Title: Low genetic diversity and limited genetic structure across the range of the critically endangered Mexican howler monkey (*Alouatta palliata mexicana*).

Short running title: Mexican howler monkey genetic diversity

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Abstract

Genetic diversity provides populations with the possibility to persist in ever-changing environments, where selective regimes change over time. Therefore, the long-term survival of a population may be affected by its level of genetic diversity. The Mexican howler monkey (*Alouatta palliata mexicana*) is a critically endangered primate restricted to southeast Mexico. Here, we evaluate the genetic diversity and population structure of this subspecies based on 83 individuals from 31 groups sampled across the distribution range of the subspecies, using 29 microsatellite loci. Our results revealed extremely low genetic diversity (H_O = 0.21, H_E = 0.29) compared to studies of other *A. palliata* populations and to other *Alouatta* species. Principal component analysis, a Bayesian clustering method, and analyses of molecular variance did not detect strong signatures of genetic differentiation among geographic populations of this subspecies. Although we detect small but significant F_{ST} values between populations, they can be explained by a pattern of isolation by distance. These results and the presence of unique alleles in different populations highlight the importance of implementing conservation efforts in multiple populations across the distribution range of *A. p. mexicana* in order to preserve its already low genetic diversity. This is especially important given current levels of population isolation due to the extreme habitat fragmentation across the distribution range of this primate.

Key words: Conservation genetics, isolation by distance, microsatellites, neotropical primate, population structure.

Introduction

The environment is under constant change, some of which is due to natural processes while other changes are induced by anthropogenic activities. In changing environments, the selective pressures that allow some individuals to gain reproductive advantage may quickly shift, thus genetic diversity provides the foundation for populations to adequately respond to these changes and persist over time. Mutations and gene flow are sources of genetic diversity, while selective forces and genetic drift interplay to increase, decrease or maintain this diversity (Frankham, Briscoe, & Ballou, 2002). Specifically, genetic drift leads to the random rearrangement of allele frequencies from generation to generation, with a high probability of losing low-frequency alleles, particularly when populations are small in size (Allendorf, 1986). As populations lose genetic diversity, they become more susceptible to new diseases, predators, and competitors, and the probability of consanguineal mating (i.e., inbreeding) increases along with recessive diseases and other disorders related to high levels of homozygosity (Reed, & Frankham 2003). Assessing the genetic diversity of populations is thus a key component of developing appropriate conservation strategies. The distribution of the total genetic diversity within a species is not always homogeneous, but instead it may be differentially distributed among populations, creating some genetic structure across populations. Evaluating population genetic structure can inform whether groups are isolated and therefore subject to stronger genetic drift effects or are part of an interconnected metapopulation (Levins, 1969). Genetic diversity and genetic structure are closely linked parameters that have been used to inform genetic management in many taxa (e.g., Dalton, Charruaua, Boast, & Kotze, 2013; Sasaki, Hammer, Unmack, Adams, & Beheregaray, 2016)

including primates (Brown et al., 2014; Liu et al., 2015; Moraes et al., 2018). Low levels of genetic diversity may indicate that a given species is susceptible to environmental changes and its survival is at risk. On the other hand, signatures of population structure can help determine genetically distinct populations that should be targeted for conservation interventions in order to maintain the overall genetic diversity of the species (Wang, Qiao, Li, Pan, & Yao, 2017).

The mantled howler monkey (*Alouatta palliata*) is distributed from the Tumbes region in northern Peru through the Pacific coast of northern South America and into Central America to the Honduras-Guatemala border, with a disjunct population in southeast Mexico that represents a distinct subspecies, the Mexican howler monkey (*A. p. mexicana*) (Rylands, Groves, Mittermeier, Cortés-Ortiz, & Hines, 2006; Cortés-Ortiz, Rylands, & Mittermeier, 2015). This subspecies is restricted to southern Veracruz, northern Oaxaca, western Tabasco and northern Chiapas, and is considered critically endangered by the IUCN (Cuarón, Shedden, Rodríguez-Luna, de Grammont, & Link, 2008). The current distribution of *A. p. mexicana* was likely shaped by climatic and topographic variability that occurred after the closure of the Panama Isthmus, during the colonization of the species from South America into Central America and southern Mexico (Cortés-Ortiz et al., 2003). It has been proposed that there were at least four dispersal waves of primates from South America into Central America, from which the ancestor of the Mexican howler monkey might have taken part in the latest, approximately 1.6 MYA (Ford, 2006). These expansion events likely reduced the genetic diversity of the subspecies as small numbers of individuals moved northwards and populations experienced founder effect and bottlenecks.

Since the late 1960s, the forest habitat of the Mexican howler monkey has undergone severe transformations as a result of intensive agriculture and cattle ranching (Dirzo, & García, 1992; Trejo, & Dirzo, 2000; Guevara, Sánchez-Ríos, & Landgrave, 2006; Kolp, & Galicia, 2018). Forest cover has been reduced and fragmented threatening the long-term survival of this primate (Arroyo-Rodríguez, Mandujano, & Benítez-Malvido, 2008; Mandujano & Escobedo-Morales 2008; Hernández, Ellis, & Gallo, 2013).

Only a handful of studies have analyzed the genetic composition of *A. p. mexicana* (Cortés-Ortiz et al., 2003; Ellsworth & Hoelzer 2006; Dunn et al., 2014; Jasso-del Toro Márquez-Valdelamar, & Mondragón-Ceballos, 2016) and concluded that this primate harbors lower genetic diversity than other subspecies of *A. palliata*, and other Neotropical primates. These initial studies were based on a relatively low number of individuals from one or a few locations and were conducted with only one or a few markers, thus providing a limited picture of the genetic diversity of this subspecies. To generate more accurate estimates of genetic diversity it is necessary to analyze a larger number of individuals sampled from populations across the geographic distribution of this subspecies.

In this study, we evaluated the genetic diversity and population structure of *A. p. mexicana* based on 83 individuals from 31 groups from 25 localities distributed across the geographic range of the subspecies (Figure 1) and using a broad panel of neutral nuclear markers (microsatellites). Despite our relatively large sampling with broad geographical representation, our results revealed very low levels of genetic diversity for this subspecies. Moreover, we found no strong evidence for population structure, although some populations hold unique allelic diversity. The results of this study provide the necessary foundation to

112 develop conservations strategies aimed at maintaining the genetic diversity of this critically
113 endangered primate.
114
115 **Methods**
116 ***Sampling***
117 We collected blood (N=70) and fecal (N=13) samples from a total of 83 wild *A. p. mexicana*
118 individuals from 31 groups in 25 localities throughout the distribution range of this
119 subspecies in the Mexican states of Veracruz and Tabasco (Figure 1, Supplementary Table
120 S1). We assigned these individuals to three geographic groups: Western Veracruz (N=38),
121 Uxpanapa (N=13), and Tabasco (N=32). All groups occupied forest patches of different size
122 and quality and were surrounded by cattle ranches and/or farmland (see Ho et al., 2014 and
123 Dunn et al., 2014 for further details). We obtained blood samples from wild individuals that
124 were anesthetized with ketamine hydrochloride using a remote delivering system and handled
125 on-site following procedures described in Kelaita, Dias, Aguilar-Cucurachi, Canales-
126 Espinosa, & Cortés-Ortiz (2011). We tattooed restrained individuals after collecting
127 biological samples for individual identification, and all samples analyzed in this study
128 represent distinct individuals. Individuals were immediately released in the same location
129 after they recovered from anesthesia. Blood samples were preserved in lysis buffer (Seutin,
130 White, & Boag, 1991) and were refrigerated in the field until they were transported to the lab
131 and then stored at -80°C. In Uxpanapa, we collected fresh fecal samples from observable
132 individuals immediately after defecation to ensure minimal degradation and individual
133 identification (see Dunn et al., 2014 for details). We identified distinct individuals through
134 natural markings on feet and tail, typical of this species. Fecal samples were preserved in

96% ethanol and kept refrigerated until transported to the lab, where they were stored at -20°C. It is well known that Mexican howler monkeys hybridize with black howler monkeys (*A. pigra*) in Tabasco, Mexico (Cortés-Ortiz et al., 2007). To avoid possible effects of introgression on our analyses of population genetic diversity and structure we only used samples from individuals outside the hybrid zone or from individuals known to be non-admixed based on microsatellite (Cortés-Ortiz et al., 2019) and/or genomic analyses (Baiz, Tucker, & Cortés-Ortiz, 2018). The University of Michigan Committee for the Use and Care of Animals (UCUCA) approved the protocols used for animal restraint and sample collection (protocol # 09319). All samples were collected and exported with permission of Mexican authorities and legally imported into the United States.

