Genetic variability and structure of jaguar (*Panthera onca*) in Mexican zoos

Pilar Rueda Zozaya^a, Germán D. Mendoza-Martínez^a, Daniel Martínez-Gómez^{b*}, Octavio Monroy-Vilchis^c, José Antonio Godoy^d, Armando Sunny^c, Francisco Palomares^d, Cuauhtémoc Chávez^e, José Herrera-Haro^f,

^aLaboratorio de Ensayos Metabólicos, Universidad Autónoma Metropolitana-Unidad Xochimilco, Calz. del Hueso 3100, Distrito Federal, 04960, México.

^bLaboratorio de Microbiología Agropecuaria, Universidad Autónoma Metropolitana-Unidad Xochimilco, Calz. del Hueso 3100, Distrito Federal, 04960, México.

^cCentro de Investigación en Ciencias Biológicas Aplicadas, Facultad de Ciencias, Universidad Autónoma del Estado de México, Instituto Literario 100, Colonia Centro, Toluca, Estado de México, 50000, México.

^dEstación Biológica de Doñana, CSIC. c/ Américo Vespucio s/n, 41092 Isla de la Cartuja, Sevilla, España.

^eDepartamento de Ciencias Ambientales, Universidad Autónoma Metropolitana-Unidad Lerma, Hidalgo Pte. 46, Col. La Estación, Lerma, Estado de México, 52006, México.

^fPrograma de Ganadería, Colegio de Postgraduados-Campus Montecillo, Carretera México-Texcoco km 36.5, Montecillo, Texcoco, 56230, México.

Corresponding author: Daniel Martínez Gómez, Tel.: +521 54837000 ext. 3060; e-mail address: dmartin@correo.xoc.uam.mx

Running title: population genetics of jaguar in Mexican zoos.

Abstract

Genealogical records of animals (studbook) are created to avoid reproduction between closely related individuals, which could cause inbreeding, particularly for such endangered species as the *Panthera onca* (Linnaeus, 1758). Jaguar is the largest felid in the Americas and is considered an important ecological key species. In Mexico, wild jaguar populations have been significantly reduced in recent decades, and population decline typically accompany decreases in genetic variation. There is no current census of captive jaguars in Mexico, and zoos do not follow a standardized protocol in breeding programs based on genetic studies. Here, we emphasise the importance of maintaining an adequate level of genetic variation and propose the implementation of standardised studbooks for jaguars in Mexico, mainly to avoid inbreeding. In addition, achieving the aims of studbook registration would provide a population genetic characterisation that could serve as a basis for *ex situ* conservation programmes.

Key words: *Panthera onca*, studbook, genetic variability, microsatellite, population genetics.

Introduction

The jaguar (*Panthera onca*), the largest felid in the Americas, is considered an important and emblematic species for many pre-Columbian cultures (Campos 2002). As most large carnivores, this felid is currently cited in the Red List (IUCN 2013) as Near Threatened (Caso et al. 2008), in CITES Appendix I (2015) and as Endangered in Mexican laws (SEMARNAT 2010). It is estimated that there are approximately 4000 jaguars in the wild throughout the country (Fig. 1), approximately 70 % less than in the 1960s (Chávez et al. 2009; Ceballos et al. 2011). The decline in jaguar population is mainly due to habitat loss and fragmentation, and fur trade is still a threat to wild populations (Quigley and Crawshaw 1992; Eizirik et al. 2001; Silver et al. 2004; O' Brien and Johnson 2005; Ruiz-García et al. 2006; Eizirik et al. 2008; Ruiz-García 2013; Roques et al. 2014); it is estimated that only 16 % of the current territory in Mexico is suitable for the increasingly diminished and isolated jaguar populations (Rodríguez-Soto et al. 2011).

Genetic diversity is progressively lost by the action of drift in small populations, and this effect is further intensified by the reduction of gene flow when these populations become effectively isolated. (Frankham 1995; Hedrick and Kalinowski 2000; Keller and Waller 2002; Martínez-Cruz et al. 2004), and this can seriously compromise the survival of a species already at risk of extinction (Hedrick 2001). Previous characterization of genetic variation in jaguar populations revealed moderate to high global genetic diversity in the wild, with some variation among populations and studies (Table 1). Most recent studies have reported a reduced genetic diversity at the periphery of the species range as a consequence of isolation by distance and recent isolation due to fragmentation (Haag et al. 2010; Roques et al. 2014). Mexican jaguar populations in particular show a reduced genetic diversity and a high differentiation to those in Brazil (Roques et al. 2015).

