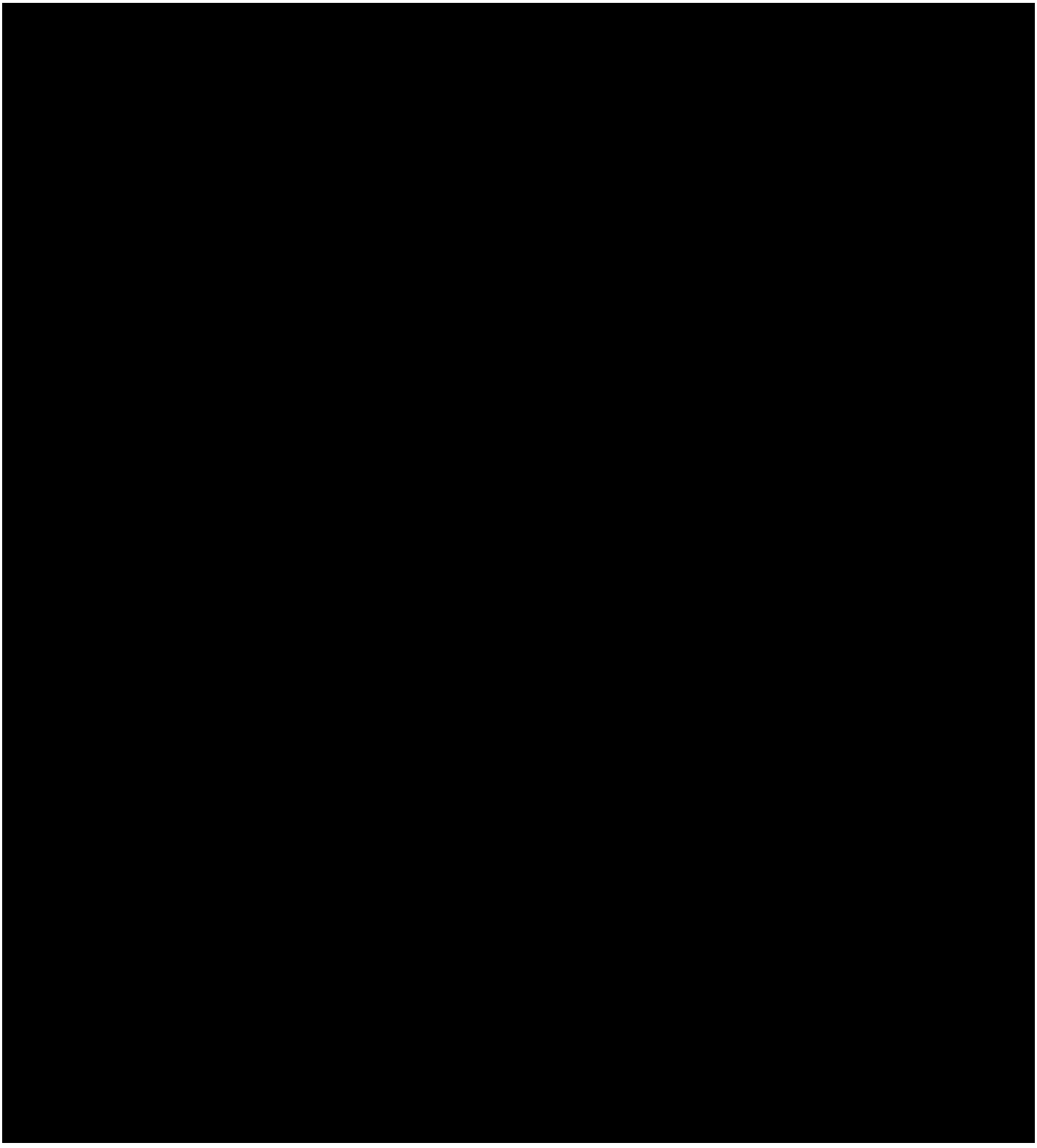

Introduction

Severe anthropogenic changes have caused worldwide loss and fragmentation of natural habitats, contributing significantly to the decline and isolation of wild populations, thus increasing their risk of extinction (Frankham *et al.* 2002; Schipper *et al.* 2008). In this context, large carnivores such as the jaguar (*Panthera onca*) are thought to be particularly sensitive to population decline and local extinction (Gittleman *et al.* 2001). The jaguar is the largest wild felid in the Americas (Nowell & Jackson

1996), historically ranging from the southwestern USA to the Argentinean Patagonia. In the last 100 years, however, it has lost approximately half of its original range because of severe habitat loss and fragmentation, associated with a declining prey base and direct human persecution (Sanderson *et al.* 2002; Zeller 2007). As a consequence, the current jaguar distribution from Mexico to northern Argentina comprises a mosaic of remnant populations of variable size and increasing geographical isolation. For this reason, it is critical to characterize the historical and current genetic structure of these populations, so as to allow the assessment of possible effects of fragmentation on levels of diversity and non-adaptive differentiation. So far, two studies have analysed the genetic structure of jaguars (Eizirik *et al.* 2001; Ruiz-Garcia *et al.* 2006), both of which reported evidence of historical connectivity across broad geographical areas, and only some inferred barriers to gene flow on a continental scale (e.g. the Amazon river, the Andean mountain chain and an additional barrier affecting Central American populations). Although the genetic structure of local jaguar populations has not yet been investigated, it may be thus hypothesized that jaguars have effectively moved across the various types of habitat contained in their historical range, so that little differentiation should be observed on a regional scale.

One of the most extreme examples of habitat fragmentation is the Atlantic Forest biome of South America, all of which was formerly included in the jaguar range, and where the species is now severely endangered (Fig. 1). In the Upper Paraná Atlantic Forest (UPAF) Ecoregion (Fig. 1), spanning southwestern Brazil, northeastern Argentina and eastern Paraguay, resident jaguars are essentially restricted to semi-connected protected areas that possibly form a metapopulation structure (Cullen *et al.* 2005). Habitat loss in this region caused by deforestation and land conversion into livestock ranches and cropland was intensified in the second half of the 20th century, with effects on fragment connectivity already visible in satellite images from the 1970s (De Angelo 2009). However, most of the deforestation in areas separating present-day fragments occurred from the 1980s onward and was compounded by the flooding of large dams that also led to important habitat loss.

Conservation units in the UPAF have been categorized as bearing highest priority because they correspond to the last viable populations of jaguars left in

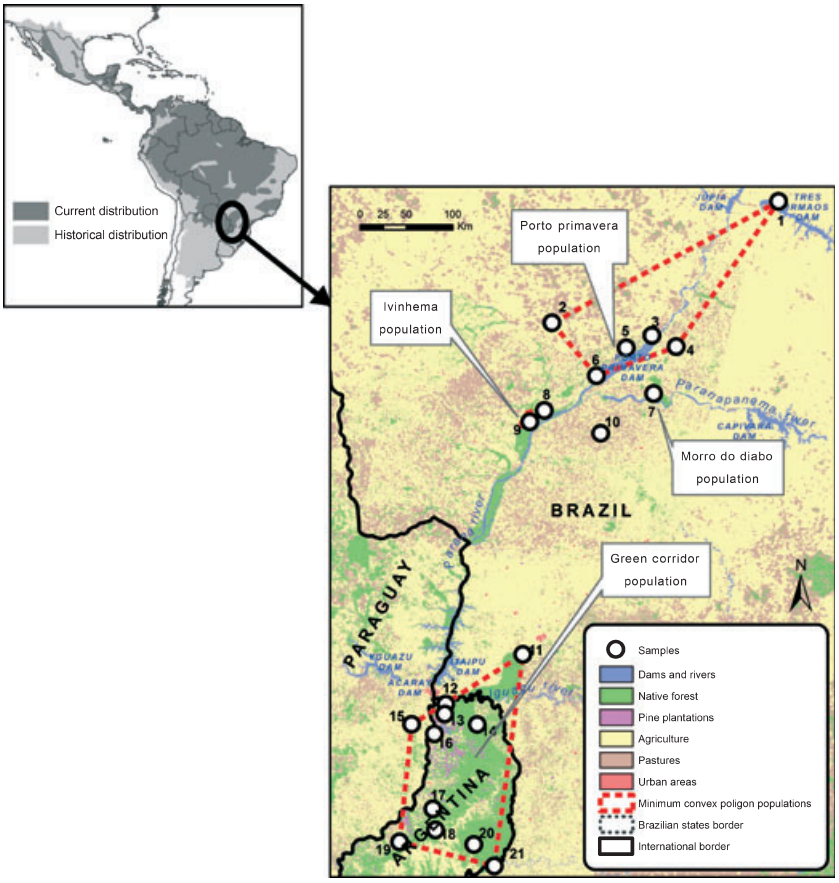

Fig. 1 Detailed map of the Upper Paraná Atlantic Forest representing the studied populations. Each circle represents the geographical origin of one or more samples (see Table S1 in Supporting information). Point 10 refers to one stray individual (bPon24) captured in Alto Paraná [PR] and genetically identified as originating from the Porto Primavera (see text for details). The smaller map on top depicts the historical and current distribution of the jaguar (modified from Sanderson *et al.* 2002 and Zeller 2007) and indicates the location of the UPAF ecoregion.

this type of ecosystem (Zeller 2007). However, recent estimates of these populations indicated that there are only 25–53 adult jaguars in the 'Green Corridor' of Argentina (Misiones Province) and Brazil (Iguaçu National Park, Paraná [PR] state, and Turvo State Park, Rio Grande do Sul [RS] state) (Paviolo *et al.* 2008), in addition to 9–15 adults in Morro do Diabo State Park (São Paulo [SP] state, Brazil) (Cullen 2006), and 10 in Ivinhema State Park (Mato Grosso do Sul [MS] state, Brazil) (D. A. Sana, unpublished data). A fourth jaguar population from this region (located in the riverine marshes that have now been flooded by the Porto Primavera dam [MS/SP states]) was estimated to contain 10–20 individuals in 1993 (P. G. Crawshaw Jr., unpublished data), but is currently considered to be extinct (see below). Moreover, no core area in the UPAF is sufficiently large to sustain viable populations of this species (Di Bitetti *et al.* 2003; Galindo-Leal & Câmara 2003).

