Genes Genet. Syst. (2006) **81**, p. 115–127

DNA microsatellite characterization of the jaguar (*Panthera onca*) in Colombia

Manuel Ruiz-Garcia*, Esteban Payán, Andrea Murillo and Diana Alvarez

Laboratory of Molecular Population Genetics (Grupo de Genética de Poblaciones-Biología Evolutiva). Unidad de Genética. Departamento de Biología. Facultad de Ciencias. Pontificia Universidad Javeriana. Cra 7A No 43-82. Bogotá DC., Colombia

(Received 8 March 2005, accepted 26 February 2006)

The Colombian jaguar population is thought to contain two different subspecies, *Panthera onca centralis* and *Panthera onca onca*. The genetic structure of this population was evaluated using 12 microsatellite loci (n = 62 samples). In addition, 22 jaguar DNA samples from Guatemala, Paraguay, Perú, Bolivia, Venezuela and Brazil were analyzed for these microsatellite loci (n = 84 samples). The results of this study indicate six primary themes. First, the levels of gene diversity were very high. Second, the majority of the loci analyzed showed an absence of Hardy-Weinberg equilibrium, probably due to the Wahlund effect (= population subdivision). Third, several microsatellite loci showed significant heterogeneity between the two supposed subspecies in the country. Nevertheless, gene flow was present between them, and heterogeneity was relatively low, although the assignment analyses showed good classification of the jaguars studied into their respective subspecies. Fourth, the long-term historical effective population sizes were calculated through a maximum likelihood procedure for single and multi-step mutation models. Fifth, seven out of twelve DNA microsatellites studied significantly deviated from a single-step mutation model. However, the overall mean multi-step mutation percentage for these 12 DNA microsatellites was only 6%. Therefore, 94% of mutations were uni-step. Sixth, no bottleneck events were detected in the Colombian jaguar population overall.

Key words: *Panthera onca*, jaguar, microsatellite loci, molecular population genetics, Colombia

INTRODUCTION

The jaguar (*Panthera onca*) is the largest feline predator in the Americas. Its beauty and its extraordinary ability as a predator have made it a mythological reference and a religious icon for the Amerindian indigenous people for thousands of years. Although commercial exploitation for their skins is no longer a threat (Nowell and Jackson, 1996), jaguars still face local extirpation by deforestation, cattle ranchers and extensive agriculture. IUCN ranked the jaguar as a Near Threatened species and CITES listed it in Appendix I (Nowell and Jackson, 1996, IUCN 2004). It is the only representative of the *Panthera* genus in the American hemisphere, and appears to have entered from Asia through the Beringia strait in the early Pleistocene, after it diverged from its common ancestor at least 1.5 million years ago. Fossil jaguars in North America date to the middle Pleistocene (Seymour, 1989), and the immediate ancestor of modern jaguars appears to be the *Panthera onca augusta,* which was 15–20% larger.

Colombia contains two of eight currently accepted morphologically proposed subspecies (Pocock, 1939). *Panthera onca onca* ranges northwards from the central Amazon towards the Colombian Llanos and to the eastern Cordillera. *Panthera onca centralis* ranges from central Colombia northwards to Central America, encompassing the Chocó region in the Pacific area.

Only two papers have considered jaguars from a molecular population genetics standpoint (Eizirik et al., 2001 and Ruiz-García, 2001). In the former, the authors analyzed 40 specimens covering a considerable part of the jaguar's distribution and employed 715 base pairs from the mtDNA control region and 29 microsatellite loci. The samples were largely provided by zoos in Guatemala, Nicaragua, México, Venezuela and Brazil. The most outstanding result was a high microsatellite genetic diversity

Edited by Hidenori Tachida

* Corresponding author. E-mail: mruiz@javeriana.edu.co

(H = 0.739), suggesting a recent demographic expansion and mtDNA lineages originating some 280,000 to 510,000 years ago. The molecular analysis did not find any strong geographic structure throughout the jaguar distribution. The second paper analyzed five microsatellite markers in the six wild Felidae present in Colombia (*Leopardus pardalis, L. wiedi, L. tigrinus, Herpailurus yaguarondi, Puma concolor* and *Panthera onca*). A multiple regression analysis between the expected heterozygosity for the six species and 54 ecological, demographic, morphological and reproductive traits showed a positive and significant relationship among the genetic measure and the other 54 variables. The average gene diversity for the jaguar was H = 0.76, a similar value to that discovered in *P. concolor* (H = 0.75) and in *L. tigrinus* (H = 0.78), slightly higher than that detected in *H. yaguarondi* (H = 0.62) and slightly lower than that found in *L. pardalis* (H = 0.84) and in *L. wiedi* (H = 0.85). However, in a new study of the ocelot (n = 133 individuals), Ruiz-García et al., (2005b) showed a H = 0.92 for this species. The demographic population size of jaguars in Latin America was calculated using diverse population genetics measures and the values ranged from 105,000 to 310,000 animals.

A molecular study at a finer geographical scale (Colombia) is necessary to assess the molecular genetic population structure and to detect possible genetic differences between the two subspecies proposed for this country (*P. o. onca* and *P. o. centralis*).

In the present paper we examine DNA microsatellite from samples of diverse origin, covering all the Colombian regions inhabited by jaguars. Additionally, samples from other Latin American areas covered by two other proposed morphological subspecies are also analyzed. The main aims were as follows: 1- To determine gene diversity levels in the Colombian jaguar population. 2- To estimate possible Hardy-Weinberg equilibrium in the jaguar samples analyzed. 3- To determine genetic heterogeneity and gene flow estimates between the two supposed Colombian jaguar subspecies, as well as to apply assignment analysis to each one of the subspecies studied. 4- To estimate long-term historical effective population sizes for jaguars in Colombia. 5- To determine the evolutionary mutation model of the 12 microsatellites employed in this study and 6- To determine possible bottleneck events for the Colombian jaguar population.

METHODS

Eighty-four (n = 84) jaguar DNA samples were analyzed (Fig. 1). Sixty-two (n = 62) DNA samples were from Colombian jaguars. These samples were partitioned as follows: 47 were from the Eastern Llanos and the Amazon [Caquetá (Araracuara), Meta (La Macarena), Caguán, Vaupés (Miraflores- Itilla River-, Yuruparí, Apaporis river), Guaviare (Tucanema), Guainia (Pto.Inirida) and Amazon (Leticia) Departments, representing *P. onca onca* (Pocock, 1939)]. Fifteen (15) samples of *P. o. centralis* (Pocock, 1939) were obtained from North of Santander Department, Colombian Atlantic coast, Darién area, Antioquia Department and Colombian Pacific region (Riosucio, Chocó). The remaining 22 DNA samples were from the following Latin America countries: one sample was obtained in the Petén area of Guatemala representing *P. onca goldmani*, one sample from Venezuela representing *P. onca onca*, two samples from the Paraná River in Paraguay (*P. onca paraguensis*), seven samples from the Loreto Department in the Peruvian Amazon (Napo and Curaray rivers and Puinahua Channel in Ucayali river) (*P. o. onca* following Pocock, 1939; *P. onca ucayalae* following Nelson and Goldman, 1933), five samples from the Bolivian Amazon (Villa Bella, St. Ana de Yacumo, St. Rosa de Vigo in the Mamoré River) (*P. o. onca* following Pocock, 1939; *P. onca boliviensis* following Nelson and Goldman 1933), and six samples from the Central Brazilian Amazon, approximately 100km from Manaus (*P. o. onca*).

DNA was isolated from blood, hair, muscle biopsies, pieces of skins, bones and teeth of animals collected from the field or killed directly in the wild by colonists or Indians. Skulls and teeth of jaguars were obtained from several native Indian tribes across diverse regions of Colombia, Perú and Bolivia.

Blood samples (0.5–4 ml) were collected in tubes with disodium EDTA. Hair with roots and muscle tissues obtained in the field were immediately stored in absolute alcohol prior to transportation to the Bogotá laboratory. Three different methods were used to extract DNA from the blood samples (phenol-chloroform, DTAB-CTAB and Chelex resin). For muscle tissues, pieces of skins, teeth and bones, the phenol-chloroform procedure was employed. DNA extraction from the hairs with follicle roots was carried out using 10% Chelex resin, modified from Walsh et al. (1991). The DNA concentration extracted from the blood samples ranged from 17 to 1014 ng/µl.

