RESEARCH ARTICLE

Estimating genetic and demographic parameters relevant for the conservation of the Neotropical otter, *Lontra longicaudis,* in Mexico

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Abstract

Habitat deterioration and fragmentation increase the risk of wildlife extirpation as they have strong impacts on population size, genetic diversity and gene flow. Small populations are more susceptible to these factors because the loss of genetic diversity by drift and inbreeding occurs at faster rates. Therefore, estimates of genetic diversity and population sizes of threatened and small wildlife populations in deteriorated landscapes are critical for managing and conservation. Here, we used a non-invasive sampling approach in combination with eleven microsatellite loci to evaluate genetic diversity, genetic structure, and demographic parameters of the Neotropical otter (*Lontra longicaudis*) in three river basins (Actopan, La Antigua and Jamapa), which are priority conservation areas for Veracruz, Mexico. Our results revealed moderate genetic diversity and genetic structure among river basins. However, we detected first-generation migrants among basins, suggesting current gene flow. Effective population size for each basin was considerably lower than the value ($N_e < 100$) suggested to maintain genetic variation of populations in the short-term. Similarly, census population size was lower than estimates reported for *L. longicaudis* in a conserved region in Mexico. We did not find evidence of recent genetic bottlenecks for any basin. Our genetic and demographic results suggest that *L. longicaudis* in the three river basins could be experiencing genetic isolation and erosion, with La Antigua being the most vulnerable basin. Natural fragmentation and habitat deterioration seem to be shaping the observed patterns of genetic variation in the Neotropical otter.

Keywords *Lontra longicaudis* · Effective population size · Genetic structure · Gene flow · Freshwater ecosystems · Non-invasive genetic sampling

Introduction

Genetic diversity is essential for the adaptation of natural populations to changes in their landscape and environment. Populations that have suffered genetic bottlenecks or that have low genetic diversity are more vulnerable to extirpation as they have less potential to cope with changes resulting from climate change and habitat fragmentation (Crow and Kimura 1970; Frankham et al. 2017). Smaller populations are more susceptible to the erosion of genetic diversity because genetic drift and inbreeding occur at faster rates (Frankham 1996; Franklin and Frankham 1998; Allendorf et al. 2013). Several empirical studies in plants and animals have shown that population size is positively correlated with genetic diversity (e.g., Soulé 1976; Frankham 1996; Hague and Routman 2016).

Habitat loss and fragmentation can have strong impacts on population size, genetic diversity and gene flow

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(Frankham et al. 2002; Manel et al. 2003; Keyghobadi 2007; Frankham 2010; Cushman et al. 2013). While habitat loss mainly reduces the resources and suitable conditions for the reproduction and growth of individuals (Vergeer et al. 2003; Morris et al. 2008), habitat fragmentation can reduce dispersal and consequently gene flow among remnant populations (Manel et al. 2003; Frankham et al. 2017). The effects of habitat loss and fragmentation are expected to be stronger in threatened species because they are characterized by having several generations of reduced population size and genetic diversity (Spielman et al. 2004; Palstra and Ruzzante 2008; Frankham et al. 2015). Therefore, the estimation of genetic diversity and population sizes in threatened species inhabiting fragmented landscapes are imperative.

The identification of bottlenecks, as well as the estimation of census (N_c) and effective (N_e) population sizes allow generating essential genetic and demographic information, which is key for developing conservation and management plans. Genetic bottlenecks reduce the possibility of survival of individuals from populations that have been subjected to significant declines (Peery et al. 2012). N_c is the number of living individuals in a population, and it is an important demographic parameter for evaluating the conservation status of a species (Knaepkens et al. 2004; Hague and Routman 2016). N_e provides information on the size of an ideal population that would lose genetic diversity as the result of inbreeding or genetic drift, at the same rate as the population under consideration (Wright 1940). The estimation of N_e is essential to warrant the maintenance of genetic diversity and the evolutionary potential of populations (Bouzat 2010).

The Neotropical otter, *Lontra longicaudis*, is an ecological important species (i.e. top predator and umbrella species) that inhabits freshwater ecosystems. Although *L. longicaudis* has a wide distribution from northern Mexico to northern Argentina (Larivière 1999), it is classified as a "near threatened" species by the International Union for the Conservation of Nature (IUCN), mainly because of the decrease in its population sizes. Between 1950 and 1970, *L. longicaudis* was hunted for fur trade (Rheingantz and Trinca 2015), which dramatically reduced its population sizes. A further population reduction of 25% has been estimated to occur in the next 30 years (Pacifici et al. 2013).

In Mexico, *L. longicaudis* is classified as a threatened species due to its habitat deterioration mainly by pollution, fisheries overexploitation and deforestation (NOM-059 SEMARNAT 2010; Gallo-Reynoso 2007). Despite these threats, population sizes (N_e and N_c) of *L. longicaudis* have not yet been estimated in Mexico, and there are few studies that have evaluated the genetic diversity and population structure in the species (Ortega et al. 2012; Guerrero et al. 2015; Hernández et al. 2017). Most studies of *L. longicaudis* in Mexico have estimated abundance employing indirect counting methods (i.e. spraints) (Larivière 1999; Arellano

et al. 2012, González-Christen et al. 2013) and more recently molecular markers (Ortega et al. 2012). Only one study has assessed genetic variation using microsatellites, finding moderate levels of genetic diversity ($Ho = 0.55$), lack of genetic structure, and a maximum displacement of individuals of 17 km in a conserved river system in Chiapas (Ortega et al. 2012). Studies in South American populations of *L. longicaudis* reported low to high genetic diversity ($Ho = 0.322—0.83$; Weber et al. 2009, Trinca et al. 2013, 2016). Contemporary genetic structure, gene flow and migration rates have not yet been assessed for *L. longicaudis*, but for the Eurasian otter (*Lutra lutra*), studies suggest that gene flow among basins is restricted by physical characteristics of the basins, such as slope and altitude (Janssens et al. 2008; Pagacz 2016).

The main objectives of this study were to estimate genetic diversity and demographic parameters of the Neotropical otter in three river basins in central Veracruz, Mexico: Actopan, La Antigua, and Jamapa. These river basins are classified as highly human perturbed and are priority targets for conservation (Cotler-Ávalos et al. 2010). Specifically, we used non-invasive sampling and eleven microsatellite loci to: (i) evaluate genetic diversity of the Neotropical otter in each basin; (ii) identify patterns of genetic structure within and among basins; (iii) detect contemporary migration events among basins; and (iv) estimate population sizes and test for evidence of genetic bottlenecks. Because the three basins have been subject to a high degree of human disturbance, we expect to find low genetic diversity and small population sizes. Similarly, we anticipate finding strong genetic structure and low contemporary migration rates among basins due to habitat deterioration and to the hierarchical organization of the rivers, which creates a natural fragmented habitat.