The role of zoos in recent decades as conservation centers has been key to the successful development of conservation programmes for several endangered species: Speke's gazelle (*Gazella spekei*), California condor (*Gymnogyps californianus*), bighorn sheep (*Ovis Canadensis*), Florida panther (*Puma concolor coryi*), European bison (*Bison bonasus*), Mexican grey wolf (*Canis lupus baileyi*) are noticeable examples (Willis and Wiese 1997; Templeton and Read 1998; Meretsky et al. 2000; Whittaker et al. 2004; Hedrick 1994; Olech and Perzanowski 2002; Fredrickson and Hedrick 2002; Fredrickson et al. 2007). Captive populations of endangered species can be used as a genetic reservoir and as a source of individuals for reintroductions and for the demographic and genetic reinforcement of extant populations (Frankham 2015). However, captive populations often begins with a small number of individuals distributed in several breeding centers, partly due to spatial constraints (Laikre 1999; Boakes et al. 2007), and if left unmanaged genetic drift and non-random matings results in a rapid loss of genetic diversity and accumulation of inbreeding (Jiménez et al. 1994; Laikre 1999; Keller and Waller 2002; Charlesworth and Willis 2009), with likely reduction of fitness and adaptive potential that can seriously limit their conservation value

(Frankham 2015). The erosion of genetic diversity in captive population can however be minimized with the implementation of proper genetic management program that establish the breeding priority and the optimal breeding schemes within and among centers. Such a program is often based on the minimization of average kinship and thus requires exhaustive and reliable genealogical records and/or molecular marker data for kinship estimation.

The genetic status of captive jaguars is mostly unknown. Moreno et al. (2006) assessed genetic variability of jaguars in Brazilian zoos and reported moderate to high levels of polymorphism (suited genetic variability in this species), but genetic data on Mexican captive jaguars is currently lacking. Some Mexican zoos do not exhaustively record births, deaths, or translocations and do not carry a standardized studbook, this difficult an appropriate genetic management in these populations (Ralls et al. 1988; Laikre 1999). Considering these circumstances, we carried out a research in order to: 1) To evaluate some parameters of genetic variation on 56 captive jaguars, 2) To assign individuals to genetic clusters approximating the breeding processes carried out in zoos, and 3) To establish a baseline information useful to implement measures that unify and standardize a prospective studbook in Mexico based on genetic and genealogical information of jaguars.

Materials and methods

Sampling

Fourteen Mexican zoos provided fresh blood samples of 56 jaguars with unknown origin and practically no information on their pedigree. The handling of all individuals was carried out according to the protocols established in each zoo, based on NOM-126-SEMARNAT-2000 and collecting permit No. SGPA/DGVS/01685/11. Blood was collected in tubes with EDTA and stored at 4 °C until processing.

Molecular methods

Total genomic DNA was extracted from blood samples according to the protocol of Sambrook and Russell (2001). DNA samples were visualized by agarose gel electrophoresis. A set of 11 labeled microsatellite markers developed by Menotti-Raymond et al. 1999 and optimized by Roques et al. (2014) was used for individual genotyping (Table 2). PCR conditions were: a first denaturation step at 95 °C for 5 minutes, followed by 40 cycles of denaturation at 95 °C, lasting 30 seconds, annealing at 57-60 °C, lasting 90 seconds and extension at 72 °C, lasting 30 seconds, and a final extension step of 30 minutes at 72 °C. PCR reactions consisted of 3 µl of DNA extract (15 ng/µl) in a final volume of 20 µl containing Type-it Multiplex PCR Master Mix 1X (QIAGEN®), BSA 0.01 %, and 0.2 µM of each primer (Sigma-Aldrich®) and 0.4 U of Taq polymerase (Bioline®). Allele sizes were determined using the ABI 3130xl Genetic Analyzer System (Applied Biosystems), based on the size standard GS-600 LIZ (Life Technologies Inc.) using GENEMAPPER software v4.0 (Applied Biosystems®) and manually checked to assure reproducibility and correct misreading.