In this study, we investigated the magnitude and spatial distribution of genetic diversity in remnant jaguar populations of the UPAF ecoregion. We assessed the occurrence of genetic differentiation among local population fragments and measured demographic connectivity by identifying migrants and inferring patterns of recent gene flow. Our results indicate that these populations are losing diversity and undergoing rapid genetic differentiation induced by genetic drift, as a consequence of anthropogenic isolation and very small population sizes in individual forest fragments.

Materials and methods

Study sites

The study area included a network of large protected areas located in the Paraná River Basin (Fig. 1) including Morro do Diabo State Park (37,000 ha; SP state), Ivinhema State Park (73 300 ha; MS state), Iguaçu National Park (185 262 ha; PR state), and Turvo State Park (17 491 ha; RS state) in Brazil. In addition, there are smaller forest fragments that may be able to sustain individuals in connection to the protected areas. Another surveyed field site included the area of influence of the Porto Primavera hydroelectric dam (MS/SP states; Fig. 1) where jaguar monitoring was conducted before, during and after the filling of the reservoir. This region suffered a strong environmental impact in 1998, when the filling of the dam flooded an area of ~220 000 ha, submerging the riverine marshes and semi-deciduous forests that were the jaguar's strongholds in that location. The last radio-collared jaguar in that area was found dead in 2003, and there are currently only occasional reports of scattered animals roaming in this region (D. A. Sana, unpublished data), suggesting that the species is essentially extinct locally. Further south, another large dam (Itaipu Binacional, on the border between Paraguay and Brazil; Fig. 1) in 1982 flooded an area of ~135 000 ha containing primary forests, likely leading to partial or complete demographic isolation between the jaguar populations located in the northern and southern sectors of the UPAF ecoregion.

In the Misiones Province in Argentina, the largest and most continuous remnant of the UPAF, the Green Corridor (Fig. 1), encompasses 1 100 000 ha and spans 200 km linking Iguaçu National Park to Turvo State Park in Brazil, through a patchwork of intervening protected and unprotected areas in the province of Misiones. In adjoining eastern Paraguay, deforestation has occurred at a very high rate in recent years, and most protected areas are now isolated and cover <10 000 ha (Di Bitetti *et al.* 2003).

Sample collection and laboratory procedures

Biological samples were obtained from remnant areas in the UPAF, where field projects addressing jaguar ecology and conservation have been carried out over the last two decades. Samples were subdivided on the basis of their origin and proximities to four pre-defined geographical groups (referred to as 'populations'; Table S1, Supporting information; Fig. 1), which have been suggested by radio-telemetry and camera-trapping data, and habitat suitability models for jaguars in this region (L. Cullen Jr. and D. A. Sana, unpublished data; De Angelo 2009). Blood samples from eight individuals were obtained from Morro do Diabo State Park and its surroundings (SP) between 1998 and 2004; 23 samples including blood, tissue and pelts were obtained from the area affected by the Porto Primavera dam (MS/SP) between 1993 and 2004; eight blood and tissue samples were collected between 2002 and 2007 from Ivinhema State Park and its surroundings (MS); and 11 samples including blood, tissue, serum, pelt and hair were obtained between 1992 and 2007 from the Green Corridor (encompassing areas in Brazil [PR and RS] and several forested locations in the Misiones Province, Argentina).

In addition to the samples mentioned earlier, faeces were collected opportunistically in the field, and two scats were collected from captive animals whose geographical origin was known (both from the Green Corridor; Table S1, Supporting information). All faecal samples, as well as those consisting of pelts and hairs, were subjected to a rigorous procedure to confirm the species source using a short segment of the mtDNA *ATP synthase subunit 6* (*ATP6*) gene (Haag *et al.* 2009). Four faecal samples were obtained from Ivinhema, two

of which were confirmed as originating from jaguars. We also included six faecal samples collected in the Green Corridor that had been identified in a previous study (Haag *et al.* 2009) as belonging to jaguars. More recently, 12 additional scats were collected in the Green Corridor, five of which could be identified as originating from jaguars, so that a total of 11 scats from the Green Corridor were included in this study.

Blood samples were preserved with EDTA, and in some cases with an equal volume of a salt saturated solution (100 mM Tris, 100 mM EDTA, 2% SDS). Pelts, tissues and hairs were preserved in 96% ethanol. Faecal samples were stored in sterile vials containing silica gel at a ratio of 4 g silica/g faeces (Wasser *et al.* 1997). All samples were stored at −20 °C prior to DNA extraction. Genomic DNA was extracted from blood and tissue samples using a standard phenol-chloroform protocol (Sambrook *et al.* 1989). Extractions from pelt and hair samples were performed with the Puregene DNA Purification Kit (GENTRA) or using the ChargeSwitch® Forensic DNA Purification Kit (Invitrogen). DNA from scats was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN), following the manufacturer's instructions. Each batch of faecal DNA extraction ($n = 10$) included one negative control. All pre-PCR procedures were carried out in a separate laboratory area, within a UV-sterilized laminar flow hood and employing dedicated pipettes with aerosol-resistant tips to prevent the occurrence of contamination.

Jaguar DNA extracts were screened for 13 microsatellite loci: one containing a dinucleotide repeat (FCA742), two with trinucleotide repeats (F146 and F98), and 10 with tetranucleotide repeats (FCA741, FCA740, FCA723, FCA453, FCA441, FCA391, F124, F85, F53 and F42). These primers were originally developed for the domestic cat (*Felis catus*) by Menotti-Raymond *et al.* (1999, 2005) and have been optimized and standardized for use with jaguar samples (Eizirik *et al.* 2001, 2008). Every forward primer was 5′-tailed with an M13 sequence (5′-CACGACGTTGTAAAACGAC-3′) (Boutin-Ganache *et al.* 2001) and used in combination with an M13 primer that had the same sequence but was dye-labelled (with 6-FAM, HEX or NED) on its 5′ end. PCR reactions were performed in a 10-μL volume containing 0.5–3 μl of empirically diluted DNA, 1× PCR Buffer (Invitrogen), 1.5–2.5 mM MgCl2, 200 μM of each dNTP, 0.2 μM of the reverse and M13-fluorescent primers, 0.0133 μM of the M13-tailed forward primer, and 0.25 or 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen). The reaction profile was as follows: 10 cycles (Touchdown) of 94 °C for 45 s, 60–51 °C for 45 s, 72 °C for 1.5 min, followed by 30 cycles of 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1.5 min and a final extension at 72 °C for 30 min. 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. In the case of faecal samples, we empirically improved the PCR yield by including additives such as 0.2% Triton X-100, 3% DMSO or 0.5 × PCR enhancer solutions (Invitrogen). Microsatellite genotyping was performed using a MegaBACE 1000 automated sequencer and the ET-ROX 550 size standard (GE Healthcare), and then analysed utilizing the accompanying Genetic Profiler 2.2 software. Negative controls were run for each batch of PCR reactions and genotyped to monitor the presence of any exogenous DNA. Microsatellite data have been deposited in the Dryad Digital Repository and can be found at http://datadryad.org/handle/10255/dryad.1884.

As the use of non-invasive samples may induce genotyping problems, we adopted the multiple-tube approach (Taberlet *et al.* 1996) for scats and hairs. In such cases, we only considered genotypes that had been sufficiently replicated, by establishing an *a priori* threshold for inclusion (heterozygotes were identified by a minimum of two independent scores of each allele, and homozygotes were considered to be correct if the same allele was detected by at least five independent PCR experiments). In addition, we genotyped paired faecal and blood samples obtained from five different individuals to verify the congruence between the two types of material (Table S1, Supporting information).

The pre-established threshold of the multiple-tube approach was reached for both hair samples, as well as for the two scats collected from captive animals from the Green Corridor (bPon91 and bPon141—see Table S1, Supporting information), and the two field scats collected in Ivinhema. However, for the 11 field-collected jaguar scats from the Green Corridor, only six could be reliably genotyped. Therefore, a total of 10 faecal samples were included in the study as potentially representing distinct individuals (see Results).

Data analysis

The existence of possible genotyping errors due to stuttering, short allele dominance, and null alleles was tested using MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.* 2004). Searches for identical genotypes were performed using the program GENECAP (Wilberg & Dreher 2004) to compare each multilocus genotype with all others within the data set. To quantify the discriminatory power of our microsatellite data set, we calculated the probability of identity ($P_{(ID)}$) index, using two different approaches implemented in GENECAP and the full set of 59 distinct individuals (see Results and Table S1, Supporting information). One of them assumed Hardy–Weinberg equilibrium (HW $P_{(ID)}$), and

the other assumed that the individuals are siblings (Sib $P_{(ID)}$).