Materials and methods

Study area

We conducted the study in three river basins of the state of Veracruz in Mexico (Fig. 1). Actopan is the smallest of the river basins, with an area of 1999 km^2, followed by La Antigua consisting of 2190 km^2 and Jamapa of 3918 km^2. In the last decades, human population growth and changes in land use have altered the functional dynamics of the basins (e.g., hydrological filtration processes, evapotranspiration, sediment movement; Cotler et al. 2010). The main economic activities conducted along the river shores of the basins include fishing, livestock, and agriculture (INEGI 2011). Along these basins *L. longicaudis* is generally found from 0 to 1200 m a.s.l. (Macias-Sánchez and Aranda 1999; Latorre-Cardenas 2013 unpublished data, Hernández-Romero et al. 2017), but it has been reported at up to 2500 m a.s.l.,
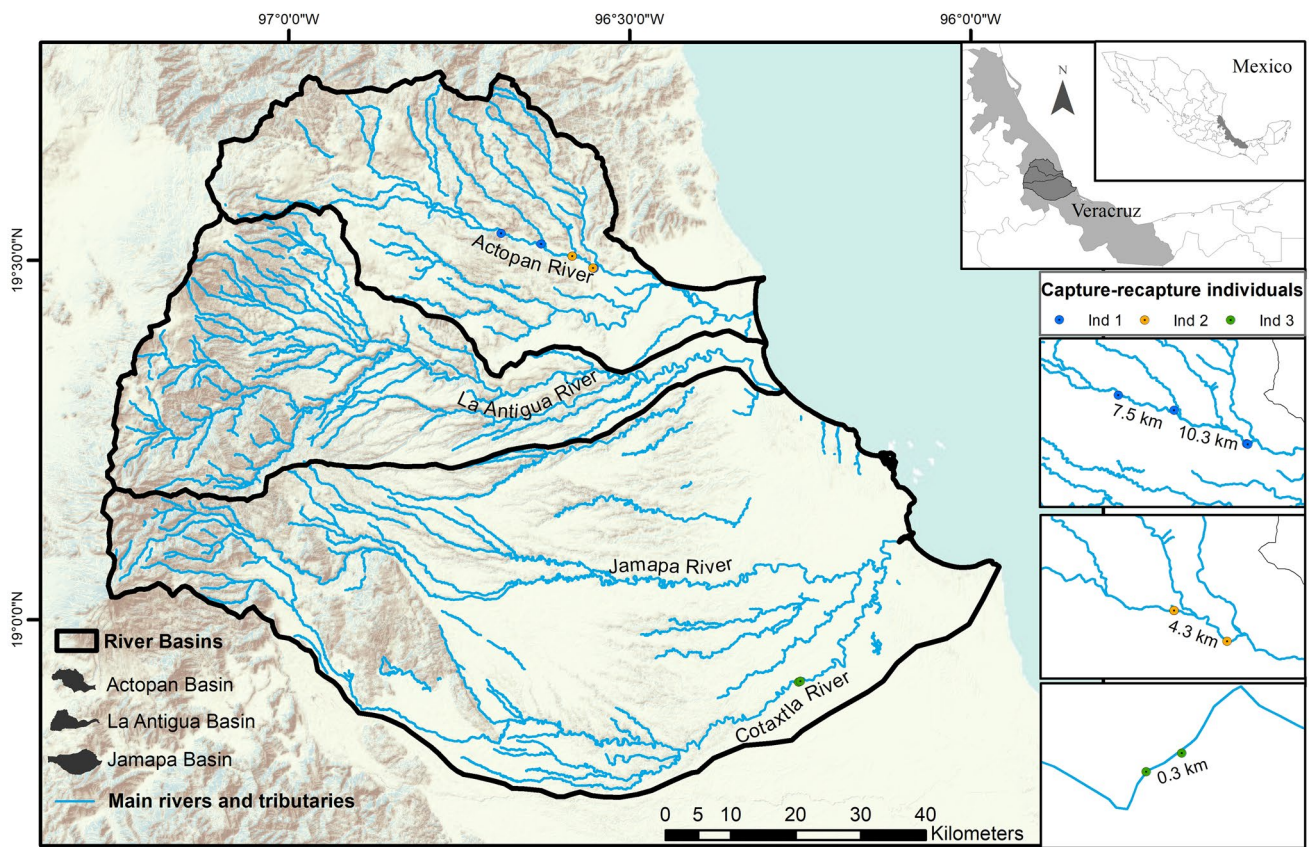
Fig. 1 Map of the studied area including the rivers and tributaries of the three basins where the sampling of the Neotropical otter was conducted, and the location of the studied area within the state of Veracruz in Mexico (inset in the top right). Color dots in the map indicate the position of the samples of the three individuals that were capture and recapture. The insets on the right show the position of the samples of each capture-recapture individual and the distance between them is indicated

probably due to the presence of aquaculture ponds at these higher altitudes (Hernández-Romero et al. 2018).

Sample collection

Due to the elusive behavior of *Lontra longicaudis*, we used a non-invasive approach for sample collection. We collected fresh spraints and anal glands secretions deposited in latrines along the main rivers and third or higher order tributaries of the Actopan, La Antigua and Jamapa basins (Fig. 1). We restricted sampling collection to third-order or higher rivers because they have enough water flow for the otter to inhabit them. We used a hydrological stream layer (hydrological network; INEGI 2016) to identify the rivers and tributaries to be surveyed, employing the spatial tools in ArcGIS v.10.2.1. (ESRI 2013).

We conducted sample collection by raft in the main rivers of Actopan and La Antigua, along 45 and 64 km, respectively. In order to detect as many individuals as possible, we carried out surveys on periods of two months from: May to June 2016, November to December 2016 and March to April 2018. Because of the low water level of the Jamapa river, we only performed one survey by raft or walking along the rivers, from November 2017 to February 2018, covering a total of 105 km. We performed all surveys in the morning to obtain fresh otter samples (Lerone et al. 2014), which were collected using sterile sticks, preserved in 1.25 ml of RNA later buffer (Sigma Aldrich), and stored at − 20 °C until genetic analyzes were performed. We registered the geographic coordinates of all collected samples.

We also included blood samples of six captive individuals from the Veracruz aquarium ("Acuario de Veracruz, A.C."), which were rescued from Jamapa in the same time period of our field work. Veterinarians of the aquarium took blood samples following the ethical protocol "NOM-135-SEMARANT-2004" and the "Code of ethics and welfare animal of the World Association of Zoos and Aquariums (WAZA)". We preserved blood samples in EDTA at − 20 °C until DNA extractions were performed.

DNA extraction and amplification

We extracted genomic DNA from anal secretions and feces using the ZR Fecal DNA MiniPrep kit (Zymo Research) and from blood samples with the DNeasy Blood & Tissue kit (Qiagen) following the manufacturer instructions. We amplified eleven microsatellite loci: two (RIO 2 and RIO11) previously designed for *L. canadensis* (Beheler et al. 2004, 2005) and nine (Lolo13, Lolo18, Lolo19, Lolo29, Lolo30, Lolo37, Lolo39, Lolo41 and Lolo48) developed for *L. longicaudis* (Latorre-Cardenas et al. 2020) using the polymerase chain reaction (PCR). We provide details on DNA extractions and PCR conditions in Latorre-Cardenas et al. (2020) and in Online Resource 1. We pooled PCR products of the eleven microsatellite markers in four multiplex panels, consisting of three loci each (Table S1). Fragment analyses were conducted in a 3730XL sequencer (Applied Biosystems) using 600 LIZ size standard (GeneScan™) at Macrogen, South Korea. We assigned allele size with the aid of the software GeneMapper 4.0 (Thermofisher).

