Monitoring jaguar populations *Panthera onca* with non-invasive genetics: a pilot study in Brazilian ecosystems

S. Roques, M. Furtado, A. T. A. Jácomo, L. Silveira, R. Sollmann
N. M. Tôrres, J. A. Godoy and F. Palomares

Abstract The global population of jaguars *Panthera onca* has decreased significantly since the beginning of the 20th century. Given the scarcity of demographic and biological information, estimating population parameters is critical for the design of conservation measures. The jaguar's elusive behaviour makes it impossible to estimate and monitor populations by direct observation. We propose a non-invasive genetic sampling approach and demonstrate its potential for large-scale monitoring. Sex identification was optimized for faecal samples of jaguars and other felids. We also optimized a set of 11 microsatellite markers for reliable identification of individuals. We estimated the effectiveness of faecal sample genotyping in two distinct Brazilian biomes: the Pantanal and the semi-arid Caatinga. Almost 90% of the samples that were molecularly identified as jaguar (n = 90) were successfully genotyped and were assigned to 30 individuals. Genetic diversity was generally high but was significantly lower in the Caatinga population. We show that non-invasive genetic sampling can be a reliable tool to study population parameters and to monitor the genetic status of jaguar populations in different habitats. It may also be useful for future surveys of jaguars that address ecological, behavioural and conservation issues, and could provide a baseline for non-invasive genetic studies of other wild felid populations.

Keywords Brazil, faeces, felid, genetic monitoring, jaguar, non-invasive sampling, *Panthera onca*

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S. Roques (Corresponding author) and F. Palomares Department of Conservation Biology, Estación Biológica de Doñana, CSIC, Avenida Americo Vespuccio s/n, Isla de la Cartuja, Sevilla, Spain. E-mail severine@ebd.csic.es

M. Furtado*, A.T.A. Jácomo, L. Silveira*, R. Sollmann† and N.M. Tôrres Jaguar Conservation Fund, Mineiros, Goiás, Brazil

J.A. Godoy Department of Integrative Ecology, Estación Biológica de Doñana, CSIC, Sevilla, Spain

* Also at: Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Brazil

† Also at: Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany

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Introduction

The jaguar *Panthera onca* is categorized as Near Threatened on the IUCN Red List (Caso et al., 2008). Since the beginning of the 20th century its distribution has decreased, mainly as a result of anthropogenic influences (Sanderson et al., 2002; Zeller, 2007). Monitoring tools that facilitate reliable estimates of jaguar range and population size are essential for the design of conservation strategies and the assessment of management plans. The elusive nature of this felid makes it difficult to obtain information on distribution and status, and populations can only be estimated using indirect methods. Camera trapping is one of the most frequently used methods and has provided repeatable estimates of densities and sex ratios of jaguars in several areas (see Maffei et al., 2011 for review). Faeces are a reliable source of biological material for elusive wild carnivores and are often used as evidence of species presence in monitoring programmes (De Angelo et al., 2011) as they are usually deposited in visible places to mark territories (Davison et al., 2002). Non-invasive genetic sampling of faeces has been used to monitor other elusive felids (Bhagavatula & Singh, 2006; Perez et al., 2006; Herring, 2008; Mondol et al., 2009; Borthakur et al., 2011). This method has been applied to wild jaguar populations for species identification (Haag et al., 2009; Roques et al., 2011) and for the study of melanism (Haag et al., 2010a,b) but not for individual identification.

Genetic identification of jaguars has been limited to studies using high quality samples such as blood and tissue (Moreno et al., 2006; Ruiz-Garcia et al., 2006; Soares et al., 2006; Eizirik et al., 2008), with only one example using faecal samples as a complement (Haag et al., 2010b). However, non-invasive genetic sampling can be expensive, time consuming, and error prone, and thus it is necessary to assess the reliability of protocols for each species and biological setting (Taberlet et al., 1999; Taberlet & Luikart, 1999; Broquet et al., 2007; Valière et al., 2007).