DNA amplification and genotyping

We extracted DNA from blood samples with the DNeasy tissue kit (Qiagen, Valencia, CA), following manufacturer's protocol for tissue samples, with the exception that we used 100 µl of preserved sample instead of tissue. For DNA extraction of fecal samples, we used the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA), following manufacturer's protocol. We repeated DNA extraction of fecal samples up to three times, depending on the amount of DNA yield, to improve DNA quantity and quality. We amplified 29 microsatellite loci that had previously been polymorphic for *A. palliata* or that were polymorphic in this study (Supplementary Table S2). We used fluorescently (FAM, TET, or HEX) labeled primers in single- or multiplex reactions (Cortés-Ortiz, Mondragón, & Cabotage, 2010; Nidiffer & Cortés-Ortiz, 2015). We carried out PCR amplifications in a 10 µl reaction containing 0.25 µl of each primer (at 10 µM), 1.0 µl of 10X Buffer, 1.0 µl dNTPs (at 2 mM each), 0.8 µl of

158 $MgCl_2$ (50 mg/ml), 5.7 µl of ultrapure H_20, 0.045 µl of Platinum-Taq (Invitrogen, Waltham,
159 MA) and 1.0 µl of DNA. PCR thermal cycle comprised 2 minutes at 94°C of initial
160 denaturation and 35 cycles of 94°C for 30 seconds, annealing temperature (Supplementary
161 Table S2) for 30 seconds and 72°C for 45 seconds, with a final extension of 72°C for 10
162 minutes.

163 We assessed the quality of the PCR products through electrophoresis, to confirm
164 fragment size and used negative controls in all reactions to ensure no contamination. To
165 genotype each individual, we diluted PCR products according to the intensity of the band and
166 the type of fluorophore, mixed them with HI-DI formamide, ultrapure water and GeneScan
167 500 LIZ size standard, and submitted them for genotyping at the University of Michigan
168 Sequencing Core facilities, where PCR products were separated via capillary electrophoresis
169 on an ABI 3730xl DNA Analyzer. Allele calls were done manually using GeneMarker
170 version 1.4 (SoftGenetics, State College, PA).

171 We repeated amplifications for all samples extracted from feces at least three times,
172 following procedures described in Morin, Chambers, Boesch, C., & Vigilant (2001). Due to
173 intrinsic difficulties of amplifying DNA from fecal samples (Morin et al., 2001; Van Belle,
174 Estrada, & Di Fiore, 2014), we were only able to confidently genotype seven microsatellite
175 loci (D6S260, D14S51, Apm1, Apm4, Api08, 157 and TGMS2) for those samples. We
176 used Micro-Checker (Van Oosterhout, Hutchinson, Wills, & Shipley, 2004) to determine
177 genotyping errors associated with null alleles, large allele dropout or stuttering. We then
178 used *ARLEQUIN 3.5* (Excoffier, Laval, & Schneider, 2005) to analyze deviations from
179 Hardy-Weinberg equilibrium (HWE) and assess linkage disequilibrium (LD) in the
180 populations of Western Veracruz and Tabasco (where we genotyped all loci), and

181 implemented a sequential Bonferroni correction (Holm, 1979) to account for multiple
182 comparisons. Two microsatellite loci in each population (Apm4 and HAM80, in Veracruz,
183 and D8S165 and AC45 in Tabasco) showed deviation from HWE and signs consistent with
184 null alleles. It is likely that these deviations are due to a Wahlund effect (Wahlund, 1928),
185 which creates an excess of homozygosity as a consequence of analyzing individuals from
186 different populations or breeding units together, which is common when analyzing natural
187 populations (Waples, 2015). When we excluded loci out of HWE our general genetic
188 diversity results were not affected (Supplementary Table S3). Analyses of LD by
189 population showed signatures of linkage between a few microsatellite pairs (Ab06 and
190 Ab07, and TGMS2 and Ham 80 in Veracruz, and TGMS1, TGMS2 and 1118 in Tabasco).
191 The fact that we found different pairs of microsatellites showing LD in each population
192 suggests that the LD pattern found across all individuals is not caused by physical
193 proximity. Removing microsatellite loci that were in LD from our analyses did not produce
194 different qualitative results than those using the entire dataset (Supplementary Table S3), so
195 we report our findings including all microsatellite loci.
196
197 ***Analysis of genetic diversity and inbreeding***
198 To estimate genetic diversity and population-level inbreeding we used *ARLEQUIN* 3.5
199 (Excoffier et al., 2005) to analyze genotypes of 29 microsatellite loci for 70 individuals from
200 the Western Veracruz and Tabasco populations. We calculated the number of alleles per locus
201 per population, population private alleles (i.e., alleles that are only present in a single
202 population but not in other), observed heterozygosity (H_O, per locus and total), and expected
203 heterozygosity (H_E, per locus and total). We compared H_O to that reported for other howler

204 monkey (*Alouatta*) populations. We estimated the inbreeding coefficient (F_{IS}) to determine
205 if there was evidence of significant consanguineal mating among individuals in each
206 population.
207
208 ***Statistical Analysis of population differentiation***
209 To determine the number of genetically distinct populations, we used three different
210 approaches. First, we analyzed individuals of Western Veracruz and Tabasco with a Bayesian
211 clustering method implemented in the software *STRUCTURE* 2.3.4 (Pritchard, Stephens, &
212 Donnelly, 2000) using the default parameters. Burn-in was set to 5,000 iterations, followed
213 by 500,000 MCMC iterations and replicated 15 times for each value of the number of genetic
214 clusters (K) from 1 to 10.
215 We then used *adegenet* (Jombart, 2008), which performs exploratory multivariate
216 analysis of genetic markers not relying on HWE or LD, and implemented a principal
217 component analysis (PCA) to visualize possible clusters and identify the components that
218 better explain allelic variation across individuals. In this analysis we included the population
219 of Uxapanapa, which is geographically located in between our samples from Western
220 Veracruz and Tabasco (Figure 1). Since we only have data for 7 microsatellite loci for
221 individuals in Uxpanapa, we assessed the effect of the reduction in the number of
222 microsatellites on estimates of population structure by analyzing the 70 individuals from
223 Western Veracruz and Tabasco with all 29 microsatellite loci and with only the seven
224 microsatellite loci present in Uxpanapa, and confirmed that these seven loci provided the
225 same qualitative result (i.e., the grouping of individuals is corresponding in both cases and
226 the variance explained by the two principal components is low) as our entire dataset

(Supplementary Figure S1). We performed a PCA on all 83 individuals using these 7 microsatellite loci.

