UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE BIOLOGÍA

GENÉTICA DE LA CONSERVACIÓN DE LA NUTRIA NEOTROPICAL: PARÁMETROS POBLACIONALES, VARIACIÓN GENÉTICA Y CONECTIVIDAD FUNCIONAL

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS BIOLÓGICAS

PRESENTA:

M. en C. MARÍA CAMILA LATORRE CÁRDENAS

TUTORA PRINCIPAL DE TESIS: DRA. CARLA GUTIÉRREZ RODRÍGUEZ
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Ciudad Universitaria, CD. MX., Agosto, 2020

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POSGRADO CIENCIAS BIOLÓGICAS

UNAM POSGRADO

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ASUNTO: Oficio de Jurado

M. en C. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que en la reunión del Subcomité por Campo de Conocimiento de Biología Experimental y Biomedicina del Posgrado en Ciencias Biológicas, celebrada el día 1° de junio de 2020, se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la alumna **LATORRE CARDENAS MARIA CAMILA** con número de cuenta **516491215** con la tesis titulada: **"Genética de la conservación de la nutria neotropical: parámetros poblacionales, variación genética y conectividad funcional"**, realizada bajo la dirección de la **DRA. CARLA GUTIÉRREZ RODRÍGUEZ,** quedando integrado de la siguiente manera:

Presidente:	DR. ANTONIO GONZÁLEZ RODRÍGUEZ
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Suplente	DR. JESÚS ALEJANDRO ESPINOSA DE LOS MONTEROS SOLIS

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Cd. Universitaria, Cd. Mx., a 05 de agosto de 2020

COORDINADOR DEL PROGRAMA

POSGRADO EN CIENCIAS BIOLOGICAS
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llevaré en el alma.

Ustedes me demuestran que…

"El amor es lo único que somos capaces de percibir que trasciende las dimensiones del tiempo y del espacio"

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RESUMEN

El conocimiento del tamaño de las poblaciones biológicas, su diversidad, estructura y conectividad genética es fundamental para comprender su estado de conservación y potencial evolutivo. La acelerada pérdida de hábitat y los cambios en la configuración del paisaje reducen los recursos y las condiciones que las poblaciones requieren para mantener su diversidad y conectividad genética, incrementado el riesgo de extinción local. La genética de la conservación se centra en el estudio de especies con poblaciones pequeñas y aisladas, o que se encuentran amenazadas, ya que son más susceptibles a los cambios en su ambiente.

La nutria neotropical, *Lontra longicaudis*, es una especie semiacuática que se encuentra amenazada debido al declive de sus poblaciones y al deterioro ambiental de los ecosistemas dulceacuícolas. Por lo cual, es imprescindible estimar parámetros poblacionales y genéticos de la nutria neotropical para formular estrategias de conservación que mantengan los procesos ecológicos y evolutivos que ocurren en sus poblaciones. Los objetivos principales de esta investigación fueron: evaluar el tamaño poblacional, diversidad genética, estructura genética y flujo génico contemporáneo de la nutria neotropical, así como determinar los elementos del paisaje que moldean la estructura genética y el flujo génico a una escala local (parches de hábitat) y del paisaje (configuración de la matriz). El estudio se llevó a cabo en un sistema de tres cuencas hidrográficas: Actopan, La Antigua y Jamapa, en el estado de Veracruz, México. Estas cuencas tienen un alto deterioro ambiental y son prioritarias para la conservación de la biodiversidad en el país. En este estudio se empleó un muestreo no invasivo junto con un conjunto de microsatélites desarrollados *de novo* para *L. longicaudis*.