Our main aim was to optimize and validate a reliable, efficient and standardized non-invasive genetic sampling protocol for monitoring jaguar populations, based on individual identification and gender determination using faecal samples. Techniques were first optimized using fresh faecal samples, and their efficacy was later demonstrated using samples collected in two Brazilian biomes differing in

habitat and climate: the large seasonal floodplain of the Pantanal and the semi-arid Caatinga of north-eastern Brazil.

Study area

The Caatinga is a unique semi-arid biome in the extreme south-east of the distribution of the jaguar in Brazil (Fig. 1; Sanderson et al., 2002) and is one of the largest and most diverse regions of dry forest in South America. Almost 50% of the Caatinga has been completely or partially transformed (Casteleti et al., 2000). One of its most important protected areas, the Serra da Capivara National Park (130,000 ha) is believed to support an important jaguar population (Silveira et al., 2009). The Pantanal is the world's largest wetland and has a characteristic annual wet–dry cycle in which floods occur during and after an extended summer rainy period (Hamilton et al., 1996). It is home to the second largest jaguar population (Sanderson et al., 2002). The Caiman Ecological Refuge (a cattle ranch and ecotourism centre) lies in the southern Pantanal.

FIG. 1 Locations of the Pantanal and Caatinga biomes in Brazil.

Methods

Sample collection and preservation

Matched blood and fresh faecal samples (four replicates) from three captive jaguars (Zoological Park of Pajanosas, Seville, Spain) were used to select markers. Faecal samples were collected in sterilized plastic vials containing c. 30 ml of absolute alcohol. They were subsequently transferred to 100-ml plastic jars containing silica pellets (Wasser et al., 1997; Nsubuga et al., 2004; Roeder et al., 2004) and stored at room temperature prior to DNA extraction. Blood samples were mixed with Longmire's buffer solution (Longmire et al., 1988) at a ratio of 1 : 4 immediately after extraction and stored at 4 °C. Genetic analysis was conducted on faecal samples collected in 2008 in the Serra da Capivara National Park (n = 81) and the Caiman Ecological Refuge (n = 98). Blood samples from 21 jaguars captured at the Refuge were also analysed as reference samples and for comparison with genotypes obtained from faeces collected at the same site.

DNA extraction for species and sex identification

To extract DNA from blood samples we followed standard phenol–chloroform extraction protocols (Sambrook et al., 1989). To extract DNA from faecal samples we used protocols based on the GuSCN/silica method (Boom et al., 1990; Höss & Pääbo, 1993; Frantz et al., 2003). We purified and concentrated the extracted DNA by ultrafiltration, using a Microcon-30 device (Millipore Corporation, Billerica, USA). To monitor for contamination each set of 16 faecal samples included an extraction control. All steps were performed in a laboratory free from PCR (polymerase chain reaction) products and specially designated for the manipulation of non-invasive material. All scat samples collected in the wild were first screened for species identity using species-specific primers (Roques et al., 2011).

For sex identification we used a method described by Pilgrim et al. (2005), which we optimized for use on faecal samples from jaguars and other American felid species (puma *Puma concolor*, ocelot *Leopardus pardalis*, margay *Leopardus wiedii*). This method (see full details in Supplementary Material 1) is based on the size difference of the Amelogenin gene between males and females.

Optimization of microsatellite markers for individual identification

In the faecal samples from captive animals we tested 21 microsatellite markers originally developed for the domestic cat *Felis catus* (Menotti-Raymond et al., 1999). Some of the microsatellites had previously been redesigned for faecal samples from the Iberian lynx *Lynx pardinus*, and these are referred to with *a* or *b* following the original locus name (J.A. Godoy, pers. comm.). Four independent PCR replicates were performed for the faecal samples, and the genotypes obtained from blood and faecal samples from the same individuals (n = 4) were compared to evaluate the reliability of each microsatellite marker. We then selected 11 loci for use in the individual identification of faecal samples, based on the genetic variability indices (probability of identity, allelic diversity, observed and

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TABLE 1 Summary of non-invasive genetic analysis of faeces from the Caiman Ecological Reserve, in the Pantanal, south-western Brazil, and the Serra da Capivara National Park, in the Caatinga, north-eastern Brazil (Fig. 1).