We grouped individuals by general geographic location (Western Veracruz, Uxpanapa, Tabasco) to calculate pairwise F_{ST} values in *ARLEQUIN 3.5* (Excoffier et al., 2005), using 1000 permutations to assess statistical significance. We also used *ARLEQUIN 3.5* to conduct an analysis of molecular variance (AMOVA) to test if different data partitions provided different support for geographic structure. Because AMOVA requires a hierarchical organization of the data, we first grouped individuals based on sampling locality and then by general geographic location to evaluate the percentage of variation between the three main groups. We used 10,000 permutations to assess statistical significance. Given that the Uxpanapa individuals are geographically located in between individuals from Western Veracruz and Tabasco we included Uxpanapa individuals as part of either population (i.e., including them with individuals from Western Veracruz in one analysis, and with individuals from Tabasco in another) and calculated pairwise F_{ST} between these groups to measure the effect of our *a priori* groupings on the overall population structure.

Finally, we used *GENALEX* (Peakall & Smouse 2006; 2012) to conduct a Mantel test on all individuals based on the seven microsatellite loci to evaluate any possible correlation of genetic and geographic distances, under the isolation by distance hypothesis (IBD). We constructed two pair-wise distance matrices for all individuals in our sample, one for Nei's genetic distance and another for geographic distance based on straight-line distances between sampling locations, and performed a paired Mantel test to detect any correlation.

Results

250 ***Genetic diversity and inbreeding***

251 Analyses of 29 microsatellites across 70 individuals revealed low observed (H_O=0.21) and
252 expected (H_E=0.29) heterozygosity. The mean number of alleles per locus was 3.19 (Table
253 1). The Tabasco population showed private alleles at 14 microsatellite loci, while the Western
254 Veracruz population only had private alleles at 8 microsatellite loci (Table 1). Wright's
255 inbreeding coefficient *(*F_{IS}) averaged across all loci was F_{IS}=0.204 ($P < 0.001$). F_{IS} for
256 Western Veracruz and Tabasco populations separately were 0.199 ($P < 0.001$) and 0.217 (P
257 < 0.001), respectively. Comparisons of genetic diversity estimates in this study to other
258 *Alouatta* species show that *A. p. mexicana* harbors the lowest genetic diversity, both among
259 populations of *A. palliata* and when compared to populations of other *Alouatta* species (Table
260 2).

261

262 ***Population structure***

263 Based on the subset of 70 individuals from Western Veracruz and Tabasco, results of
264 *STRUCTURE* analyses did not detect any patterns of population structure *in A. p. mexicana*.
265 Ln P(D) values ranged between -1050.7 (K=1) and -930.1 (K=10). Since *STRUCTURE*
266 cannot directly test for "no-structure" (i.e., K=1), this conclusion is based on the expectation
267 that likelihood values show a steep increase followed by a plateau when the number of
268 clusters is reaching a "true" value (Rosenberg et al., 2001). However, we did not observe
269 either a steep or a continuous increment in the likelihood values Ln P(D) as the number of
270 clusters (K) increased (Supplementary Figure S2). Furthermore, our multivariate analysis
271 (PCA) with *adegenet*, did not recover strong clusters based on geography (Figure 2). The

variance explained by the two principal components was less than 8% and no exclusive aggregation patterns were observed when plotting these two principal components.

AMOVA results showed that most of the genetic variation was recovered within populations, irrespective of the grouping scheme using to analyze our data (Table 3). Pairwise F_{ST} values between the three populations (Western Veracruz, Uxpanapa, Tabasco) show significant differences between Western Veracruz and Tabasco (0.135, $P < 0.001$) and between Western Veracruz and Uxpanapa (0.039, $p < 0.05$), but no differences between Tabasco and Uxpanapa (0.003, $P > 0.05$). When individuals from Uxpanapa and Western Veracruz were grouped together F_{ST} between this group and Tabasco was 0.106 ($P < 0.001$) and when individuals from Uxpanapa were included in the Tabasco population F_{ST} between this group and Western Veracruz was 0.114 ($P < 0.001$). The paired mantel test showed a slight correlation between genetic distance and geographic distance (Figure 3), with a Mantel $r = 0.173$ ($P = 0.001$). All together these results show low genetic diversity in this subspecies and indicate no strong genetic structure across the different sampled sites and only a small differentiation between populations consistent with an IBD pattern.

Discussion

This study represents the most comprehensive genetic analysis conducted to date for the Mexican howler monkey, both in terms of geographical representation of our samples and in the number of microsatellite loci analyzed. Our analysis of genetic diversity for 70 *A. p. mexicana* individuals from Western Veracruz and Tabasco using 29 microsatellite loci revealed low levels of genetic diversity (H_O= 0.21) when compared to other *Alouatta*

295 species (Table 2). Our results are consistent with those of other studies that used a small
296 number of samples and/or genetic markers for specific populations of the Mexican howler
297 monkey. The degree of polymorphism across microsatellites is variable and thus using
298 distinct sets of markers can yield different estimates of genetic diversity. The use of a larger
299 number of loci, most of which are polymorphic, may slightly have inflated our estimates,
300 but the fact that we still found low levels of genetic diversity confirms the reduced genetic
301 diversity previously observed for this subspecies (Table 2). The seven markers (Ap68,
302 Ap74, D5S111, D6S260, D8S165, D14S51, and D17S804) most commonly used in
303 previous studies present low levels of polymorphism in our study population and if we
304 restrict our analyses to this set of markers our estimate of genetic diversity considerably
305 decreases (Supplementary Table S4). Therefore, direct comparisons of genetic diversity
306 across studies that use different markers should be interpreted with caution. However,
307 comparing results across studies that use different sets of markers can be informative to
308 select the most useful markers for future studies of the species.

309 When comparing the genetic diversity of Mexican and Central American *A. palliata*
310 populations (Table 2) a latitudinal reduction in genetic diversity is apparent, with greatest
311 diversity at lower latitudes. This pattern is consistent with the inferred evolutionary history
312 of *A. palliata*, which originated in South America and has had a relatively recent expansion
313 into Central America (Cortés-Ortiz et al., 2003; Ford, 2006), with the Mexican population
314 currently representing an isolated population at the forefront of the expansion wave that
315 likely experienced strong founder effect. This pattern of lower genetic diversity at higher
316 latitudes is also observed in other North and Central American mammals of South

317 American origin, such as the nine-banded armadillo (Arteaga, Piñero, Eguiarte, Gasca, &
318 Medellín, 2012) and brown-throated sloth (Moss et al., 2012).

319 None of our analyses support strong population structure in Mexican howler
320 monkeys. When testing different possible partitions of our dataset, the importance of
321 obtaining a thorough sampling across the distribution range becomes evident. Our result of
322 the AMOVA analyses showed that most of the molecular variation occurs within populations
323 irrespective of how individuals are grouped. However, F_{ST} values are slightly higher if we
324 only include individuals from the populations at the extreme ends of the subspecies'
325 distribution in our analyses (i.e., Western Veracruz and Tabasco, Figure 1). However, the
326 inclusion of individuals from Uxpanapa (the population in between Western Veracruz and
327 Tabasco) in AMOVA and PCA showed that the genetic variation in *A. p. mexicana* has some
328 level of differentiation, possibly as a result of geographic distance. The weak but significant
329 correlation between genetic and geographic distances in our sample, as evidenced in our IBD
330 analyses, supports this hypothesis. Our results of pairwise F_{ST} and between-populations
331 percentage of molecular variation using different partitions of our dataset suggest that
332 Tabasco and Uxpanapa populations are more closely related to each other than to the Western
333 Veracruz population (Table 3).

