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SHORT COMMUNICATION

Is it possible to identify four small Neotropical felids (Carnivora: Felidae) based on hair microstructure?

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ABSTRACT. The microscopic characters of the hair can be used to indirectly identify species that are either uncommon or are difficult to see, for instance small Neotropical felids of the southern Brazilian Atlantic Forests. This widely used technique, which has not yet been standardized, involves the identification of hairs collected from feces. We tested the hypothesis that this tool is effective to identify four species of small Neotropical felids: *Leopardus guttulus*, *Puma yagouaroundi*, *L. wiedii* and *L. pardalis*. To accomplish that, we used measurements of the length, width and area of the cuticular scales in the guard hairs and calculated the relationship between width and length (quotient) for each species. A multiple discriminant analysis was conducted on the measurements and the percentage of correct identification was obtained. We found a high overlap in the quotients of these species, which indicates that this technique does not identify Neotropical felids accurately. This inefficiency was also confirmed by the multiple discriminant analysis, where only 74% correct identifications were obtained. Therefore we recommend that hair analysis is used only in combination with other sources of evidence, for instance molecular tools.

KEY WORDS. Diet; *Leopardus guttulus*; *Leopardus wiedii*; *Leopardus pardalis*; *Puma yagouaroundi*.

In the southern Brazilian Atlantic Forest, the guild of small Neotropical felids includes four species: *Leopardus guttulus* (Hensel, 1872), recently separated from *L. tigrinus* (Schreber, 1775), *Puma yagouaroundi* (É. Geoffroy Saint-Hilaire, 1803), *L. wiedii* (Schinz, 1821) and *L. pardalis* (Linnaeus, 1758) (Nowell & Jackson 1996). The separation of *L. guttulus* from *L. tigrinus* was based on molecular and morphological data that demonstrated that populations of *L. tigrinus* in southern Brazil constitute a separate species (Trigo et al. 2013, F.O. Nascimento unpubl. data). Thus, in this report we recognize all samples of oncilla from the southern Brazilian Atlantic Forest as *L. guttulus*.

These four small Neotropical felids occur in low densities and are difficult to find in their natural environment (Chiarello 1999, Silver et al. 2004, Di Bitetti et al. 2010). Furthermore, they have large home ranges and territories, and have nocturnal/twilight activity, which reduce the efficiency of direct sampling techniques *in-situ* and results in insufficient ecological data in the literature (Downey et al. 2007, Abreu et al. 2008, Di Bitetti et al. 2010).

Indirect detection techniques, such as the identification of guard hairs ingested during self-cleaning and excreted in feces (Eckstein & Hart 2000), have been used to collect information about Neotropical felids in the wild. This technique is commonly used in feeding studies (see Guerrero et al. 2002, Wang 2002, Ludwig et al. 2007, Tófoli et al. 2009, Silva-Pereira et al. 2011) and also provides data on the distribution and occurrence of these animals.

Nevertheless, the usefulness of species identification based on guard hairs in the stool has not escaped criticism. In a study that included all Brazilian felid species, Vanstreels et al. (2010) indicated that they were able to successfully identify only 75% of the samples. Because this indirect tool is so important and so widely used (e.g., Wang 2002, Martins et al. 2008, Tófoli et al. 2009, Bianchi et al. 2011, Silva-Pereira et al. 2011), and considering the flaws in the diagnostics elaborated by Vanstreels et al. (2010) (their method is circular: they used only qualitative characters for species determination, which in turn are dependent on the experience and the skills of the observer), we endeavored to evaluate metrically its usefulness to identify *L. guttulus*, *P. yagouaroundi*, *L. wiedii*, and *L. pardalis*.

The current method used for the identification of these four species follows three procedures: identification of the medullar pattern, the cuticular pattern, and the relationship between width and length of the cuticular scales of the hair shaft (Quadros & Monteiro-Filho 2010, Vanstreels et al. 2010). The medullar pattern, called Trabecular with fringed margins, is similar in all small Brazilian Neotropical felids (Vanstreels et al. 2010). The cuticular character, however, divides them into two pairs of species: the Losangic pattern clusters *L. guttulus* with *P. yagouaroundi*, and the Folidaceous pattern clusters *L. wiedii* with *L. pardalis* (Quadros & Monteiro-Filho 2010). Each small Neotropical Brazilian felid can be identified using the relationship between width and length of the scales on the shaft of the guard hairs (Quadros & Monteiro-Filho 2006, 2010). In the species displaying the Losangic pattern, the scales are wider than long in *P. yagouaroundi* and longer than wide in *L. guttulus*. In the pair characterized by the Folidaceous pattern, the scales are as wide as long in *L. pardalis* and longer than wide in *L. wiedii* (Quadros & Monteiro-Filho 2006, 2010).