	Caiman Ecological Reserve, Pantanal	Serra da Capivara National Park, Caatinga
Total area (km^2)	530	524
No. of faeces collected	98	81
No. identified to species (%)	79 (81)	57 (70)
Puma *Puma concolor*	32 (40)	4 (7)
Ocelot *Leopardus pardalis*/Margay *Leopardus wiedii*[1]	10 (13)	0 (0)
Jaguar *Panthera onca*	37 (47)	53 (93)
No. identified to individual (%)		
Sample through filter quality	32 (87)	49 (92)
Genotype quality after filtering		
Total no. of genotypes	26	48
No. of genotypes at $\geqslant 7$ loci (%)	23 (88)	46 (94)
No. with quality index $\geqslant 0.5$ (%)	23 (88)	43 (90)
No. of individuals with sex identification	37	53
No. of males (%)	20 (54)	40 (76)
No. of females (%)	7 (19)	11 (20)
Sex ratio (M/F)	2.9	3.6
No. of individuals without sex identification (%)	10 (27)	2 (4)
No. of jaguars detected	14	16
No. of males (%)	10 (71)	9 (56)
No. of females (%)	4 (29)	7 (44)
Sex ratio (M/F)	2.5	1.3
No. of individuals (Chessel)	13–27	13–21
Genetic diversity		
Expected heterozygosity (H_E)	0.70	0.67
Observed heterozygosity (H_O)	0.73	0.76
Identity index	0.346	0.462[2]

[1]Because of the recent divergence between ocelot and margay (Johnson et al., 2006), it was not possible to differentiate scat from these species with the approach used (Roques et al., 2011).
[2]Highly significant, $P \leqslant 0.0001$ (permutation test, *IDENTIX*)

expected heterozygosity) calculated using *GENETIX v. 4.05* (Belkhir et al., 2004) and *GIMLET v. 1.3.3* (Valière, 2002). For the selection of markers we also took into account PCR success rate (proportion of positive PCRs; Broquet et al., 2007), genotype reliability (error rates; i.e. allelic dropout, ADO and false alleles, computed using *GIMLET*) and multiplexing compatibilities (Table 1). For full details of the methodology see Supplementary Material 1.

Genetic parameters and estimation of population size

After assigning consensus genotypes to individuals we estimated genetic diversity at each site based on the following indices: probability of identity, mean number of alleles per locus, allelic richness, and observed and expected heterozygosities. We tested deviation from the Hardy–Weinberg equilibrium at each locus, and linkage disequilibrium, using *GENEPOP v. 3.3* (Raymond & Rousset, 1995). We evaluated the extent of genetic differences between jaguars from the Caiman Ecological Refuge and the Serra da Capivara National Park based on the F_{ST} estimator θ_{ST} (Weir & Cockerham, 1984), using *GENETIX* (Belkhir et al., 2004). We also investigated the level of relatedness between jaguars by calculating the mean identity index (Mathieu et al., 1990), using *IDENTIX* (Belkhir et al., 2002). We used a Monte Carlo resampling procedure (1,000 permutations) to compare the observed distributions of identity index to that expected under the null hypothesis of random mating (i.e. the expected unrelated and full-sibling pairwise relatedness). An excess of inbred individuals in the population could cause deviation of the means and variance values from random expectation. We further evaluated the relatedness composition within each population by analysing the observed distribution of genotypic differences between individuals and comparing it with the expected distributions for three different relatedness categories (unrelated, parent–offspring and sibling). Finally, to define the minimum number of loci necessary for individual discrimination in each area, we calculated the cumulative probability of genotype identity between unrelated individuals and full siblings for different sets of loci (incorporating loci in order of increasing probability). We considered a cumulated probability of identity over loci of $< 10^{-3}$ as sufficient for individual identification.