334 The habitat of Mexican howler monkeys has been severely affected by anthropogenic
335 activities isolating populations in fragments of different sizes and compositions (Arroyo-
336 Rodríguez & Dias, 2010; Dunn et al., 2014; Cristóbal Azkarate, Dunn, Domingo Balcells, &
337 Veà Baró, 2017). These dramatic habitat alterations are relatively recent. Major changes date
338 back to the 1960s when different government initiatives for agricultural and cattle ranching
339 were implemented, depleting more than 400,000 ha of rainforest (Toledo, Carabias, Mapes,

& Toledo, 1985; Guevara et al., 2006). Currently, most populations of the Mexican mantled howler monkey live in small and isolated fragments of forest surrounded by human settlements and heavily affected by human activities, such as cattle ranching, the oil industry, and extensive monoculture crops (Arroyo-Rodríguez & Mandujano 2006; Cristóbal Azkarate et al., 2017). These and other human-related activities such as the pet trade have significantly affected populations of this subspecies, placing it in grave danger of extinction and listed as Critically Endangered by the IUCN (Cuarón et al., 2008). Our analyses of 29 microsatellite loci showed that Western Veracruz, Uxapana and Tabasco populations each have private alleles. Therefore, even when no strong genetic differentiation was observed between populations, it is likely that given the current conditions of isolation (with no current gene flow among these geographical locations) these subpopulations will continue to lose genetic diversity that may not be found in other populations. The reduction of gene flow also increases the chances that deleterious genetic variants arise in frequency by genetic drift, and the small size of these populations amplify the probability of inbreeding, which may directly affect reproductive and individual fitness (Ralls, Ballou, & Templeton, 1988; Hedrick, & Kalinowski, 2000). Thus, the low level of genetic diversity, the observed level of inbreeding ($F_{IS} > 0$), and the anthropogenic factors that continue to drive habitat loss and fragmentation pose a strong threat to the long-term viability of populations of Mexican howler monkeys. The loss of some of these isolated populations of *A. p. mexicana* would imply the loss of unique genetic diversity, and consequently, a potential reduction in the ability of the subspecies to face environmental change.

In summary, given the low genetic diversity and levels of inbreeding currently observed, the long-term viability of *A. p. mexicana* may be at risk. Although we did not detect

strong population structure, the current levels of habitat fragmentation greatly limit or completely prevent gene flow among the different locations, reducing the effective size of each population. From a genetic point of view, conservation efforts should focus on increasing the connectivity and quality of the habitat where *A. p. mexicana* lives to allow gene flow between populations. This would allow increasing the population size of the subspecies as a whole and consequently reducing the effects of genetic drift and inbreeding. Constant monitoring of the genetic diversity of isolated populations should be considered to ensure that levels of genetic diversity are not locally declining. In cases where genetic diversity is extremely low, management strategies to increase genetic diversity should be considered to preserve the adaptive potential of the subspecies. This study provides a baseline to evaluate the genetic diversity and establish long-term genetic monitoring of populations of Mexican howler monkeys.

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388
389 **Data Availability Statement**
390 The data that support the findings of this study are available from the corresponding author
391 upon reasonable request.
392

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606

607 **Figure legends**

608 **Figure 1.** Sampling localities of *A. palliata mexicana* individuals included in this study.
609 Colored circles represent the locations of groups sampled by region: Western Veracruz
610 (blue circles) = 38 individuals from 5 groups, Uxpanapa (red circles) = 13 individuals from
611 9 groups, and Tabasco (gold circles) = 32 individuals from 11 groups. Gray shading
612 represents the distribution range of *A. p. mexicana*. Inset map shows the general location of
613 the sampling region in southern Mexico and the zoomed area shows the aggregation of
614 sampled groups in Tabasco, each of which lived in a distinct forest fragment. Due to
615 concerns of poaching activities we do not disclose the exact coordinates for each group, but
616 the corresponding author can provide exact locations to researchers on reasonable request.
617

618 **Figure 2.** Principal component analysis (PCA) using the *adegenet*, based on 83 individuals
619 genotyped for 7 microsatellites. Plot is based on the first two principal components (PC1 and
620 PC2). Colored circles represent individuals from Tabasco (gold), Uxpanapa (red), and
621 Western Veracruz (blue). PC1+PC2 explain less than 8% of the total variation.
622

623 **Figure 3.** Mantel test correlation plot between Nei genetic distance and geographical
624 distance. Mantel $r = 0.173$ $P = 0.001$), suggesting weak, but significant, isolation by distance.
625

626 **Tables**

627 **Table 1.** Genetic diversity estimates per locus per population and total, for 70 individuals of Western Veracruz (WVe) and Tabasco
628 (Tab). N = sample size, Na = number of alleles, H_O = observed heterozygosity, H_E = expected heterozygosity. Mono = monomorphic
629 locus.

Locus	**N**	**Na**	**Private alleles**	**H_O**	**H_E**	**N**	**Na**	**H_O**	**H_E**
	WVe \ Tab	**WVe \ Tab**	**WVe \ Tab**	**WVe \ Tab**	**WVe \ Tab**	(total)	(total)	(total)	(total)
157	38 \ 32	5 \ 5	2 \ 2	0.66 \ 0.66	0.65 \ 0.78	70	7	0.66	0.75
1110	38 \ 32	2 \ 3	0 \ 0	0.34 \ 0.28	0.32 \ 0.33	70	3	0.31	0.33
1118	31 \ 29	1 \ 3	0 \ 2	Mono \ 0.10	Mono \ 0.16	60	3	0.05	0.08
Ab06	38 \ 32	2 \ 3	0 \ 1	0.13 \ 0.16	0.21 \ 0.15	70	3	0.14	0.18
Ab07	38 \ 31	2 \ 2	0 \ 0	0.29 \ 0.26	0.38 \ 0.48	69	2	0.28	0.43
Ab09	37 \ 31	2 \ 2	0 \ 0	0.14 \ 0.35	0.13 \ 0.37	68	2	0.24	0.25
Ab16	38 \ 32	2 \ 1	1 \ 0	0.03 \ Mono	0.03 \ Mono	70	2	0.01	0.01
Ab17	38 \ 32	1 \ 1	0 \ 0	Mono \ Mono	Mono \ Mono	70	1	Mono	Mono
Ab20	38 \ 32	1 \ 2	0 \ 1	Mono \ 0.06	Mono \ 0.06	70	2	0.03	0.03
AC45	38 \ 31	5 \ 7	0 \ 2	0.66 \ 0.23	0.73 \ 0.60	69	7	0.47	0.75
Ap68	38 \ 32	1 \ 1	0 \ 0	Mono \ Mono	Mono \ Mono	70	1	Mono	Mono
Ap74	38 \ 32	1 \ 2	0 \ 1	Mono \ 0.06	Mono \ 0.06	70	2	0.03	0.03
Api06	38 \ 32	2 \ 1	1 \ 0	0.08 \ Mono	0.08 \ Mono	70	2	0.04	0.04
Api07	38 \ 32	2 \ 2	0 \ 0	0.58 \ 0.41	0.49 \ 0.50	70	2	0.50	0.50
Api08	38 \ 32	2 \ 2	0 \ 0	0.37 \ 0.25	0.39 \ 0.22	70	2	0.31	0.32
Api09	38 \ 32	2 \ 1	1 \ 0	0.21 \ Mono	0.23 \ Mono	70	2	0.11	0.13
Api11	38 \ 32	2 \ 3	0 \ 1	0.03 \ 0.03	0.03 \ 0.09	70	4	0.03	0.06
Api14	38 \ 32	1 \ 2	0 \ 1	Mono \ 0.06	Mono \ 0.06	70	2	0.03	0.03
Apm1	38 \ 32	3 \ 2	1 \ 0	0.21 \ 0.53	0.19 \ 0.51	70	3	0.36	0.42
Apm4	38 \ 32	3 \ 7	0 \ 4	0.16 \ 0.56	0.52 \ 0.67	70	7	0.34	0.60