Genotyping

After assigning consensus genotypes to jaguar individuals we tested for genotyping errors with the software MICROCHECKER 2.2.3 (Van Oosterhout et al. 2004), with a 95 % confidence interval and 1,000 repetitions; this program can help identify null alleles and accordingly adjust genotype frequencies. FCA115, FCA547 and FCA566 showed a heterozygote deficit consistent with the presence of null alleles. Genotypes were also evaluated with the software FREENA (Chapuis and Estoup 2007), a computer program that allows several statistical treatments on microsatellite datasets with null alleles. Based on INA-corrected allele frequencies we checked whether there are significant differences between values of F_{st} due to the presence of null alleles (Chapuis and Estoup 2007), using a t-

test implemented in GRAPHPAD QUICK CALCS to assess the statistical significance of differences.

Genetic structure

With STRUCTURE 2.3.4 (Pritchard et al. 2000; Falush et al. 2003; Hubisz et al. 2009) we assessed a Bayesian cluster analysis for inferring the probability of individual assignment to a varying number of distinct populations. The procedure uses a Markov Chain Monte Carlo (MCMC) approach to estimate the data fit to each range of potential K clusters. The simulations was performed using 1,000,000 burn in periods and 1,000,000 MCMC iterations, with correlated allele frequencies and an admixture model without prior information on population origin. We selected the most likely number of clusters based on the maximum value of ΔK , following the Evanno method as implemented in STRUCTURE HARVESTER 0.6.92 (Earl and von Holdt 2012). We calculated the distribution of the genetic variance between and within clusters and individuals using an analysis of molecular variance (AMOVA) based on F_{st} as implemented by GENALEX 6.5. (Peakall and Smouse 2006). A phylogenetic tree was constructed from the genetic distances in accordance with the stepwise mutation model (SMM): Nei's genetic distance (Nei 1972) with all individuals using POPULATIONS 1.2.30 (Langella 2002). These distances were used to construct a NJ tree to cluster individuals by genetic similarity with FIGTREE 1.4.2. (Rambaut and Drummond 2010). Finally in GENETIC STUDIO (Dyer 2009) we estimated Nei's genetic distance (D_{Nei}) between clusters.

Genetic variability

Allelic frequencies were used to calculate some genetic parameters including the mean number of alleles per locus (A), the mean number of effective alleles per locus (A_e), observed and expected heterozygosities (H_o , H_e), and the allelic fixation index (F_{st}), using GENALEX 6.5. The data set were evaluated to detect deviations from the Hardy-Weinberg Equilibrium

(HWE) in the global captive population and in each specific population with ARLEQUIN 3.5.1.2 (Excoffier and Lischer 2010) and GENEPOP (Raymond and Rousset 1995) using 100,000 Markov chain steps and 100,000 dememorisation steps. The significant values inferred to LD or HWE deviations were corrected for multiple comparisons with a False Discovery Rate (FDR) approach according to Benjamini and Hochberg (1995) as implemented by the QVALUE software (Storey 2002) for R (version 3.0.1; R Development Core Team, 2013).

Effective population size, genetic bottlenecks, and relatedness

Effective population size was estimated with the software LDNE (Waples 2006; Waples and Do 2008), based on the linkage disequilibrium method (Hill 1981). We used the software BOTTLENECK 5.1.26 (Cornuet and Luikart 1996; Piry et al. 1999) to test for a trace of genetic bottlenecks events. We estimated the observed and expected heterozygosity under the infinite allele model (IAM), SMM and the two-phase model (TPM), with settings at 90 % SMM, 10 % IAM, and 10 % variance and default values (70 % SMM, 30 % IAM, and 10 % variance). Both settings were calculated with 10,000 replicates and excess of heterozygosity was tested with Wilcoxon test. We evaluated relatedness among individuals with the program ML-RELATE (Kalinowski et al. 2006), which takes into account null alleles and is based on maximum likelihood tests. Also we presented a summary of the of relationships assigned with the highest likelihood, consistent with the genetic data at the 0.05 level of significance; a graphical comparison of the number of pairs of individuals probably related (ML RELATE) and the number of pairs of individuals having a relationship according to the information provided by the veterinarians of each zoo.