Genotyping quality control

The low amount and quality of the DNA contained in anal secretions and feces could yield low quality of PCR reactions and could increase genotyping errors. Quality control of PCR reactions was assessed by verifying the genotypes of each sample using a multi-tube approach (Taberlet et al. 1996). This approach consists on repeating the PCR reactions of all samples using the same conditions to assure the same alleles are obtained in each replicate (Taberlet et al. 1996). We considered a sample to be heterozygote if the same two alleles were obtained in two identical repetitions of the PCR, and a sample was a homozygote if the same single allele was obtained in three PCR replicates. We assessed genotyping error per locus, based on the number of mismatches between resulting genotypes of the PCR replicates (Pompanon et al. 2005). We also used MICROCHECKER (Van Oosterhout et al. 2004) to determine the presence of large allelic dropout and null alleles.

Individual identification and microsatellite power of discrimination

When employing a non-invasive sampling approach, several samples of the same individual could be collected during the same or in different sampling sessions. We identified unique multilocus genotypes (i.e., individuals) using the ALLELEMATCH package (Galpern 2012) in R v.3.4.2. (R Core Team 2017). This package identifies unique multilocus genotypes in data sets in which the number of individuals is unknown, considering the genotyping error and missing data. We performed all subsequent analyses using the unique multilocus genotypes. To assesses the discriminating power of microsatellites in identifying individuals, we assessed the probability of identity (P_{ID}) and the probability of identity between siblings (P_{IDSIBS}) (Waits et al. 2001) for each locus and combining all loci in GIMLET. P_{ID} calculates the probability that two unrelated individuals and P_{IDSIBS} that two siblings drawn at random from a population will have the same genotype with a given set of loci.

Genetic diversity

To assess genetic diversity for each basin, we calculated rarefied allelic richness (AR) in FSTAT v.2.9.3.2. (Goudet 2001), rarefied private allelic richness (PAR) in HP-rare (Kalinowski 2004, 2005) and the effective number of alleles (ENA) in GENEALEX v.6.5.02 (Peakall and Smouse 2012). We also calculated observed (H_O) and expected (H_E) heterozygosity and significant deviations from Hardy–Weinberg equilibrium (HWE) by means of the inbreeding coefficient (F_{IS}), with 10,000 permutations in FSTAT. We tested for significant differences in AR and H_O among basins, using 1000 permutations and two-sided tests in FSTAT. To identify which of the basins differed, we performed pairwise comparisons. We also tested for significant differences in H_E across basins by means of an ANOVA in R. We used FSTAT to assess linkage disequilibrium (LD) between all pairs of loci. We performed corrections for multiple comparisons with the sequential Bonferroni method (Rice 1989).

Population genetic structure

To assess genetic structure, we identified the most likely number of genetic clusters by employing a Bayesian clustering analysis in STRUCTURE (Pritchard et al. 2000). We used the admixture model with correlated allele frequencies. We ran 10 independent chains for each K (from K = 1 to K = 6). The length of the burn-in was 500,000 followed by 1,000,000 Markov Chain Monte Carlo iterations. We determined the most likely number of genetic clusters in STRUCTURE HARVESTER Web v.0.6.94 (Earl and VonHoldt 2012) using the ΔK statistic (Evano et al. 2005) and the likelihood of the data (ln P(D); Pritchard et al. 2000). We used the software CLUMP v.1.1.2 (Jakobsson and Rosenberg 2007) to average the results of the 10 replicates with the highest ΔK value, and DISTRUCT v.1.1 (Rosenberg 2004) to visualize the genetic clusters. In order to assess genetic substructure, we ran STRUCTURE separately in each of the genetic clusters detected by the initial analysis (see "Results"). We performed these subsequent analyses as described above.

We also performed a Discriminant Analysis of Principal Components (DAPC) in the ADEGENET package v.2.1.1 in R (Jombart et al. 2008) to further evaluate genetic

structure. DAPC is a multivariate multilocus analysis designed to maximize genetic variance among groups, while minimizing within group variance (Jombart et al. 2008). DAPC does not assume HWE or LD (Jombart et al. 2008) and it is not sensitive to the presence of null alleles. We assessed the most likely number of clusters from K = 1 to K = 6, with the *find.clusters* function and selected the K with the lowest BIC. To identify the number of PCs to be retained, we used the *dapcCross* validation function that selects the optimal number of PCs based on the highest successful assignment with the lowest mean squared error (Jombart et al. 2010).

We calculated genetic differentiation between basins using pairwise F_{ST} comparisons and assessed their significance with 1000 permutations in FSTAT. We used the excluding null allele correction method to calculate pairwise F_{ST} (Chapuis and Estoup 2007) in FREENA v.1.0. This method prevents overestimation of F_{ST} values due to the presence of null alleles. To avoid bias associated to the high polymorphism characteristic of microsatellites, we calculated pairwise D_{EST} (Jost 2008) in the DEMEtics package (Gerlach et al. 2010) implemented in R. We obtained confidence intervals and significance values by performing 1000 bootstrap replicates.

We estimated fine-scale genetic structure among basins using spatial autocorrelation analysis in the program GENEALEX. This analysis allows relating individual pairwise genetic distances, estimated through the autocorrelation coefficient (r) proposed by Smouse and Peakall (1999), with the geographical distances of individuals that fall within a specified distance class (Epperson 2005; Smouse et al. 2008). We used 10 km as the size distance class, 10,000 permutations and 1,000 bootstrap replicates to estimate 95% confidence intervals (CI) around r at each distance class.

Migration estimates

We used a Bayesian assignment method implemented in GENECLASS v.2.0 (Piry et al. 2004) to identify putative first-generation migrants (individuals that were not born in the sampled population), and their most probably population of origin. We used the L = L_home/L_max likelihood criteria and 1000 simulations (Rannala and Mountain 1997). This criterion is the ratio of the likelihood computed for the population where the individual was sampled (L_home) over the highest likelihood value among samples of all populations, including the population where the individual was sampled (L_max). We also employed the USEPOPINFO option and the MIGPRIOR set at 0.05 in STRUCTURE to identify individuals for which the population of origin was different to the one they were sampled from.

Population size and bottleneck estimations

We assessed population sizes by estimating N_c for each basin using the CAPWIRE package (Pennell et al. 2013) implemented in R. This method is based on the capture-recapture of multilocus genotypes collected from non-invasive genetic sampling, during single or multiple sampling sessions and performs well for small populations (< 100 individuals). CAPWIRE considers that individuals may be sampled multiple times per sampling session and uses the assumption of individual replacement. For this analysis, we considered all the performed surveys as a single sampling session. We first obtained the maximum likelihood estimate (MLE) of the population size, using the 'equal capture model' (ECM) and then estimated the 'two-innate rates model' (TIRM). The later model assumes heterogeneity in the capture probabilities that are related to sex, age and social status of the individuals (Miller et al. 2005). This is particularly important for otters as the scent-marking behavior differs among sexes and age, affecting the number of feces present in the studied area (Arrendal et al. 2007). To select between ECM and TIRM models, we conducted a likelihood ratio test (LRT) and performed 100 parametric bootstraps to estimate 95% confidence intervals for the MLE. We also estimated the density of otters by dividing the number of detected individuals by the total number of kilometers surveyed in each basin (Actopan: 45 km, La Antigua: 64 km and Jamapa: 105 km) and the total studied area (214 km).