El tamaño efectivo de las poblacionales de la nutria neotropical fue bajo y la diversidad genética moderada. Ambos parámetros variaron entre cuencas, lo que sugiere diferentes estados de vulnerabilidad de las poblaciones de la nutria. Se encontró estructura genética, la cual es explicada por la jerarquización de los ríos y por la idoneidad del hábitat de la nutria a lo largo del paisaje. A pesar de existir aislamiento genético, el flujo génico entre las poblaciones es mantenido por migrantes de primera generación. A una escala local, la pendiente, el índice de vegetación de diferencia normalizada (NDVI) y el orden de los ríos explicaron la diferenciación genética de la nutria. A una escala de paisaje, la idoneidad del hábitat, la integridad del ecosistema y la acumulación de agua en los ríos facilitaron su flujo génico. La importancia de estas variables para mantener la conectividad funcional de la nutria fue diferente entre las zonas altas, medias y bajas de las cuencas. Estos

resultados tienen implicaciones relevantes para la conservación de la especie, incluyendo que la cuenca de Actopan presenta las áreas más idóneas para mantener la conectividad funcional de la nutria, y que se necesita mejorar la conectividad en Jamapa.

ABSTRACT

Assessing population size, the genetic diversity, genetic structure, and connectivity of natural populations is essential for understanding their conservation status and their evolutionary potential. The accelerated loss of habitat and changes in the configuration of the landscape reduce the resources and conditions necessary for populations to maintain their genetic diversity and connectivity, increasing the risk of local extinction. Conservation genetics focuses on the study of threatened species or with small and isolated populations, since they are particularly susceptible to changes in their environment.

The Neotropical otter, *Lontra longicaudis*, is a semiaquatic species that is threatened with exctinction due to the decline of its populations and the environmental deterioration of freshwater ecosystems. Therefore, estimating demographic and genetic parameters of the Neotropical otter is essential to inform conservation strategies that can maintain the ecological and evolutionary processes. The main objectives of this study were to evaluate the population size, genetic diversity, genetic structure and contemporary gene flow of the Neotropical otter, as well as to determine the elements of the landscape that shape the genetic structure and gene flow of the species *at-local sites* (habitat patches) and *between sites* along the landscape (configuration matrix). This study was carried out in a hydrographical system of three basins: Actopan, La Antigua and Jamapa, located in the state of Veracruz, Mexico. These basins are highly impacted by human activities and are a priority for the conservation of biodiversity in the country. To accomplish these objectives, I carried out a non-invasive sampling, coupled with a set of microsatellite markers developed de *novo* for *L. longicaudis*.

The effective population size of the populations of the Neotropical otter was low and the genetic diversity moderate. Both parameters varied among basins, suggesting different states of vulnerability of otter populations. I detected genetic structure in *L. longicaudis*, which was explained by the hierarchical organization of the streams and the environmental suitability across the landscape. Despite that the Neotropical otter experiences genetic isolation, first-generation migrants maintain gene flow among populations. Slope, the normalized difference vegetation index (NDVI) and stream order were the landscape variables that explained the genetic differentiation *at-local sites*, while environmental suitability, ecosystem integrity and water accumulation of streams enhanced gene flow of the Neotropical otter *between sites*. The importance of these landscape-riverscape variables to maintain the functional connectivity of the otter differed between the upper, middle, and

lower areas of the basins. These results have important implications for the conservation of the species, including the maintenance of larger suitable areas in Actopan and the necessity to improve connectivity in Jamapa.

INTRODUCCIÓN GENERAL

La diversidad genética es la materia prima para la evolución y la adaptación de las poblaciones naturales frente a los cambios ambientales en su hábitat. Varios estudios teóricos y empíricos han demostrado que la diversidad genética se ve reducida en poblaciones con tamaños pequeños (p.ej. Soulé 1976, Frankham 1996, Hague y Routman 2016), ya que los efectos de la deriva génica y la endogamia fijan o eliminan alelos a una tasa más rápida que en poblaciones más grandes (Frankham 1996, Franklin y Frankham 1998, Allendorf et al. 2013). Debido a esto, las poblaciones pequeñas son más vulnerables a los cambios estocásticos demográficos y ambientales (p.ej., pérdida de hábitat, cambio climático), lo cual incrementa su riesgo de extinción local (Crow y Kimura 1970, Frankham *et al*. 2017). La estimación del tamaño poblacional censal (N_c) y efectivo (N_e) son por lo tanto esenciales para el manejo y conservación de poblaciones de especies amenazadas o de las que se sospecha que sus tamaños están en declive. N_c es el número de individuos en una población, y es un parámetro demográfico clave para evaluar el estado de conservación de una especie (Knaepkens et al. 2004, Hague y Routman 2016). N_e es el tamaño de una población ideal, la cual pierde diversidad genética a la misma tasa que lo haría la población considerada (Wright 1939). En otras palabras, N_e es el número de individuos reproductivos que contribuyen al acervo genético de cada generación (Hedrick 2005) y, por ende, es esencial para mantener la diversidad genética y el potencial evolutivo de las poblaciones (Bouzat 2010).