Genetic diversity was measured by the number of alleles per locus (A), observed heterozygosity (H_o) and expected heterozygosity (H_e) under Hardy–Weinberg assumptions (Nei 1978). These analyses were performed using FSTAT 2.9.3.2 (Goudet 2002) and GENEPOP 3.4 (Raymond & Rousset 1995). FSTAT was also used to calculate allelic richness (AR), a measure of the observed number of alleles per locus independent of sample size (Petit *et al.* 1998), as well to statistically compare levels of genetic diversity among populations. The number of private alleles was computed for each population following a rarefaction method that compensates for uneven sample sizes, as implemented in the software HP-Rare (Kalinowski 2004, 2005).

Global and population-specific tests for deviations from the Hardy–Weinberg equilibrium (HWE) were performed with ARLEQUIN 3.11 using an exact test based on the procedure described by Guo & Thompson (1992), with 10 000 dememorization steps (Excoffier *et al.* 2005). An assessment of linkage disequilibrium (LD) among loci was conducted using FSTAT. Significance levels ($\alpha = 0.05$) for inferred LD or departures from HWE were corrected for multiple simultaneous comparisons, with the sequential Bonferroni approach (Rice 1989).

The degree of genetic differentiation among the predefined geographical groups was investigated with pairwise F_{ST} measures as implemented in ARLEQUIN (Weir & Cockerham 1984), as well as the related R_{ST} index (Slatkin 1995). The statistical significance of F_{ST} and R_{ST} values was tested using 10 000 permutations. We also calculated the F_{ST} and R_{ST} between different time periods in the Green Corridor (eight samples from 1992 to 1999 vs. 10 samples from 2004 to 2007) and Porto Primavera populations (five samples from 1993 to 1994 vs. 16 samples from 1998 to 2002), to test for temporal changes in allele frequencies, as these populations were sampled over a longer time frame than the remaining localities.

As the traditional F_{ST} may have undesirable attributes in some situations when estimated from highly polymorphic markers such as microsatellites (Jost 2008; Heller & Siegismund 2009), we also calculated a recently developed alternative measure, D_{EST} (Jost 2008), using the software SMOGD 1.2.5 (Crawford 2010). The overall value of D_{EST} for each pairwise population comparison was calculated as the arithmetic mean across loci, following the strategy outlined by Heller *et al.* (2010). Estimates based on D_{EST} were compared to those obtained with F_{ST}, so as to assess the potential impact of heterogeneous levels of genetic diversity among population fragments on estimates of differentiation.

A Bayesian clustering analysis for inferring population structure was performed in STRUCTURE 2.2 (Pritchard *et al.* 2000), which uses a Markov chain Monte Carlo (MCMC) procedure to estimate the posterior probability that the data fit the hypothesis of K clusters [Pr(X/K)]. In the first step, we estimated the number of genetic clusters by performing 10 independent runs for each K between 1 and 10 using 1 000 000 MCMC iterations and a burn-in period of 500 000 steps. We checked for consistency among replicate runs for the same K value and then computed the arithmetic mean among the 10 runs. We ran the program without supplying any prior information on the sampling locations, using correlated allele frequencies and assuming the admixture model. The optimal value of K was selected as the one that maximized the probability of the data (averaged across different runs). In the second step of the analysis, we incorporated prior population information (assuming $K = 4$) to identify which individuals were not residents of their sampled location (i.e. were migrants) and those that had admixed ancestry. Individuals were considered residents if $q > 0.8$ for the area where they were sampled. Individuals with q-values from 0.2 to 0.8 were considered to be potentially admixed, as they could not be readily assigned as residents or migrants (Lecis *et al.* 2006; Bergl & Vigilant 2007). Burn-in and run length were the same as described earlier.

Analyses were also performed with another Bayesian method for the inference of population genetic structure as implemented in BAPS 5.2 (Corander *et al.* 2003, 2004) which uses stochastic optimization to infer the posterior mode of the number of populations. The program was used to cluster individuals using both spatial and non-spatial mixture options. We ran the software with a predefined maximum of $K = 2$–10 and repeated the runs five times in order to check the stability of the results. For each run, the program reports the probabilities for different numbers of subpopulations, $K \leq$ maximum K, and we averaged probabilities over the five runs.

Assignment/exclusion of individuals using predefined subpopulations was performed using GENECLASS 2 (Piry *et al.* 2004), which does not assume that all potential source populations have been sampled. The program was also used to detect first-generation migrants. We employed the Bayesian criterion (Rannala & Mountain 1997) applying the Monte Carlo resampling method with 10 000 simulated individuals and an alpha of 0.01 (Paetkau *et al.* 2004). We computed a likelihood ratio test comparing the population where the individual was sampled over the highest likelihood value among all available populations (L = L_home/L_max).

The subprogram ISOLDE within GENEPOP was used to test for a relationship between geographical and genetic (F_{ST} and D_{EST}) distances among populations, with the statistical significance assessed using a Mantel test with 10 000 permutations. Geographical distances among populations were measured as the Euclidean distance between the 'mean centre' of each population, constructed from the average x and y coordinates of each population sample using ArcMap 9.1 software (ESRI Inc., Redlands, CA, USA).

The program LDNE (Waples & Do 2008) was used to estimate N_e from genotypic data based on the LD method and implementing the bias correction of Waples (2006). We used the jackknife method and assumed a random mating model. The program calculates separate estimates using different criteria for excluding rare alleles, and we tested the following critical values (P_{crit}): 0.05; 0.02; 0.01. In addition, N_e was also estimated with the program ONESAMP 1.1 (Tallmon *et al.* 2008), incorporating an approach that uses summary statistics and approximate Bayesian computation. The upper and lower bounds of the prior distribution for N_e were 2 and 100, respectively. Priors for N_e of 2–50 and 2–200 were also tested to verify if the results were robust to changes in these assumed values. In both programs, we also carried out tests in which we changed the number of individuals analysed for each population to assess the effects of sample size.

Results

Discrimination of individuals and genetic variability

Probability of identity calculations showed that our panel of microsatellites had considerable power to discriminate among individuals. The HW $P_{(ID)}$ was 1.86×10^{-13} and the more conservative measure Sib $P_{(ID)}$ was 1.06×10^{-5}, indicating that even related individuals would have a very low probability of bearing identical genotypes. All samples were subjected to a search for genotype identity using GENECAP, and only two produced identical genotypes. These samples were scats collected in Uruguaí Provincial Park (Green Corridor), 2 km apart from each other, and were thus considered to originate from the same individual (bPon140). This result indicated that the 10 evaluated faecal samples comprised nine distinct individuals, leading to a total of 59 jaguars included in this study (Table S1, Supporting information).

Paired faecal and blood samples obtained from five different individuals were amplified for nine microsatellite loci and produced identical genotypes, lending confidence to the reliability of genotypes generated from scat samples. Global evaluation of the microsatellite data set using MICRO-CHECKER revealed no evidence of genotyping errors due to stuttering or large allele dropout, but indicated that null alleles might be present at loci FCA441, FCA723 and FCA741. However, no evidence of non-amplifying alleles was detected when local populations were analysed separately. The results suggested that the inference of null alleles based on the pooled sample was more likely due to genetic structure among sites. Moreover, significant deviations from HWE induced by heterozygote deficiency were observed at two loci (F42 and F85) after the Bonferroni correction ($\alpha = 0.05$) when all samples were treated as a single population. Nevertheless, no evidence was found for deviations from Hardy–Weinberg equilibrium (HWE) within each population after Bonferroni correction. These results are consistent with a Wahlund effect (1928). All pairwise locus combinations were in linkage equilibrium for global and population-specific analyses ($\alpha = 0.05$, after Bonferroni correction for 78 comparisons).

When samples from all four populations were pooled, UPAF jaguars showed a mean observed (H_o) and expected (H_e) heterozygosity of 0.682 and 0.732, respectively (Table 1). The number of alleles per locus ranged from 3 (FCA741) to 14 alleles (FCA742), with a mean of 7.23. All loci were polymorphic in all populations except locus FCA741 in the Morro do Diabo. Individual analysis of each population separately (Table 1) showed expected heterozygosity ranging from 0.497 (Morro do Diabo) to 0.737 (Green Corridor), whereas observed heterozygosity ranged from 0.548 (Morro do Diabo) to 0.782 (Porto Primavera). Allelic richness was lowest in Morro do Diabo (3.2) and highest in the Green Corridor (5.05). Statistical testing of these three measures of genetic diversity showed that the Morro do Diabo population contained significantly lower values ($P < 0.05$) of H_e, H_o and AR when compared to Porto Primavera, and significantly lower ($P < 0.01$) H_e and allelic richness (AR) when compared to the Green Corridor (Table S2, Supporting information). Unique alleles could be observed in all populations (Table S3 and Fig. S1, Supporting information) except Morro do Diabo, with the largest and most distant fragment (Green Corridor) presenting the largest number of unique alleles (18), followed by the Porto Primavera (3) and Ivinhema (2). A similar pattern was observed when the rarefaction approach was applied, yielding the following estimates of private alleles per populations: Green Corridor (1.43), Porto Primavera (0.29), Ivinhema (0.24) and Morro do Diabo (0.15).