We also estimated N_e for each basin employing the linkage disequilibrium information method (LD; Waples and Do 2008) implemented in NeESTIMATOR v.2.0 (Do et al. 2014). NeESTIMATOR and LDne (Waples and Do 2008) are the most robust and accurate single sample genetic estimators of N_e (Gilbert and Whitlock 2015). We chose NeES-TIMATOR over LDne as the former allows to set a minimum allele frequency cutoff value to estimate N_e, minimizing the effect of the presence of rare alleles on the estimations (Do et al. 2014). NeESTIMATOR is based on the fact that as N_e decreases, genetic drift and inbreeding generate nonrandom associations among alleles at different loci (Waples 1991; Luikart et al. 2010). We processed the data as a "single sample time" because our sample collection is likely to contain a single generation based on previous reports on *Lutra lutra* and *Lontra canadensis*, for which one generation comprises between 2 and 2.5 years (Kruuk 2006; Reed-Smith 2008). We assumed random mating and screened out alleles with frequencies below 0.02.

We inferred the possibility that the Neotropical otter underwent bottlenecks in the software BOTTLENECK v.1.2.02 (Piry et al. 1999), using the two-phase mutation model (TPM), and the Wilcoxon signed-rank test with 1000 replicates. This algorithm evaluates whether the expected heterozygosity of a population is larger than the expected

heterozygosity under drift-migration equilibrium (Luikart et al. 1998).

Results

Genotyping quality control, individual identification and microsatellite power of discrimination

We collected a total of 130 spraints and anal secretion samples along the three river basins. DNA amplification of these samples in at least nine loci, yielded a successful amplification rate of 42%. Genotyping error per locus ranged from 0 to 0.19, with a mean across loci of 0.08 (La Antigua), and 0.09 (Actopan and Jamapa) (Table 1). We did not detect allelic dropout at any loci. We obtained 60 genotypes (54 from non-invasive samples and six from the captive individuals). Among the genotypes of non-invasive samples ALLEMATCH identified 49 unique multilocus genotypes (i.e. individuals). Twelve individuals were from La Antigua, 25 from Actopan, and 18 (including the six captive individuals) from Jamapa. We detected samples of three individuals in different surveys (i.e. recaptures); all recaptures were in the same basin in which individuals were captured. In Jamapa, one individual was recaptured once, and in Actopan one individual was recaptured once and another individual was recaptured in two different occasions. The geographic distance between the position of the

Table 1 Genetic diversity of the Neotropical otter per basin at each microsatellite locus and combining all 11 loci

Locus	La Antigua Basin ($n=12$)										Actopan Basin ($n=25$)									
	N	Na	AR	PAR	ENA	H_O	H_E	F_{IS}	FNA	GE	N	Na	AR	PAR	ENA	H_O	H_E	F_{IS}	FNA	GE
RIO2	11	3	2.82	0.51	1.97	0.55	0.49	−0.06	−0.04	0.11	24	4	2.97	0.42	1.60	0.29	0.38	0.24	0.06	0.13
RIO11	11	3	3.00	0.01	1.77	0.36	0.43	0.21	0.05	0.10	25	6	4.68	0.55	3.43	0.44	0.71	**0.40**	0.16	0.10
Lolo13	11	6	5.40	1.81	2.05	0.45	0.51	0.16	0.04	0.17	23	10	7.00	2.71	5.29	0.65	0.81	0.22	0.09	0.09
Lolo18	11	4	3.79	0.23	2.60	0.64	0.62	0.01	−0.01	0.14	25	5	4.05	0.91	2.48	0.48	0.60	0.22	0.07	0.03
Lolo19	9	4	4.00	0.63	2.35	0.56	0.57	0.09	0.01	0.08	20	6	4.35	0.99	3.03	0.45	0.67	0.35	0.13	0.17
Lolo29	12	6	5.43	0.94	2.88	0.75	0.65	−0.11	−0.06	0.09	25	8	4.55	0.69	2.44	0.60	0.59	0.00	−0.01	0.04
Lolo30	11	4	3.97	0.01	3.51	0.55	0.71	0.28	0.10	0.04	21	4	3.82	0.00	3.08	0.38	0.68	**0.46**	0.18	0.13
Lolo37	12	5	4.24	0.98	1.71	0.42	0.42	0.04	0.00	0.03	24	3	2.95	0.03	2.06	0.38	0.51	0.29	0.09	0.03
Lolo39	10	3	2.90	0.51	1.36	0.30	0.27	−0.08	−0.03	0.00	20	6	4.45	0.73	2.38	0.50	0.58	0.16	0.05	0.05
Lolo41	12	5	4.98	0.40	4.36	0.92	0.77	−0.15	−0.08	0.14	25	5	4.64	0.10	3.47	0.60	0.71	0.18	0.07	0.08
Lolo48	10	4	3.80	0.25	2.04	0.60	0.51	−0.13	−0.06	0.04	24	7	5.66	1.62	3.81	0.42	0.74	**0.45**	0.18	0.14
Overall	11	4.27	4.03	0.57	2.42	0.55	0.54	0.03	–	0.08	23	5.82	4.46	0.8	3.01	0.47	0.63	**0.28**	–	0.09
SE	0.28	0.33	0.28	0.16	0.27	0.05	0.04	0.04	–	0.02	0.60	0.60	0.24	0.17	0.30	0.03	0.04	0.03	–	0.01

Locus	Jamapa Basin ($n=18$)									
	N	Na	AR	PAR	ENA	H_O	H_E	F_{IS}	FNA	GE
RIO2	13	3	2.99	0.74	2.05	0.38	0.51	0.29	0.08	0.15
RIO11	14	5	4.60	0.66	3.04	0.64	0.67	0.08	0.02	0.19
Lolo13	18	11	8.56	3.81	6.82	0.83	0.85	0.05	0.01	0.17
Lolo18	18	7	5.86	0.95	3.31	0.56	0.70	0.23	0.08	0.05
Lolo19	17	7	4.90	1.31	2.88	0.29	0.65	**0.57**	0.22	0.06
Lolo29	18	8	6.23	1.25	4.73	0.67	0.79	0.18	0.07	0.08
Lolo30	17	4	3.96	0.01	3.40	0.41	0.71	0.44	0.17	0.11
Lolo37	16	6	5.28	1.29	3.66	0.25	0.73	**0.67**	0.28	0.03
Lolo39	18	4	3.84	0.58	2.08	0.39	0.52	0.28	0.09	0.00
Lolo41	18	8	6.39	1.76	4.32	0.50	0.77	0.37	0.15	0.00
Lolo48	17	5	4.57	1.06	3.40	0.47	0.71	0.36	0.14	0.13
Overall	17	6.18	5.20	1.22	3.61	0.49	0.69	**0.32**	–	0.09
SE	0.52	0.70	0.37	0.24	0.40	0.05	0.03	0.05	–	0.02

We show the number of total individuals detected in each basin (n), number of individuals that successfully amplified and that were used in the analyses (N), number of alleles per locus (Na), allelic richness (AR), private allelic richness (PAR), effective number of alleles (ENA), observed (H_O) and expected (H_E) heterozygosity, inbreeding coefficient (F_{IS}), frequency of null alleles (FNA) and genotyping error (GE). F_{IS} values in bold indicate significant deviations from HWE after Bonferroni corrections (adjusted P-value $= 0.0023$)

capture-recapture individuals was 0.3 km in Jamapa, 4.3 km for one of the individuals in Actopan, and 7.5 and 10.3 km (a total of 17.8 km) for the first and second recaptures of the other individual (Fig. 1).