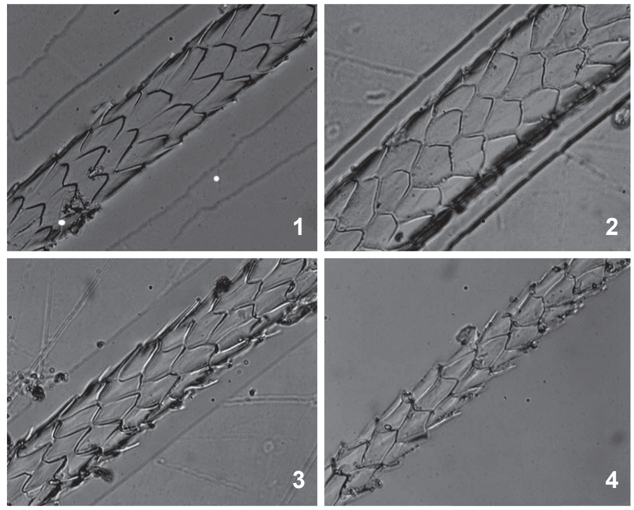
We collected morphometric data from animals kept at the Bela Vista Biological Sanctuary located at the ITAIPU Binational area and Center for Conservation of Neotropical Felids, Associação Mata Ciliar. Hairs were collected from 10 specimens of *L. guttulus*, seven of *P. yagouaroundi*, 10 of *L. wiedii* and 10 of *L. pardalis*. All samples of *L. guttulus* were collected from southeastern and southern Brazil (Appendix 1). In this study, we used only secondary guard hairs with a straight shaft, as described by Quadros & Monteiro-Filho (2010).

We measured the length, width and area of 888 scales from *L. guttulus* (mean to specimens 88.80 ± 12.6 SD scales), 669 (66.90 ± 22) from *P. yagouaroundi*, 717 (71.70 ± 23.8) from *L. wiedii* and 548 (78.28 ± 9.6) from *L. pardalis*. This information was taken from the following total number of hairs per specimen: *L. guttulus* = 9.20 ± 1.03, *P. yagouaroundi* 8.30 ± 1.5, *L. wiedii* = 7.70 ± 2.1, and *L. pardalis* = 8.14 ± 1.09.

To measure the hairs, we applied thin Entellan (Merck©) resin layers onto microscope slides. After ten minutes, we placed the hairs onto the Entellan resin, pressed them and subsequently removed them. The resulting impression in the resin was then identified with the number of the specimen and abbreviation of the species name, facilitating individualization. Subsequently, microscopic images were obtained from the impressions using a digital camera coupled to a microscope, and the program MIAS 2.2. The images were taken at 400× magnification. The following measurements were taken from the images, using the program Image Tool (Wilcox et al. 2002): length, width and area of each scale. The area of the scale was included as a variable in the analysis due to irregularities in the shape of scales (Figs. 1-4).

To demonstrate the efficiency of the quotient between the width and length of guard hair scales, indicated in the dichotomous key of Quadros & Monteiro-Filho (2006, 2010), we prepared a blox-plot of quotient for each species in the pair. An overlap of quotients within a pair of small Neotropical felids suggests the inefficiency of the dichotomous key proposed.

Multivariate Linear Discriminant Analysis (LDA) was performed to test the hypothesis that it is possible to discriminate between pairs of species. The discriminant function used to calculate the scores of the samples was $Z_k = \alpha + {}_iX_{1k}+...+W_iX_{ik}$, where:



Figures 1-4. Cuticular patterns on the shaft of the secondary guard hairs of four species of Neotropical felids. Losangic: (1) *Leopardus guttulus* and (2) *Puma yagouaroundi*; and Imbricate Folidaceous: (3) *L. wiedii* and (4) *L. pardalis*.

Z_{jk} is the score obtained by the score function for the sample *k*, α is the intercept, *Wi* is the discriminant coefficient for the independent variable *i*, and X_{ik} is the real value of the independent variable *i* for the object *k* (Hair et al. 2005). After randomization of the original data, the matrices of the two species were divided into two sub-matrices; one was called the analysis matrix and the other, the testing matrix. The latter consisted of 100 samples and was used to validate the discriminant function. The remaining data were included in the analysis matrix and were used for the preparation of the discriminant function.