Population structure

In the genetic clustering analysis computed with STRUCTURE, the lowest likelihood value of the data

Table 1 Measures of diversity at 13 microsatellite loci in the four jaguar populations of the UPAF investigated in this study

	Green Corridor (n = 18)					Morro do Diabo (n = 8)					Ivinhema (n = 10)					Porto Primavera (n = 23)					Global population* (n = 59)				
Locus	N	A	$AR^{\dagger}$	H_o	$H_e^{\ddagger}$	N	A	$AR^{\dagger}$	H_o	$H_e^{\ddagger}$	N	A	$AR^{\dagger}$	H_o	$H_e^{\ddagger}$	N	A	$AR^{\dagger}$	H_o	$H_e^{\ddagger}$	N	A	$AR^{\S}$	H_o	$H_e^{\ddagger}$
FCA742	15	8	6.97	0.733	0.831	8	4	4.00	0.625	0.589	10	8	7.31	0.800	0.844	23	10	7.64	0.957	0.881	56	14	13.9	0.821	0.877
FCA723	16	5	4.00	0.500	0.708	8	2	2.00	0.250	0.232	10	2	2.00	0.400	0.511	23	6	4.71	0.696	0.707	57	6	6.00	0.526	0.682
FCA740	17	5	4.32	0.765	0.730	8	3	3.00	0.250	0.348	9	3	2.99	0.667	0.514	23	4	3.81	0.913	0.696	57	5	4.93	0.737	0.705
FCA441	18	4	3.79	0.444	0.639	8	2	2.00	0.125	0.125	10	4	3.77	0.700	0.656	23	4	3.79	0.696	0.660	59	5	4.99	0.542	0.687
FCA391	16	7	5.98	0.750	0.808	8	4	4.00	0.750	0.723	10	5	4.74	0.800	0.728	23	6	4.93	0.826	0.728	57	8	7.99	0.789	0.775
F98	17	3	2.86	0.588	0.517	8	3	3.00	0.625	0.482	10	4	3.97	0.800	0.717	23	3	2.83	0.609	0.552	58	4	3.99	0.638	0.586
F53	16	9	6.95	0.688	0.856	8	4	4.00	1.000	0.732	10	7	6.53	0.900	0.839	23	8	5.73	0.913	0.798	57	10	9.92	0.860	0.840
F124	17	7	5.50	0.824	0.761	8	4	4.00	0.750	0.741	10	6	6.53	0.800	0.811	23	7	5.72	0.826	0.803	58	8	7.91	0.810	0.803
F146	18	4	3.66	0.444	0.629	8	3	3.00	0.500	0.509	10	3	2.99	0.800	0.639	23	4	3.34	0.739	0.658	59	5	4.89	0.627	0.643
F85	14	10	7.55	0.643	0.860	8	3	3.00	0.625	0.607	9	5	4.88	0.778	0.708	22	6	4.99	0.818	0.769	53	12	12.0	0.736	0.821
F42	17	8	6.38	0.647	0.838	8	5	5.00	0.875	0.714	10	3	2.97	0.600	0.478	23	6	4.42	0.696	0.725	58	9	8.98	0.690	0.775
FCA453	13	5	4.81	0.538	0.792	8	3	3.00	0.750	0.661	10	3	2.80	0.400	0.350	23	5	4.25	0.783	0.723	54	5	5.00	0.648	0.742
FCA741	15	3	2.99	0.333	0.610	8	1	1.00	0.000	0.000	10	3	2.80	0.400	0.550	23	3	2.90	0.696	0.602	56	3	3.00	0.446	0.576
Overall	—	6	5.05	0.607	0.737	—	3.2	3.2	0.548	0.497	—	4.3	4.2	0.680	0.642	—	5.5	4.54	0.782	0.716	—	7.2	7.19	0.682	0.732

Sample size (N), observed number of alleles (A), allelic richness (AR), observed (H_o) and expected (H_e) heterozygosities.

*Samples from all four population fragments pooled.

†Allelic richness is calculated using minimum sample size of eight diploid individuals.

‡Unbiased gene diversity (corrected for sampling bias in FSTAT).

§Allelic richness based on a minimum sample size of 53 diploid individuals.

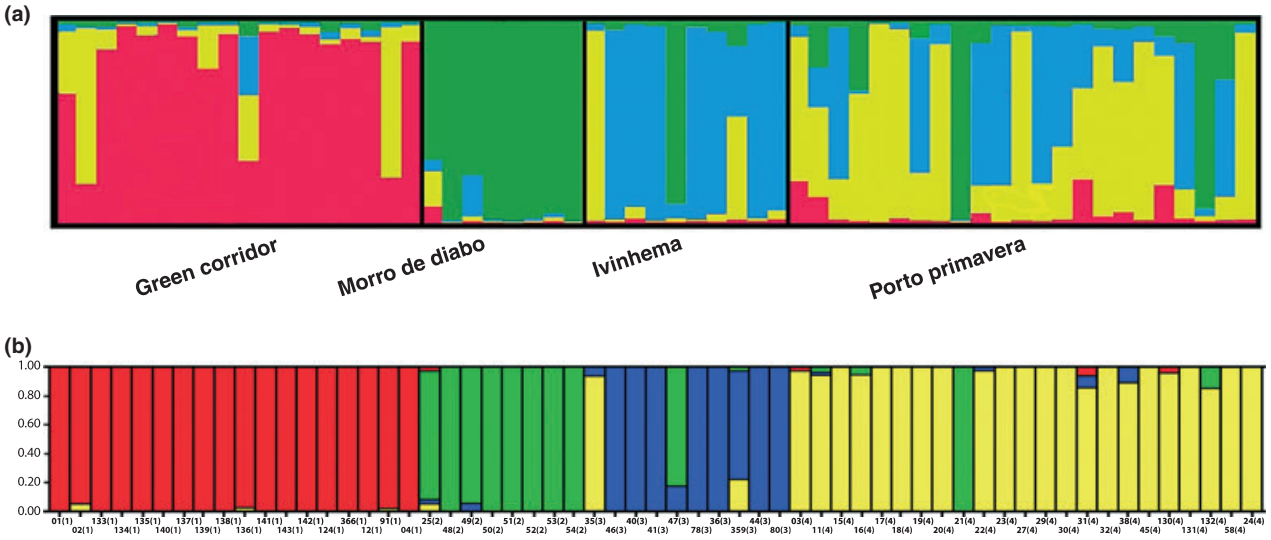

Fig. 2 (a) Proportional membership (q) of each jaguar in the genetic clusters inferred by STRUCTURE with $K = 4$, without use of prior population information (USEPOPINFO = 0). Alternative allocations with $K = 5$ and $K = 6$ are shown in Fig. S3 (Supporting information). Each individual is represented by a vertical bar, and the length of each bar indicates the probability of membership in each cluster [Green Corridor (red); Morro do Diabo (green); Ivinhema (blue); Porto Primavera (yellow)]. (b) Proportional membership (q) of each jaguar in the genetic clusters inferred by STRUCTURE with $K = 4$, utilizing prior population information (USEPOPINFO = 1). Numbers below the horizontal axis are sample identification (number after 'bPon' in Table S1, Supporting information) of *Panthera onca* individuals in each area. Colours are as in panel 'a', and the locality of origin is indicated in parentheses: (1) Green Corridor; (2) Morro do Diabo; (3) Ivinhema; (4) Porto Primavera.

was observed with $K = 1$ [mean Ln P (D) = −2318.18], indicating the presence of population subdivision. Two modes were observed, one at $K = 4$ [mean Ln P (D) = −2106.02] and another at $K = 6$ [mean Ln P (D) = −2075.63], with declining likelihood at higher K values (Fig. S2, Supporting information). Detailed analyses of results with $K = 4$, $K = 5$ and $K = 6$ revealed that the former value led to the clearest allocation of individuals to distinct population groups (Figs 2a and S3, Supporting information). At $K = 4$, genetic clusters mostly corresponded to the four pre-defined geographical groups (Cluster 1 = Green Corridor; Cluster 2 = Morro do Diabo; Cluster 3 = Ivinhema; Cluster 4 = Porto Primavera), with a few exceptions largely consisting of individuals subsequently identified as migrants or admixed. At $K = 6$, samples from Morro do Diabo and Ivinhema retained the same allocations as in $K = 4$, but those from Porto Primavera were separated into two different groups (cluster 4a, $n = 8$ [only Porto Primavera samples]; and cluster 4b, $n = 10$ [including 8 Porto Primavera samples plus bPon02 and bPon35—see Table S1, Supporting information and text below]). The Green Corridor population was allocated into a main cluster ($n = 14$), while a sixth unit was formed with three samples from this site (bPon91, 136 and 139) plus four from Porto Primavera (bPon03, 11, 38, 130). There was no obvious biological interpretation for these subdivisions affecting the Porto Primavera and Green Corridor groups, as the subgroups did not systematically correspond to different geographical locales within each region, nor did individuals cluster chronologically. This result may thus stem from an overestimate of the optimal K, which may have been influenced by the use of the correlated allele frequencies model (as pointed out by Pritchard & Wen 2004), and possibly also by the presence of related individuals in the sample. To investigate this possibility, we further analysed the data from Porto Primavera using KINSHIP 1.2 (Goodnight *et al.* 1998). Within each of the two clusters (4a and 4b), the mean relatedness coefficient (R) was 0.21 and 0.18, respectively, while that between these clusters was 0.10. In addition, almost all pairs of individuals from Porto Primavera showing $R > 0.25$ were grouped in the same STRUCTURE cluster (22 of 24 pairs). These results are consistent with the hypothesis that related individuals (at least in the Porto Primavera population) may have induced an overestimate of the optimal K. Based on this inference, along with the recommendations by Pritchard & Wen (2004), we concluded that $K = 4$ seems to best represent the genetic structure of jaguars in this region, largely corresponding to the four pre-defined geographical groups that can be circumscribed in the UPAF.