P_{ID} and P_{IDSIB} values were 8.180×10^{-11} and 1.391×10^{-04}, respectively, suggesting a high power of these markers to discriminate individuals. Five loci are enough to distinguish between unrelated individuals and 11 to differentiate among siblings with a probability of identity of < 0.0001 (Fig. S1).

Genetic diversity

Genetic diversity estimates per locus and combining the 11 loci are shown in Table 1. All loci were polymorphic, the number of alleles per locus ranged from 3 to 11; the total number of alleles for all loci was 91: 47 were found in La Antigua, 64 in Actopan and 68 in Jamapa. AR per basin varied from 4.04 (La Antigua) to 5.20 (Jamapa), PAR from 0.57 (La Antigua) to 1.22 (Jamapa) and NEA from 2.42 (La Antigua) to 3.61 (Jamapa). H_O and H_E ranged from 0.47 (Actopan) to 0.55 (La Antigua), and from 0.54 (La Antigua) to 0.69 (Jamapa), respectively. AR ($P = 0.007$) and H_E ($P = 0.025$) were significantly different between La Antigua and Jamapa, but H_O did not differ significantly among basins ($P > 0.05$; Fig. 2a–c).

F_{IS} values indicated significant deviations from HWE in Actopan and Jamapa, suggesting inbreeding and/or the presence of null alleles. Further inspection of the loci indicated that F_{IS} values were positive and significant for five loci (RIO11, Lolo19 Lolo30, Lolo37, Lolo48) but deficits

Fig. 2 Mean and standard deviations of genetic diversity of the Neotropical otter in the three studied river basins. **a** Rarefied allelic richness (AR), **b** expected heterozygosity (H_E) and **c** observed heterozygosity (H_O). The stars (***) indicate significant differences (P < 0.01)

for a particular locus were not consistently detected across basins (Table 1). MICROCHECKER suggested the presence of null alleles in these five loci and in locus Lolo41, but the frequency of most of them was lower than 0.20 (Table 1), which is considered not to introduce significant biases in the results (Chapuis and Estoup 2007). The exceptions were loci Lolo19 and Lolo37 in Jamapa, for which the frequency of null alleles was slightly higher than 0.20 (Table 1). We included the five loci (RIO11, Lolo19 Lolo30, Lolo37, Lolo48) in the analyses because if null alleles were causing the deviations from HWE we would expect the other two basins (La Antigua and Actopan) to have homozygote deficits at these loci. To evaluate the possible effect of null alleles in the loci with null allele frequencies higher than 0.20 (Lolo19 and Lolo37), we performed estimates of genetic diversity excluding those loci. Excluding loci Lolo19 and Lolo37 did not bias the results, as genetic diversity estimates with and without these two loci were similar (Tables 1, S2). There was no evidence of LD for any of the 55 paired loci comparisons after the Bonferroni correction (P < 0.0009).

## Population genetic structure

Bayesian clustering analysis in STRUCTURE, including all individuals, showed K = 4 as the most likely number of clusters. This corresponded to the highest value of ΔK (Fig. S2). Individuals from Jamapa and La Antigua formed clusters K1 and K2, respectively; while Actopan individuals were clustered into two additional groups (K3 and K4) (Fig. 3a). Nonetheless, all individuals showed shared ancestry (Fig. 3a). Subsequent analyses for each of the clusters separately did not detect additional substructure in Jamapa and La Antigua (Figs. 3b, S3, S4). However, two genetic clusters were recognized in Actopan (Figs. 3b, S5), supporting the results of the analysis that included all samples. Mixed ancestry was evident in individuals from the two Actopan groups (Fig. 3b). Pie charts of membership scores for each basin suggest that in average the majority of Jamapa individuals were assigned to cluster K1 (blue), while most assignments of La Antigua individuals were to group K2 (green). In Actopan a large proportion of membership assignments were to groups K3 (orange) followed by groups K1 (blue) and K4 (yellow) (Fig. 3c). DAPC identified three genetic clusters (BIC = 3), each one corresponding to a river basin, but some individuals from the three groups overlapped (Fig. 4). To build the plot, ten PCs were retained, constituting 66% of the cumulative variance as predictors of the discriminant analysis.

The three estimators of genetic differentiation (F_{ST}, $F_{ST\text{-}FREENA}$ and D_{EST}) indicated low to moderate significant genetic differentiation among all basins (Table 2). The comparisons between Actopan and La Antigua showed the
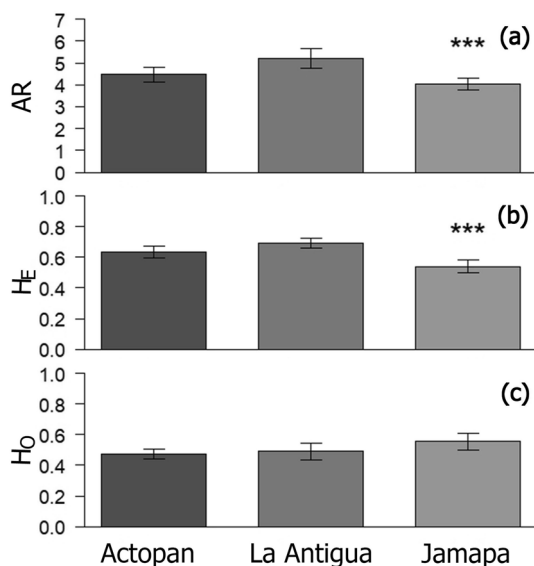
Fig. 3 Assignment of Neo-tropical otter individuals to genetic groups (K) based on 11 microsatellite loci performed in STRUCTURE. **a** Probability of membership of 55 analyzed individuals to belong to an optimal number of genetic clusters K = 4. **b** Probability of belonging to a group when analyzing separately each of the resulting groups from the initial analysis. Different colors indicate the proportion of membership to a genetic group and the basin where the individuals were collected is indicated at the bottom of the graph. **c** Geographic location in the basins of each of the 55 individuals identified from the non-invasive samples, indicating with colors their probability of membership to each of the four inferred genetic clusters of the analysis using the entire dataset. Pie charts show averages of cluster assignments for each basin using the complete data set
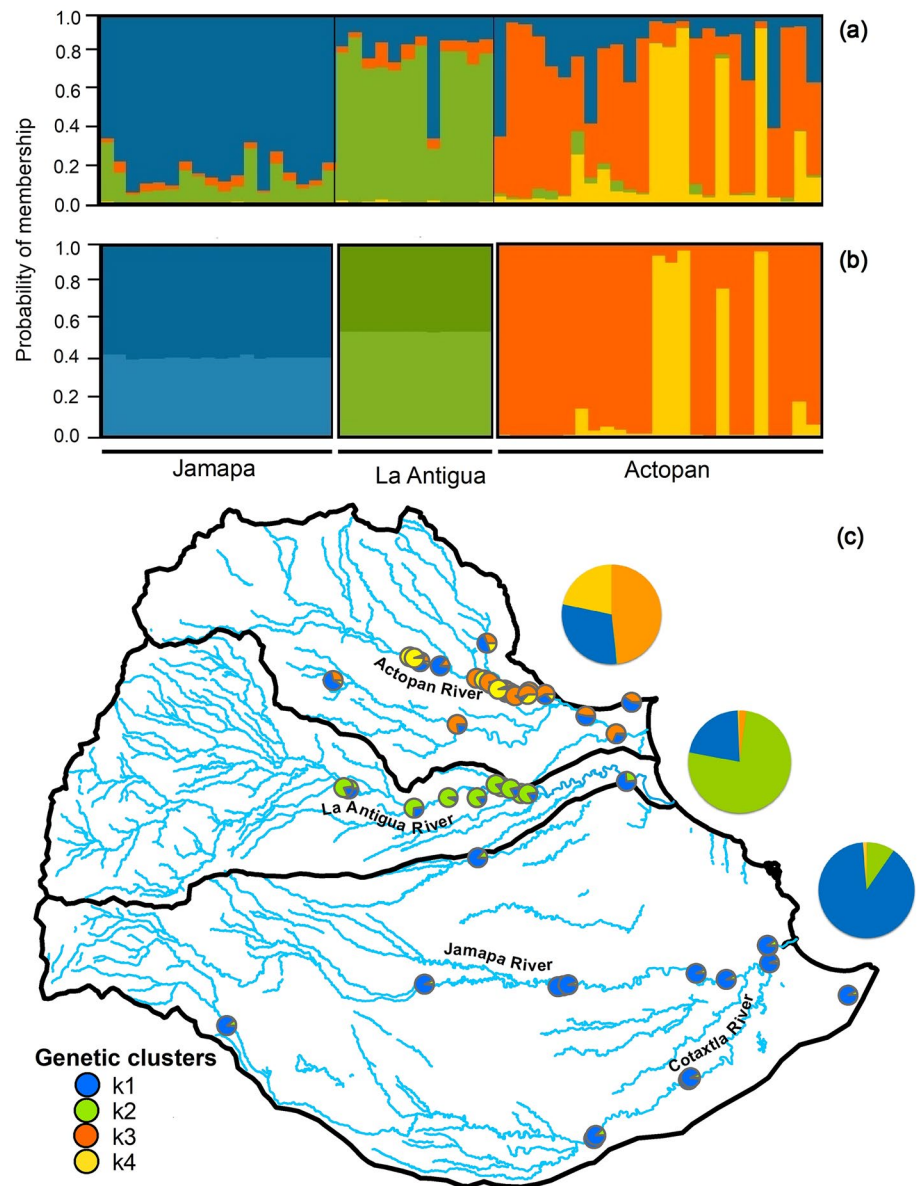