Molecular markers. Twelve (12) microsatellite markers (*Fca 08*, *Fca 24, Fca 43, Fca 45, Fca 96, Fca 126, Fca 136, Fca 176, Fca 225, Fca 294, Fca 391* and *Fca 506*) were examined.

All loci are dinucleotide repeats $(CA)_n$ or $(GT)_n$, with the unique exception of *Fca 391*, which is a tetranucleotide repeat locus. The sequences of these microsatellite primers were obtained from Menotti-Raymond and O'Brien (1995) and Menotti-Raymond et al., (1999). Polymerase chain reaction (PCR) was performed in a 25-µl volume. When DNA was extracted from blood, skin, teeth and bones, PCR reaction mixtures included 2.5 µl of 2.5 mM $MgCl_2$, 2.5 µl of 10x Buffer, 1 µl of 1 mM dNTPs, 10 pmol of each primer, 14.5 µl of H_20, 2–3 µl of DNA (50–100 ng/

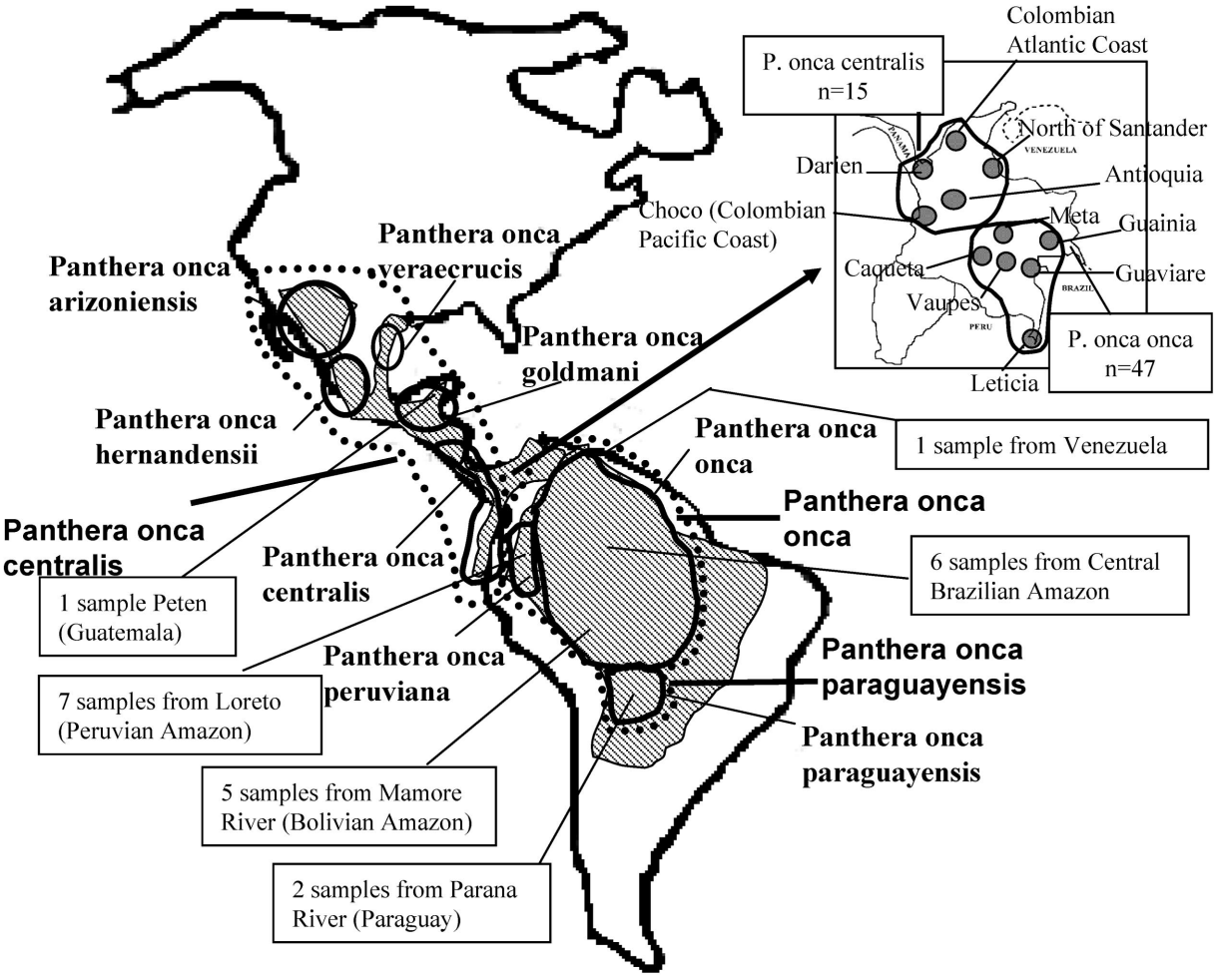


Fig. 1. Map with the geographic distribution of the 84 jaguars analyzed. The eight jaguar subspecies defined by Pocock (1939) are shown in bold letters. The three jaguar groups defined by Seymour (1989) in larger bold letters. The detailed map is that of Colombia, where 47 jaguars were sampled in the area of *P. onca onca*, in the regions of 1- Meta, 2- Caguán, 3- Vaupés, 4- Guaviare, 5-Guainia, 6- Leticia (Amazon), 7- Caquetá, and 15 jaguars were sampled in the area of *P. o. centralis*, concretely in the regions of 8- North of Santander, 9- Colombian Atlantic coast, 10- Darien, 11- Antioquia and 12- Chocó, Pacific Colombian coast. Other sample points were as follows: 13- 1 jaguar from Petén (Guatemala), 14- 1 jaguar from Venezuela, 15- 2 jaguars from Paraná River (Paraguay), 16- 7 jaguars from Loreto Department in the Peruvian Amazon (Perú) on the Napo, Curaray and Ucayali rivers, 17- 5 jaguars from the Bolivian Amazon (Mamoré River) and 18- 6 jaguars from central Brazilian Amazon near Manaus (Brazil).

µl) and one unit of Taq polymerase. DNA extracted from hair was subjected to PCR in a 50-µl PCR reaction volume containing double the quantities of all the above reagents and 20 µl of DNA. PCR reactions were carried out in a Geneamp PCR System 9600 thermocycler (Perkin Elmer, Wellesley, Massachusetts). The temperatures employed were as follows: 95°C for 5 minutes; 35 cycles of 1 minute at 95°C, 2 minutes at 55°C for all the markers used, and 2 minutes at 72°C. A final extension for 5 minutes at 72°C was used. Amplification products were kept at 4°C until used. PCR products were electrophoresed in denaturing 6% polyacrylamide gels and visualized with a Hoefer SQ3 sequencer vertical camera. Staining was performed with silver nitrate. Allele sizes were obtained by comparison with the molecular weight marker, ϕX174 DNA digested with *Hind* III and *Hinf* I. A molecular weight marker was loaded every four lanes. The PCR amplifications were performed in triplicate to ensure the accuracy of the genotypes obtained. In 96% of cases, the observed genotypes were the same in the three replicates. Therefore, preferential allele amplifications had minimal effects on the results obtained.

Population genetics analysis. The respective allele frequencies at each locus and average number of alleles per locus were calculated. Genetic diversity was measured with the unbiased expected heterozygosity (Nei, 1978). The Hardy-Weinberg equilibrium (H-W E) was tested using the Weir and Cockerham (1984) F statistic to calculate the degree of excess or deficit of homo- and/or heterozygosity. To measure the exact probabilities of these statistics the program Genepop (Raymond and

Rousset, 1995, v. 3.1) was employed with the Markov chain method.

Colombian samples of known geographic origin, genotyped for all 12 microsatellites (enabling classification to subspecies *P. o. onca* and *P. o. centralis*), were analyzed by means of the GENECLASS program (Cornuet et al., 1999) in order to assign individuals to populations. Likelihood-based (Paetkau et al., 1995; Rannala and Mountain, 1997) and genetic distance-based methods were applied. The distance-based methods assign individuals to the "closest" population and require the definition of a distance between the individuals and the populations considered. The ones used here were those employed by Nei (1978), Cavalli-Sforza and Edwards (1967), DAS (shared allele distance; Chakraborty and Jin, 1993) and the $\delta\mu^2$ genetic distance (Goldstein et al., 1995). The "leave one out" and the "as is" procedures were applied to the above assignment analysis.