La pérdida de la diversidad genética también puede ser el resultado de la disminución del flujo génico, es decir, el intercambio de genes entre poblaciones que es promovido por la dispersión de los individuos o de sus gametos (Allendorf 2013). La disminución del flujo génico conlleva al aislamiento genético y a la subdivisión de la variación genética, dando lugar a la estructuración genética (Wright 1951, Mayrs 1963). En general, la pérdida y la fragmentación del hábitat tienen efectos negativos sobre los tamaños poblacionales, la diversidad genética y el flujo génico de las poblaciones (Frankham et al. 2002; Manel et al. 2003, Keyghobadi 2007, Frankham 2010, Cushman et al. 2013).

La genética del paisaje se centra en investigar el efecto del paisaje sobre los patrones de diversidad genética y de dispersión de las poblaciones, lo cual es crucial para entender y preservar procesos evolutivos y ecológicos, como la adaptación y la conectividad funcional (Manel et al. 2003, Storfer et al. 2007, Manel et al. 2013). El paisaje es entendido como un mosaico de parches de hábitat,

arreglados en una matriz, la cual contiene elementos que facilitan o impiden la dispersión (Taylor et al. 1993). El paisaje puede analizarse como una red de nodos y enlaces, en donde los *nodos* son los parches de hábitat que contienen los recursos y las condiciones necesarias para el crecimiento y reproducción de los individuos; y los *enlaces* están conformados por los elementos de la matriz que favorecen o limitan el movimiento de los individuos entre parches de hábitat (Tischendorf y Fahrig 2000, Ewers y Didham 2006). Mientras que la pérdida de hábitat usualmente reduce el tamaño de los parches, la fragmentación suele incrementar la distancia entre los parches de hábitat (Kindlmann y Burel 2008, DiLeo y Wagner 2016). Tanto la pérdida de hábitat como la fragmentación reducen la conectividad funcional del paisaje, es decir, el grado en que los elementos del paisaje permiten el movimiento de los individuos y mantienen el flujo génico (Fahrig 2005, Cushman et al. 2013, Auffret et al. 2015). Tradicionalmente, la conectividad funcional se ha evaluado midiendo la influencia de los elementos de la matriz del paisaje sobre la dispersión de los individuos. Sin embargo, la inclusión del efecto de las características locales en los parches de hábitat permite tener un mejor entendimiento de los eventos de dispersión y conectividad. Esto sucede porque localmente estos elementos pueden atraer o repeler a individuos migrantes que están en la búsqueda de hábitats donde establecerse, y últimadamente afectar la estructura genética de las poblaciones (Murphy et al. 2010, Dyer et al. 2012).

Existen diferentes modelos empleados por la genética del paisaje para poner a prueba el efecto de los elementos que componen la matriz del paisaje sobre la estructura y flujo génico de las especies. El aislamiento por distancia (IBD por sus siglas en inglés; Wright 1943), postula que la diferenciación genética entre poblaciones o individuos incrementa con la distancia geográfica. Este modelo no considera el efecto de los elementos del paisaje, y por ello se emplea como el modelo nulo en los estudios de genética del paisaje. Por el contrario, el modelo de aislamiento por resistencia (IBR, por sus siglas en inglés), propone que la diferenciación genética incrementa con la resistencia que generan los elementos del paisaje, es decir el costo al movimiento que representa para los individuos (McRae 2006). Poner a prueba ambos modelos nos permite identificar si los elementos del paisaje tienen una mayor influencia sobre la conectividad funcional, a comparación de la distancia geográfica.