Results

Population sampling and potential scoring errors

Genotypes from fifty-six blood samples from Mexican zoos were obtained by microsatellite analysis with 11 loci (Roques et al. 2014). The analysis of genotypes with MICRO-CHECKER showed three loci with potential null alleles: FC115, FC547 and FC566. However, since the adjusted F_{st} values did not significantly differ from the unadjusted values ($F_{st-INA} = 0.096$, $F_{st} = 0.095$; $t = 0.015$, $P = 0.99$, $gl = 20$) we kept all loci for further analyses.

Genetic structure

Three genetic clusters were obtained with STRUCTURE software ($\text{LnPr}(k = 3) = -1716.35$ Fig. 2), according to the Evanno method (based on the maximum likelihood of k clusters; Fig. A1). At least 85 % of jaguars were assigned with a probability of 95 % to the clusters obtained (Table 3), several individuals were assigned partially to one, two or all three clusters (Figure 2). Results of AMOVA showed that most of the genetic variation resided within individuals (76 %; $P = 0.001$), then between individuals within clusters (16 %; $P = 0.001$) and finally between clusters (8 %; $P = 0.001$, Table 4). The F_{st} and Nei's genetic distances were slightly moderate among Clusters, moderate between Cluster 1 to Cluster 2 ($F_{st} = 0.051$ Nei's = 0.2142), moderate between Cluster 1 to Cluster 3 ($F_{st} = 0.113$ Nei's = 0.4857), and high between Cluster 2 to Cluster 3 ($F_{st} = 0.151$ Nei's = 0.5425). In agreement with AMOVA, the NJ tree showed the Clusters 1 and 2 are more similar between them, instead the Cluster 3 is the most different from all (Fig. A2). Also the results of the NJ tree and STRUCTURE showed signs of admixture (Fig. A2 and Fig. 2).

Genetic variability

After FDR correction, no significant overall linkage disequilibrium was detected in any Cluster, so we considered the eleven loci as independent markers. We measured H_o , H_e , A_e , Shannon's Information Index (I) and F_{st} values per locus for each of the three inferred clusters and for the overall captive jaguar population (CJP), see Table 4. In the genetic Cluster

1, the following values were obtained: A ($\bar{x} = 7$), Ae ($\bar{x} = 4.4$), Ho and He were the same ($\bar{x} = 0.72$) and, Fst ($\bar{x} = -0.01$); Cluster 2: A ($\bar{x} = 4.4$), Ae ($\bar{x} = 3.13$), Ho ($\bar{x} = 0.53$) He ($\bar{x} = 0.62$) and, Fst ($\bar{x} = 0.12$) and Cluster 3: A ($\bar{x} = 3.63$), Ae ($\bar{x} = 2.8$), Ho ($\bar{x} = 0.7$) He ($\bar{x} = 0.59$) and, Fst ($\bar{x} = -0.2$).

In summary, levels of genetic diversity were moderate to high (0.53-0.72) with an overall mean of 0.65 (Table 4). Ho was higher in genetic Cluster 1 (0.72), but considering that genetic Cluster 3 contained only eight individuals, it would be proportionately higher (0.71), while the Cluster 2 has a moderate heterozygosity (0.53). In addition, the values of the inbreeding coefficient were low to moderate: in the captive jaguar population CJP (Fis; from -0.12 to 0.21), the Cluster 1 (Fis; from -0.12 to 0.14), the Cluster 2 (Fis; from -0.13 to 0.35), and Cluster 3 (Fis; from -0.49 to 0.25; Table A2).

Effective population size, genetic bottlenecks, and relatedness

The effective population size was estimated in 17.3 (95% CI: 13.4-22.7), with a harmonic mean sample size of 51.9 (Table 5). Evidence of a recent genetic bottleneck associated with a heterozygote excess (BOTTLENECK results) was observed for all captive jaguar population CJP with a variance of 30 % and a probability of 70 %, under the IAM and TPM models ($P = 0$), also with a variance of 10 % and a probability of 90 %, under the IAM model ($P = 0$). When analyzed independently each cluster the same phenomenon was observed, all Clusters showed possible bottlenecks under the two models used for this analysis (Table A3).