The genetic heterogeneity among the *P. o. onca* and the *P. o. centralis* samples was obtained using exact probabilities with Markov chains and the exact test applied to the F_{ST} statistic. A hierarchical Wright *F* analysis was also done by the Michalakis and Excoffier (1996) procedure. The significance of these F statistics was measured using 2,000 jackknife and 2,000 bootstrap permutations over loci, independently.

Indirect theoretical gene flow estimates (Nm) between two jaguar subspecies were obtained using the F_{ST} statistic, with an infinite island model (Wright, 1951; Ruiz-García, 1993, 1998; Ruiz-García and Alvarez, 2000) and a *n*-dimensional island model (Takahata, 1983; Crow and Aoki, 1984).

An estimate of the long-term historical effective population size (N_e) of jaguars in Colombia was calculated using the Nielsen (1997) procedure. This technique estimates historical effective population sizes by using a maximum likelihood procedure with a Markov chain recursion method to calculate the values more probable of θ (= $4N_e\mu$), N_e being the effective population size and μ being the mutation rate per generation. The MISAT program (Nielsen, 1997) was employed, which estimates the likelihood surfaces for θ. A grid size of 40 with a previous θ calculated with the method of the moments (θ_0) in a single-step mutation model with 1,000,000 Markov chains was employed. The estimate of the maximum likelihood θ was that with the minor negative log likelihood value. From this value, N_e was calculated for the Colombian jaguar population, assuming several mutation estimates generated for other mammals. The selected rates ranged from 2.5×10^{-4} (Rooney et al., 1999) to 5.6×10^{-4} (Weber and Wong, 1993).

In addition, the most probable multi-step mutation percentages were calculated through the maximum likelihood method by means of 3,000,000 Markov chains. We examined differences of mutation rates between the microsatellite loci studied for the Colombian jaguar samples. For this, we started from the hypothesis $\theta_1 = \theta_2 = \theta$ (the values of θ for two different microsatellites) and we tested it with a likelihood ratio, which approximately follows a χ^2 distribution with one degree of freedom. A probability lower than $\alpha = 0.05$ indicates that both microsatellites have different mutation rates. Likewise, we measured if the multi-step mutation models estimated were really significantly better than the single-step mutation models within the Colombian jaguar sample. Considering the maximum likelihood multi-step $\underline{p}$ percentage obtained, one may apply a likelihood ratio, which for large samples follows approximately a χ^2 distribution with one degree of freedom under the null hypothesis that $p = 0$. A probability lower than $\alpha = 0.05$ indicates that the multi-step mutation model is significantly better than the single-step mutation model. Note that long-term effective population sizes represent effective sizes that have characterized the species historically and do not necessarily represent current effective or censused population sizes.

Using the BOTTLENECK program (Piry et al., 1999), the following tests were performed to detect possible recent bottleneck events on the Colombian jaguar population: sign test, standardized difference test, Wilcoxon's signed rank test and a graphical descriptor of the shape of the allele frequency distribution. These are based on the theory by Cornuet and Luikart (1996), Luikart and Cornuet (1998) and Luikart et al., (1998). The second procedure used to detect possible reduction events in the population size was that developed by Garza and Williamson (2001). This procedure is based on the ratio $M = k/r$, where k is the total number of alleles detected in a locus given and r is the spatial diversity, the distance between the smallest and largest alleles in number of repeats. When a population is reduced in size, this ratio will be smaller than in equilibrium populations. The program will simulate an equilibrium distribution of M in a constant size population assuming values for three parameters ($\theta = 4N_e\mu$, $\underline{p_s}$ = mean percentage of mutations that add or delete only one repeat unit, and Δ_g = mean size of larger mutations). The observed M is ranked relative to the equilibrium distribution. Using conventional criteria, there is evidence of a significant reduction in population size if less than 5% of the replicates are below the observed value. The parameter values employed in this analysis were obtained from the outputs of the MISAT program by Nielsen in 1997 (average value of θ = 24.084, whereas p_s and Δ_g were 0.9396 and 3.5, respectively). This analysis was carried out with the M-P-Val and Critical-M programs from Garza and Williamson (2001).

RESULTS

Alleles and molecular polymorphisms. Figure 2 shows

the histograms of the microsatellite allele frequencies of all the Colombian jaguar samples combined. The loci with the highest number of alleles were *Fca 96* (14 alleles), *Fca 08* (13 alleles), *Fca 391* (13 alleles), *Fca 176* (11 alleles), *Fca 225* (11 alleles) and *Fca 43* (11 alleles). The loci with the lowest number of alleles were *Fca 24* (6 alleles) and *Fca 136* (6 alleles). Several alleles were present only in one of the proposed Colombian jaguar subspecies. For instance, *Fca 96* showed alleles of 185, 187, 189, and 195 base pairs (bp) only present in *P. onca onca* and absent in *P. o. centralis*. Conversely, the latter possessed an allele of 219 bp which was absent in the *P. o. onca* samples. *Fca 45* showed allele sizes of 139, 147, 149, 151, 153, and 155 bp exclusive to the *P. o. onca* subspecies. On a higher geographic level, several alleles were found only in some alien jaguars, even though the Colombian sample size was considerably larger than the foreign one (62 vs. 22). At *Fca 08*, an allele of 124 bp was only detected in jaguars of the Central Brazilian Amazon, which is near Manaus. At *Fca 24*, 212 and 220 bp alleles were only found in jaguars from Bolivia. At *Fca 45*, a 159 bp allele was only discovered in a jaguar from Guatemala. At *Fca 96*, an allele of 183 bp was detected solely in a sample from Guatemala. For *Fca 136*, an allele of 231 bp and another of 221 bp were only detected in animals from Bolivia and Venezuela, respectively. For *Fca 176*, a 233 bp allele was detected solely in Bolivian jaguars, as were two alleles of 218 and 222 bp for *Fca 294*. At *Fca 391*, an allele of 205 bp was detected only in Peruvian jaguars, while 199 and 233 bp alleles were solely found in jaguars from Bolivia. Finally, at *Fca 506*, an allele of 206 bp was detected only in jaguars from Perú and Bolivia, and an allele of 208 bp was exclusively found in a Bolivian jaguar.

In Table 1, the average number of alleles per locus (*napl*) and the expected heterozygosity (*H*) for each sample analyzed is shown. The genetic diversity estimates were high. The total jaguar sample presented the highest *napl* and *H* values. The *H* values of *P. o. centralis* ($H = 0.8281 \pm 0.0831$) and *P. o. onca* ($H = 0.8269 \pm 0.1179$) were not significantly different. The *napl* is slightly higher in *P. o. onca* ($napl = 7.71 \pm 2.43$) than in *P. o. centralis* ($napl = 6.25 \pm 1.58$), although the difference is not significant ($P = 0.1578$). These results show that the two Colombian jaguar subspecies contain a major fraction of the gene diversity of the species as a whole, revealing a high degree of historical gene flow among Colombian jaguar populations.

Table 1. Mean expected levels of heterozygosity (*H*) and average number of alleles per locus (*napl*) at different geographical levels of jaguar populations.