La conectividad funcional de especies semiacuáticas que habitan en ecosistemas dulceacuícolas ha sido poco estudiada, en parte por la complejidad de incluir elementos del paisaje terrestre, ribereño y acuático, por ejemplo, la organización espacial de las redes de ríos, y la variabilidad espacial y temporal de algunas características de los ríos (Selkoe et al. 2016; Rico 2019).

Los paisajes dulceacuícolas se caracterizan por tener una estructura de redes de ríos que están organizadas jerárquicamente dentro y entre cuencas. Este arreglo jerárquico se refiere a la anidación de secciones de río dentro de ríos, los cuales se agrupan en sub-cuencas, y éstas últimas en cuencas (Davis et al. 2018). Estos paisajes también están fuertemente influenciados por el flujo unidireccional de las corrientes de agua y la cantidad de agua que contienen los ríos (Selkoe *et al*. 2016, Paz-Vinas y Blanchet 2015, Thomaz et al. 2016). Se han propuesto diferentes modelos para explicar el flujo génico de las especies que habitan ecosistemas dulceacuícolas (Hughes et al. 2003, 2013). El modelo de jerarquización de ríos (SHM, por sus siglas en inglés) es el más común y plantea que el arreglo jerárquico (anidamiento) de los ríos o ramas dendríticas, afecta la estructuración genética, puesto que un menor anidamiento implica mayor conectividad entre las secciones del río (Meffe y Vrijenhoek 1988). La elevación también afecta el anidamiento de las ramas dendríticas y puede generar un flujo génico diferencial entre las partes altas y bajas de las cuencas (Selkoe et al. 2016). Sin embargo, el efecto de la estructura de las redes de ríos sobre el flujo génico de los individuos dependerá de la capacidad de dispersión de éstos (Hugues et al. 2009, 2013). Por tanto, se espera que en especies que tienen una baja movilidad terrestre o que están restringidas al ambiente acuático (p.ej. peces, anfibios, reptiles y macro-invertebrados), la estructuración genética ocurra dentro y entre cuencas (Todd et al. 2014, Pisa et al. 2015, Unger et al. 2013, Cole et al. 2016, Brauer et al. 2016). En el caso de especies que tienen una capacidad de dispersión moderada a alta, tanto en tierra como en el medio acuático (p. ej. mamíferos pequeños y medianos), se espera que la estructura genética ocurra a escalas espaciales más grandes (sistemas de cuencas; McGlashan y Hugh 2002, Hopken et al. 2013). La evaluación de la conectividad funcional en ecosistemas dulceacuícolas es fundamental, ya que estos ecosistemas se encuentran entre los más amenazados a causa del deterioro del hábitat y el cambio climático (Storfer et al. 2010; Poff et al. 2012, Martínez-Meyer et al 2014).

La nutria neotropical, *Lontra longicaudis*, es una especie semiacuática que tiene un papel ecológico como depredador tope y especie sombrilla en los sistemas dulceacuícolas (Larivière 1999). A pesar de su amplia distribución geográfica, desde el norte de México hasta el norte de Argentina, se encuentra clasificada por la Unión para la Conservación de la Naturaleza (UICN) como una especie "casi amenazada". Esto se debe al deterioro de su hábitat, y al declive de sus poblaciones (Rheingantz y Trinca 2015). Se estima que sus poblaciones sufrirán una reducción del 25% durante los próximos 30 años (Pacifici et al. 2013). En México, la nutria se distribuye en las cuencas de las vertientes del Pacífico y del Golfo de México (Gallo-Reynoso 1997), y se encuentra clasificada como una especie amenazada debido a la contaminación, la sobreexplotación pesquera y a la deforestación en su