The relatedness analysis based on the maximum likelihood (ML) estimator, according to the amount of shared alleles, in Cluster 1 revealed 8 full siblings (FS), 44 half siblings (HS) and 9 parent/offspring (PO) relationships; we also evaluated the differences between the Ln Likelihood (LnL) for the other relationships categories (Table 7). There were significant differences between FS and PO comparisons, however in HS relationships there were no

differences compared with unrelated (U) and FS. In Cluster 2 revealed 7 FS, 22 HS and 7 PO relationships; there were significant differences between all the relationships categories compared but in FS with PO. In Cluster 3 revealed 6 FS, 17 HS and 5 PO relationships assigned by ML; in this cluster all comparisons with other categories of relationships categories had significant differences (Table 7).

Discussion

Genetic structure and genetic variability

Although captive jaguars are not a natural population, genetic analysis could be useful for conservation on this endangered species. The analysis of fifty-six blood samples from captive animals showed three genetic clusters based on the Δk estimated by STRUCTURE (Fig. 2). Cluster 1 (red) with 29 individuals, Cluster 2 (green) with 19 individuals and Cluster 3 (blue) only 8 individuals. This software infers population structure, assigning individuals to the closest population, identifying migrants and admixed individuals. We start from the premise that captive jaguar population have some degree of structure because many animals from highly differentiated populations have been reproduced each other (Brazilian, Venezuelans with individuals from Mexican origin), as mentioned by zookeepers. More than 85% of the jaguars were assigned to one of three clusters ($P = 95\%$), in several cases it was observed that some individuals were partially assigned to more than one cluster (Table 3), probably these characteristics of variable proportions of membership to the clusters within individuals was due to the mate of jaguars from very different populations, a common situation in the sampled animals.

AMOVA results revealed that there was moderate genetic differentiation between the three clusters formed according to allelic frequencies; it may result from different founder individuals (presumably from differentiated populations), in turn this difference is probably to

267 be a result of the diverse geographical origin of jaguars and the effects of different selective
268 breeding. We attribute these results to the variation within individuals (76%) and are
269 concordant with the NJ tree: Clusters 1 and 2 were more similar instead cluster 3 was the
270 most distant. This assignment probably was originated from the selective breeding, chiefly
271 South American individuals mated with Mexicans, for example reproduction of melanistic
272 jaguars have been increased because they are very attractive to the visitors (pers. obs); in
273 Mexico there is no evidence of black jaguars in wild, the northernmost record being from
274 Costa Rica (Meyer 1994; Eizirik et al. 2003; Cartín-Núñez and Carrillo 2009), and few
275 sightings are reported in Belize without further solid evidence. Both F_{st} analysis and
276 AMOVA showed significant genetic differentiation among clusters, although the F_{st} value
277 between Cluster 1 and 2 was small, it is statistically significant (0.051). The values of Nei's
278 distances were also significant to consider the clusters with a degree of differentiation.

279 Despite limitations in space and number of individuals, in this research we found moderate to
280 high levels of heterozygosity (0.53 to 0.72; Table 5). Because this captive population is
281 probably formed by individuals of several origins, levels of heterozygosity could be raised as
282 a result of the mixture of individuals from populations with some degree of differentiation
283 (Luo et al. 2008). The highest record of heterozygosity in jaguars reported by Ruiz-García et
284 al. 2006 for Colombian jaguars ($H = 0.84$), is perhaps derived from the population subdivision
285 that they assessed (Boecklen 1986; Swindell and Bouzat 2006). Other research about genetic
286 diversity in jaguar was developed by Haag et al. 2010, the average $H_o = 0.73$ was high,
287 nonetheless they reported allelic loss, differentiation between populations isolated and low
288 effective sizes, this processes possibly caused by recent genetic drift. In other large felids
289 genetic diversity has been reported low levels, cheetah (*Acinonyx jubatus*) $H_e = 0.64$ to 0.70
290 in Namibia (Marker et al. 2008); Luo et al. (2004) obtained a mean observed heterozygosity
291 in several samples of tiger subspecies (*P. tigris*) ranged from 0.40 (H_o minimum in (*P. t.*

altaica) to 0.66 (H_o maximum in *P. t. corbetti*). In contrast, Indian leopard (*P. pardus*) showed high levels of $H_o = 0.74$ (Dutta et al. 2013).