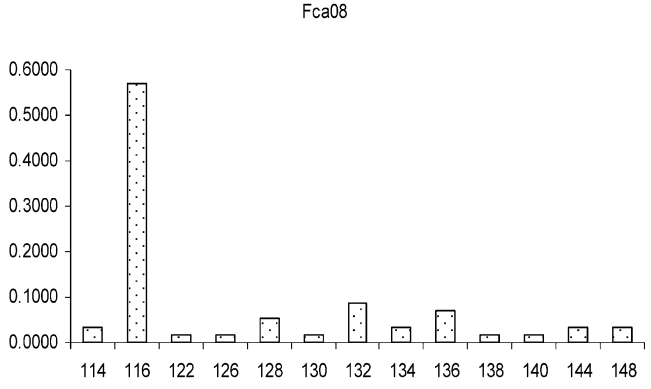
	napl	*H*
Overall jaguar sample	11.330 ± 2.570	0.846 ± 0.066
Total Colombian jaguar sample	10.083 ± 2.571	0.835 ± 0.083
P. o. onca sample	7.714 ± 2.429	0.826 ± 0.117
P. o. centralis sample	6.250 ± 1.581	0.828 ± 0.083

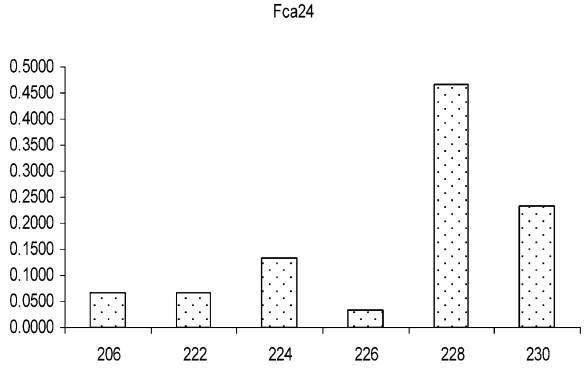
Hardy-Weinberg disequilibrium. For the total Colombian sample, *Fca 08, Fca 45, Fca 96, Fca 126* and *Fca 225* showed positive significant values of the Weir and Cockerham *F* statistic. For all loci, the overall homozygote excess was highly significant (χ^2 = infinity, 16 df, $P < 0.00001$) using Fisher's method. A multi-locus test also indicated a significant homozygote excess ($P = 0.0000 \pm 0.0000$); all these analyses were carried out with the Genepop program. Independent analyses of samples separated as subspecies gave the following: *P. o. centralis* showed that 4 loci, *Fca 96* ($P = 0.0000 \pm 0.0000$), *Fca 126* ($P = 0.0000 \pm 0.0000$), *Fca 43* ($P = 0.0013 \pm 0.0013$) and *Fca 225* ($P = 0.0026 \pm 0.0013$), yielded a significant homozygote excess. *P. o. onca* showed significant homozygote excess at four loci, *Fca 45* ($P = 0.0000 \pm 0.0000$), *Fca 43* ($P = 0.0255 \pm 0.0050$), *Fca 08* ($P = 0.0005 \pm 0.0005$) and *Fca 176* ($P = 0.0203 \pm 0.0046$). Consequently, homozygote excess is present in the overall sample and also within subspecies. As will be discussed later this could be some evidence of the Wahlund effect, by subdivision of jaguar populations.

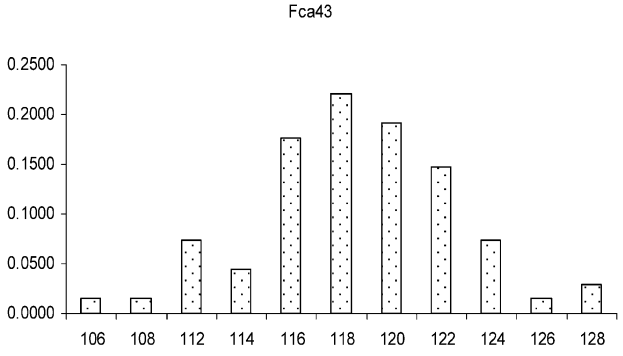
Population assignment. Population assignment using likelihood-based and genetic distance-based methods are presented in Table 2 and were undertaken with the Geneclass program. This analysis was done with 39 Colombian jaguars of known origin and genotyped for all 12 microsatellites studied. The "as is" method classified the jaguars studied into their respective subspecies (*P. o. centralis* and *P. o. onca*) more accurately than the "leave one out" procedure. The best classifications were obtained with the frequency method and the "as is" procedure (100% of individuals correctly classified, 39/39) and with the Bayesian method and the "as is" procedure (97.44%, 38/39). The worst assignments were obtained with the genetic distance-based method with the Nei and DAS genetic distances and with the "leave one out" procedure (64.10%, 25/39, for both distances). Therefore, the above methods appropriately classify individuals belonging to different populations. However, there is no extreme genetic differentiation, which indicates that a certain degree of gene flow has been present between both supposed jaguar subspecies, or that the time separation among them is relatively short.

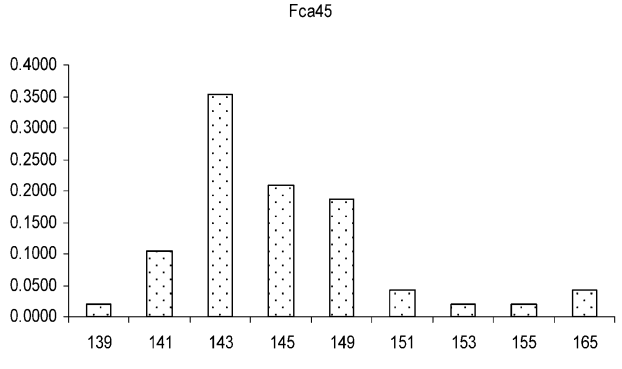
Genetic heterogeneity and gene flow between *P. o. centralis* and *P. o. onca* in Colombia. Several loci showed significant differences between the two Colombian jaguar populations. The genic differences (using the exact test) showed that the *Fca 96, Fca 45* and *Fca 391* loci presented significant differences between *P. o. centralis* and

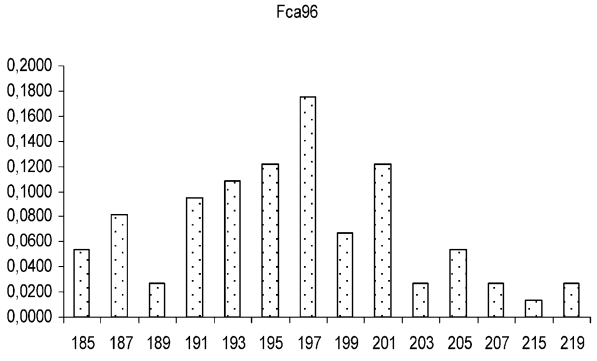
P. o. onca. The combination test using Fisher's method with the twelve loci also revealed a globally significant heterogeneity between the two subspecies (χ^2 = 42.419, 16 df, P = 0.0004). Nevertheless, the G_{ST} and G_{ST}' statistics (not shown) ranged from 0.01 to 0.02, which suggests relatively low global genetic heterogeneity between the two supposed subspecies. The Michalakis and Excoffier (1996) F statistics showed the F_{ST} estimates with the highest values at *Fca 45* (F_{ST} = 0.078) and at *Fca 391* (F_{ST} = 0.064) loci, while *Fca 126* and *Fca 43* showed insignificant degrees of heterogeneity. Jackknifing and bootstrapping of loci also showed small levels of heterogeneity and significant evidence of homozygous excess at the total and subpopulation levels (F_{IT}, F_{IS}). When alleles where randomized 2,000 times in the overall sample, all loci showed significant positive F_{IT} values at $P < 0.05$. With the standard Bonferroni test ($\alpha' = 0.00625$) all loci presented a significant homozygote excess, except at *Fca 96* and at *Fca 391*. A similar situation was found for F_{IS}. For the F_{ST} significance, two procedures were employed. Two thousand randomizing allele overall samples assuming random mating with the exact G-test (Goudet et al., 1996), and $P < 0.05$, showed significant heterogeneities at *Fca 96, Fca 45, Fca 391* and *Fca 176*. However, when