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The number of jaguars in the Caatinga and the Pantanal was estimated using individual profiles and asymptotic curve methods. In this approach the estimated number of individuals corresponds to the projected asymptote of the function of number of samples analysed vs the cumulative number of unique genetic profiles. We used the ACM–Chessel approach, which according to Petit & Valière (2006) performs best for a low sampling effort of n = 50 (close to our actual sampling size in the Caiman Ecological Refuge and the Serra da Capivara National Park).

Results

Species and sex identification based on faecal samples

Species identification success was 81% for the Caiman Ecological Refuge and 70% for the Serra da Capivara National Park. Of the faecal samples identified, 37 (47%) were from jaguars in the Refuge and 53 (93%) were from jaguars in the Park. The remaining samples were from pumas, ocelots or margays (Table 1). Our method for sex determination proved to be robust for low quantities of template DNA (between 10 pg and 50 ng; results not shown). Sex identification success was significantly different between the Refuge (73%) and the Park (96%; Table 1; $\chi^2 = 2.832$, P = 0.005). In both areas the sex ratio was significantly biased towards males (Serra da Capivara National Park, $z = 3.202$; P = 0.001; Caiman Ecological Refuge, $z = 2.885$; P = 0.004; Table 1).

Identification of individual jaguars from faeces

The 11 microsatellite markers used in this study were selected for their high discriminatory power between individuals, high PCR success (89–100%) and low error rates (allelic dropout and false alleles) in reference faecal samples (Supplementary Tables S1 & S2). The observed number of alleles per locus was 4–13 and observed heterozygosity 0.50–0.92 across markers. The accumulated probability of identity of the set of 11 loci for the Caiman Ecological Refuge reveals that the one or two most informative loci (Fca115, Fca547b) would be sufficient to discriminate among unrelated individuals, whereas seven loci would be necessary to reach a sufficiently high discriminatory power among related individuals (Fig. 2). Of the 90 faeces samples assigned to jaguar, we successfully amplified the microsatellite marker Fca82b in 81 (90%), with similar percentages of success for samples from the Refuge and the Park, and these were selected for further genotyping (Table 1). Six of the 11 loci (Fca26, Fca90a, Fca82b, Fca77, Fca126 and Fca566b) showed amplification success rates > 90% in both areas. Although rates varied for the remaining markers they were always > 65%, and the overall success rate was similarly high for both populations (Supplementary Table S2). Twenty-three samples from the Refuge and 46 samples from the Park were genotyped at seven or more loci, with QI ⩾ 0.5, and were considered reliable for individual assignment. Of the jaguar faeces collected, 77% of samples yielded high-quality genotypes. After grouping consensus incomplete genotypes with the complete ones, 16 unique genotype profiles were identified in the Caatinga and 14 in the Pantanal. We used probability of identity curves based on the 11 loci (Fig. 2) to estimate the minimum number of loci necessary in each area to reliably discriminate between any two individuals. Based on the 10^{-3} threshold we observed that two loci would be enough to discriminate between any two unrelated individuals in both areas and seven loci (Fca115, Fca566b, Fca126, Fca176, Fca90a, Fca547b, and a 7th locus varying among sites and depending on the relatedness scenario) would be enough to reach a sufficiently high discriminatory power among relatives.

Estimates of population size

In the Caatinga our estimate of the jaguar population using rarefaction methods was 16.57 ± 1.37, equivalent to the number of observed genotypes. In the Pantanal the asymptotic population size was 19.30 ± 2.46, higher than the 14 unique profiles obtained.