Effective population size, genetic bottleneck and relatedness

There is no a regular update of the census of jaguars in Mexican zoos, the most recent data is from 2003, published in the workshop "*El papel de los zoológicos de Mesoamérica y el Caribe en la conservación de los jaguares*" (The role of zoos in Mesoamerica and the Caribbean in the conservation of jaguars) developed by the Conservation Breeding Specialist Group (CBSG). They estimated 135 jaguars (62 males, 67 females and 7 unknown), however, it has not been updated a census to verify whether this number has increased or decreased. In several zoos where we collected blood samples, veterinarians mentioned that they try to avoid the breeding between close-related individuals, however, in the absence of an updated and standardized jaguar studbook, consanguineous matings cannot be completely avoided and an effective genetic management cannot be implemented, what will adversely affect genetic variation (Witzenberger and Hochkirch 2011). On the contrary, breeding programs that are genetically managed based on mean kinship, retained genetic diversity and delayed the effects of inbreeding, even when there is scarce information about the pedigree of individuals, these programs can still be implemented by using molecular marker data to estimate kinship among individuals. The data generated by this study provides thus a starting point for the implementation of genetic management program of captive jaguars in Mexico, since in more than half of the relationships assigned, it was verified that there was a high probability of consistency with the information provided by zoo keepers. Also, it could be detected that some families are formed due to the limited interchange that has occurred in four zoos having proximity (data not shown). Accordingly, this may also be reflected in the small effective population size obtained ($N_e = 17$) considering there have been no reproductions guided in a program to maintain genetic variation, for example, random mating that includes the greatest

number of jaguars (Table 6). In the three clusters a recent genetic bottleneck was detected (Table A3), it is possible that this result indicated a restricted reproduction between few individuals (founder members) and furthermore, the captive jaguar population has recently been formed (it is estimated that in the 1980's). To avoid the loss of genetic diversity caused by of the lack of accurate planning in the genetic management of captive jaguar populations, this research would contribute to be the starting point for further establishment of appropriate genetic management for Mexican zoos.

Although we had a moderate number of loci, the relatedness analysis was mostly consistent with the other results. Cluster 1 showed the largest number of possible relationships (Total = 61), of which 2 were known (1 parent/offspring and 1 full siblings). In Cluster 3 it was found only one parent/offspring relationship, however this two melanistic jaguars formed one of the confirmed relationships. The relationship with the largest possibilities in the three clusters was HS (Table 7), it is expected to be ambiguous because of the challenge of calculating and assigning individuals that share only 25 % of the genetic material and may actually be categorized in other relationships such as cousins, uncles/nephews, grandparents/grandchildren. Hence, we emphasize the necessity for further analysis of parentage based on genetic information, preferably using a high number of molecular markers to improve the precision of the estimates. In three of the four known relationships we observed consistency on the assignment of the individuals into the same cluster, one of full siblings (Figure 2, see bars 14 and 38) and two of parent/offspring (Figure 2, see bars 15 and 17; 16 and 22). The fourth relationship of parent/offspring was not very clear (bars 56 and 39 of the Figure 2), there is the possibility that parents and offspring were assigned in different clusters, the most likely cause that we consider is because these parents have different origins, and each parent could be assigned to a different cluster meanwhile offspring are assigned to the proportion inherited from each parent. In general, it is likely that our results show a medium level of relatedness among individuals; which suggests that litters have been

distributed in zoos that have maintained a limited exchange of individuals. Following the protocol of reproduction based on the mean kinship (MK) as suggested by Willoughby et al. (2015) is strongly recommended to avoid the loss of genetic variation, if there is the purpose to establish a studbook for captive jaguars, it is important to have an intensive relatedness analysis prior to start a breeding program.

Conclusions

Captive breeding has become an important tool in animal conservation, but the lack of a well-executed management program aimed at minimizing the impact of drift and inbreeding can reduce their usefulness for species conservation. Wild Mexican jaguars have declined and show signs of genetic erosion, a situation that could eventually demand supplementation from a healthy captive genetic stock, there was no information about genetic status of captive jaguars, and our research is the first to provide basic information on genetic variation of captive jaguars in Mexico. They need to be genetically managed based on genealogical and molecular information to guarantee the appropriate management to retain genetic diversity for jaguar conservation programmes. This paper is the first in genetically evaluate captive populations of jaguars in Mexico and provides the basis to designing breeding programs that conserve the genetic diversity of these populations.

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