In the BAPS analysis of individual clustering without spatial information, the highest posterior probability was observed for $K = 5$ [mean log (marginal likelihood) = −2259.89], considering the minimum threshold

of three individuals per cluster suggested by Latch *et al.* (2006). The analysis partitioned individuals into four clusters that mostly corresponded to the predefined geographical groups, with the main exception that the Green Corridor was divided into two clusters ($n = 11$ and $n = 5$). When the BAPS analysis was run incorporating spatial information (Fig. S4, Supporting information), four clusters were observed as the best partition (mean log [marginal likelihood] = −2330.35). In this analysis, BAPS formed essentially the same groups, also dividing the Green Corridor into two clusters, but grouping samples from Porto Primavera and Ivinhema into a single cluster (see Fig. S4, Supporting information).

Using the GENECLASS assignment/exclusion test, 43 of 59 (72.9%) jaguars were assigned with the highest probability to the location at which they had been sampled. Many of the individuals that were 'misassigned' with respect to their sampling locale were the same that had been assigned to a different genetic cluster in the STRUCTURE and/or BAPS analyses, and some of them were identified as migrants or admixed using conservative criteria.

An initial analysis of pairwise F_{ST} and R_{ST} comparing samples collected at different time periods supported the temporal stability of allele frequencies in both the Green Corridor ($F_{ST} = 0.025$; $P = 0.067$) and Porto Primavera ($F_{ST} = -0.005$; $P = 0.296$) indicating that it was valid to pool our full data set for each locality. However, it is interesting to note that the temporal variation in allele frequencies in the Green Corridor appears to have been substantial in this period (leading to an F_{ST} that approached significance), an observation that may be further investigated with additional sampling in the area.

The F_{ST} among all locations was 0.089 ($P < 0.001$) and the R_{ST} was 0.075 ($P = 0.003$), while the overall value of D_{EST} was 0.25. Pairwise F_{ST} values were significant for all comparisons (Table 2). The highest differentiation was between the Green Corridor and Morro do Diabo ($F_{ST} = 0.198$; $P < 0.001$), and the lowest differentiation was between Porto Primavera and Green Corridor ($F_{ST} = 0.048$; $P < 0.001$). On the other hand, R_{ST} values were significant only when other populations were compared with the Green Corridor (Table 2). The highest observed differentiation was also between the Green Corridor and Morro do Diabo populations ($R_{ST} = 0.112$; $P = 0.001$). Differentiation between the Green Corridor and Porto Primavera was low but still significant ($R_{ST} = 0.036$; $P = 0.030$). A similar pattern was observed with D_{EST} (see Table 2), with the highest values of differentiation estimated for the comparison between the Green Corridor and Morro do Diabo (0.313). Interestingly, this measure indicated a substantially higher level of differentiation (0.104) between Porto Primavera and the Green Corridor than that estimated with the other two indices. The association between populational geographical distance (measured in kilometers) and values of genetic differentiation was non-significant using either F_{ST} or D_{EST} ($P = 0.77$ and 0.54, respectively), although the latter did indicate a pattern more compatible with some influence of isolation by distance on this system (Fig. S5, Supporting information).

Identification of migrants and admixed individuals in the UPAF populations

To better investigate the genetic composition of our data set and to detect migrants as well as admixed individuals, we performed a second set of analyses with STRUCTURE, incorporating geographical sampling as prior population information (Fig. 2b). We considered the four geographical groups as separate populations and observed that 88% (52/59) of the individuals had high probability of being residents, with $q > 0.8$ for the locality in which they had been sampled (Table S4, Supporting information; Fig. 2b). The analysis identified seven individuals (bPon21, bPon25, bPon31, bPon35, bPon47, bPon132 and bPon359; Tables 3 and S4, Supporting information) as potential migrants or bearers of admixed ancestry ($q < 0.8$ for the sampling locale). These samples were assigned to a different cluster relative to their sampling site by one or more of the assignment tests (STRUCTURE, BAPS, or GENECLASS). Two of those individuals (bPon21 and bPon35) were strongly assigned ($q > 0.9$) to a different cluster and were thus considered to be migrants. The sample bPon21, from Porto Primavera, was estimated to have a

Table 2 Pairwise F_{ST} (left number above the diagonal), D_{EST} (right number above the diagonal) and R_{ST} values (below the diagonal) for the four jaguar populations of the UPAF

	Green Corridor	Morro do Diabo	Ivinhema	Porto Primavera
Green Corridor	—	0.198***/0.313	0.122***/0.211	0.048***/0.104
Morro do Diabo	0.112**	—	0.120***/0.132	0.073***/0.143
Ivinhema	0.081**	−0.010	—	0.060***/0.052
Porto Primavera	0.036*	−0.007	0.024	—

Significant values *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ for F_{ST} and R_{ST}.

Table 3 Results of migrant detection analyses performed with STRUCTURE and GENECLASS

Sample	Sex	Geographical origin	Year of collection	STRUCTURE (*q*-values) (Green Corridor/ Morro do Diabo/ Ivinhema/Porto Primavera clusters)	GENECLASS F_0 migrant: LOD value†	GENECLASS (*P*- value)‡	GENECLASS [-log (L)] (Green Corridor/Morro do Diabo/Ivinhema/ Porto Primavera clusters)
bPon-02	M	Green Corridor	1993	**0.853**/0.000/0.000/0.020	2.260	*0.005*	18.57/24.10/23.75/**16.31**
bPon-21**	F	Porto Primavera	1998	0.000/**0.984**/0.000/0.000	6.263	*0.000*	19.64/**8.23**/14.23/14.50
bPon-25*	F	Morro do Diabo	1998	0.000/**0.678**/0.000/0.000	0.000	0.506	18.05/**15.06**/17.16/15.42
bPon-31*	M	Porto Primavera	2000	0.002/0.000/0.009/**0.698**	2.603	*0.008*	19.02/26.12/**15.81**/18.42
bPon-35**	F	Ivinhema	2005	0.000/0.000/0.004/**0.906**	8.430	*0.000*	23.80/28.48/24.03/**15.60**
bPon-47*	M	Ivinhema	2002	0.000/**0.753**/0.085/0.001	3.060	0.028	18.60/**10.44**/13.50/11.86
bPon-132*	F	Porto Primavera	1993	0.000/0.087/0.000/**0.795**	0.282	0.050	20.23/12.95/15.81/**12.90**
bPon-136	?	Green Corridor	1992	**0.897**/0.000/0.000/0.010	1.764	*0.007*	15.93/18.19/16.05/**14.16**
bPon-359*	M	Ivinhema	2007	0.000/0.000/**0.422**/0.022	0.261	0.132	19.62/18.23/13.50/**13.24**

Individuals marked with ** were identified as migrants with both methods and thus interpreted as such (see text). Individuals marked with * were identified as potentially admixed on the basis of the STRUCTURE results. The most likely source population for each individual is shown in bold.

†LOD = −log (L_home/L_max).

‡*P*-value refers to the test aimed to detect first-generation migrants in GENECLASS, $P < 0.01$ (italic) indicates a potential F0 migrant.

98.4% probability of belonging to Morro do Diabo. Likewise, the individual bPon35, captured in Ivinhema, had a 90.6% probability of originating in the Porto Primavera region. All other individuals had *q*-values ranging from 0.4 to 0.8 (see Tables 3 and S4, Supporting information) and were defined as potentially admixed, because they could not be classified as migrants, but were not clearly assigned as residents either. In particular, the sample bPon47, collected in Ivinhema, had a 75.3% probability of belonging to Morro do Diabo. In the GENECLASS analysis designed to detect first-generation migrants, bPon47 showed the highest probability of belonging to Morro do Diabo, but could not be considered a migrant because it failed to reach the established threshold ($P = 0.028$; Table 3).

In the analysis aimed at detecting first-generation migrants, GENECLASS identified five individuals with a probability below the threshold (bPon02; bPon21; bPon31; bPon35; bPon136; $P < 0.01$; Table 3). As with STRUCTURE, GENECLASS strongly assigned bPon21 and bPon35 to Morro do Diabo and Porto Primavera, respectively. The sample bPon31 (classified as migrant by GENECLASS) was considered an admixed individual in the STRUCTURE analysis ($q = 0.698$). The other two individuals, bPon02 and bPon136, from the Green Corridor, were assigned to Porto Primavera, but showed similar probabilities for both localities. In STRUCTURE, both individuals were not considered migrants or admixed ($q = 0.85$ and $q = 0.90$ for the Green Corridor, respectively). We thus used a conservative approach and classified only bPon21 and bPon35 as migrants (Table 3).