With the scores obtained from the analysis matrix, we calculated the centroid of each species, which we used to obtain the cut-off scores calculated by the weighted mean of centroids (Hair et al. 2005). We used the Wilk's Lambda statistics to evaluate the significance and power of the discriminant function and the relative contribution of each variable to the model. The results of these statistics varied from zero to one, with a result close to zero indicating greater discrimination. To check which percentage of the overall variance was explained by the model, we used the square of the canonical correlation (Hair et al. 2005). We also calculated the accuracy of reclassification for each species, and the rate of success of the discriminant function as a percentage. We adopted the criterion of proportional chances to exclude the possibility that the correct determinations were due to chance. The discriminant function was validated after confirming that the correct identifications were equal or greater than the sum of the values explained by chance, with the addition of a quarter of the same-value. Finally, we compared the success ratios obtained in the analysis matrix with those obtained in the testing matrix using the Chi-squared test for two samples. The premise of homoscedasticity was fulfilled. Independence between variables was tested using the Pearson's correlation test (Hair et al. 2005), using R (R Development Core Team 2011) and observing the predominance of low correlations (< 0.5, Table I). The proportionality of the sample size and the minimum size of the group per independent variable were observed (Hair et al. 2005).

Table I. Pearsons's correlation coefficient between the cuticular variables: width, length and area of the cuticular scales of the secondary guard hairs of small felids in the Brazilian Neotropical forest.

Variável	*L. guttulus*	*P. yagouaroundi*	*L. wiedii*	*L. pardalis*
Length and Width	-0.42*	-0.55*	-0.31*	-0.43*
Area and Length	0.37*	0.10*	0.57*	0.46*
Area × Width	0.45*	0.63*	0.34*	0.37*

* Variables have a normal distribution. Correlation criteria: 0 to ± 0.3 = reduced, ± 3.1 to ± 6.9 = moderate, ± 7 to ± 10 = high.

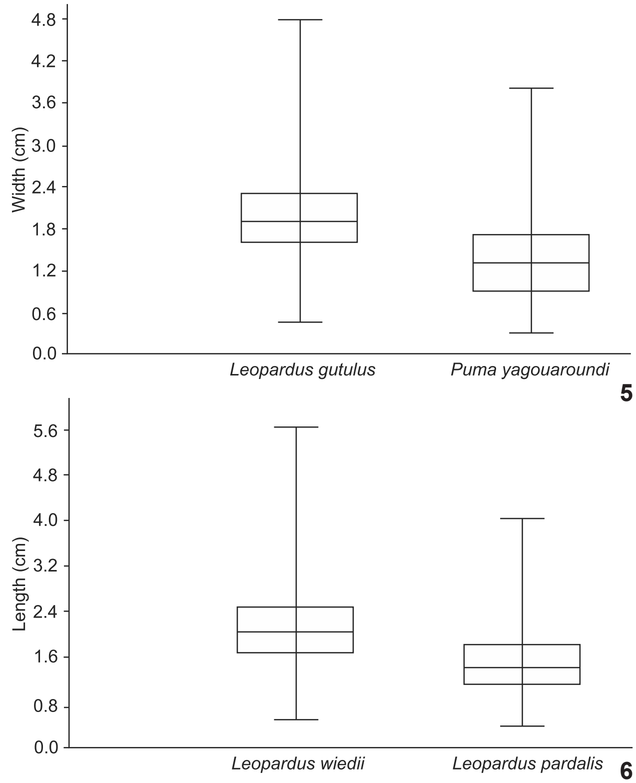
There is strong overlap in the width\length quotient obtained for the scales of the guard hairs of each pair of small Neotropical felids. Therefore, this quotient is not adequate to separate these species (Figs. 5 and 6).

The success rate in determining species using LDA was 80% and 73% for the pairs *L. guttulus* and *P. yagouaroundi*, and *L. wiedii* and *L. pardalis*, respectively. The precision of the identification was 91% for *L. guttulus*, 60% for *P. yagouaroundi*, 76% for *L. wiedii* and 70% for *L. pardalis*.

Despite the apparent success, the discriminatory power of the technique in the identification of the pairs *L. guttulus* and *P. yagouaroundi* (Wilkin's Lambda = 0.65, $p < 0.001$) and *L. wiedii* and *L. pardalis* (Wilkin's Lambda = 0.74, $p < 0.001$) was low and failed to explain the variance (34% and 25%, respectively). The variable length contributed the most to the discrimination of both pairs.

