Jaguar Conservation Genetics

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Information on genetic aspects of jaguar populations is still scarce. Initial studies have surveyed genetic diversity parameters and assessed the geographic differentiation among individuals on a continental or sub-continental scale, but so far little has been accomplished with respect to investigating regional or local jaguar populations. Moreover, different studies have employed different sets of molecular markers, posing potential problems for the future development of comparative analyses across study sites and ecosystems. Here we review the current status of jaguar genetic studies, present a new set of microsatellite markers that may be useful for jaguar population genetic studies, and survey the molecular diversity of two adjacent wild jaguar populations, sampled in the Brazilian Pantanal region. Our results suggest that this set of markers is highly efficient for jaguar genetic studies, and that moderate to high levels of variability are present in wild jaguar populations, at least in the surveyed areas of the Pantanal. This contribution may be useful as a review of jaguar genetics, as well as a baseline empirical work that might support future in-depth investigations of these and other free-ranging populations of this felid.

The use of molecular tools to investigate genetic, ecological and behavioral aspects of wildlife populations has gained immense popularity in recent years, allowing unprecedented probing into multiple components of organismal biology which were previously inaccessible. In addition to its scientific relevance, knowledge of such aspects is often a critical component for the design of adequate conservation strategies on behalf of species and ecosystems. Genetic data are required to understand long-term demographic history and dynamics, and to characterize social structure and patterns of dispersal and territoriality. They are also useful for assessing evolutionary potential and inferring census and effective population sizes, which are important components of Population Viability Analyses. The field of Conservation Genetics encompasses a diverse array of methodological approaches involving the use of genetic information to tackle these and other issues of conservation concern.

Jaguars (Fig. 1) are an elusive species whose population biology has been historically difficult to study, and only recently has been the focus of in-depth investigation made possible by technological and analytical innovations. If ecological investigations of jaguars are now the focus of multiple studies at various field sites, genetic analyses of this species are still in their infancy, having been severely limited by the practical difficulty in sampling biological materials representative of natural populations. A range-wide assessment of genetic diversity and evolutionary history has been performed, and studies addressing regional or local-level issues are starting to become feasible, as improved methods for biological sampling become incorporated in this scientific discipline. Here we (i) review the history of jaguar conservation genetics and the current state of the field, (ii) discuss the advantages and prospects of developing a set of molecular markers that can become standardized for jaguar population genetics, and (iii) present novel preliminary data describing the levels of microsatellite diversity in a natural jaguar population, that of the southern Brazilian Pantanal.

Although the jaguar had been included in previous genetic studies addressing phylogenetic questions with the use of molecular markers (e.g. Johnson & O'Brien 1997), its intraspecific levels of diversity had not been investigated until 2001. In that year, a study employing mitochondrial DNA (mtDNA) sequences encompassing a segment of the control region (CR) and 29 nuclear microsatellite loci addressed the genetic diversity and demographic history of jaguars, based on 44 individuals sampled from Mexico to southern Brazil (Eizirik *et al*. 2001). That study revealed that this species exhibits a shallow mtDNA structure, compared to other felids, with low differentiation among geographic regions. The shallow structure, with low inter-regional differentiation, was inferred to have been caused by a rather recent population expansion, *ca.* 300,000 years ago, followed by a history of demographic connectivity over a continental scale. No support was observed for the classically recognized jaguar subspecies, a finding that had also been reported on the basis of morphological data (Larson 1997). The major pattern that emerged from that data set was a phylogeographic partition between the northern and southern portions of the range, likely a function of reduced historical gene flow across the Amazon River. The levels of diversity detected in the hypervariable microsatellite loci were quite high and also indicative of large scale gene flow across the range of the species. No ma-

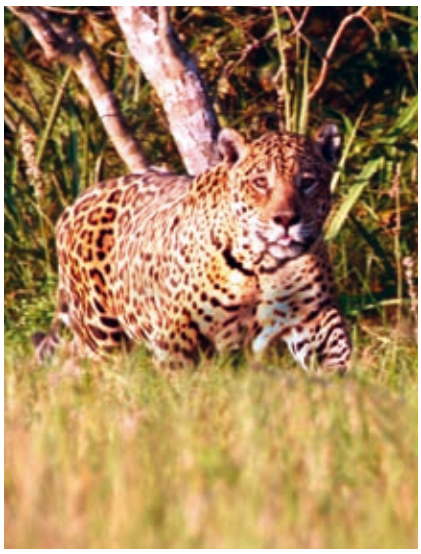

Fig. 1. Female wild jaguar in its natural habitat in the Pantanal (Photo L. Leuzinger, Fazenda Barranco Alto).