hábitat (NOM-059 SEMARNAT 2010; Gallo-Reynoso 2007). Pese a su estado de conservación, no hay estudios que estimen el tamaño poblacional efectivo. Una aproximación para evaluar los tamaños censales de la nutria ha sido mediante métodos de conteo de rastros (principalmente heces), los cuales sugieren rangos de abundancia relativa de 0.3 a 2 nutrias por km^{-1} (Lariviere 1999, Casariego-Madorell et al. 2006, Arellano et al. 2012, González-Christen et al. 2013). Mediante el uso de marcadores moleculares y la técnica de captura-recaptura de genotipos, se ha reportado una abundancia de 2 individuos por km^{-1} en ríos de una región conservada de la Selva Lacandona (Ortega et al. 2012). La combinación de métodos moleculares y de muestreos no-invasivos (p.ej., colecta de heces, secreciones anales, pelos) ha demostrado alcanzar estimaciones confiables de los tamaños poblacionales, y ser más eficiente en tiempo y costo que otros métodos, como el conteo directo de individuos o el uso de cámaras trampa (Mumma et al. 2015, Biffi et al. 2017, Ferreira et al. 2018).

De forma similar, pocos estudios han evaluado aspectos genéticos de *L. longicaudis* en México (Ortega et al. 2012, Guerrero et al. 2015, Hernández et al. 2017) y dos de estos han utilizado marcadores mitocondriales, que proporcionan información sobre la historia evolutiva de la especie. Únicamente el estudio de Ortega y colaboradores (2012) ha empleado marcadores microsatélites, los cuales proporcionan información sobre patrones contemporáneos de las poblaciones. En dicho estudio se reportó una diversidad genética moderada (*Ho* = 0.55) y ausencia de estructura genética en el sistema hidrológico del río Lacantún, Chiapas. Otros estudios a una escala regional en Suramérica reportan valores de diversidad genética contemporánea de bajos a altos (*Ho* = 0.322 - 0.83; Weber et al. 2009, Trinca et al. 2013, Trigila et al. 2016).

La conectividad funcional y los patrones de dispersión de la nutria Neotropical han sido poco estudiados. A partir de estudios moleculares, se sabe que la nutria puede moverse entre 3 y 17 km dentro de los ríos (Ortega et al. 2012, Trinca et al. 2013), lo que sugiere una capacidad de dispersión moderada. Se han reportado movimientos de hasta 40 km para la nutria euroasiática, *Lutra lutra*, y para la nutria de río de Norteamérica, *Lontra canadensis* (Janssens et al. 2008, Spinola et al. 2008). Aunque no se ha evaluado directamente la influencia del paisaje sobre los patrones genéticos de la nutria neotropical, algunos elementos del paisaje han sido asociados con el uso del hábitat y con eventos de recolonización en varias especies de nutrias. Los ríos son elementos del paisaje indispensables para las actividades diarias de la nutria, y se ha observado que los ríos más grandes y con una mayor cantidad de agua, son preferidos por la nutria, ya que tienen mayor disponibilidad de presas (Ruiz-Olmo 1998, Holland y van der Merwe 2016, Smith et al. 2020). Además, los ríos facilitan

el desplazamiento de la nutria, ya que ésta es más ágil en agua que en tierra (Kruuk 2006). La vegetación ribereña conservada y densa provee de refugios para el descanso y la reproducción, y también brinda cobertura de protección cuando las nutrias se desplazan por tierra (Mayor-Victoria y Botero-Botero 2010, Loy et al. 2009). Los atributos topográficos de los ríos y de las zonas terrestres que están contiguas a los ríos, como la elevación y la pendiente, pueden limitar el flujo génico (Janssens et al. 2008). Algunos elementos antropogénicos del paisaje, como la contaminación, el cambio de uso del suelo, la presencia de asentamientos humanos y caminos, tienen efectos negativos sobre las poblaciones de las nutrias (Ramos-Rosas et al. 2013, Rheingantz et al. 2014, Trigila et al. 2016). La idoneidad del hábitat es otra característica del paisaje que puede influir la conectividad funcional, ya que ésta refleja la presencia local de condiciones favorables para la supervivencia y el éxito reproductivo de los individuos (Drake y Richards 2018, Lunghi et al. 2018). A lo largo de la distribución geográfica de *L. longicaudis* se reportó una relación positiva entre la diversidad genética y la idoneidad ambiental (Trigila et al. 2016). Para la nutria euroasiática se ha documentado que la alta conexión entre los ríos, la cobertura ribereña conservada, la baja densidad humana y de carreteras, así como pendientes bajas y moderadas son elementos del paisaje que mantienen la conectividad estructural de la especie (Loy et al. 2009, Carranza et al. 2012, Van Loy et al. 2013, 2014).