Genetic diversity, differentiation and relatedness structure

No significant overall linkage disequilibrium was found in consensus genotypes for individuals in Serra da Capivara National Park (P ⩾ 0.01), whereas for individuals in Caiman Ecological Refuge we found significant linkage disequilibrium at a single locus pair (Fca43-Fca566b; $\chi^2 = 45.84$, df = 25, P < 0.0001). We did not find any significant overall deviation from Hardy–Weinberg equilibrium proportions in either of the two samples ($\chi^2 = 26.57$, df = 22, P = 0.223, for the Park; $\chi^2 = 33.07$, df = 22, P = 0.07, for the Refuge), although observed heterozygosity in the Park tended to be higher than expected and a heterozygote excess test did not reach significance (P = 0.2495). We found a relatively high and significant divergence in allele frequencies between populations ($F_{st} = 0.178$, P ⩽ 0.01). Genetic diversity was generally higher in the Refuge than in the Park, although not significantly so (Table 1). Mismatch distributions in the Park differed from the expected distribution for the unrelated kinship class, in that a given proportion of individuals differed only at a few loci, meaning that this population may be composed of both unrelated and closely related individuals (Fig. 1). The mean and variance of the relatedness index distributions differed significantly from the null expectation for the Park (permutation test,

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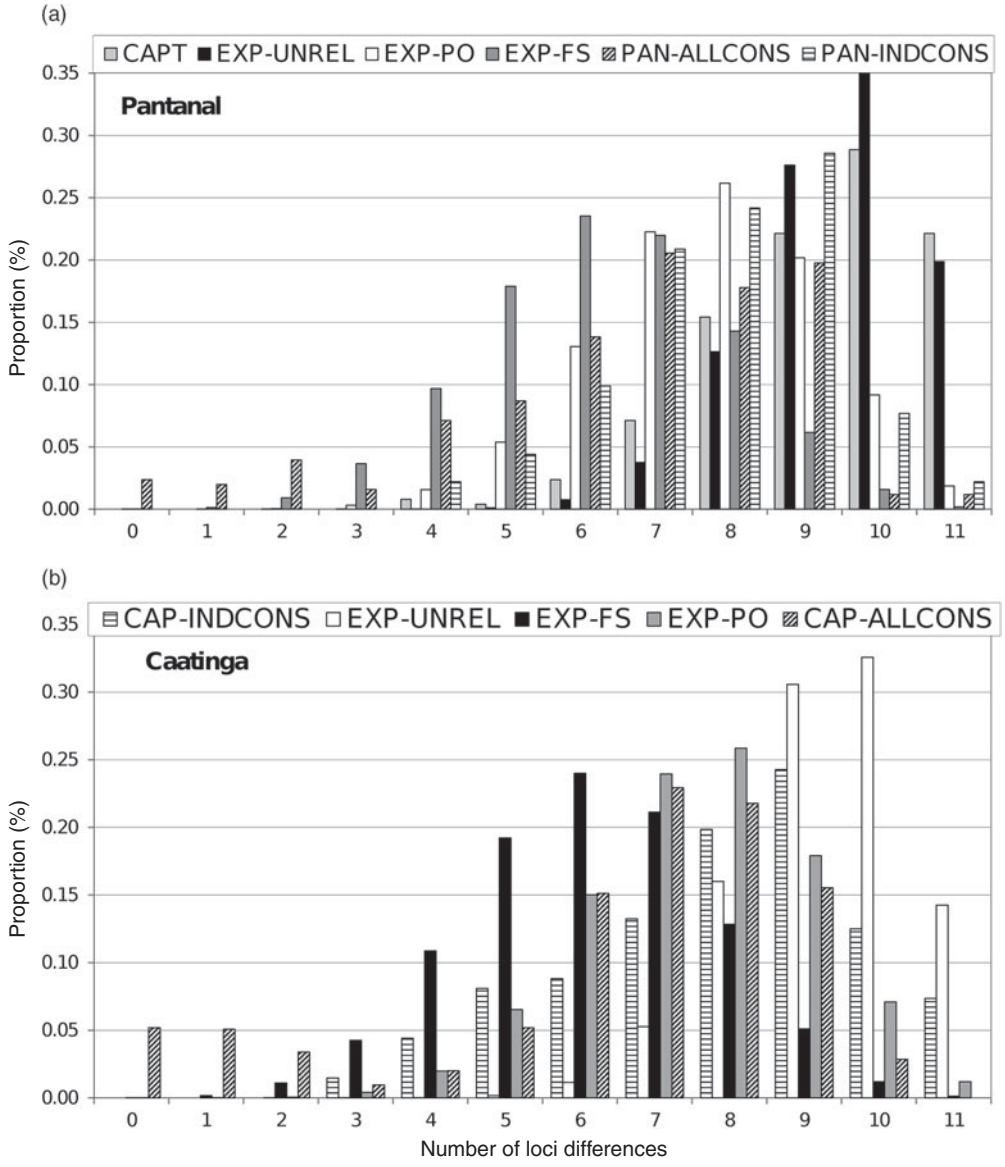