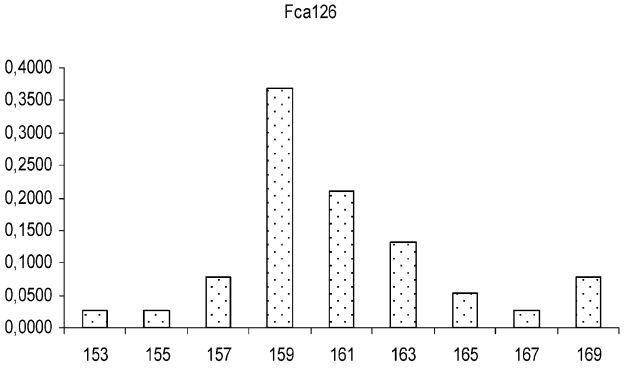












Continued

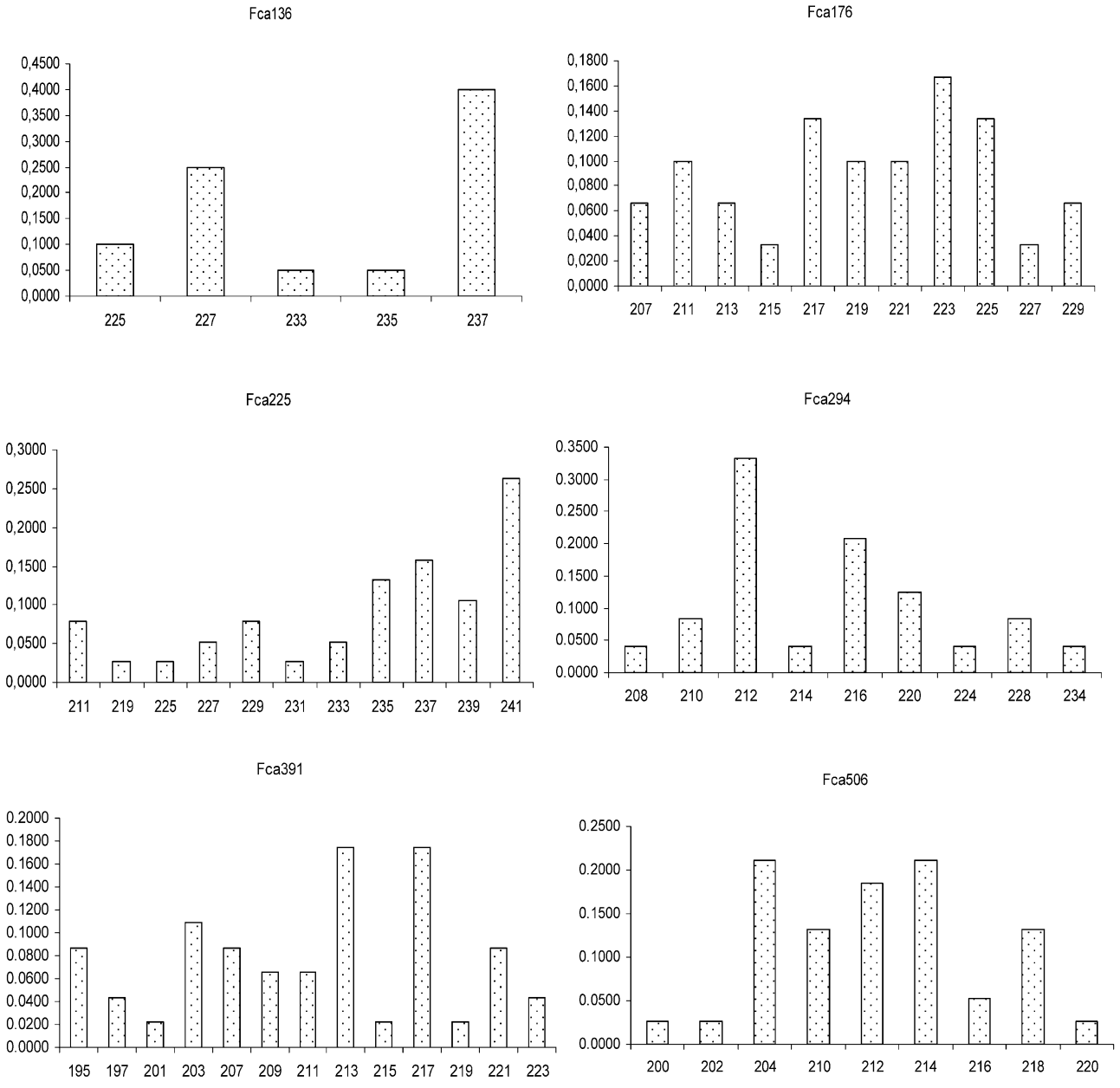


Fig. 2. Histograms of the allele frequencies of the 12 microsatellites studied in 62 Colombian jaguars.

using the standard Bonferroni correction ($\alpha' = 0.00625$), only *Fca 45, Fca 391* and all the loci analyzed simultaneously showed significant heterogeneity. Therefore, the genetic heterogeneity between the two Colombian jaguar populations, although significant for certain loci, was relatively small.

The *Nm* estimate from F_{ST} with an island model was 12.13, whereas with an island n-dimensional model it was 3.09. In general, these values show relatively high gene flow and incomplete isolation between the two proposed subspecies.

Long-term historical effective population sizes and microsatellite mutation models for the Colombian jaguar population. The historical effective population sizes (N_e) were obtained by means of the maximum likelihood method of Nielsen (1997) with the MISAT program. Several important observations result (Table 3): 1-The average maximum likelihood θ estimates were similar for the single-step mutation model and for the multi-step mutation model (22.68 ± 13.36 vs. 24.08 ± 11.77, respectively). Therefore, the effective population size estimates were approximately the same for each one of the mutation models considered. Also the N_e values from the θ estimate with the moments method were sim-

Table 2. Population assignment results for two jaguar subspecies (*P. o. onca* and *P. o. centralis*) using the microsatellites *Fca 08, Fca 43, Fca 45, Fca 96, Fca 126, Fca 176, Fca 200* and *Fca 391*.

Method	% correct assignment	Individuals correctly identified out of 39
Frequency "As is"	100	39
Bayesian "As is"	97.44	38
Nei standard distance "As is"	92.31	36
Cavalli-Sforza distance "As is"	94.87	37
Goldstein et al. (1995) distance "As is"	82.05	32
DAS distance "As is"	89.74	35
Frequency "Leave one out"	69.23	27
Bayesian "Leave one out"	69.23	27
Nei standard "Leave one out"	64.10	25
Cavalli-Sforza "Leave one out"	66.67	26
Goldstein "Leave one out"	76.92	30
DAS distance "Leave one out"	64.1	25

ilar to those obtained with Nielsen's (1997) procedure. 2-The mutation rate of 5.6×10^{-4} offered more realistic effective numbers than those obtained with the mutation rate of 2.5×10^{-4}. With the first mutation rate, the long-term historical effective population size for the single-step mutation model was estimated to be 10,127, whereas for the multi-step mutation model it was 10,752. 3-Seven of the 12 microsatellites employed presented a significant multi-step mutation model (*Fca 08, Fca 45, Fca 96, Fca 176, Fca 225, Fca 294* and *Fca 391*) with a percentage of multi-step mutation ranging from 5% to 20%. Overall, the average multi-step mutation percentage was 6.04%, which means that 94% of mutations affecting the microsatellites studied were single-step. 4-In general, the diverse microsatellites used here have significantly different mutation rates (Table 4). For the single-step model, the mutation rate order from high to low values was: *Fca 08 > Fca 96 > Fca 391 > Fca 225 > (Fca 43, Fca 45, Fca 294) > (Fca 506, Fca 24, Fca 176) > Fca 126 > Fca 136*. For the multi-step mutation model, the order was: *Fca 08 > Fca 391 > (Fca 96, Fca 225) > Fca 43 > (Fca 45, Fca 176, Fca 294, Fca 506) > Fca 24 > Fca 126 > Fca 136*. Therefore, for both models, *Fca 08* was the marker with the

Table 3. Maximum likelihood estimates of θ (= $4N_e\mu$) at 12 DNA microsatellites assuming a single-step and a multi-step mutation model. θ_0 is calculated with the moment procedure. **SIG** = microsatellites which showed significant multi-step mutation models. Long-term historical effective population sizes (N_e) were calculated assuming two extreme mutation rates per generation (5.6×10^{-4} and 2.5×10^{-4}) for the Colombian jaguar population.