Los objetivos principales de este estudio fueron: 1) estimar el tamaño poblacional (N_e y N_c) de la nutria neotropical y sus patrones de diversidad genética, estructuración genética y flujo génico, y 2) determinar los elementos del paisaje que promueven o limitan la conectividad funcional de la especie. El estudio se llevó a cabo en un sistema de tres cuencas hidrográficas que presenta un alto deterioro ambiental: Actopan, La Antigua y Jamapa, ubicadas en el estado de Veracruz, México. Dichas cuencas son consideradas prioridades para la conservación, puesto que albergan una alta biodiversidad, pero al mismo tiempo se caracterizan por tener uno de los valores más bajos de calidad e integridad ecológica en el país, lo cual denota una alta intensidad de actividades humanas a lo largo del paisaje (Cotler et al. 2010, Equihua et al. 2014, Mora 2019). El alto deterioro ambiental sugiere que la calidad del hábitat de la nutria neotropical debe ser baja en este sistema de cuencas, por lo que se espera que el tamaño poblacional, la diversidad genética y el flujo génico sean bajos.

El estudio se divide en cuatro capítulos. En el primer capítulo se describen el tamaño poblacional censal y efectivo, la diversidad y estructura genética, y los eventos de migración contemporánea de la nutria neotropical en las tres cuencas estudiadas. En el segundo capítulo, se evalúa el efecto del paisaje sobre los patrones de estructura genética y flujo génico de la nutria; y se

identifican los elementos de los parches de hábitat y de la matriz del paisaje que facilitan o limitan la conectividad funcional de la especie. En el capítulo tres se describe el aislamiento y la caracterización de trece nuevos loci de microsatélites, desarrollados específicamente para *L. longicaudis*, empleando técnicas de secuenciación de la siguiente generación. Con estos marcadores se obtuvo la información genética de la nutria de este estudio. El cuarto capítulo comprende el diseño de marcadores moleculares que permiten identificar el sexo de individuos de *L. longicaudis*, a partir de un fragmento del gen zinc finger, ZFY/ZFX. Los microsatélites y los marcadores para el sexado constituyen el desarrollo de herramientas moleculares que facilitan la obtención de datos genéticos para estudiar la variación genética, el comportamiento, la ecología y evolución de la nutria, la cual es información clave para su conservación.

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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.,

