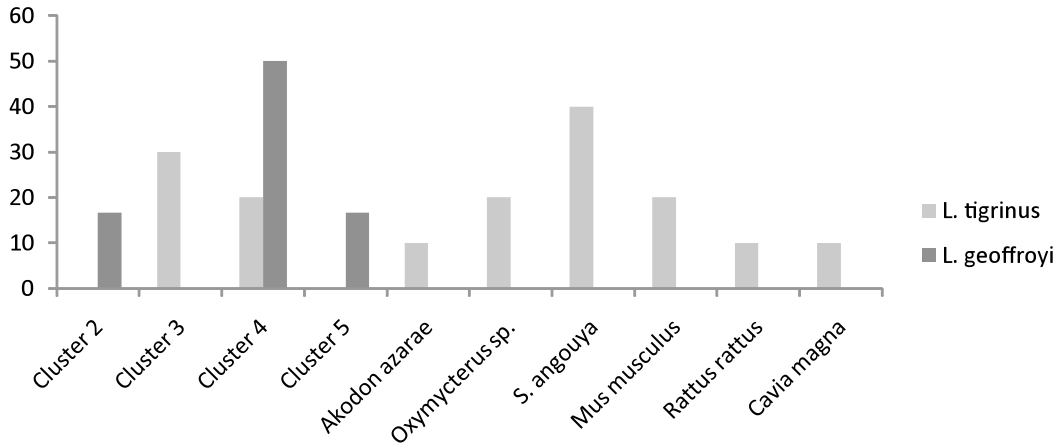

Figure 3

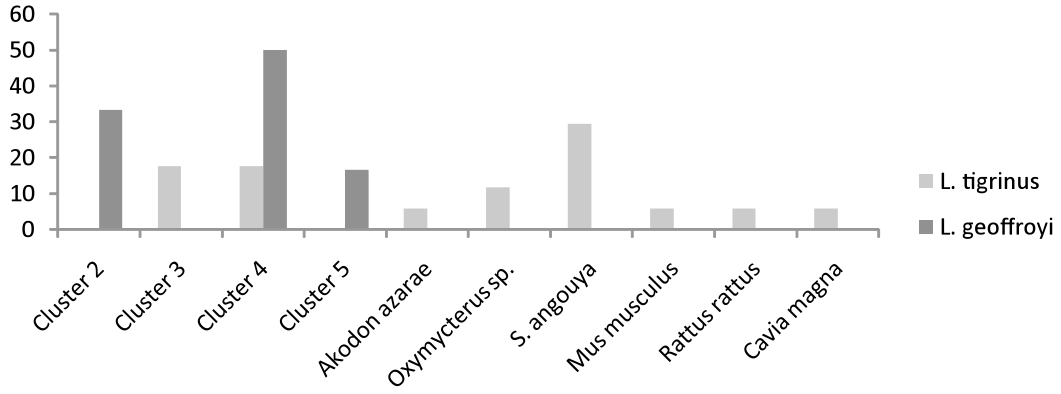

Figure 4

Table 1 – List of rodent species sampled for this study.
List of all species used in the dietary content identification with its respective source. The species marked with an asterisk are those shown in the neighbor-joining tree (fig. 2).

Species (40)	N	Source	Species (38)	N	Source
Akodon	6	BOLD	*Nectomys squamipes*	4	UFRGS
*Akodon azarae**	3	UFRGS	*Nephlomys albigularis*	2	GenBank
*Akodon cursor**	1	UFRGS	*Oecomys sp.*	1	GenBank
*Akodon montensis**	21	UFRGS	*Oecomys auyantepui*	5	GenBank
*Akodon paranaensis**	2	UFRGS/PUCRS	*Oecomys bicolor*	3	GenBank
Akodon reigi	4	UFRGS	*Oecomys cf.rex*	2	GenBank
Akodon sp	2	UFRGS	*Oecomys roberti*	1	GenBank
Akodon sp2	9	UFRGS	*Oecomys rutilus*	3	GenBank
Akodon urichi	1	UFRGS	*Oligoryzomys flavescens**	7	UFRGS
Calomys expulsus	8	UFRGS	*Oligoryzomys fulvescens**	2	BOLD
*Calomys sp**	2	UFRGS	*Oligoryzomys nigripes**	24	UFRGS
Calomys sp.nov.	1	UFRGS	*Oligoryzomys vegetus**	1	BOLD
*Calomys tener**	1	UFRGS	*Oryzomys couesi*	2	UFRGS
Caluromys philander	5	BOLD	*Oryzomys melanotis*	1	UFRGS
Cavia sp.	1	BOLD	*Oxymycterus sp.**	5	PUCRS
*Cavia aperea**	2	PUCRS	*Oxymycterus nasustus**	7	PUCRS
*Cavia fulgida**	2	BOLD	*Oxymycterus quaestor**	2	PUCRS
*Cavia magna**	2	PUCRS	*Rattus norvegicus**	3	GenBank
Cerradomys subflavus	6	UFRGS	*Rattus rattus**	3	GenBank
Chaetomys subspinosus	3	UFRGS	*Rhagomys rufescens*	2	UFRGS
Delomys dorsalis	13	UFRGS	*Rhipidomys leucodactylus*	1	GenBank
Delomys sublineatus	3	UFRGS	*Rhipidomys mastacalis*	3	GenBank
Deltamys kempi	5	UFRGS	*Rhipidomys nitela*	3	GenBank
Euryoryzomys macconnelli	1	GenBank	*Rhipidomys scandens*	1	GenBank
Euryoryzomys russatus	2	UFRGS	*Rhipidomys wetzeli*	2	GenBank
Euryzygomatomys	3	UFRGS	*Scapteromys sp**	5	PUCRS
Euryzygomatomys spi	3	UFRGS	*Scapteromys tumidus**	2	PUCRS
Handleyomys alfaroi	1	UFRGS	*Sciurus vulgaris*	2	GenBank
Holochilus brasiliensis	1	UFRGS	*Sooretamys angouya**	3	UFRGS
Hylaeamys laticeps	3	GenBank	*Sphiggurus insidiosus*	3	BOLD
Hylaemys megacephalus	5	GenBank	*Sphiggurus villosus*	1	BOLD
Hylaemys yunganus	4	GenBank	*Thaptomys nigrita*	3	UFRGS
Juliomys ossitenuis	2	UFRGS	*Transandinomys bolivaris*	1	GenBank
Juliomys pictipes	3	UFRGS	*Transandinomys talamancae*	2	UFRGS
Kannabateomys amblyonyx	4	BOLD	*Trinomys sp.*	9	BOLD
*Mus musculus**	2	BOLD	*Trinomys albispinus*	1	BOLD
Neacomys guianae	6	BOLD	*Trinomys denigratus*	1	BOLD
Neacomys paracou	5	BOLD	*Trinomys gratiosus*	1	BOLD
Neacomys spinosus	1	BOLD			
Necromys lasiurus	1	UFRGS			
TOTAL = 150					