Apm9	38 \ 32	2 \ 1	1 \ 0	Mono \ Mono	0.05 \ Mono	70	2	0.00	0.03
D14S51	38 \ 29	4 \ 3	1 \ 0	0.11 \ 0.28	0.15 \ 0.25	67	4	0.18	0.20
D17S804	38 \ 32	1 \ 2	0 \ 1	Mono \ 0.16	Mono \ 0.15	70	2	0.07	0.07
D5S111	38 \ 32	1 \ 1	0 \ 0	Mono \ Mono	Mono \ Mono	70	1	Mono	Mono
D6S260	38 \ 32	3 \ 6	0 \ 3	0.32 \ 0.31	0.52 \ 0.42	70	6	0.31	0.53
D8S165	36 \ 32	1 \ 2	0 \ 1	Mono \ 0.06	Mono \ 0.22	68	2	0.03	0.11
Ham80	38 \ 31	3 \ 3	0 \ 0	0.22 \ 0.33	0.54 \ 0.59	69	3	0.27	0.58
TGMS1	31 \ 28	1 \ 3	0 \ 2	Mono \ 0.43	Mono \ 0.51	59	3	0.20	0.41
TGMS2	31 \ 28	3 \ 3	0 \ 1	0.55 \ 0.43	0.49 \ 0.56	59	4	0.49	0.65
Average	37.17 \ 31.38	2.68 \ 3.14	0.28 \ 0.79	0.27 \ 0.27	0.32 \ 0.35	68.55	2.97	0.21	0.29

630

631 **Table 2.** Observed heterozygosity (H_O) in *Alouatta palliata mexicana* in this study compared to
632 other studies of populations of *A. palliata* and other *Alouatta* species.

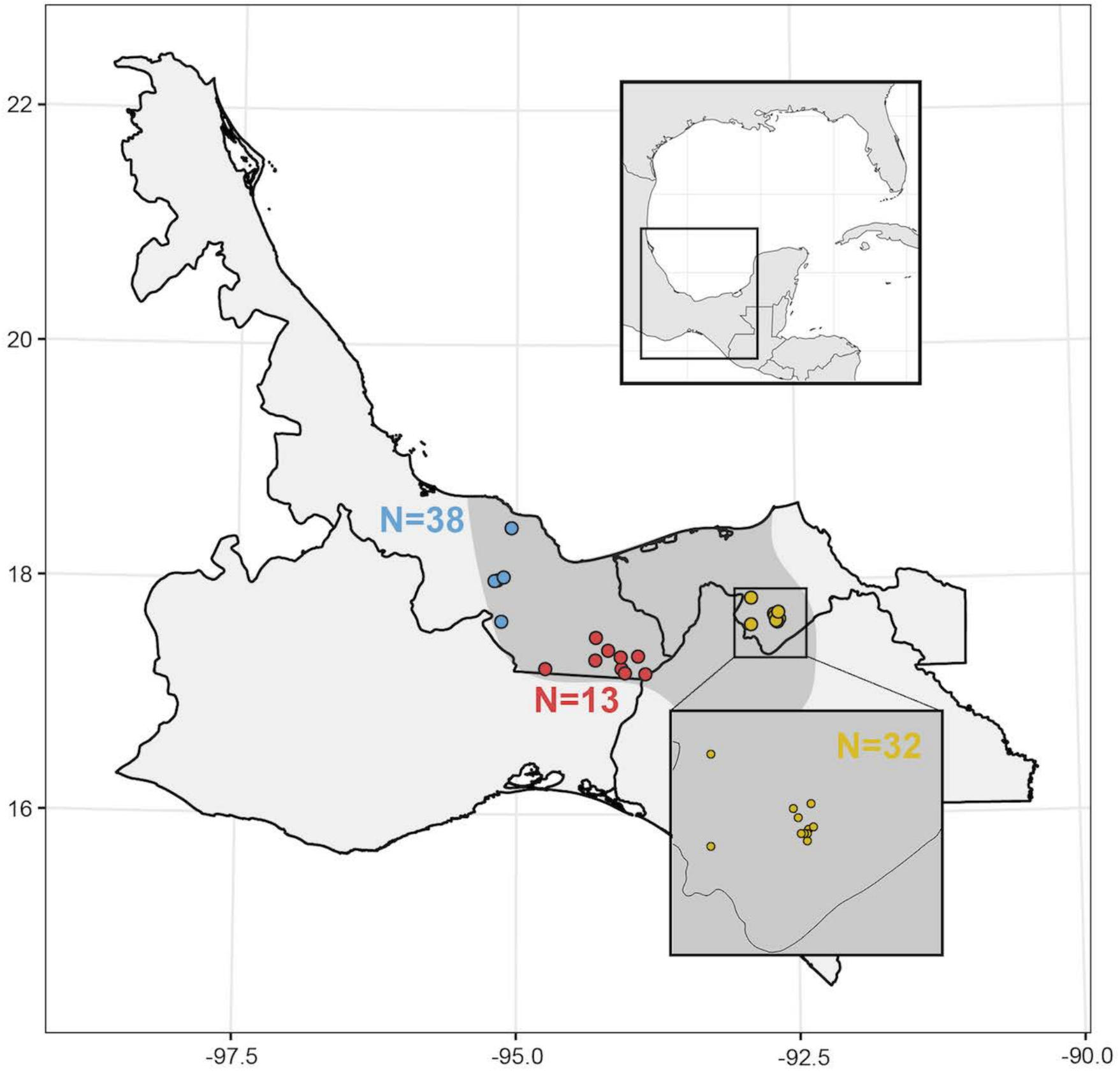
Taxa	N	H_O	Sampling locations	Reference
A. p. mexicana	70	0.21	25 locations in Veracruz and Tabasco, Mexico	This study
A. p. mexicana	8	0.14	2 locations, Veracruz, Mexico	Ellsworth & Hoelzer 2006
A. p. mexicana	61	0.14	1 location, Veracruz, Mexico	Jasso-del Toro et al., 2016
A. p. palliata	29	0.16	1 location, Ometepe Island, Nicaragua	Winkler et al., 2004
A. p. palliata	89	0.33	1 location, La Pacifica, Costa Rica	Ellsworth & Hoelzer 2006
A. p. aequatorialis	20	0.51	1 location, Barro Colorado Island, Panama	Ellsworth & Hoelzer 2006
A. p. aequatorialis	76	0.47	1 location, Barro Colorado Island, Panama	Milton et al., 2009
A. palliata	48	0.45	Multiple locations in Costa Rica, 2 in Mexico & 1 in Colombia	Ruiz-García et al., 2007
A. belzebul	92	0.62	5 locations, Tucurui, Brazil	Bastos et al., 2010
A. pigra	10	0.43	1 location, Scotland Half Moon, Belize	Winkler et al., 2004
A. pigra	28	0.45	1 location, Bermuda landing, Belize	Ellsworth & Hoezler 2006
A. pigra	107	0.59	1 location, Palenque National Park, Mexico	Van Belle et al., 2014
A. caraya	20	0.55	2 locations in Argentina and multiple in Bolivia	Ruiz-García et al., 2007
A. caraya	138	0.44	10 locations, Argentina	Oklander et al., 2017
A. seniculus	84	0.64	Multiple locations in Colombia	Ruiz-García et al., 2007
A. maconelli	7	0.57	3 locations, French Guiana	Ruiz-García et al., 2007

633

634 **Table 3.** Analysis of molecular variance (AMOVA) and pairwise F_{ST} estimates between *A. p.*
635 *mexicana* geographical populations based on seven microsatellite loci and using three partition
636 schemes (i.e., with Uxpanapa individuals grouped as a separate population or merged with either
637 Western Veracruz or Tabasco populations). * $P < 0.05$, ** $P < 0.001$, $^{n.s.}$ not significant ($P > 0.05$).
638 WVe = Western Veracruz, Tab = Tabasco, Uxp = Uxpanapa.