	UNI-STEP			MULTI-STEP			
Markers	θ_0	θ	log likelihood	θ	log likelihood	%multi-step	χ^2
Fca08	51.547	34.7945	–59.56936	29.8975	–63.0069	20%	**6.87 SIG**
Fca24	17.517	10.1600	–21.72314	20.1448	–20.5300	2.50%	2.39 NS
Fca43	8.634	18.1311	–28.70809	16.4907	–28.1399	2.50%	1.14 NS
Fca45	14.042	16.1479	–27.80836	10.8121	–25.4404	10%	**4.74 SIG**
Fca96	25.191	38.5417	–42.38675	48.1142	–36.7579	5%	**11.26 SIG**
Fca126	6.528	13.7082	–18.75456	14.9485	–18.3959	0%	0.72 NS
Fca136	14.374	6.9712	–14.43525	13.7987	–16.0956	0%	3.32 NS
Fca176	18.374	12.6015	–20.90673	25.0164	–24.3505	5%	**6.89 SIG**
Fca225	36.768	49.2693	–32.48042	42.2833	–38.1362	5%	**11.31 SIG**
Fca294	21.507	30.8629	–26.08909	16.5606	–23.8856	7.50%	**4.41 SIG**
Fca391	33.194	28.7129	–37.90330	31.8664	–41.8561	12.50%	**7.91 SIG**
Fca506	14.238	12.3155	–23.46632	19.0783	–24.0518	2.50%	1.17 NS
average	21.8507	22.6847		24.0843		6.0416%	
	±12.9949	±13.3657		±11.7709		±5.7857%	
Mutation rate per generation	N_e			N_e			
5.6×10^{-4}	9,755	10,127		10,752			
2.5×10^{-4}	21,851	22,685		24,084			

Table 4. Chi-square values from a likelihood ratio test with the expression *-2 log* $[L_1(\theta)L_2(\theta)]/[L(\theta_1,\theta_2)]$ to detect differences of mutation rates per generation at microsatellite pairs.

Markers	Fca08	Fca24	Fca43	Fca45	Fca96	Fca126	Fca136	Fca176	Fca225	Fca294	Fca391	Fca506
Fca08												
Fca24	75.69**											
Fca43	61.72**	13.97**										
Fca45	63.52**	12.17**	1.80NS									
Fca96	34.36**	41.33**	27.36**	29.16**								
Fca126	81.63**	5.94**	19.91**	18.11**	47.26**							
Fca136	90.27**	14.58**	28.55**	26.75**	55.90**	8.64**						
Fca176	77.32**	1.63NS	15.60**	13.80**	42.96**	4.30*	12.94**					
Fca225	54,18**	21.51**	7.55**	9.34**	20.96**	27.45**	36.09**	23.15**				
Fca294	66.96**	8.73**	5.24*	3.43NS	32.59**	14.67**	23.71**	10.36**	12.78**			
Fca391	43.33**	32.36**	18.39**	20.19**	8.97**	38.30**	46.97**	33.99**	10.86**	23.63**		
Fca506	72.21**	3.48NS	10.48**	8.68**	37.84**	9.42**	18.06**	5.12*	18.03**	5.25*	28.87**	

*P < 0.05, **p < 0.01 Significant differences between microsatellite mutation pairs.
NS = Not significant.

highest mutation rate, while *Fca 136* was that with the lowest mutation rate.

Bottleneck events in the Colombian jaguar population. For the total Colombian jaguar sample, the stepwise mutation model (SMM) does not support a recent bottleneck event (BOTTLENECK program). The sign test, the standardized differences test, the Wilcoxon test and the graphical mode-shift showed a lack of evidence to support a bottleneck event. The graphical mode-shift showed an L-shaped distribution typical of a population with a constant effective size. Additionally, the standardized difference test with the SMM yielded a significantly negative value (T_2 = –2.926, P = 0.00172) contrary to the expectation from a recent bottleneck event, which could imply population expansion or gene flow. The occurrences of these events could obliterate the detection of bottleneck events. Also, the *P. o. onca* sample did not show evidence of bottleneck events. The situation for *P. o. centralis* is different and at least two of the different tests applied detected a possible bottleneck event in this subspecies (Standardized difference test, T_2 = 1.945, P = 0.02589; Wilcoxon test, P = 0.00586). Therefore, its conservation situation could be more critical than that of *P. o. onca*.

The Garza and Williamson (2001) procedure (with the M-P-Val and Critical-M programs) was performed with the following three parameters: θ = 24.08, P = 0.9396 and Δ_g = 3.5 obtained from the Nielsen (1997) analysis. The observed average M ratio was 0.7669. Simulating 10,000 replicates, the expected average M ratio should be 0.7388 and 95% of the M values in equilibrium should be over a critical value (M_c) of 0.6578. Three out of 12 values obtained were under this critical M_c value (*Fca 24*, M = 0.4615; *Fca 45*, M = 0.6429; *Fca 294*, M = 0.6429). The possible bottleneck event detected in the overall Colombian jaguar population was not very strong because not all loci studied showed its effects.

DISCUSSION

The total jaguar sample showed a high genetic diversity (H = 0.846) and a high average number of alleles per locus. The expected heterozygosity in the two Colombian jaguar subspecies was not significantly different (H = 0.828 and 0.827) from the total H value. *P. o. centralis* had the lower average number of alleles per locus but it was a statistically insignificant difference. These results show a species with a highly diverse genome and without strong microsatellite differences between the subspecies considered. Jaguar gene diversity levels and the average number of alleles per locus found here were slightly higher than those obtained by Eizirik et al., (2001) for the same species using 29 microsatellites (H = 0.622 to 0.739). It is noteworthy to mention that our estimate of the jaguar gene diversity is significantly higher than those obtained by Culver et al. (2000) and Walker et al. (2000) for pumas (*Puma concolor*)(0.42–0.71, including different subspecies), another wide ranging big cat found across the Americas. Similar heterozygosity levels were discovered in leopards from Tanzania, with H = 0.77 ± 0.03 (Spong et al., 2000). This is not surprising since leopards and jaguars share similar ecological traits.

Hardy-Weinberg analysis showed a homozygote excess for several microsatellites. Possible explanations for the heterozygote deficiencies (Rooney et al., 1999; Spong et al., 2000) include: population subdivision causing homozygote excess (Wahlund effect), strong genetic drift and

endogamy, hitchhiking, null alleles, synteny or natural selection in favor of homozygosity. The most plausible explanation in this case is population subdivision. Strong genetic drift and elevated consanguinity can be discarded as explanations since the genetic diversity levels were very high for all the jaguar samples studied. If genetic drift was important we would expect low levels of gene diversity, such as those found for the cheetah (*Acinonyx jubatus*) (H = 0.39; Menotti-Raymond and O'Brien, 1995), the Ethiopian wolf (*Canis simensis*) (H = 0.21–0.36; Gotelli et al., 1994) and the Andean bear (*Tremarctos ornatus*) (H = 0.3–0.5; Ruiz-García, 2003; Ruiz-García et al., 2005a). Endogamy affects all loci in the same way. Hitchhiking and synteny are ruled out since the loci studied were deliberately chosen because they are distributed in different chromosomes, and the possibility of all loci being affected identically is remote. The same explanation could be used to discard natural selection in favor of homozygosity. Null alleles also seem unlikely to produce high and similar levels of homozygote excess simultaneously for a large fraction of the loci studied. Therefore, the most probable explanation is the Wahlund (= subdivision) effect. In fact, when both jaguar subspecies were separately analyzed some loci presented homozygote excess, which could indicate that even within the subspecies the subdivision process is still relatively important.

The classification of individuals with likelihood-based (frequentist and Bayesian techniques) and genetic distance-based methods showed a very high percentage of correct assignment with the "as is" procedure (95–100% of correct assignments), whereas with the "leave-one-out" procedure the percentages of adequate classification of the individuals was less accurate (64–67%). It is possible that the separation between the two proposed subspecies has been recent and thus no striking genetic differences have emerged between them, although some significant genetic heterogeneity is present. For this reason, the genetic distance-based methods did not discriminate jaguars from the two populations perfectly. The estimates of *Nm* with the infinite and the n-dimensional island models support the above idea. Eizirik et al. (2001) also found values of *Nm* around or above 1. The jaguar's high migration capacity has been reported before (Crawshaw and Quigley, 1991; Schaller and Crawshaw, 1980) and this explains why populations of jaguars in Colombia are relatively similar. However, comparing our gene flow estimates with those found for Tanzanian leopard populations, jaguars experience considerably less gene flow in Colombia than leopards in Tanzania. In comparison to leopard dispersal in Tanzania, jaguar dispersal in Colombia is probably more limited due to the rugged Andean landscape.