Table 2 – List of wildcat individuals analyzed in the present study.

Species	Sample ID	Geographic origin	Geographic coordinates	
			Latitude	Longitude
Leopardus geoffroyi	bLge074	Pinheiro Machado - RS	31°34'36.95"S	53°23'6.19"O
	bLge076	Arroio Grande - RS	32°13'44.64"S	53° 4'39.71"O
	bLge008	Cachoeira do Sul - RS	30° 0'44.56"S	52°55'10.98"O
	MCNU880	Dom Pedrito - RS	30°58'56.13"S	54°40'44.58"O
	MCNU896	Dom Pedrito - RS	30°58'56.13"S	54°40'44.58"O
	bLge096	Pelotas - RS	31°46'20.12"S	52°21'14.11"O
Leopardus tigrinus	bLti001	Triunfo - RS	29°56'34.28"S	51°43'6.44"O
	bLti068	Montenegro - RS	29°41'21.13"S	51°28'1.17"O
	bLti069	Santa Cruz - RS	29°42'47.31"S	52°25'53.79"O
	bLti079	Guaíba - RS	30° 6'50.82"S	51°19'41.22"O
	bLti122	Arroio do Sal - RS	29°32'7.41"S	49°54'56.58"O
	bLti208	Unknown		
	bLti137	Morro Reuter - RS	29°32'2.53"S	51° 5'11.37"O
	bLti160	Urubici - SC	28° 1'38.75"S	49°36'45.07"O
	bLti143	Unknown		
	bLti149	Forquetinha - RS	29°22'53.98"S	52° 5'29.49"O
Leopardus wiedii	bLwi62	Unknown		
	TC13	Nova Araçá - RS	28° 38.708'S	51° 43.618'O
Puma yagouaroundi	bPya073	Cerrito - RS	31°51'6.60"S	52°48'38.33"O
	bPya076	RS		

Table 3 – Frequencies of the dietary items found in each species of felids

Number of identified items for each species (*L. tigrinus*, *L. geoffroyi*, *L. wiedii* and *P. yagouaroundi*) with their respective frequency of occurrence (FO) and relative frequency (RF). For more details regarding the frequencies for *L. tigrinus* and *L. geoffroyi*, see figs 3 and 4.

Prey Items		*L. tigrinus* (n=10)			*L. geoffroyi* (n=6)			*L. wiedii* (n=2)			*P. yagouaroundi* (n=2)		
Family	Specific group	N	FO(%)	RF(%)	N	FO(%)	RF(%)	N	FO(%)	RF(%)	N	FO(%)	RF(%)
Cricetidae	*Akodon paranaensis*	0	0	0	0	0	0	0	0	0	1	50	33.33
	Akodon azarae	1	10	5. 88	0	0	0	0	0	0	0	0	0
	Sooretamys angouya	5	40	29.41	0	0	0	0	0	0	0	0	0
	Oligoryzomys nigripes	0	0	0	0	0	0	0	0	0	2	50	66.66
	Oxymycterus quaestor	0	0	0	0	0	0	1	50	20	0	0	0
	Oxymycterus sp.	2	20	11.76	0	0	0	0	0	0	0	0	0
Muridae	*Rattus rattus*	1	10	5.88	0	0	0	0	0	0	0	0	0
	Mus musculus	2	20	5.88	0	0	0	1	50	60	0	0	0
Caviidae	*Cavia magna*	1	10	5.88	0	0	0	0	0	0	0	0	0
Unidentified	cluster 1	0	0	0	0	0	0	1	50	20	0	0	0
	cluster 2	0	0	0	2	16.67	33.33	0	0	0	0	0	0
	cluster 3	3	30	17.65	0	0	0	0	0	0	0	0	0
	cluster 4	3	20	17.65	3	50	50	0	0	0	0	0	0
	cluster 5	0	0	0	1	16,67	16.67	0	0	0	0	0	0