highest degree of differentiation, according to the tree statistics; while La Antigua *vs.* Jamapa comparison showed the lowest, as indicated by D_{EST} and F_{ST} values. Pairwise F_{ST} and $F_{ST\text{-}FREENA}$ values were very similar and lower than D_{EST} values, suggesting that the relatively high frequency of null alleles of loci Lolo19 and Lolo37 is not resulting in an overestimation of population differentiation.

Spatial autocorrelation analysis identified a significant relationship between genetic and geographic distances at the first two distance classes (within 20 km; r = 0.068, P = 0.001). The autocorrelation coefficient (*r*) reached a value of zero at 28 km, suggesting that spatial autocorrelation is lost at such distance (Fig. 5).

Migration estimates

GENECLASS identified 10 individuals (P < 0.001) as putative first-generation migrants (Table 3). A total of five individuals migrated from La Antigua, three of them migrated to Jamapa and two to Actopan. Four individuals migrated from Jamapa, three to Actopan and one to La Antigua; one individual migrated from Actopan to Jamapa. STRUCTURE identified seven first generation migrants (Table 3). Six were individuals collected in Actopan and assigned to genetic clusters K1 (Jamapa) or K4 (one of the Actopan groups), and the other one was collected in La Antigua and assigned to group K3 (one of the Actopan groups). Only two
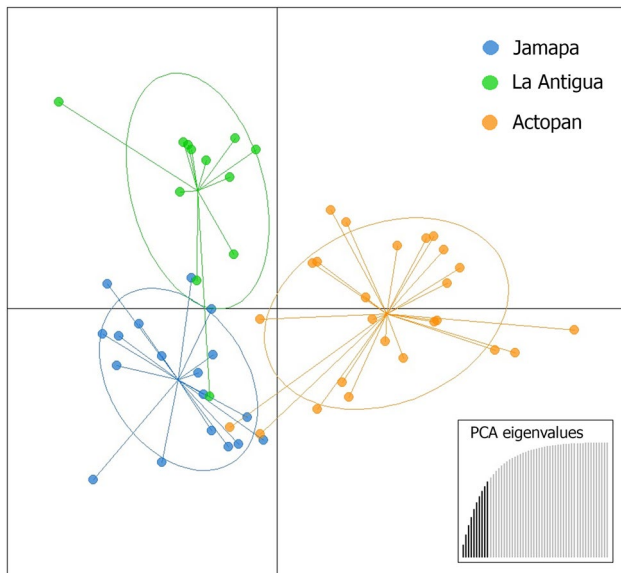
Fig. 4 Scatterplot of discriminant analysis of principal component (DPCA) of the Neotropical otter performed on 11 microsatellites loci. We show genetic clusters in different colors, matching the three basins and the 95% inertia ellipses. Axes correspond to the first two discriminant functions and the circles represent individuals. The lower right inset shows the 10 obtained PCA eigenvalues

Table 2 Pairwise F_{ST}, $F_{ST-FREENA}$ and D_{EST} comparison values among the three river basins based on 11 microsatellite loci. All comparisons were significant (P < 0.001)

Pairwise comparison	F_{ST}	$F_{ST-FREENA}$	D_{EST}
Actopan vs. La Antigua	0.053	0.080	0.159
Actopan vs. Jamapa	0.052	0.043	0.148
La Antigua vs. Jamapa	0.047	0.067	0.136

individuals (A140 and L58) were identified as migrants by the two analyses.

Population size and bottleneck estimation

All N_c estimations fitted the TIRM model ($P = 0$) and calculated 75 individuals in Actopan, 92 in La Antigua and in Jamapa, which sum a total of 259 individuals for the entire studied area (Table 4). According to the 95% confidence intervals N_C values did not significantly differ among basins. The density of *L. longicaudis* was 1.66 individuals km^{-1} in Actopan, 1.44 individuals km^{-1} in La Antigua and 0.88 individuals km^{-1} in Jamapa. The total density of the otter in the three basins was 1.21 individuals km^{-1}, corresponding to 259 individuals in 214 km of rivers. N_e estimates significantly differed among basins, with values being 19.6 for Actopan, 6.4 for La Antigua, and 37.3 for Jamapa (Table 4). According to the Wilcoxon test, we did not find evidence of bottlenecks under the two phase mutation model (Tables 4, S3).

Discussion

Genetic diversity

Genetic diversity of the Neotropical otter was moderate (Mean: $H_O = 0.50$, $H_E = 0.62$, AR = 4.56) and comparable to values reported for the conserved Lacantun river in Mexico (Ortega et al. 2012) and for the human-perturbed Paraná river in Argentina (Trigila et al. 2016). However, it was lower than the genetic diversity of otter populations inhabiting the conserved rivers of the Maquiné Valley in Brazil (Trinca et al. 2013). Similar patterns of lower genetic diversity in Mexican than in South American populations of the Neotropical otter have been detected with mitochondrial makers, and have been attributed to the higher river interconnection and degree of conservation of Amazon basins compared to Mexican basins (Guerrero et al. 2015; Hernández et al. 2017).