Clearly, the *Fca 96, Fca 45* and *Fca 391* microsatellites and the total sample set showed significant differences between *P. o. centralis* and *P. o. onca*. There are no molecular studies intended to resolve the phylogenetic relationships among the eight subspecies of jaguar recognized by Pocock (1939), which are separated on the basis of morphology and pelage characters. Seymour (1989) groups these eight subspecies in three geographic clusters: a central American group composed of *P. o. goldmani*, *P. o. centralis*, *P. o. arizonensis*, *P. o. veraecrucis* and *P. o. hernandesii*, all of them under the designation of *P. onca centralis*; a northern South American group composed of *P. o. onca* and *P. o. peruviana* under the designation of *P. onca onca*, and a southern South America group represented by *P. o. paraguayensis*. The two subspecies considered here, and present in Colombia, form part of the Central American (*P. o. centralis*) and the northern South American (*P. o. onca*) groups. Nevertheless, the genetic heterogeneity we found is considered low (F_{ST} = 0.02), and is practically non-existent if the Bonferroni correction is applied without assuming random mating to calculate the log-likelihood G statistic, for these two subspecies. Therefore, the relatively small genetic heterogeneity found between these populations casts some doubt on the morphologically proposed subspecies separation.

Walker et al. (2000) found slightly higher values of genetic differentiation in the Texas pumas (global mean for 10 microsatellite loci, F_{ST} = 0.107) at a smaller geographic scale than that covered here. Other Neotropical cats have shown extremely high F_{ST} values, such as ocelots (0.602–0.748) and margays (0.622–0.634) (Eizirik et al., 1998). Thus, gene flow in other neotropical cats seems substantially less than in jaguars and/or the divergence times between populations are larger than those of jaguar populations studied here. Although we found evidence of significant genetic heterogeneity among the Colombian jaguar populations, this differentiation is limited by effects of gene flow or a recent divergence. The latter agrees quite well with the results obtained by Eizirik et al. (2001), who estimated that extant jaguar mtDNA lineages arose 280,000–510,000 years ago (95% confidence interval, 137,000–830,000 years ago). Additionally, with mtDNA haplotypes, they obtained a star-phylogeny, with short and compact branches, a pattern related to recent population divergence, (see Lavery et al. (1996)). Therefore, and considering our results, jaguars do not show strong geographic structure. Complementary to this, they found an ancestral widespread haplotype, evidencing extensive gene flow between the northern Amazon jaguar populations. They detected only two strong geographic barriers, which did not completely separate jaguar gene pools: the Amazon river and Darien straits. However, we have not found any differences between Colombian and Brazilian jaguars from the northern side of the Amazon river and Peruvian jaguars from the southern side of the Amazon river, but we suggest that the Andean mountain chains act as a barrier

separating the Colombian populations to a certain limited degree.

Thus far there have been no estimates of the size of the jaguar population in Colombia and the estimates presented here are the first long-term historical population estimates generated for this country. Population genetics theory predicts that genetic variability is correlated with effective population size (Kimura, 1983, 1986), and empirical observations have strongly supported this idea (Frankham, 1996). The maximum likelihood method of Nielsen (1997) based on coalescence, produced estimates of jaguar population ranging from 9,000 to 11,000, assuming 5.6×10^{-4} for the microsatellite mutation rate, and values ranging from 22,000 to 24,000 jaguars were produced when assuming 2.5×10^{-4} for the mutation rate. The long-term historical effective population size differences between the single-step and the multi-step mutation model were minimal. Thus, the real mutation model was not extremely important to estimate effective population sizes with the microsatellites employed. The global multi-step mutation percentage in the jaguar (6%) is very similar to the multi-step mutation percentage found in other felids investigated in our laboratory, such as the ocelot (8.4%), the puma (7.8%) and the Spanish wild cat (7.2%) for the same microsatellites (Ruiz-García et al., 2005c). Some microsatellites showed a general trend to follow a multi-step mutation model in all the felid species studied (*Fca 45*, *Fca96, Fca225* and *Fca 391*), meanwhile others were only multi-step in the jaguar. It is important to mention that each microsatellite has different mutation rates within each felid species studied and between the different species analyzed.

The bottleneck detection tests gave negative evidence for demographic reduction events in the total Colombian jaguar sample. The only case showing this trend was that detected for the *P. o. centralis* population using the standardized differences and the Wilcoxon tests. Therefore, this population seems in a more critical situation than the other Colombian jaguar population studied. A possible explanation for the overall result found has to do with the absence of a decrease in size in the last several generations, although commercial hunting and trapping of jaguars for their pelts during 1960s and mid-1970s was dramatically high. In the Peruvian Amazon alone about 2,000 jaguars were annually killed in the years 1968–1970 in the Loreto region. Furthermore, the United States of America imported 13,516 jaguar skins in 1968 and 9,831 in 1969. The majority of these skins came from Bolivia, Brazil, Colombia, México, Paraguay and Perú. A second possible explanation is that jaguars have recently undergone important demographic reductions but the population genetics procedures could not detect these events. The probable Wahlund (= subdivision) effect detected here implies that neither the Colombian jaguar population as a whole nor each of the two subspecies in this country represent the real minimum genetic population units and so the subdivision effect could counteract the detection of bottlenecks. The high levels of expected heterozygosity detected and the relatively high long-term historical effective population sizes are contrary to the expectation of this latter scenario. On the other hand, it is likely that the existence of gene flow between the two Colombian subspecies might mitigate the probability of bottleneck detection. Indeed, the allele distributions studied were highly continuous without the presence of predominant alleles, which agrees quite well with a historically stable population.

These types of molecular studies are very important in order to understand past events which could affect the present genetic variation in any population, and therefore its potential to respond to evolutionary changes (Frankham, 1996), such as changes related to disease (O'Brien, 1994). Knowledge of genetic structure and the gene diversity levels are "musts" when planning wildlife management and conservation. Jaguars exemplify typical carnivore ecology; large home range, low population density and threatened by hunting and habitat loss, thus presenting the ultimate challenge for conservation efforts. For this reason, genetic analysis at a finer geographic scale in different jaguar and other Neotropical wild felid populations is required to adequately preserve these amazing predators in the Latin American forests.

The authors strongly thank the Vicerectory of the Pontificia Universidad Javeriana for approving the grant (905) to the first author to study the molecular genetic structure of the six wild feline species in Colombia. Thanks also go to the Instituto Alexander von Humboldt (IAvH) at Villa de Leyva for providing access to its jaguar pelt collection. These acknowledgments are mainly directed to the curator of the mammal collection, mss. Yaneth Muñoz-Sabas and the directors of IAvH, Dr. Cristián Samper and Dr. Fernando Gast. We also thank Hugo Gálvez, Connie Stelle, Roberto Munevar, the Huitoto, Ticuna and Yaguas Indian communities in Colombia, the Movima, Moxeño, Sirionó, Canichana, Cayubaba and Chacobo Indian communities in Bolivia, the Ocaina, Shipigo-Comibo, Capanahua, Angoteros, Orejón, Cocana, Kishuarana and Alamas Indian communities in Perú and all the people and facilities that helped collect the biological material studied here. Thanks also go to Julieta Vargas from the Colección Boliviana de Fauna (La Paz, Bolivia). Joe Smith helped with the English.

REFERENCES

Cavalli-Sforza L. L., and Edwards, A. W. F. (1967). Phylogenetic analysis: models and estimation procedures. Evolution **21**, 550–570.

Chakraborty, R., and Jin, L. (1993). A unified approach to study hypervariable polymorphisms: Statistical considerations of determining relatedness and population distances. Pp 153–175. In DNA Fingerprinting: State of the Science (Ed. S. D. J. Pena, R. Chakraborty, J. T. Epplen, A. J. Jeffreys). Birkhauser, Basel.

Cornuet, J. M., and Luikart, G. (1996). Description of power analysis of two tests for detecting recent population bottle-

necks from allele frequency data. Genetics **144**, 2001–2014.

Cornuet, J. M., Piry, S., Luikart, G., Estoup, A., and Solignac, M. (1999). New methods employing multilocus genotypes to select or exclude populations as origins of individuals. Genetics **153**, 1989–2000.

Crawshaw, P. G. Jr., and Quigley, H. B. (1991). Jaguar spacing, activity and habitat use in a seasonally flooded environment in Brazil. Journal of the Zoological Society of London **223**, 357–370.