Effective population size

The two methods used to determine N_e provided rather congruent estimates (Table 4). The results obtained with ONESAMP were robust to changes in the prior (data not shown). At the same time, separate estimates using different criteria for excluding rare alleles, which

Table 4 Effective population size estimates and their approximate confidence limits for each UPAF jaguar population based on two different methods (LDNE and ONESAMP; see text for details)

	LDNE ($P_{crit} = 0.05$)		ONESAMP (Prior = 2–100)	
	N_e	Confidence limits (95%)	N_e	Confidence limits (95%)
Green Corridor	51.4	23.5–11004.5	30.3	23.4–45.8
Morro do Diabo	4.6	2.3–12.8	7.8	6.8–9.8
Ivinhema	12.3	6.9–27.1	10.3	9.2–12.5
Porto Primavera	13.5	10.4–18.0	21.7	19.4–26.6

may overestimate N_e values in LDNE (Waples & Do 2008), also produced similar results (data not shown). The highest value of N_e was obtained for the Green Corridor utilizing both LDNE (N_e = 51.4) and ONESAMP (N_e = 30.3). The lowest effective size was estimated for Morro do Diabo (N_e = 7.8 with ONESAMP and 4.6 with LDNE). Estimates for Ivinhema and Porto Primavera yielded intermediate values. We observed that the N_e estimates produced with ONESAMP were affected by sample size, showing a strong correlation with it. For example, ONESAMP estimated a N_e of 21.7 individuals for Porto Primavera (n = 23); but this value decreased to only 11.2 when the sample was reduced twofold (n = 11). Likewise, when 10 individuals were analysed for Ivinhema, the estimated N_e was 10.3, while a N_e of 5.6 was estimated utilizing a sample of 6 individuals. In contrast, the N_e estimates calculated with LDNE seemed to be robust to changes in sample size, as the values remained very similar in any of the tests which varied the number of included animals per population.

Discussion

Genetic variability

The overall genetic variation of jaguars in the UPAF is still high (H_e = 0.732 and a mean of 7.23 alleles per locus) and comparable to that estimated for the species throughout its geographical distribution (H_e = 0.739 and a mean of 8.31 alleles per locus; Eizirik *et al.* 2001). However, the genetic diversity found here was somewhat lower than that obtained by Ruiz-Garcia *et al.* (2006) using mainly samples from Colombia, with some individuals from Guatemala, Paraguay, Peru, Bolivia, Venezuela, and Brazil (H_e = 0.846 and a mean of 11.33 alleles per locus). Nevertheless, no strict comparison among these studies should be attempted, given that they employed different markers.

Even though the genetic variation observed in the UPAF jaguar populations may be considered to be high, our results indicate that a relevant portion of this diversity has been locally lost and is now spatially subdivided. This is illustrated by the observation that estimates of genetic diversity and private alleles were lowest in the smallest populations (Morro do Diabo and Ivinhema). Particularly, Morro do Diabo exhibited reduced genetic variation relative to the other locations (which was statistically significant when compared to the largest populations of Porto Primavera and Green Corridor), and it was the only area that showed an allele fixed for a microsatellite locus. So far, Morro do Diabo has the lowest level of genetic diversity reported for the species (Eizirik *et al.* 2001, 2008; Ruiz-Garcia *et al.* 2006). Although the Green Corridor, Porto Primavera and Ivinhema populations exhibited moderate to high levels of expected heterozygosity, this diversity is likely lower than what was present in the original population (as can be inferred if we assume that the pool of local populations harbours the original allelic diversity of the UPAF).

As expected in the presence of recent historical gene flow among these populations, we can observe that alleles that have been lost within each population (e.g. those of intermediate size within the sampled range) are present in another (Table S3 and Fig. S1, Supporting information). Moreover, the Green Corridor and Porto Primavera regions shared the greatest number of alleles, even though they are more distant (~500 km) than the other pairs. This could be explained by the fact that both populations were considerably larger than the others at the time of sampling, thus retaining greater allelic diversity in the face of fragmentation.

Population structure

Our results clearly indicated that jaguars in the UPAF are currently not a panmictic population. Non-spatial analyses in BAPS and STRUCTURE indicated that the four geographical groups are differentiated and form distinct genetic clusters. However, the BAPS spatial analysis suggested that Porto Primavera and Ivinhema might be considered a single population. A recent debate has addressed the issue of whether clusters identified by non-spatial Bayesian algorithms were artificially defined because of uneven sampling along clines, or were in fact real genetic units (Serre & Pääbo 2004; François *et al.* 2006). However, studies that assess the performance of spatial algorithms and compare them to non-spatial approaches are scarce. Chen *et al.* (2007) compared the STRUCTURE results and those of three spatially oriented clustering programs, verifying that STRUCTURE performs very well even along a cline of variation, countering previous claims (Serre & Pääbo 2004). Field data indicate that there was likely demographic continuity between the Porto Primavera and Ivinhema populations until very recently, which was almost certainly interrupted by the flooding of Porto Primavera hydroelectric dam in 1998 (D. A. Sana, unpublished data.). The flooding itself may have induced the movement of individuals downstream to the Ivinhema region, increasing the genetic contribution of Porto Primavera animals in this latter area. However, after the flooding, the Porto Primavera population has essentially gone extinct, with few animals sighted in the area in the last few years (D. A. Sana, unpublished data), making it very unlikely that any current gene flow with Ivinhema remains.

In agreement with the clustering analyses, frequency-based assessments based on F_{ST} and D_{EST} detected substantial population structure in the UPAF, which was also observed to some extent with R_{ST}. Pairwise F_{ST} values were significant among all populations and ranged from 0.048 to 0.198 (see Table 2). D_{EST} values tended to be higher than F_{ST}'s, ranging from 0.052 to 0.313. R_{ST} values were lower than F_{ST}'s, suggesting that genetic drift has been more important than mutation in creating differences between these populations. R_{ST} values were significant only when other populations were compared to the Green Corridor. This population is located further south (~380 km from the nearest population, Ivinhema) and probably was the first to be partially or completely isolated from the others. This separation process may have been ongoing for decades, having been accelerated in the second half of the 20th century because of large-scale agricultural changes to the landscape (see Introduction). However, it may have become complete owing to the flooding of the huge Itaipu Binacional hydroelectric dam in 1982, which submerged thousands of hectares of riverine habitat, likely severing the connectivity between the northern and southern UPAF ecoregion.

The observed genetic differentiation among populations (as assessed by F_{ST} and D_{EST} values) was remarkably high, given the geographical proximity of the areas (their pairwise distances ranging from ~69 km to 500 km), the ability of this species to disperse over broad areas (Oliveira 1994), as well as the short time frame (30–40 years, approximately 6–8 generations) in which these populations have been isolated or semi-isolated (Di Bitetti *et al.* 2003; De Angelo 2009). These levels of genetic differentiation imply a strong impact of local genetic drift, indicating that the effective population sizes are very small in each fragment and that current gene flow among them is likely very low.

The highest F_{ST} and D_{EST} values were observed between the most distant population (Green Corridor) and the smallest fragments (Morro do Diabo and Ivinhema). At the same time, high F_{ST}'s were observed between Morro do Diabo and Ivinhema, while both of these populations showed lower differentiation relative to Porto Primavera (see Table 2). These results are not surprising, given the field-based knowledge on the current and historical landscape connectivity between these areas (De Angelo 2009), as well as on jaguar dispersal patterns in this region inferred from radio-telemetry data (Cullen 2006; D. A. Sana, unpublished data). The estimates based on D_{EST} were similar, also pointing to high differentiation between Morro do Diabo and Ivinhema, but in this case also indicating considerable differentiation between the former and Porto Primavera. This measure also strengthened the interpretation of more recent connectivity between Porto Primavera and Ivinhema than between any other pair of population fragments (see above), as the estimated value was even lower than the respective F_{ST}.

An interesting observation was that the Green Corridor and Porto Primavera populations, which are quite distant from one another (~500 km), exhibited the lowest F_{ST} values. The equivalent comparison performed with D_{EST} indicated a higher level of differentiation between these populations (possibly reflecting more accurately the current connectivity between them), but still lower than most other comparisons spanning a similar geographical distance (see Table 2). Probably, these populations exhibited relatively low differentiation not because of ongoing gene flow between them, but because they have lost fewer alleles via genetic drift (given their larger N_e) during this recent fragmentation process. Finally, the relationship between geographical and genetic distances(F_{ST} and D_{EST}) among populations was not significant, suggesting that the observed subdivision could not be explained by geographical distances between populations only, and that genetic drift may be the primary force affecting differentiation among them.

Identification of migrants and admixed individuals among UPAF populations

Assignment tests indicated that most jaguars had originated from the populations where they had been sampled supporting the view that current migration among populations is very low, and consistent with the observation of high differentiation among areas. Additional support for this conclusion derives from extensive radio-telemetry data (including 10 individuals equipped with GPS collars and 23 animals with VHF collars, and encompassing a total period of *c.* 15 years), which detected no dispersal among these sites (Cullen 2006; D. A. Sana, R. G. Morato, P. G. Crawshaw Jr., unpublished data). Although some adult jaguars did show long distance movements within their home range (up to 30 km in 3–4 days through the fragmented landscape), they significantly avoided the use of areas modified by agriculture, pasture and human settlement (Cullen 2006).