FIG. 2 (a) Distribution of genotypic differences (mismatches or difference in number of loci) between captured individuals from the Pantanal (CAPT) and field-collected faeces from the Pantanal (considering all consensus genotypes, PAN-ALLCONS, n = 23 and only unique genotypes, PAN-INDCONS, n = 14), and (b) the Caatinga (all consensus genotypes, CAP-ALLCONS, n = 43 and unique genotypes, CAP-INDCONS, n = 16) biomes of Brazil. Expected probability distributions of locus differences between genotypes are shown for three different relationship scenarios: parent-offspring (EXP-PO), full siblings (EXP-FS) and unrelated (EXP-UNREL).

$P \leqslant 0.0001$ and $P \leqslant 0.01$, respectively, indicating that jaguars in this population are more related than expected. Differences were not significant for the Refuge.

Discussion

An efficient protocol for the genetic analysis of jaguar faecal samples

Jaguar populations are found in highly variable environmental conditions that can influence DNA quality, amplification success, and thus the possibility of successful genetic analysis of faecal samples. This is especially true in tropical environments (a large part of the species' range), where faecal samples generally decompose much faster and DNA is more likely to degrade rapidly because of high levels of humidity. However, in this study we had similarly high success rates for DNA extraction and PCR amplification for species, sex and individual identification from jaguar faecal samples in two areas with contrasting environmental conditions. Furthermore, species identification rates based on our previously developed method (Roques et al., 2011) were higher (70% for Serra da Capivara National Park and 81% for Caiman Ecological Refuge) than those reported for sequencing methods for faeces of the same species collected in the field (59%, Farrell et al., 2000; 55%, Haag et al., 2009).

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The use of a single pair of Amelogenin-based primers for sex determination also proved to be reliable and a more efficient technique (73–96% success rates) than those previously reported, which were usually based on amplification of the Y-chromosome-linked sex-determining region gene, using a combination of two pairs of conserved primers (e.g. 71%, tiger *Panthera tigris*, Borthakur et al., 2011). As primers were purposely designed to prevent amplification of human Amelogenin sequences they should be less prone to false results arising from human contamination. Our sexing protocol was efficient for jaguar samples from both areas and also for puma, ocelot and margay and could therefore be a good approach for sex identification based on non-invasive felid DNA samples in general.

For individual identification, stringent laboratory conditions, a previous DNA quality filtering step, an optimal panel of reliable markers and sufficient PCR replicates allowed us to maximize the amplification rate and minimize genotyping errors, which resulted in a high proportion of reliable genotypes in both areas (62–87%; Supplementary Table S2), higher than in other recent non-invasive genetic sampling studies of felids based on a similar number of loci (c. 70% for the Bengal tiger *P. tigris tigris*, Borthakur et al., 2011, and the snow leopard *Panthera uncia*, Janecka et al., 2008; 50% for the tiger, Mondol et al., 2009). Furthermore, the selected set of markers showed a considerable discriminatory power and we found that any two siblings will differ at at least two loci. Consequently we found that only seven or eight loci were necessary to reliably discriminate even among close relatives in both areas. Given that large populations are generally composed of a pool of mostly unrelated individuals, the use of only seven markers will be sufficiently conservative and still useful in most situations. However, while it is generally true that fewer loci reduce both costs and error rates (Kalinowski, 2006), the selection of the number of loci used may depend upon the question to be addressed. Using a reduced set of loci may be appropriate for standard population monitoring (i.e. identification and number of individuals) but a larger number of loci is more suitable when analyses include other aspects of population genetics relevant to conservation (e.g. genetic diversity and structure, or relatedness among individuals), which are often relevant at regional and local geographical scales. Using a larger set of markers may be particularly critical for monitoring jaguar populations, as the probability of 'capturing' individuals from the same family is expected to be non-negligible at the scale that populations are usually sampled.