Crow, J. F., and Aoki, K. (1984). Group selection for a polygenic behavioural trait: Estimating the degree of population subdivision. Proceedings of National Academy of Sciences of United States of America **81**, 6073–6077.

Culver, M., Johnson, W. E., Pecon-Slattery, J., and O'Brien, S. J. (2000). Genomic ancestry of the American puma *(Puma concolor)*. The Journal of Heredity **91**, 186–197.

Eizirik, E., Bonatto, S. L., Johnson, W. E., ET AL. (1998). Phylogeographic patterns and mitochondrial DNA control region evolution in two Neotropical cats (Mammalia, Felidae). Journal of Molecular Evolution **47**, 613–624.

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

Frankham, R. (1996). Relationship of genetic variation to population size in wildlife. Conservation Biology **10**, 1500–1508.

Garza, J. C., and Williamson, E. G. (2001). Detection of reduction in population size using data from Microsatellite loci. Molecular Ecology **10**, 305–318.

Goldstein, D. B., Ruiz-Linares, A., Cavalli-Sforza, L. L., and Feldman, M. W. (1995). An evaluation for genetic distances for use with Microsatellite loci. Genetics **139**, 463–471.

Gotelli, D., Sillero-Zubiri, C., Applebaum, G. D., Roy, M. S., Girman, D. J., Garcia-Moreno, J., ET AL. (1994). Molecular genetics of the most endangered canid-the Ethiopian wolf *Canis simenis*. Molecular Ecology **3**, 301–312.

Goudet, J., Raymond, M., Demeeus, T., and Rouset, F. (1996). Testing differentiation in diploid populations. Genetics **144**, 1933–1940.

IUCN (2004). IUCN Red List of Threatened Species. <www.iucnredlist.org>. Downloaded on 02 January 2006.

Kimura, M. (1983). The neutral theory of molecular evolution. Cambridge University Press, Cambridge.

Kimura, M. (1986). DNA and the neutral theory. Philosophical Transactions of the Royal Society of London, series B **312**, 343–354.

Lavery, S., Moritz, C., and Fielder, D. R. (1996). Genetic patterns suggest exponential population growth in a declining species. Molecular Biology and Evolution **13**, 1106–1113.

Luikart, G., Allendorf, F., Sherwin, B., and Cornuet, J. M. (1998). Distortion of allele frequency distribution provides a test for recent population bottleneck. Journal of Heredity **86**, 319–322.

Luikart, G. and Cornuet, J. M. (1998). Empirical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. Conservation Biology **12**, 228–237.

Menotti-Raymond, M. A., David, V. A., Lyons, L. A., Sch‰ffer, A. A., Tomlin, J. F., Hutton, M. K., and O¥Brien, S. J. (1999). A genetic linkage map of microsatellites in the domestic cat (*Felis catus*). Genomics **57**, 9–23.

Menotti-Raymond, M. A., and O'Brien, S. J. (1995). Evolutionary conservation of ten Microsatellite loci in four species of Felidae. Journal of Heredity **86**, 319–322.

Michalakis, Y., and Excoffier, L. (1996). A genetic estimation of population subdivision using distances between alleles with special reference to Microsatellite loci. Genetics **142**, 1061–1064.

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

Nelson, E. W., and Goldman, E. A. (1933). Revision of the jaguars. Journal of Mammalogy **14**, 221–240.

Nielsen, R. (1997). A likelihood approach to populations samples of Microsatellite alleles. Genetics **146**, 711–716.

Nowell, K., and Jackson, P. (1996). Wild Cats, Status survey and conservation action plan. IUCN/SSC Cat Specialist Group. IUCN Gland, Switzerland.

O'Brien, S. J. (1994). A role for molecular genetics in biological conservation. Proceedings of the National Academy of Sciences of the United States of America **91**, 5748–5755.

Paetkau, D., Calvert, W., Stirling, I., and Strobeck, C. (1995). Microsatellite analysis of population structure in Canadian polar bears. Molecular Ecology **4**, 347–354.

Piry, S., Luikart, G., and Cornuet, J. M. (1999). BOTTLENECK: A computer program for detecting recent reductions in the effective population size using allele frequency data. Journal of Heredity **90**, 502–503.

Pocock, R. I. (1939). The races of the jaguar, *Panthera onca*. Novitates Zoology **41**, 406–422.

Rannala, B., and Mountain, J. L. (1997). Detecting immigration by using multilocus genotypes. Proceedings of the National Academy of Sciences USA **94**, 9197–9201.

Raymond, M., and Rousset, F. (1995). GENEPOP version 3.1 Population genetics software for exact tests and ecumenicism. Journal Heredity **86**, 248–249.

Rooney, A. P., Honeycutt, R. L., Davis, S. K., and Derr, J. N. (1999). Evaluating a putative bottleneck in a population of bowhead whales from patterns of microsatellite diversity and genetic disequilibria. Journal of Molecular Evolution **49**, 682–690.

Ruiz-García, M. (1993). Analysis of the evolution and genetic diversity within and between Balearic and Iberian cat populations. Journal of Heredity **84**, 173–180.

Ruiz-García, M. (1998). Genetic structure of different cat populations (*Felis catus*) in Spain, Italy and Argentina at a microgeographical level. Acta Theriologica **43**, 39–64.

Ruiz-García, M. (2001). Diversidad genética como herramienta de zonificación ambiental: Estudios moleculares (microsatélites) en el caso de Primates y Félidos neotropicales comportan una nueva perspectiva. In: Zonificacion ambiental para el ordenamiento territorial en la Amazonia Colombiana (eds. T. Defler and P. A. Palacios), Instituto Amazónico de Investigaciones, pp. 84–110.

Ruiz-García, M. (2003). Molecular population genetic analysis of the spectacled bear (*Tremarctos ornatus*) in the Northern Andean Area. Hereditas **138**, 81–93.

Ruiz-García, M., and Alvarez, D. (2000). Genetic microstructure in two Spanish cat populations I: Genic diversity, gene flow and selection. Genes and Genetic Systems **75**, 269–280.

Ruiz-García, M., Orozco-TerWengel, P., Castellanos, A., Arias, L. (2005a). Microsatellite analysis of the Andean bear (*Tremarctos ornatus*) across its range distribution. Genes and Genetic Systems **80**, 57–69.

Ruiz-García, M., Corrales, C., Payán, E.,and Castellanos, A. 2005b. Hierarchical genetic structure of *Leopardus pardalis* in Colombia, Ecuador, Perú, Bolivia and Brazil: molecular gene diversity and geographical patterns. Molecular Ecology (submitted).

Ruiz-García, M., García-Perea, R., Corrales, C., Murillo, A., and Alvarez, D. (2005c). Determination of DNA microsatellite mutation rates per generation and mutation models in four main Felidae lineages (European wild cat, *F. silvestris*; ocelot, *Leopardus pardalis*; puma, *Puma concolor;* jaguar, *Panthera onca*). Molecular Biology and Evolution (submitted).

Schaller, G. B., and Crawshaw, P. G., Jr. (1980). Movement patterns of jaguar. Biotropica **12**, 161–168.

Seymour, K. L. (1989). *Panthera onca*. Mammalian Species **340**, 1–9.

Spong, G., Johansson, M., and Björklund, M. (2000). High genetic variation in leopards indicates large and long-term stable population size. Molecular Ecology **9**, 1773–1782.

Takahata, N. (1983). Gene identity and genetic differentiation of populations in the finite island model. Genetics **104**, 497–512.

Walker, C. W., Harveson, L. A., Pittman, M. T., Tewes, M. E., and Honeycutt, R. L. (2000). Microsatellite variation in two populations of mountain lions (*Puma concolor*) in Texas. The Southwestern Naturalist **45**, 196–203.

Walsh, P. S., Metzger, D. A., and Higuchi, R. (1991). Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques **10**, 506–513.

Weber, J. L., and Wong, C. (1993). Mutation of human short tandem repeats. Human Molecular Genetics **2**, 1123–1128.

Weir, B. S., and Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population structure. Evolution **38**, 1358–1370.

Wright, S. (1951). The genetical structure of populations. Ann. Eugen. **15,** 323–354.