The result obtained from the reclassification matrix tests were not significantly different from those obtained in the matrix analysis of *L. guttulus* and *P. yagouaroundi* ($\chi^2 = 0.14$, d.f. = 1, $p > 0.05$), and of *L. wiedii* and *L. pardalis* ($\chi^2 = 0.16$, d.f. = 1, $p > 0.05$), validating the results of the discriminant function. The centroid, critical cutoff scores, sample size and discriminant functions obtained, which are essential for the computation of new samples, are available in Table II.

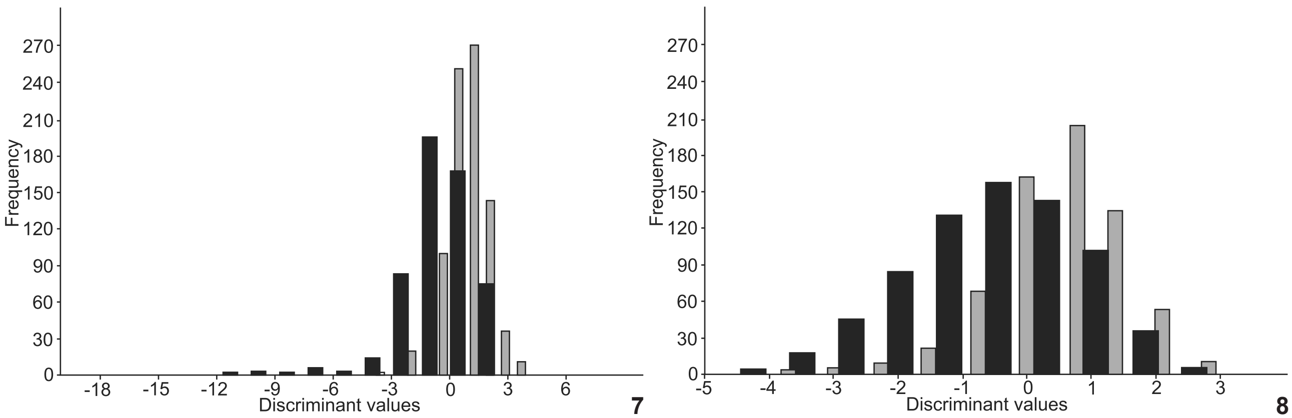
Our knowledge of the biodiversity in Neotropical ecosystems is insufficient and the limited information concerning these small Neotropical felids exemplifies this (Wang 2002, Bianchi et al. 2011, Silva-Pereira et al. 2011). Indirect techniques such as the identification of stool samples are therefore important to remediate this situation, as they allow the determination of records *in situ*. This technique, however, is not without shortcomings. The results obtained illustrate that specific determination by visual comparison of scale widths (Quadros & Monteiro-Filho 2010, Vanstreels et al. 2010) is subjective and cannot be used to identify the species of these small Neotropical felids, even with the use of metric variables (see the overlap of metrics of hair scales in Figs. 7 and 8).



Figures 5-6. Quotient of width and length (cm) of guard hairs scales for each pair of small Neotropical felids: (5) *Leopardus guttulus* and *Puma yagouaroundi*; (6) *L. wiedii* and *L. pardalis*.

Furthermore, the metric evaluation of stool guard hairs might be complemented by molecular techniques (Zuercher et al. 2003, Michalski et al. 2011) to solve specific identification, since cuticular characteristics divide these four species into two pairs, Losangic pattern for *L. guttulus* and *P. yagouaroundi*; and Folidaceous pattern for *L. wiedii* and *L. pardalis* (Quadros & Monteiro-Filho 2010 and this study).

Due to the scarcity of information concerning the four species studied here and their conservation status, ecological studies *in situ* are fundamental, especially in ecosystems where fragmentation rates and habitat loss are high (Myers et al. 2000). Reliable information can help implement correct management actions that may prevent the extinction of these small predators and the consequent adverse effect of their extinction on the entire community (see Van Jaarsveld et al. 1998, Groves et al. 2002, Wang 2002, Moreno et al. 2006). Therefore, the standardization of existing methods and the search for new tech-



Figures 7-8. Overlap frequency of the multivariate metric (length, width and area of hair scales) for: (7) *Leopardus guttulus* (grey) and *Puma yagouaroundi* (black); (8) *L. wiedii* (grey) and *L. pardalis* (black).