Fig. 5 Plot of the spatial genetic autocorrelation coefficient (r) of the Neotropical otter individuals from the three river basins using distance classes of 10 km. The upper and lower 95% confidence intervals (CI) are indicated with dashed lines and represent the null hypothesis of a random geographic distribution of otter individuals
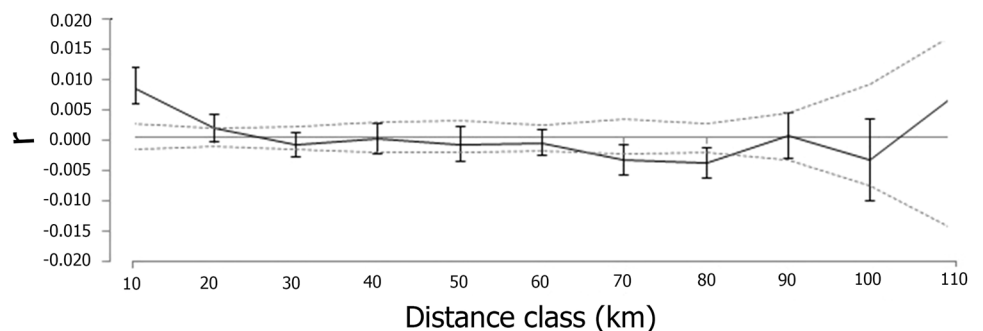
Table 3 Detection of first-generation migrants in the three basins as indicated by STRUCTURE and GENECLASS

Migrant ID	Sampling location	STRUCTURE Q (K = 4)				GENECLASS	
		K1	K2	K3	K4	Log	Origin
		Jamapa	La Antigua	Actopan	Actopan		
J23	Jamapa	–	–	–	–	16.41	La Antigua
J30	Jamapa	–	–	–	–	13.37	Actopan
T1	Jamapa	–	–	–	–	12.91	La Antigua
Tina	Jamapa	–	–	–	–	13.54	La Antigua
L58	La Antigua	0.04	0.16	0.79	0.01	17.23	Jamapa
A8	Actopan	–	–	–	–	15.53	Jamapa
A29	Actopan	–	–	–	–	12.93	Jamapa
A54	Actopan	–	–	–	–	10.67	La Antigua
A128	Actopan	–	–	–	–	8.64	La Antigua
A140	Actopan	0.42	0	0.58	0	16.46	Jamapa
A118	Actopan	0.01	0	0	0.99	–	–
A119	Actopan	0.31	0.01	0.02	0.66	–	–
A121	Actopan	0	0	0	1	–	–
A131	Actopan	0.04	0	0.04	0.92	–	–
A139	Actopan	0	0	0	1	–	–

We include sampling location of Neotropical otter individuals, Q values for each inferred genetic group (K) in STRUCTURE, and the Log (L_home)/(L_Max) significant (<0.001) probabilities estimated in GENECLASS. Origin refers to the genetic group to which migrants were classified

Although it is well known that habitat deterioration can have important impacts on genetic diversity (Keyghobadi 2007), this was not clearly evident in our study since we observed moderate genetic diversity levels. It is plausible that genetic diversity of the Neotropical otter has been maintained through time and that not enough generations have passed to detect detrimental effects of habitat deterioration (Goossens et al. 2004; Wozney et al. 2011; Epps and Keyghobadi 2015). The lifetime of *L. longicaudis* is between 10 and 15 years (Rheingantz and Trinca 2015), which could be enough to generate a time lag that masks the effect of habitat deterioration on genetic diversity. Alternatively, habitat deterioration of the studied river basins may have not reached a threshold value at which drastic reductions of genetic diversity begin, which on average starts when 50% of the habitat has been lost (Pflüger et al. 2019).

Table 4 Census (N_C) and effective (N_e) population sizes of the Neotropical otter in the three river basins with their respective 95% confidence intervals (CI)

Basin	N_c	N_c 95% CI	N_e	N_e 95% CI	Wilcoxon test
Actopan	75	48–100	19.6	13.4–31.4	0.711
La Antigua	92	29–100	6.4	2.7–16.3	0.897
Jamapa	92	29–200	37.3	19.7–139.8	0.289

We also include the P value (for heterozygosity excesses) of the Wilcoxon test to infer bottlenecks, using the two phased mutation model (TPM)

Surprisingly, although Jamapa is the most perturbed basin it had the highest values of genetic diversity (AR and H_E). This could be because its largest area allows maintaining a larger otter population, which is supported by the largest N_e estimates for Jamapa (see below). The lowest genetic diversity was detected in La Antigua and could be the result of the extreme perturbation reported for the basin (Cotler-Ávalos et al. 2010).

Population genetic structure and migration patterns

The genetic structure observed among basins could reflect the hierarchical organization of the rivers, which can promote higher gene flow within than among basins (Meffe y Vrijenhoek 1988; Hughes et al 2009; Selkoe et al. 2015). Similar patterns of genetic structure have been reported in other semi-aquatic species, such as the capibara (Byrne et al. 2015) and the North American beaver (Crawford et al. 2009). In addition to the hierarchical structure of the basins, other landscape characteristics such as highways, the presence of towns or cities and water pollution could be constraining dispersal of *L. longicaudis* as it has been reported in other otter species (Janssens et al. 2008; Carranza et al. 2012; Pagacz 2016). A study on the Eurasian otter reported that individuals cross the drainage divides of basins located at lower altitudes (up to 1300 m a.s.l.) and with lower slopes (a maximum of 45°), resulting in a lack of genetic differentiation (Pagacz 2016). The basins included in our study are

located at altitudes up to 3180 m a.s.l. and have slopes from 0 to 80° (Latorre-Cardenas et al. pers. obs.), which could be limiting gene flow among basins. The three capture-recapture events that we obtained showed that individuals can move between 0.3 and 18 km (Fig. 1), corresponding to the moderate movement capability of the species reported in other studies (7–17 km; Ortega et al. 2012, Trinca et al. 2013). These distances are similar to the 20–25 km that the spatial autocorrelation analysis suggested (Fig. 4). Similar patterns of fine scale genetic structure were detected for the Eurasian otter, with dispersal distances varying between 21 and 38 km (Quaglieta et al. 2013; Pagacz 2016).

The number and identity of first-generation migrants were different between the two algorithms employed. STRUCTU RE identified as migrants the individuals that conformed one of the Actopan genetic groups (K4, yellow group). It is plausible that those individuals are migrating from unsampled basins contiguous (in the North) to Actopan, or that they constitute unsampled genotypes in Jamapa and La Antigua that dispersed to Actopan. Further sampling in basins northern to Actopan as well as in the three studied basins is necessary to distinguish between these possibilities. GENE-CLASS identified a total of 10 first generation migrants and suggests that La Antigua is the basin with the highest number of emigrants and Actopan with the highest number of immigrants. Several studies have reported that poor habitat quality promotes dispersal of individuals to search for better and optimal habitats (Bowler and Benton 2005; Rémy et al. 2011; Honorato et al. 2015). However, otters can disperse to poor habitats as they are not able to identify the presence of pollutants that can potentially have detrimental effect on their health and fitness (Huang et al. 2018). Based on water quality and prey availability, Actopan basin could constitute a better habitat for the Neotropical otter than Jamapa and La Antigua (Macías-Sánchez and Latorre-Cardenas unpublished data). These differences support the higher migration to Actopan from the other two basins. Further studies evaluating dispersal patterns of the species and habitat quality of the three studied basins are necessary to confirm this possibility.