Partition scheme	Percentage variation		F_{CT}	*P value*	*Pairwise* F_{ST}
	Between populations	Within populations			
WVe *vs.* Tab *vs.* Uxp	8.73	82.07	0.087	$P = 0.0005$	WVe vs. Tab = 0.135** WVe vs Uxp = 0.039* Tab vs. Uxp = $0.003^{n.s.}$
(WVe + Uxp) *vs.* Tab	8.19	81.33	0.082	$P = 0.0013$	0.106**
(Uxp + Tab) *vs.* WVe	8.33	81.42	0.083	$P = 0.0003$	0.114**

639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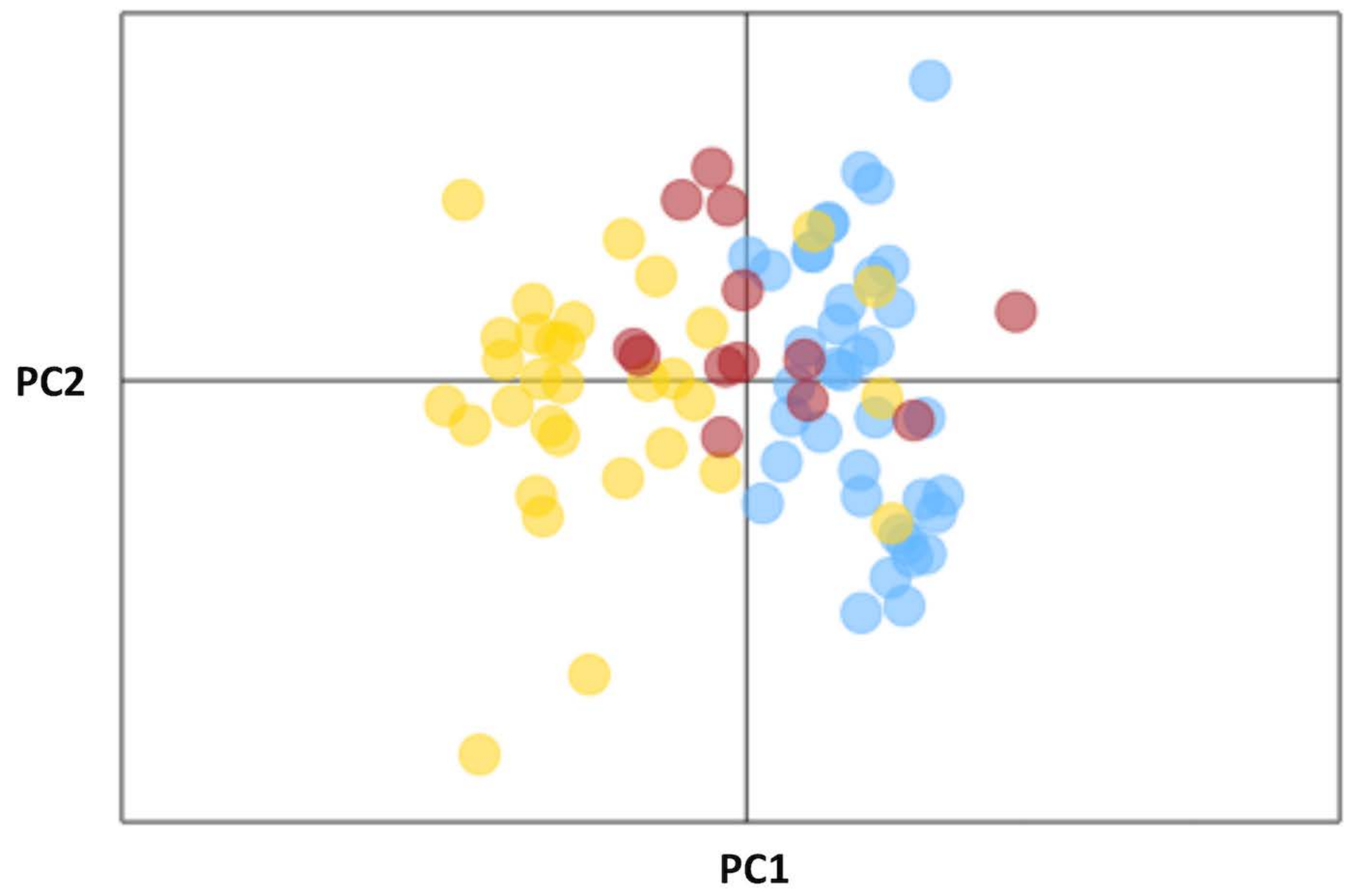
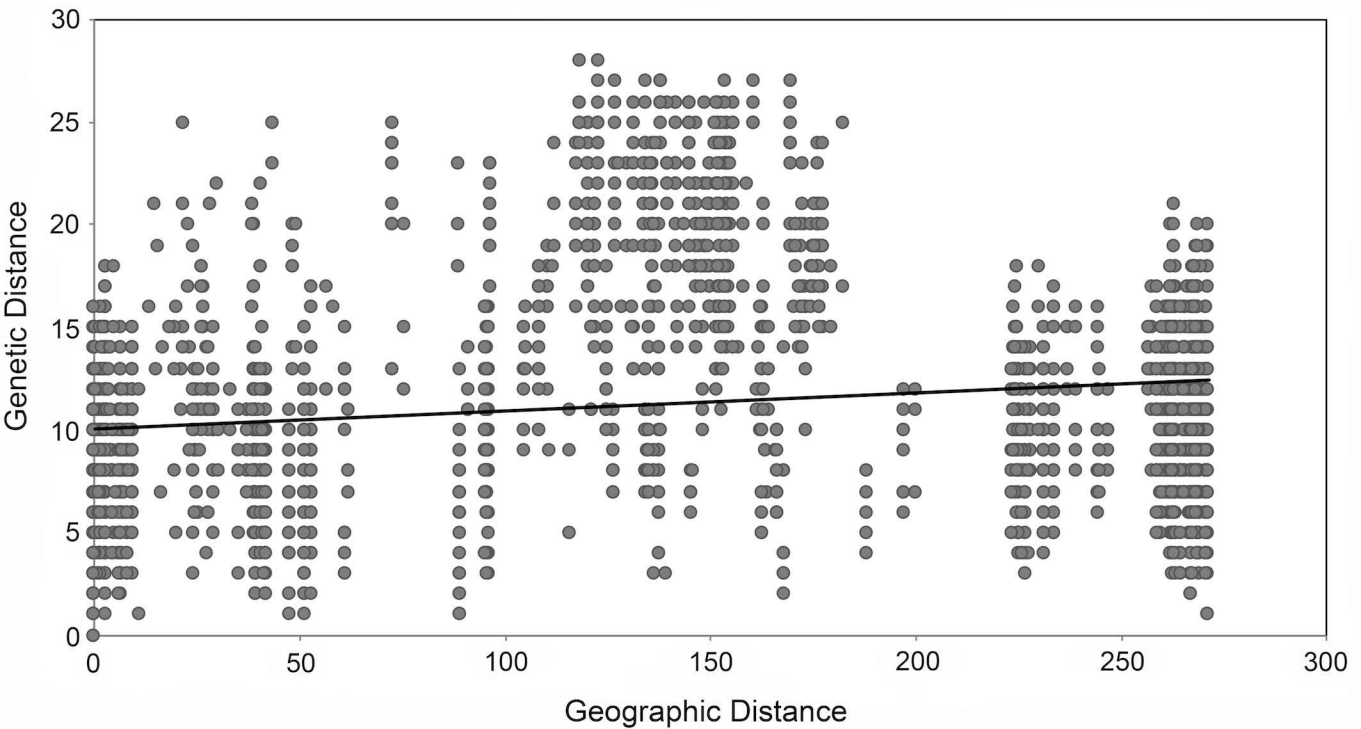
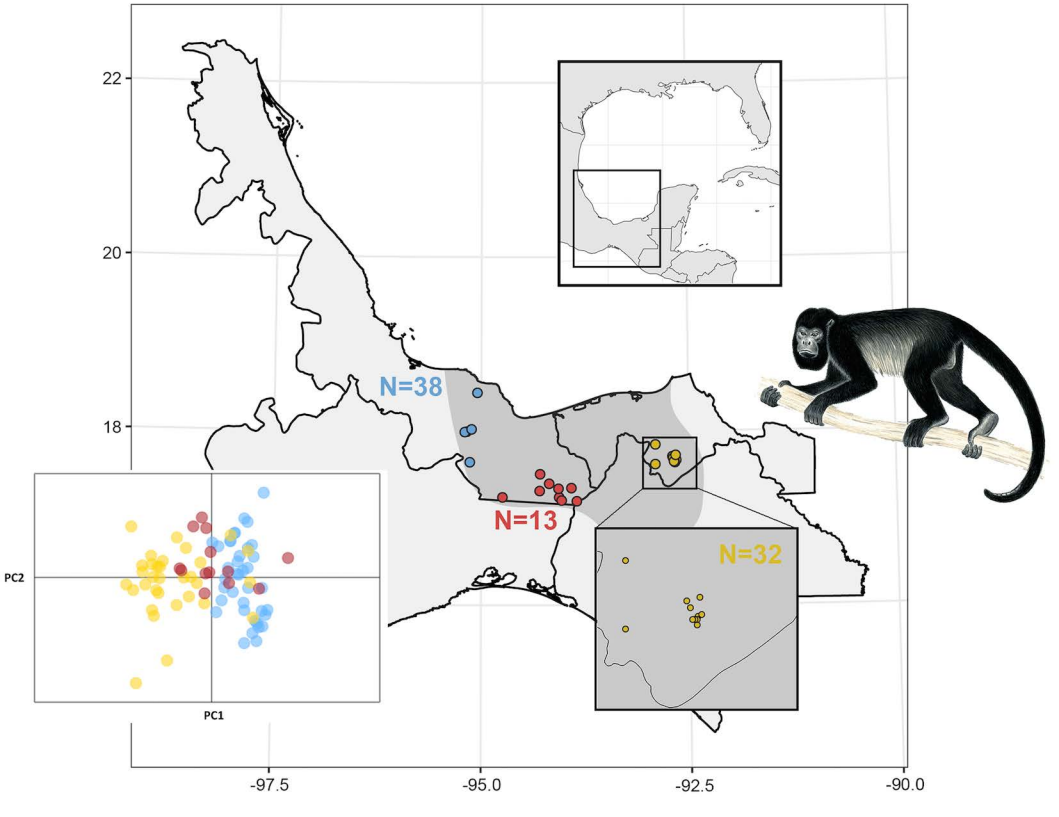