jor partitions were detected with those markers, but four moderately differentiated regional groups could be discerned. The partition likely induced by the Amazon River could still be detected, but its intensity was lower than that observed with the female-transmitted mtDNA marker, suggesting that male-mediated gene flow across the river could play a role in the historical geographic homogenization in this species. This hypothesis has so far not been thoroughly tested (but see Ruiz-Garcia *et al.* 2006), and requires more detailed sampling of local populations, particularly throughout the Amazon region. Likewise, the precise magnitude of genetic differentiation among any regional populations could not be fully tested in that study, due to the sparse sampling available for each locale, and the range-wide scope of the analyses.

Subsequent to that study, to our knowledge only three scientific papers have addressed genetic aspects of jaguar populations (Moreno *et al*. 2006, Ruiz-Garcia *et al.* 2006, Soares *et al.* 2006). All three studies have employed microsatellite loci as molecular markers, allowing an assessment of the performance of these hypervariable nuclear segments to investigate this species. These loci are currently the markers of choice for population level studies of most wildlife species, as their high mutation rates and Mendelian inheritance allow the detailed probing into demographic, behavioral and ecological questions. We will briefly review the scope and findings of these three papers, and focus on the comparison of the microsatellite loci employed, aiming to evaluate the current status of marker standardization among studies.

Moreno *et al*. (2006) analyzed 39 jaguar individuals sampled in Brazilian zoos, using four microsatellite loci, three of which had been used by Eizirik *et al*. (2001). These three loci presented high levels of allelic diversity in this captive population (no analysis of natural populations was included), with 9-12 alleles identified in each of them. Ruiz-Garcia *et al.* (2006) addressed the population genetics of Colombian jaguars, including a total of 62 individuals from that country and 22 additional samples. Twelve microsatellite loci were employed, four of which had been previously used by Eizirik *et al*. (2001), and three overlapping with those of Moreno *et al*. (2006) (one of which did not overlap with Eizirik *et al*. [2001]). They also found high levels of diversity and some evidence of genetic continuity (*i.e.* no differentiation) between areas located to the north and to the south of the Amazon River. This finding might disagree with the initial inference by Eizirik *et al.* (2001), but the sampling schemes and geographic scopes were different between the two studies, and so were most of the molecular markers employed. Further analyses with designed sampling and standardized markers are still required to test this hypothesis. Finally, Soares *et al*. (2006) employed seven microsatellite loci (all of which had previously been used by Eizirik *et al.* [2001]) to perform a paternity analysis in a jaguar population in the Brazilian Cerrado biome. Only four individuals were analyzed, and three of them were related to each other, so little inference can be made on the levels of genetic diversity in that population using these data.

An overall conclusion of this brief assessment is that still very few studies have been performed on jaguar genetics, highlighting the need for further work on this topic. Moreover, many of the employed markers were not shared among studies, precluding direct comparisons of the levels of genetic diversity identified in different areas. It would be thus important to develop a set of markers that is standardized for jaguar genetics, presenting high amplification success and allelic diversity in this species, and allowing for cross-study comparisons of variability measures. Although such rough comparisons of diversity could be made across studies as long as the loci were the same, a more refined goal would be to have data sets that could be integrated in meta-analyses.

One challenge to such integration is the lack of reproducibility of the precise allele sizes across different laboratories and genotyping devices, especially in the case of dinucleotide microsatellite markers (whose repeat unit is 2 nucleotides long). This type of locus is more difficult to score reliably, and more prone to inter-lab variation in allele assignment (E.E., personal observation). However, they are very abundant in the genome, and more frequently identified in screens for variable markers than other types of repeats. Most of the microsatellite markers originally described for the domestic cat (*Felis catus*) were dinucleotides (*e.g.* Menotti-Raymond *et al.* 1999), and this set of loci served as the basis for most population genetic studies performed with wild felids so far. As a consequence, most loci applied in the studies reviewed above were dinucleotide repeats: 27 out of 29 loci in Eizirik *et al*. (2001), four out of four loci in Moreno *et al*. (2006), 11 out of 12 loci in Ruiz-Garcia *et al*. (2006), and six out of seven loci in Soares *et al*. (2006). In spite of the variability reported for these markers in these studies, it may be better to base a standardized microsatellite set for jaguars on other types of loci, such as tetranucleotides (composed of 4-bp repeat units), whose allele scoring is more reliable and reproducible. Given that several trinucleotide and tetranucleotide loci have been reported for the domestic cat (*e.g.* Menotti-Raymond *et al.* 1999, 2005), we aimed to assess their performance in jaguars, and to test whether they may serve as a basis for a standardized panel of population-level markers for this species.