Non-invasive genetic monitoring of jaguar populations

In the southern Pantanal and the Caatinga jaguar habitat is being transformed and fragmented, and the evaluation of genetic health and population size are thus high priorities. The Serra da Capivara National Park is thought to hold one of the most important jaguar populations in the Caatinga (Silveira et al., 2009), and the southern Pantanal is one of the most important strongholds for jaguars and their prey (Sanderson et al., 2002). We found that the level of genetic diversity was similar to that estimated previously for southern populations of the species (He = 0.732 in Haag et al., 2010b and He = 0.724 in Eizirik et al., 2001), but we also report a considerable level of genetic differentiation between these areas (F_{st} = 0.178). We detected a significant level of genetic relatedness in the Park population. These results support a previous ecological evaluation that described population reductions in the Brazilian Caatinga (de Oliveira, 2002) and further indicate reduced levels of gene flow between these two areas. Our estimate of jaguar numbers in the Park is largely in agreement with a previous estimate of the number of adults in the same sampling area, based on camera-trapping (Silveira et al., 2009). This suggests that the non-invasive genetic sampling strategy used here, together with a well-planned faeces sampling design, could yield reliable population estimates and could be used in combination with, or as an alternative to, camera-trapping, especially where camera-trapping may be difficult or inefficient (tropical areas, low-density jaguar populations, Silver et al., 2004). We observed that sex ratios obtained from faeces were significantly biased towards males and this most likely reflects a lower detectability of females, as discussed elsewhere (Palomares et al., 2012).

Our study demonstrates that faeces could form the basis of a cost-effective, reliable and widely applicable method for the genetic monitoring of jaguar populations, and potentially for other felid species. Given the wide distribution of the jaguar and the importance of connectivity for the survival of this species, coordination of a common sampling strategy and molecular marker panel would facilitate the comparison of different studies.

Conclusion

Our results demonstrate the potential of non-invasive genetic sampling for population monitoring across the jaguar's range. Rigorous laboratory protocols and extensive optimization are important for reliable estimations of population and genetic parameters. Our approach could provide a tool for large-scale surveys that address ecological, behavioural and conservation-related issues such as density estimates, relatedness, social structure, patterns of gene flow and connectivity. The technique could also be used for the non-invasive study of other felid species and is particularly useful for monitoring populations or species that cannot be identified individually using camera traps.

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Biographical sketches

S. Roques develops molecular approaches to the conservation and management of threatened species. M. Furtado investigates

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the occurrence of diseases in wild carnivores and the relationship between wild and domestic animals. A.T.A. Jácomo focuses on the conservation and management of large mammals in human-dominated landscapes. L. Silveira is interested in the conservation of large predators, especially the jaguar, and researches methods for reducing predator–rancher conflict. R. Sollmann is interested in population modelling and the ecology and conservation of carnivores. N. M. Tôrres is interested in the macroecology and conservation biology of mammals, especially carnivores. J.A. Godoy uses genetic methods and theory to infer evolutionary and demographic processes acting on populations and species at different temporal and spatial scales. F. Palomares is interested in the ecology and conservation biology of vertebrates and has studied several European and American species of predators and their prey, using both invasive and non-invasive sampling techniques.

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