Although ongoing field work has not revealed any migrants among these areas, our genetic analyses did identify some individuals that indicate recent connectivity among these populations. Using conservative criteria, two individuals were inferred to be migrants, both of which were young-adult females (3–4 years old). One individual from Porto Primavera (bPon21), captured in 1998 during the wildlife rescue before the filling of the Porto Primavera dam, was identified as a migrant from Morro do Diabo. Demographic connectivity between the two areas was likely widespread in previous times, and this animal in particular was captured at a site that

was close (albeit on the opposite side of the Paraná River) to the northern end of Morro do Diabo State Park. The other migrant was captured in Ivinhema in 2004, but was genetically assigned to the Porto Primavera. This female may have dispersed southward as a result of the loss of habitat that took place in the Porto Primavera region after the flooding of its reservoir, so that it would have left the area and eventually established a home range in Ivinhema.

Another jaguar sampled in Ivinhema (bPon47) in 2002 showed more probability of belonging to Morro do Diabo, but was ultimately inferred to be of admixed ancestry, supporting the conclusion that there was recent gene flow between the two populations (see Table 3). In addition, an adult male (bPon24) captured in 1999 in the municipality of Alto Paraná (PR) (see Fig. 1, point 10) was translocated to Morro do Diabo State Park and a few days later returned to an area near the capture site. Our genetic assignment indicated that this individual originated in the Porto Primavera region. Although it is difficult to know the exact travel route of this individual, we may conclude that it was able to cross long distances across severely disturbed areas. It is possible that this animal left the Porto Primavera region because of the filling of the reservoir in 1998 and wandered through the landscape without reaching any suitable habitat fragment (L. Cullen Jr., unpublished data). This pattern may be recurrent in the region, and jointly caused by the difficulty in traversing a hostile habitat matrix and the small size of suitable patches, leading to demographic saturation that hampers the establishment of incoming dispersers.

The presence of admixed individuals in these areas suggests that jaguars have been able to move across the landscape and reproduce in their new area, at least in the recent past. However, these episodes of inferred gene flow seem to have been insufficient to avoid differentiation among areas because of intense genetic drift, and their frequency has likely decreased in recent years owing to increasing isolation of fragments and extermination of jaguars remaining in intervening forest patches. It has been proposed that one successful migrant per generation (OMPG) would be sufficient to prevent population differentiation caused by genetic drift (Wright 1931; Franklin 1980). Nevertheless, more recent studies have suggested that 1–10 migrants per generation may be necessary (Millis & Allendorf 1996) or even more than 10 migrants per generation (Vucetich & Waite 2000).

Effective population size

N_e estimates based on a single sample have been problematic, because methods based on linkage disequilibrium (LD) and heterozygote excess have proven to be imprecise or biased (Waples 1991; England *et al.* 2006). However, we used a new program (LDNE) that employs a bias correction developed by Waples (2006) for estimates of N_e based on the LD method. We also used another program (ONESAMP) that employs summary statistics and approximate Bayesian computation to estimate N_e. In our analyses, ONESAMP appeared to be biased by the sample size (see Results), an issue which was already pointed out by Sotelo *et al.* (2008). Conversely, robust results were obtained by LDNE with different sample sizes. At any rate, our results indicated a very low effective size for all populations. This inference is plausible and biologically realistic, given their very small estimated census sizes. LDNE effective sizes ranged from 51.4 in the Green Corridor to 4.6 in the Morro do Diabo. Thus, the minimum of 50 effective breeders that has been suggested as needed to prevent inbreeding depression in the short term (Franklin 1980) was only reached by the Green Corridor population. The target effective population sizes of 500–7000 recommended for securing long-term viability (Franklin 1980; Lande 1995; Reed *et al.* 2003) is clearly several times larger than those observed. In particular, the Morro do Diabo population exhibited extremely small effective size and results indicated low genetic variability. Thus, Morro do Diabo may be seriously compromised if management measures are not taken in the short term.

Conclusions

Our results, in combination with field data and satellite image analyses (e.g. Cullen 2006; De Angelo 2009), indicate that loss and fragmentation of once contiguous habitat have caused the reduction of genetic diversity in the UPAF jaguar populations, as well as drift-induced differentiation among local fragments. It is therefore important to restore gene flow among the analysed areas to avoid the negative demographic and genetic consequences of small population size, as well as to ensure the long-term viability of these groups (see Rabinowitz & Zeller 2010 for a similar conclusion based on range-wide landscape analyses).

Ecological data indicated that jaguar persistence can be achieved in this ecoregion, if these populations are managed as a metapopulation (Cullen 2006). Our genetic data support this view and argue that the restoration of connectivity between these jaguar populations should be viewed as a management priority. Habitat selection analyses have indicated that jaguars in this region (where most of the original forest cover has already been lost) exhibit a preference for riverine marshes (Cullen 2006). These are currently the only type of habitat that potentially connects the remaining

protected areas along the Paraná River basin and can serve as corridors or stepping-stones, facilitating natural dispersal and allowing genetic exchange among populations (Cullen 2006). Additionally, direct intervention via translocations or assisted reproduction could be considered as additional management strategies for jaguar conservation in the region. Techniques of assisted reproduction have been recognized as important tools in the genetic management of this species (Morato & Barnabe 2002). *In vivo* (captive individuals) and *in vitro* (semen and embryos) materials from jaguars of this ecoregion are available for this type of methodology and may be considered as a promising alternative for the future, in case habitat-oriented measures fail to achieve the targeted conservation goals in the short term. Overall, effective dispersal of jaguars through this human-dominated landscape, ultimately resulting in an increased probability of its persistence in the region, will only be successful with the mitigation of the present threats (Cullen 2006) and will require a comprehensive and effective integration of efforts from multiple disciplines.

References

Bergl RA, Vigilant L (2007) Genetic analysis reveals population structure and recent migration within the highly fragmented range of the Cross River gorilla (*Gorilla gorilla* diehli). *Molecular Ecology*, **16**, 501–516.

Boutin-Ganache I, Raposo M, Raymond M, Descepper CF (2001) M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allelesizing methods. *BioTechniques*, **31**, 1–3.

Chen C, Durand E, Forbes F, François O (2007) Bayesian clustering algorithms ascertaining spatial population strucuture: a new computer program and a comparison study. *Molecular Ecology Notes*, **7**, 747–756.

Corander J, Walmann P, Sillanpaa MJ (2003) Bayesian analysis of genetic differentiation between populations. *Genetics*, **163**, 367–374.

Corander J, Walmann P, Smarttinen P, Sillanpaa MJ (2004) BAPS2: enhanced possibilities for the analysis of genetic population structure. *Bioinformatics*, **20**, 2363–2369.

Crawford NG (2010) SMOGD: software for the measurement of genetic diversity. *Molecular Ecology Resources*, **10**, 556–557.

Cullen Jr L (2006) Jaguars as landscape detectives for the conservation of Atlantic Forests in Brazil. PhD thesis, University of Kent, Canterbury, UK.

Cullen Jr L, Abreu KC, Sana DA, Nava AFD (2005) Jaguars as landscape detectives for the upper Paraná River Corridor, Brazil. *Natureza & Conservação*, **3**, 147–161.

De Angelo C (2009) El paisaje del Bosque Atlántico del Alto Paraná y sus efectos sobre la distribución y estructura poblacional del jaguar (Panthera onca) y el puma (Puma concolor). PhD thesis, Universidad de Buenos Aires, Buenos Aires, Argentina.

Di Bitetti MS, Placci G, Dietz LA (2003) *A Biodiversity Vision for the Upper Paraná Atlantic Forest Eco-region: Designing a Biodiversity Conservation Landscape and Setting Priorities for Conservation Action*. World Wildlife Fund, Washington.

Eizirik E, Kim J, Menotti-Raymond M, Crawshaw Jr PG, O'Brien SJ, Johnson WE (2001) Phylogeography, population history and conservation genetics of jaguars (*Panthera onca*, Mammalia, Felidae). *Molecular Ecology*, **10**, 65–79.

Eizirik E, Haag T, Santos AS *et al.* (2008) Jaguar conservation genetics. *Cat News Special Issue: The Jaguar in Brazil*, **4**, 31–34.

England PR, Cornuet J-M, Berthier P, Tallmon DA, Luikart G (2006) Estimating effective population size from linkage disequilibrium: severe bias using small samples. *Conservation Genetics*, **7**, 303–308.

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47–50.

François O, Ancelet S, Guillot G (2006) Bayesian clustering using hidden Markov random fields in spatial population genetics. *Genetics*, **174**, 805–816.

Frankham R, Ballou JD, Briscoe DA (2002) *Introduction to Conservation Genetics*. Cambridge University Press, Cambridge.

Franklin IR (1980) Evolutionary change in small populations. In:*Conservation Biology: An Evolutionary-Ecological Perspective* (eds Soulé ME, Wilcox BA). pp. 135–149, Sinauer Associates, Sunderland.

Galindo-Leal C, Câmara IG (2003) *The Atlantic Forest of South America: Biodiversity Status*. Threats and Outlook, Island Press, Washington.

Gittleman JL, Funk S, Macdonald DW, Wayne RK (2001) *Carnivore Conservation*. Cambridge University Press, Cambridge.