Materials and Methods

Assessment of tetranucleotide microsatellite loci for jaguar population genetics

We tested 20 trinucleotide/tetranucleotide microsatellite loci developed for

the domestic cat (Menotti-Raymond *et al.* 1999, 2005). Two of them (FCA441, FCA453) had been previously used by Eizirik *et al*. (2001), and another (FCA391) was employed by Ruiz-Garcia *et al.* (2006). Five loci (FCA749, FCA751, FCA748, FCA732 e FCA559) did not present efficient amplification in jaguars in pilot runs, and were excluded from further testing. Another locus (FCA424) was monomorphic (*i.e.* bearing no variation) in the pilot sample, and locus FCA738 presented only two alleles; both of them were also excluded from further analyses. We thus focused on a panel of 13 loci (FCA742, FCA741, FCA740, FCA723, FCA453, FCA441, FCA391, F146, F124, F98, F85, F53 and F42) that presented good results for jaguars sampled across their range (not shown), and initiated an assessment of their performance in population-level studies. We are currently employing these markers in jaguar population genetic studies focusing on multiple sites located in the Brazilian Atlantic Forest, Pantanal and Amazon biomes. We describe below preliminary results from a screen for genetic variation in these markers in the southern Pantanal, based on samples collected at two nearby locations.

Genetic diversity of natural jaguar populations: the Brazilian Pantanal

Blood samples from 23 wild-caught jaguar individuals were obtained in two nearby areas within a seasonally flooded habitat in the southern region of Pantanal, Mato Grosso do Sul state, Brazil. The field sites were the Caiman Ecological Refuge (19.80° S / 56.27° W; n = 12) and San Francisco ranch (20.08º S / 56,60º W; n = 11) where field projects addressing jaguar ecology and conservation are currently being carried out.

Blood samples were preserved with EDTA and in some cases with a salt saturated solution (100mM Tris, 100mM EDTA, 2% SDS), and stored at 4°C or –20°C for most of the time prior to DNA extraction. Total DNA was extracted from blood samples following a standard phenol-chloroform protocol (Sambrook *et al*. 1989), and its quality and yield were assessed by analysis on an agarose gel. DNA extracts were amplified by PCR for the 13 microsatellite loci listed above. Every forward primer was 5'-tailed with an M13 sequence (Boutin-Ganache *et al*. 2001), and used in combination with an M13 primer that had the same sequence but was dye-labeled on its 5' end. PCR reactions were carried out for each locus separately, and products from 1 to 3 loci were diluted and pooled together based on yield, size range and fluorescent dye. Microsatellite genotyping was performed using a MegaBACE 1000 automated sequencer and the ET-ROX 550 size standard (GE Healthcare), and then analyzed utilizing the accompanying software Genetic Profiler 2.2.

We calculated the number of alleles, polymorphic information content (*PIC*), observed (H_O) and expected (H_E) heterozygosity for each locus, and tested for any evidence of departures from expectations of Hardy-Weinberg Equilibrium (HWE) and linkage equilibrium using CERVUS 2.0 (Marshall *et al*. 1998) and ARLEQUIN 3.1 (Excoffier *et al*. 2006). To quantify the power of individual identification with the set microsatellite markers applied here, we estimated the probability of identity (P_{ID}) index, *i.e.* the probability of any two individuals in the population randomly sharing identical genotypes for all the analyzed loci (Paetkau *et al*. 1998).

Results and Discussion

Of 13 primer pairs used, ten presented allele intervals compatible with a tetranucleotide repeat (FCA741, FCA740, FCA723, FCA453, FCA441, FCA391, F124, F85, F53, F42), two were trinucleotide repeats (F146 and F98) and one was a dinucleotide repeat (FCA742). One additional tetranucleotide locus (FCA741) was found to be monomorphic in this jaguar sample and was removed from the study.