Table II. Summary of the identification process for small Neotropical felids (*Leopardus guttulus*, *L. wiedii*, *L. pardalis*, and *Puma yagouaroundi*) using the linear discriminant analysis (ADL) of metrics of guard hair scales.

Results	Losangic	
Critical cut scores	-0.41292	
Discriminant functions	Z = 2.161+(1.173*Xlength †)+(-2.468*Xwidth†)+(-0.992*Xarea†)	
Wilk's Lambda	Wk = 0.65, F (3.1236) = 216.38, $p < 0.005$	
Centroids	*Leopardus guttulus* = 0.546509	*Leopardus wiedii* = 0.608003
Chi squared	$\chi^2 = 0.1695$, df = 1, $p > 0.05$	
	Imbricate Folidaceous	
Critical cut scores	0.05001	
Discriminant functions	Z = 2.161+(1.173* Xlength †)+(-2.468* Xwidth†)+(-0.992* Xarea†)	
Wilk's Lambda	Wk = 0.74, F (3.1185) = 134.23 $p < 0.005$	
Centroids	*Leopardus pardalis* = -0.557990	*Puma yagouaroundi* = -0.959427
Chi squared	$\chi^2 = 0.14724$, df = 1, $p > 0.05$	

†Place of insertion of the data for length, width and area of the scale for the classification of the sample.

nologies should be encouraged, with an emphasis on information gathering.

Finally, considering the results obtained in this work and the current availability of identification keys for other Neotropical mammals (e.g., rodents: Silveira et al. 2013, primates: Ingberman & Monteiro-Filho 2006, other mammals: Martin et al. 2009, Quadros & Monteiro-Filho 2010) based on the morphology of hair microstructure, we encourage the standardization of these identification keys with testable metric variables to prevent future waste of resources and possible invalid research outputs.

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Appendix 1. List of small Neotropical Felids specimens that were sampled in study of metric cuticular scale.

Number	Small Neotropical Felid	Precedence/Reference*	Sex	Precedence
1	*Leopardus guttulus*	CASIB 205	Female	São Miguel Iguaçu, PR
2		CASIB 1620	Female	Jaragua do Sul, SC
3		CASIB 1707	Female	CASIB
4		CASIB 1820	Male	CASIB
5		CASIB 1829	Male	Porto Medes, PR
6		CASIB 1830	Male	Capitão L. Marques, PR
7		CASIB 1907	Male	Santa Helena, PR
8		CASIB 2208	Male	Foz do Iguaçu, PR
9		CASIB 2284	Female	Tijucas do Sul, PR
10		CASIB 2334	Male	CASIB, PR
11	*Leopardus wiedii*	CASIB 189	Female	Foz do Iguaçu, PR
12		CASIB 595	Female	São Paulo, SP
13		CASIB 1698	Male	CASIB, PR
14		CASIB 1751	Female	CASIB, PR
15		CASIB 1801 (60)	Female	Foz do Iguaçu, PR
16		CASIB 1806	Female	CASIB, PR
17		CASIB 2185 (24)	Female	CASIB, PR
18		CASIB 2100 (38)	Female	CASIB, PR
19		CASIB 2099 (39)	Female	CASIB, PR
20		CASIB 2235 (23)	Female	CASIB, PR
21	*Puma yagouaroundi*	AMC – Study Book 005	Undetermined	Undetermined
22		AMC – Study Book 073	Undetermined	Undetermined
23		CASIB 1785	Female	Iguaçu National Park
24		CASIB 1811	Male	CASIB
25		Roadkill in Missal, PR	Male	Missal, PR
26		CASIB 1963	Male	Juvinopolis, PR
27		Roadkill in Itaipulândia, PR	Male	Itaipulândia, PR
28	*Leopardus pardalis*	CASIB 038	Female	CASIB, PR
29		CASIB 1159	Female	Sandovalina, SP
30		CASIB 1478	Female	CASIB, PR
31		CASIB 1720	Male	CASIB, PR
32		CASIB 1730	Female	CASIB, PR
33		CASIB 1897	Female	CASIB, PR
34		CASIB 2029	Female	CASIB, PR
35		CASIB 2322	Female	Campo Grande, MS
36		CASIB 2324	Male	Campo Grande, MS
37		CASIB 2325	Female	Campo Grande, MS

* CASIB = Center for Conservation of Neotropical Felids ITAIPU Binacional, AMC = Associação Mata Ciliar.

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