PC2
PC1


30
25
20
15
10
5
0
Genetic Distance
0
50
100
150
200
250
300
Geographic Distance


22
20
18
-97.5
-95.0
-92.5
-90.0
N=38
N=13
N=32
PC2
PC1

Research Highlights

- The Mexican howler monkey has low genetic diversity across its distribution range.
- There is no evidence of strong genetic structure among geographical populations.
- Current lack of gene flow may further reduce the genetic diversity and adaptive potential of this Critically Endangered taxon.

Supplementary information

Table S1. Information of sampled groups including sampling locality, number of individuals sampled per group, and general population to which they were assigned in this study based on general geographical location. Exact coordinates of the groups can be provided by the corresponding author upon request.

Locality	Group ID	Assigned population	Number of individuals included in analyses
Cascajal del Río, Ver.	A	Western Veracruz	5
Camarón, Ver.	D	Western Veracruz	6
Camarón, Ver.	I	Western Veracruz	2
Cascajal del Río, Ver.	J	Western Veracruz	2
Cascajal del Río, Ver.	K	Western Veracruz	2
Ixtal, Ver.	R	Western Veracruz	5
Cascajal del Río, Ver.	Y	Western Veracruz	5
La Flor, Ver.	78	Western Veracruz	5
Jalapilla, Ver.	79	Western Veracruz	6
Los Alamos, Tab.	24	Tabasco	1
Pochitocal, Tab.	42	Tabasco	2
Calicanto, Tab.	49	Tabasco	4
Pochitocal, Tab.	69	Tabasco	3
Pochitocal, Tab.	72	Tabasco	2
Pochitocal, Tab.	74	Tabasco	7
Carlos Green, Tab.	77	Tabasco	3
Calicanto, Tab.	80	Tabasco	2
Pochitocal, Tab.	81	Tabasco	2
Guarda Costa, Tab.	B	Tabasco	3
La Palmilla, Tab.	G	Tabasco	1
Carlos Green, Tab.	N	Tabasco	1
Carlos Green, Tab.	O	Tabasco	1
El Jaguar, Ver.	El Jaguar	Uxapanapa	1
Hueyapan, Ver.	Hueyapan	Uxapanapa	1
J Cardel, Ver.	J Cardel	Uxapanapa	1
Progreso 1, Ver.	Progreso 1	Uxapanapa	1
Rio Azul, Ver.	Rio Azul	Uxapanapa	2
Salta Barranca, Ver.	SaltaBarranca	Uxapanapa	2
Samaria, Ver.	Samaria	Uxapanapa	1
Saturnino Cedillo, Ver.	Saturnino Cedillo	Uxapanapa	3
Tenochtitlan, Ver.	Tenochtitlan	Uxapanapa	1

Table S2. Primers and PCR conditions used in this study. T°C = annealing temperatures. Superscript numbers refer to studies in which the microsatellite locus was polymorphic for *A. palliata*: [1]Winkler et al., 2004; [2]Ellsworth & Hoelzer, 2006; [3]Milton et al., 2009; [4]Cortés-Ortiz et al., 2010; [5]Jasso-del Toro et al., 2016. Asterisk indicates this study.