Population size and bottlenecks

Franklin (1980) proposed that a $N_e = 50$ is the minimum size required to avoid inbreeding depression in the short term (five generations), while a $N_e = 500$ is necessary to maintain the evolutionary potential of a population. More recently Frankham et al. (2014) suggested that N_e should be increase to $> 100/1000$ to maintain the genetic variability of populations. N_e of Neotropical otter from the three studied basins are therefore too small to maintain the genetic variation of the populations in the short and long term. It is likely that otter populations are subject to the detrimental effects of

small population sizes, including inbreeding, genetic drift and mutational meltdown (Franklin 1998; Higgins and Lynch 2001; Frankham et al. 2014).

Although we used the LD method, which is considered the most robust and accurate single sample estimator of N_e for small populations (Gilbert and Whitlock 2015), our estimations should be interpreted with caution. LD assumes random mating, lack of population structure and immigration, some of which could have been violated due to the biology of the Neotropical otter (Wang et al. 2016). For example, in this study, we detected migration among populations. Additionally, in order to obtain N_e estimates with biases lower than 10%, it is recommended to use 25–50 samples per population and 10–20 loci when the actual effective population size is small ($N_e \leq 100$; Waples and Yokota 2007; Waples and Do 2010). Population sizes of *L. longicaudis* are expected to be small due to the few offspring per litter (2 to 3 offspring; Gallo-Reynoso 2007) and our samples sizes were lower than those recommended. Therefore, it is likely that the N_e values are underestimated in a 10–50% (Waples and Do 2010; Wang, 2016). Regardless of the biases of N_e estimations for the Neotropical otter, the values for the three basins provide a good initial approximation and indicate that the studied populations are vulnerable to extirpation. Even if we doubled the obtained N_e values (assuming our values are underestimated in a 50%), the estimates would still be lower than those proposed ($N_e > 100/1000$) to maintain the viability of the populations.

Despite that the estimation of N_c is considered important to evaluate the conservation status of the species, few studies have estimated it for *L. longicaudis* using molecular markers (Rheingantz et al. 2017; Aristizabal-Duque et al. 2018). Our estimation of *Nc* for the entire studied area was 259 (75 in Actopan and 92 in La Antigua and Jamapa), which is equivalent to a density of 1.21 individuals km^{-1}. Compared to other studies, the calculated density is similar to that reported for a population of *L. longicaudis* in Brazil (1 otter km^{-1}; Trinca et al. 2013) and lower than the reported for the Lacandona jungle (1.95 individuals km^{-1}; Ortega et al. 2012). The lower density of the Neotropical otter in the studied basins compared to the Lacandona population could be associated with the lower anthropogenic activity and the higher fluvial connectivity in the Lacandona that could result in a better habitat quality and thus in larger populations (Ortega et al. 2012).

The N_c estimations of this study should be interpreted with caution as the confidence intervals were large and most individuals were captured only once (singletons), suggesting that otter populations are larger than reported here (Miller et al. 2005). A more intensive sampling of the three basins is necessary to assure more accurate N_c estimates. The use of non-invasive samples, in combination with molecular markers, have limitations as biases in N_c estimations can

result from amplification and genotyping errors (Janssens et al. 2008). Nevertheless, studies using this approximation have demonstrated that N_c estimations are similar to those obtained with other methods (Mumma et al. 2015; Biaffi et al. 2017; Ferreira et al. 2018). The genotyping error of our study was relatively low (mean = 0.08) and the amplification success (42%) was comparable to that reported in other studies (Ortega et al. 2012; Trinca et al. 2013, 2016). Nonetheless, some of the collected samples that did not amplify could correspond to recaptures or unsampled individuals, leading to the underestimation of N_c.

Even though we did not find signatures of recent bottlenecks, the low estimates of population sizes and genetic differentiation among basins suggest that the Neotropical otter could be experiencing isolation and genetic erosion. Although some migration is apparent, it could not be enough to counteract the detrimental effects of genetic drift. Further genetic erosion and isolation will occur if population size (both N_e and N_c) do not increase or if gene flow is completely interrupted as the result of habitat fragmentation and deterioration (Bouzat 2010). A previous study using mitochondrial DNA suggested that otter populations in Jamapa and La Antigua underwent recent and sudden population expansions with the N_e increasing approximately 200,000–300,000 years ago (Hernández-Romero et al. 2018). This implies that the small population sizes detected in this study are the result of recent reductions. River pollution (Ramos-Rosas et al. 2012) and hunting (Rheingantz and Trinca 2015) could have promoted decreases in the population size of *L. longicaudis*, as it has been suggested for the sea and Eurasian otters (Larson et al. 2002, 2012; Tison et al. 2015; Pigneur et al. 2019).

Conservation implications

In this study we estimated genetic and demographic parameters relevant for the management and conservation of the Neotropical otter in Mexico. Genetic diversity was similar to the reported values for other otter species that are endangered according to the UICN, including the sea otter *Enhydra lutris* ($H_O = 0.49$, $H_E = 0.47$; Gagne et al. 2018) and the giant otter, *Pteronura brasiliensis* ($H_O = 0.56$, $H_E = 0.57$; Pickles et al. 2012). This highlights the importance of monitoring genetic diversity and population sizes of *L. longicaudis* in the short and medium-term to detect possible effects of human activities. It is also essential to determine whether levels of genetic diversity in *L. longicaudis* are enough to maintain the adaptive potential of the species by performing genomic studies to detect loci under selection that are related to traits that allow the adaptation to environmental changes (Beichman et al. 2019; Mable 2019). For instance, Cianfrani et al. (2018), predicted that core areas in the distribution of *L. longicaudis* will be expose to the

negative effects of human impacts, such as illegal poaching, dams, fishing, water contamination, and tourism.

We found differences on genetic diversity among basins with contrasting degrees of habitat deterioration (Cotler-Ávalos et al. 2010). Employing Ecological Niche Modelling, Trigila et al. (2016) suggested that poor habitat suitability, characterized by high human densities, negatively affect the genetic diversity of the Neotropical otter throughout its distributional range. To evaluate those suggested effects, it would be important to conduct a landscape genetic study to explicitly test the effect of different landscape attributes, including natural (slope, altitude, vegetation coverage, density of hydrological networks) and anthropogenic (human density, presence of roads) elements on genetic diversity and connectivity.

The Neotropical otter population of La Antigua is the most threatened of the three basins. Given its small effective population size, it is likely that the genetic diversity cannot be maintained in the short term. In fact, La Antigua showed the lowest genetic diversity and it is under strong anthropogenic pressure that could continue to affect the viability of the population. Reintroduction of individuals from Actopan and Jamapa rivers into La Antigua could help reducing the loss of genetic diversity in the basin. In *L. canadensis*, reintroductions of a large number of individuals over an extended period of time resulted in the increase of genetic diversity and population sizes of the species (Mowry et al. 2015). The apparent better habitat quality of Actopan and its highest levels of gene flow with the other otter populations, highlights the importance of maintaining its connectivity with the other basins. Even though *L. longicaudis* population of Jamapa basin exhibited the highest genetic diversity and population sizes, it is important to monitor these population parameters in the short and medium term as habitat deterioration of the basin can have a negative impact. In conclusion, although genetic diversity of the Neotropical otter populations inhabiting the three river basins is moderate and migration events occur among them, it would be important to implement conservation plans for the populations of the three basins as they continue to be under anthropogenic pressure and thus have a negative effect on the maintenance of genetic variation.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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