All loci were in linkage equilibrium in both sampling locales after Bonferroni adjustments (Rice 1989 [α = 0.05]). Deviations from HWE expectations were tested for each of the two locations separately, and then combined. One locus (FCA441) was found to be out of HWE in the Caiman ranch population and another one (FCA742) in the San Francisco ranch population. In both cases, the deviation from HWE was no longer significant after application of the sequential Bonferroni correction. When both populations were combined in a joint analysis, a third locus (FCA740) appeared to depart from HWE expectations, but again the statistical significance of this result was lost after applying the sequential Bonferroni correction. These results indicate that the deviations observed prior to the correction may not bear any biological relevance, and for the present time we can infer that these markers meet HWE expectations for these populations.

The overall analysis of the 12 selected loci, employing the total sample of 23 individuals captured in both locales, revealed moderate to high levels of genetic diversity, with an average expected heterozygosity (H_e) of 0.7171, mean number of alleles per locus of 5.83, and mean Polymorphic Information Content (PIC) of 0.6592 (Table 1). Both populations exhibited considerable diversity (Table 2), a finding which will be refined with additional sampling in the

Table 1. Measures of diversity at 12 microsatellite loci characterized in this study for *Panthera onca* in the southern region of the Pantanal biome, Brazil.

Locus	*N*	No. of alleles	Allele size range	Ho[1]	He[2]	PIC[3]
FCA742	19	11	142-178	0.947	0.876	0.838
FCA740	23	5	300-316	0.652	0.739	0.681
FCA723	23	6	200-244	0.783	0.653	0.580
FCA453	22	6	192-216	0.818	0.715	0.656
FCA441	22	4	165-177	0.500	0.589	0.520
FCA391	23	6	215-243	0.870	0.776	0.727
F146	23	3	173-182	0.304	0.382	0.318
F124	23	7	203-231	0.870	0.769	0.715
F98	23	3	189-195	0.565	0.641	0.552
F85	22	7	139-183	0.773	0.834	0.790
F53	21	5	164-196	0.762	0.803	0.748
F42	22	7	251-275	0.864	0.830	0.785

[1]Observed heterozygosity; [2]Expected heterozygosity;

[3]Mean polymorphic information content.

Table 2. Measures of diversity at 12 microsatellite loci in two local populations of *Panthera onca* from the Brazilian Pantanal.

Population	*n*	Average expected heterozygosity	Average No. of alleles per locus	PIC*	No. of private alleles
Caiman E. R	12	0.6962	5.33	0.6226	17
San Francisco ranch	11	0.7088	4.42	0.6248	6

* Mean polymorphic information content

future. Since this is the first assessment of jaguar genetic diversity performed for local wild populations, and most of our molecular markers are different from those employed previously, the observed levels of variability cannot yet be directly compared to other studies. However, this scenario should change in the near future as other populations are currently being analyzed with these same markers. Given that jaguars are believed to be more abundant in the southern Pantanal region than in many other parts of their distribution, these preliminary data from this biome may serve as a baseline which may be helpful when assessing current levels of diversity in small, fragmented jaguar populations.

The estimated probability of identity (P_{ID}) using these markers in the joint Pantanal sample was 2×10^{-13}, indicating that it is extremely unlikely that any two individuals may bear the same composite genotypes at these loci (*i.e.* this estimate would imply that one would need to sample > 1 trillion jaguars to find two individuals with identical composite genotypes). This is very important in the context of allowing the individual identification of jaguars using molecular markers, such as in the case of non-invasive samples (e.g. scats, hairs) and forensic specimens, which are of direct interest to studies addressing ecological, behavioral and conservation-related issues (*e.g.* density estimates, kinship and social structure, patterns of dispersal and population connectivity). Given the power observed in this panel of 12 microsatellites, it is likely that a subset of these markers will still have very high precision in the discrimination of jaguar individuals in any local population, allowing the investigation of ecological and behavioral questions using non-invasive sampling (which often requires that one selects a smaller number of loci to minimize error rates and to facilitate thorough genotype checking via redundancy). We conclude that this set of markers holds good promise for building a standardized panel for jaguar population genetic studies, either by itself or in combination with some loci selected from previous studies.

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