Locus[a]	T°C	Allele size range*	Number of alleles*	Species from which it was originally isolated	Original reference
Ap68[1,2,3,4]	50	191	1	*Alouatta palliata*	Ellsworth & Hoelzer, 1998
Ap74[1,2,3,4,5,*]	50	148-152	2	*Alouatta palliata*	Ellsworth & Hoelzer, 1998
D5S111[1,2,3,4,5]	60	160	1	*Homo sapiens*	Ellsworth & Hoelzer, 1998
D6S260[1,2,3,4,5]	53	172-186	6	*Homo sapiens*	Ellsworth & Hoelzer, 1998
D8S165[1,2,3,4,5]	55	139-141	2	*Homo sapiens*	Ellsworth & Hoelzer, 1998
D14S51[1,2,3,4,5]	60	139-145	4	*Homo sapiens*	Ellsworth & Hoelzer, 1998
D17S804[1,2,3,*]	60	158-166	2	*Homo sapiens*	Ellsworth & Hoelzer, 1998
Ab06[3,4,5,*]	60	272-276	3	*Alouatta belzebul*	Goncalves et al., 2004
Ab07[4,*]	60	174-176	2	*Alouatta belzebul*	Goncalves et al., 2004
Ab09[*]	58	144-145	2	*Alouatta belzebul*	Goncalves et al., 2004
Ab16[3,*]	65	170-177	2	*Alouatta belzebul*	Goncalves et al., 2004
Ab17[3]	60	161	1	*Alouatta belzebul*	Goncalves et al., 2004
Ab20[*]	67	236-240	2	*Alouatta belzebul*	Goncalves et al., 2004
Apm1[4,5,*]	64	208-220	3	*A. p. mexicana*	Cortés-Ortiz et al., 2010
Apm4[4,5,*]	65	237-249	7	*A. p. mexicana*	Cortés-Ortiz et al., 2010
Apm9[4,*]	55	176-179	2	*A. p. mexicana*	Cortés-Ortiz et al., 2010
Api06[*]	64	277-279	2	*Alouatta pigra*	Cortés-Ortiz et al., 2010
Api07[4,*]	55	115-117	2	*Alouatta pigra*	Cortés-Ortiz et al., 2010
Api08[4,*]	55	277-279	2	*Alouatta pigra*	Cortés-Ortiz et al., 2010
Api09[4,*]	60	467-471	2	*Alouatta pigra*	Cortés-Ortiz et al., 2010
Api11[*]	55	253-263	4	*Alouatta pigra*	Cortés-Ortiz et al., 2010
Api14[*]	55	181-183	2	*Alouatta pigra*	Cortés-Ortiz et al., 2010
1110[*]	54	203-207	3	*Lagotrix lagotricha*	Di Fiore and Fleischer, 2005
1118[*]	53	132-134	3	*Lagotrix lagotricha*	Di Fiore and Fleischer, 2005
157[*]	54	222-246	7	*Lagotrix lagotricha*	Di Fiore and Fleischer, 2005
AC45[*]	64	331-362	7	*Alouatta caraya*	Oklander et al., 2007
Tgms1[*]	58	304-323	3	*Homo sapiens*	Tomer et al., 2002
Tgms2[*]	58	312-328	4	*Homo sapiens*	Tomer et al., 2002
Ham80[*]	53	132-138	3	*Callithrix jacchus*	Katoh et al., 2009

[a]Forward and reverse primers are referred by the locus name given in the original reference.

Table S3. Comparison of genetic diversity estimates using all loci vs. excluding loci out of Hardy-Weinberg Equilibrium (HWE) and in Linkage Disequilibrium (LD). N = sample size, Na = number of alleles, H_O = observed heterozygosity, H_E = expected heterozygosity.

All loci			
N	Na	H_O	H_E
70	29	0.21	0.28

Only loci in HWE & not in LD			
N	Na	H_O	H_E
70	29	0.19	0.21

Table S4. Comparison of genetic diversity estimates using all loci and using only the seven most commonly used loci (Ap68, D5S111, D6S260, D8S165, D14S51, D17S804 and Ap74) based on the studies reported in Table S1.

All loci			
N	Na	H_O	H_E
70	29	0.21	0.28

Most commonly used loci			
N	Na	H_O	H_E
70	29	0.12	0.19

Supplementary Figures

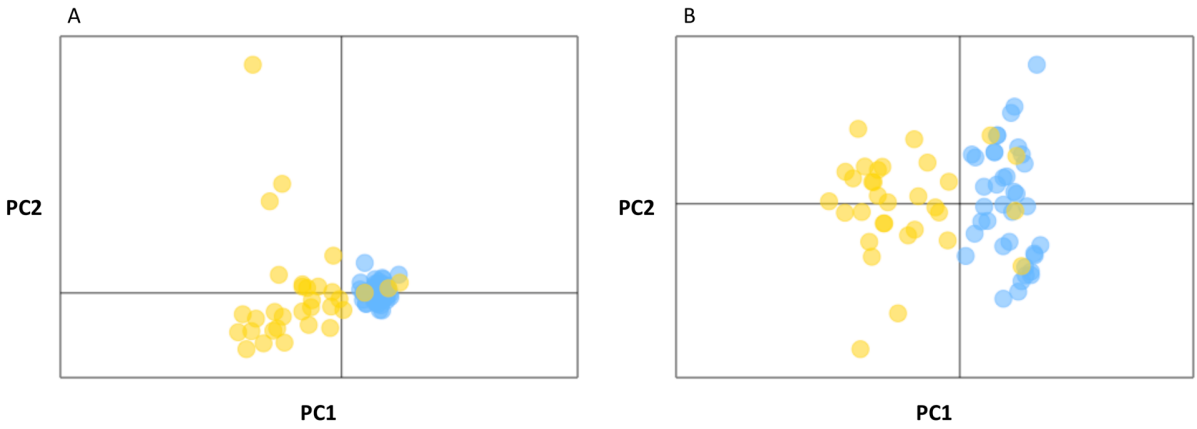


Figure S1. Principal component analysis (PCA) using *adegenet* (Jombart, 2008) for 70 individuals from Western Veracruz and Tabasco. A) PCA performed using 29 microsatellite loci showing the first two principal components (PC1 and PC2). B) PCA using the 7 microsatellite loci that were amplified in the Uxpanapa population. Blue circles represent individuals from the Western Veracruz population and yellow circles represent individuals from the Tabasco population. For both analyses PC1+PC2 is less than 6%.

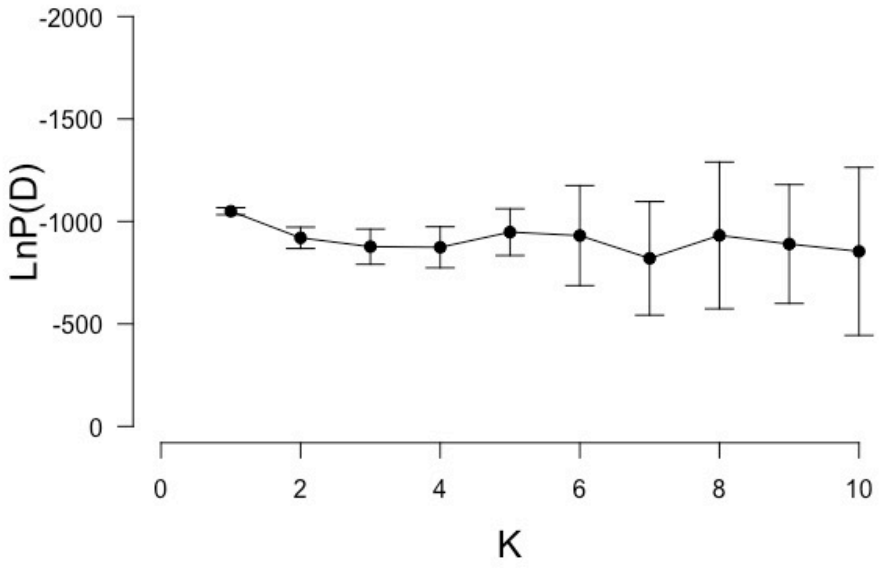


Figure S2. Likelihood plot of *STRUCTURE* (Pritchard, Stephens, & Donnelly, 2000) results for different values of K**.** Ln P(D) is the likelihood for each value of K, which represents the number of simulated clusters that better represent the data set. Black dots represent the average values of LnP(D), and vertical lines represent standard deviations. Likelihood values do not show a steep increase followed by a plateau as expected when the number of clusters (K) reaches the "true" value (Rosenberg et al., 2001).

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