Goodnight KF, Queller DC, Poznansky T (1998) *KINSHIP 1.2*. Goodnight Software, Rice University, Houston, TX.

Goudet J (2002) *FSTAT: A Program to Estimate and Test Gene Diversities and Fixation Indices*. Lausanne: Institute of Ecology, Switzerland.

Guo S, Thompson E (1992) Performing the exact test of Hardy-Weinberg proportion for multiples alleles. *Biometrics*, **48**, 361–372.

Haag T, Santos AS, De Angelo C *et al.* (2009) Development and testing of an optimized method for DNA-based identification of jaguar (*Panthera onca*) and puma (*Puma concolor*) faecal samples for use in ecological and genetic studies. *Genetica*, **136**, 505–512.

Heller R, Siegismund HRS (2009) Relationship between three measures of genetic differentiation G_{ST}, D_{EST} and G'_{ST}: how wrong have we been? *Molecular Ecology*, **18**, 2080–2083.

Heller R, Okello JBA, Siegismund H (2010) Can small wildlife conservancies maintain genetically stable populations of large mammals? Evidence for increased genetic drift in geographically restricted populations of Cape buffalo in East Africa *Molecular Ecology*, **19**, 1324–1334.

Jost L (2008) G_{ST} and its relatives do not measure differentiation. *Molecular Ecology*, **17**, 4015–4026.

Kalinowski ST (2004) Counting alleles with rarefaction: private alleles and hierarchical sampling designs. *Conservation Genetics*, **5**, 539–543.

Kalinowski ST (2005) HP-Rare: a computer program for performing rarefaction on measures of allelic diversity. *Molecular Ecology Notes*, **5**, 187–189.

Lande R (1995) Mutation and conservation. *Conservation Biology*, **9**, 782–791.

Latch EK, Dharmarajan G, Glaubitz JC, Rhodes Jr OE (2006) Relative performance of Bayesian clustering software for inferring population substructure and individual assignment at low levels of population differentiation. *Conservation Genetics*, **7**, 295–302.

Lecis R, Pierpaoli M, Biro ZS *et al.* (2006) Bayesian analyses of admixture in wild and domestic cats (*Felis silvestris*) using linked microsatellite loci. *Molecular Ecology*, **15**, 119–131.

Menotti-Raymond MM, David VA, Lyons LA *et al.* (1999) A genetic linkage map of microsatellites in the domestic cat (*Felis catus*). *Genomics*, **57**, 9–23.

Menotti-Raymond MM, David VA, Wachter LL, Butler JM, O'Brien SJ (2005) An STR forensic typing system for genetic individualization of domestic cat (*Felis catus*) samples. *Journal Forensic Science Society*, **50**, 1061–1070.

Millis LS, Allendorf FW (1996) The one-migrant-per-generation rule in conservation and management. *Conservation Biology*, **10**, 1509–1518.

Morato RG, Barnabe RC (2002) Potential de técnicas reproductivas para la conservación del jaguar. In:*El Jaguar en el Nuevo Milenio* (eds Medellín RA, Equihua C, Chetkiewicz CL, Crawshaw PG Jr, Rabinowitz A, Redford KH, Robinson JG, Sanderson EW, Taber AB). pp. 43–53, Universidad Nacional Autonoma de Mexico/Wildlife Conservation Society, México.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583–590.

Nowell K, Jackson P (1996) *Wilds Cats: Status Survey e Conservation Action Plan*. IUCN, Gland, Switzerland.

Oliveira TG (1994) *Neotropical Cats: Ecology and Conservation*. EDUFMA, São Luís, Brazil.

Paetkau D, Slade R, Burden M, Estoup A (2004) Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. *Molecular Ecology*, **13**, 55–65.

Paviolo A, De Angelo C, Di Blanco YE, Di Bitetti MS (2008) Jaguar *Panthera onca* population decline in the Upper Parana Atlantic Forest of Argentina and Brazil. *Oryx*, **42**, 554–561.

Petit RJ, Mousadik EL, Pons O (1998) Identifying populations for conservation on the basis of genetic markers. *Conservation Biology*, **12**, 844–855.

Piry S, Alapetite A, Cornuet JM, Paektau D, Baudouin L, Estoup A (2004) GeneClass2: a software for genetics assignment and first generation migrants detection. *Journal of Heredity*, **95**, 536–539.

Pritchard JK, Wen W (2004) *Documentation for STRUCTURE Software, Version 2*, Department of Human Genetics, University of Chicago, Chicago.

Pritchard JK, Stephens P, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945–959.

Rabinowitz A, Zeller KA (2010) A range-wide model of landscape connectivity and conservation for the jaguar, *Panthera onca*. *Biological Conservation*, **143**, 939–945.

Rannala B, Mountain JL (1997) Detecting immigration by using multilocus genotypes. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 9197–9221.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Reed DH, O'Grady JJ, Brook BW, Ballou JD, Frankham R (2003) Estimates of minimum viable population sizes for vertebrates and factors influencing those estimates. *Biological Conservation*, **113**, 23–34.

Rice WR (1989) Analyzing table of statistical tests. *Evolution*, **43**, 223–225.

Ruiz-Garcia M, Payán E, Murillo A, Alvarez D (2006) DNA microsatellite characterization of the jaguar (*Panthera onca*) in Colombia. *Genes & Genetic Systems*, **81**, 115–127.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning, A Laboratory Manual*, 2nd edn, Cold Spring Harbor Laboratory Press, New York.

Sanderson EW, Redford KH, Chetkiewicz C-LB *et al.* (2002) Planning to save a species: the jaguar as a model. *Conservation Biology*, **16**, 58–72.

Schipper J, Chanson JS, Chiozza F *et al.* (2008) The status of the world's land and marine mammals: diversity, threat, and knowledge. *Science*, **322**, 225–230.

Serre D, Pääbo S (2004) Evidence for gradients of human genetic diversity within and among continents. *Genome Research*, **14**, 1679–1685.

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, **139**, 457–462.

Sotelo G, Morán P, Fernández L, Posada D (2008) Genetic variation of the spiny spider crab *Maja brachydactyla* in the northeastern Atlantic. *Marine Ecology Progress series*, **362**, 211–223.

Taberlet P, Griffin S, Goossens B (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research*, **24**, 3189–3194.

Tallmon DA, Koyuk A, Luikart GH, Beaumont MA (2008) ONeSAMP: a program to estimate effective population size using approximate Bayesian computation. *Molecular Ecology Resources*, **8**, 299–301.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping erros in microsatelite data. *Molecular Ecology Notes*, **4**, 535–538.

Vucetich JA, Waite TA (2000) Is one migrant per generation sufficient for the management of fluctuating populations? *Animal Conservation*, **3**, 261–266.
Wahlund S (1928) Composition of populations from the perspective of the theory of heredity. *Hereditas*, **11**, 65–105.
Waples RS (1991) Genetic methods for estimating the effective size of cetacean populations. *International Whaling Commission (Special issue)*, **13**, 279–300.
Waples RS (2006) A bias correction for estimates of effective population size based on linkage disequilibrium at unlinked gene loci. *Conservation Genetics*, **7**, 167–184.
Waples RS, Do C (2008) LDNE: a program for estimating effective population size from data on linkage disequilibrium. *Molecular Ecology Resources*, **8**, 753–756.
Wasser SK, Houston CS, Koehler GM, Cadd GG, Fain SR (1997) Techniques for application of faecal DNA methods to filed studies of Ursids. *Molecular Ecology*, **6**, 1091–1097.
Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.
Wilberg MJ, Dreher BP (2004) Genecap: a program for analysis of multilocus genotype data for non-invasive sampling and capture-recapture population estimation. *Molecular Ecology Notes*, **4**, 783–785.
Wright S (1931) Evolution in Mendelian populations. *Genetics*, **16**, 97–159.
Zeller K (2007) *Jaguars in the New Millennium Data Set Update: The State of the Jaguar in 2006*. Wildlife Conservation Society's Jaguar Conservation Program, Takoma Park, MD.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Samples of jaguars analyzed in the present study. Numbers in parentheses next to the geographic origin represent each sample locality in Fig. 1

Table S2 Pairwise comparisons of estimates of genetic diversity (allelic richness [*AR*] and observed [H_o] and expected [H_e] heterozygosities) among the four jaguar populations remaining in the UPAF

Table S3 Observed allele frequencies at each locus in each population

Table S4 Population assignment and inferred ancestry of jaguar individuals using STRUCTURE analysis with geographical information

Fig. S1 Histograms showing the distribution of the allele frequencies in the Green Corridor (white bars), Morro do Diabo (light grey bars), Ivinhema (dark grey bars) and Porto Primavera (black bars).

Fig. S2 Results of Bayesian clustering analysis performed in STRUCTURE. For each number of population clusters (*K*) tested, the Ln P (D) is the mean of the estimated log of the probability of the data across 10 different runs.

Fig. S3 Proportional membership (*q*) of each jaguar in the genetic clusters inferred by STRUCTURE with $K = 5$ (a) and $K = 6$ (b), without use of prior population information (USEPOPINFO = 0).

Fig. S4 Graph depicting results from the BAPS analysis of population structure incorporating spatial information.

Fig. S5 Pairwise population comparison of genetic and geographical distances (in kilometers) analyzed with GENEPOP. (a) Genetic distances estimated with F_{ST}; (b) Genetic distances estimated with D_{EST}.

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