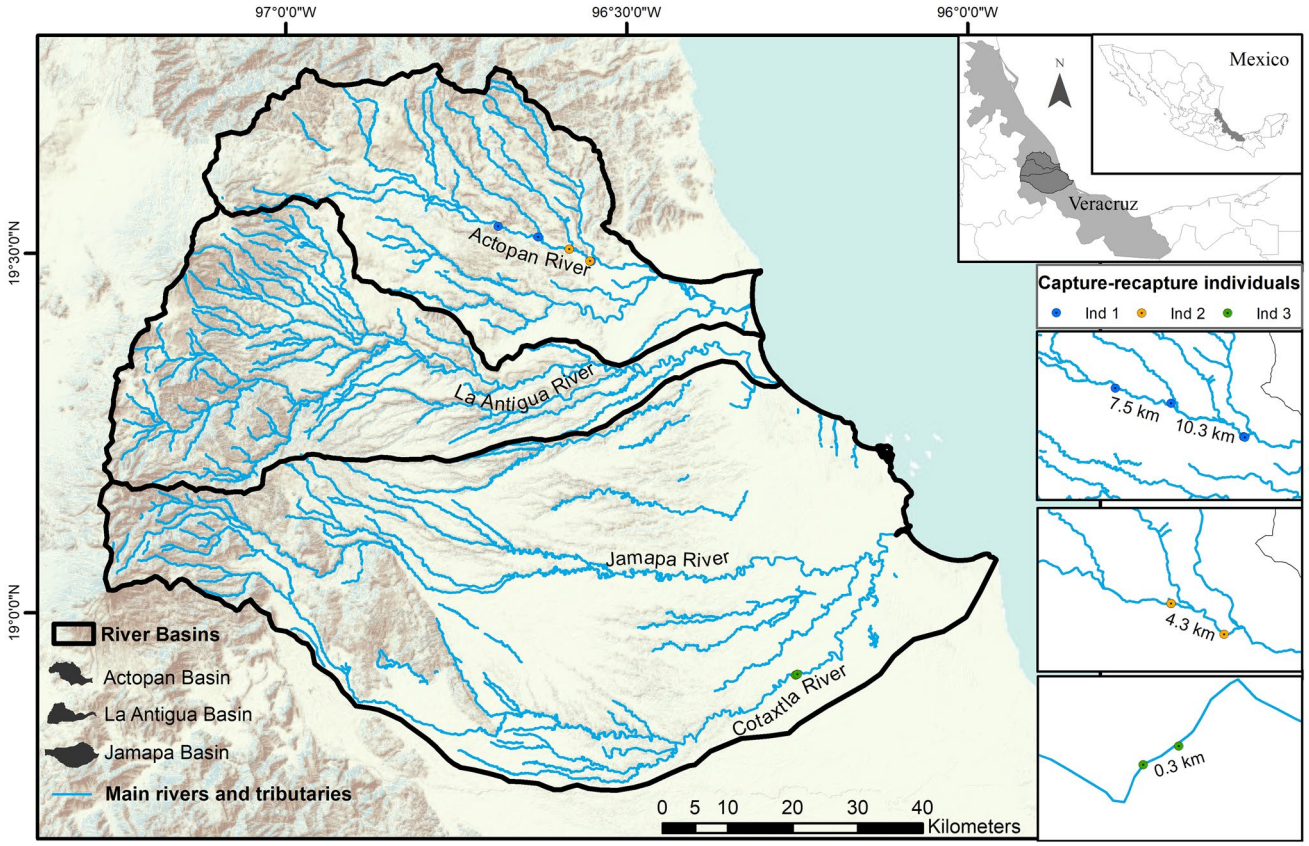

Fig. 1 Map of the studied area including the rivers and tributaries of the three basins where the sampling of the Neotropical otter was conducted, and the location of the studied area within the state of Veracruz in Mexico (inset in the top right). Color dots in the map indicate the position of the samples 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 STRU CTURE 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 NeESTIMATOR 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 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 ($P < 0.0009$).

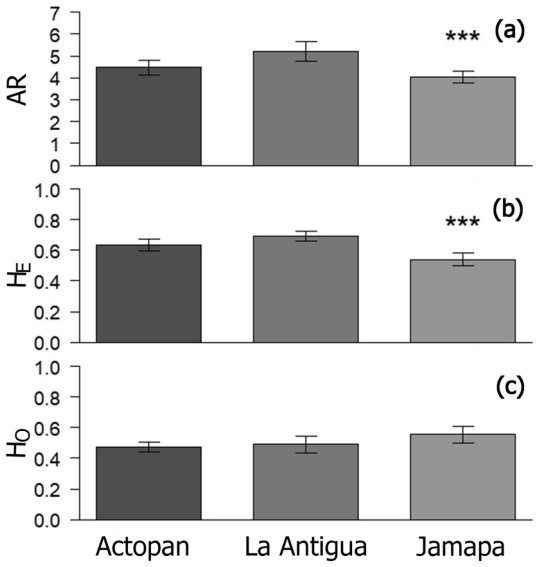

Fig. 2 Mean and standard deviations of genetic diversity of the Neotropical otter in the three studied river basins. **a** Rarefied allelic richness (AR), **b** expected heterozygosity (H_E) and **c** observed heterozygosity (H_O). The stars (***) indicate significant differences ($P < 0.01$)

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

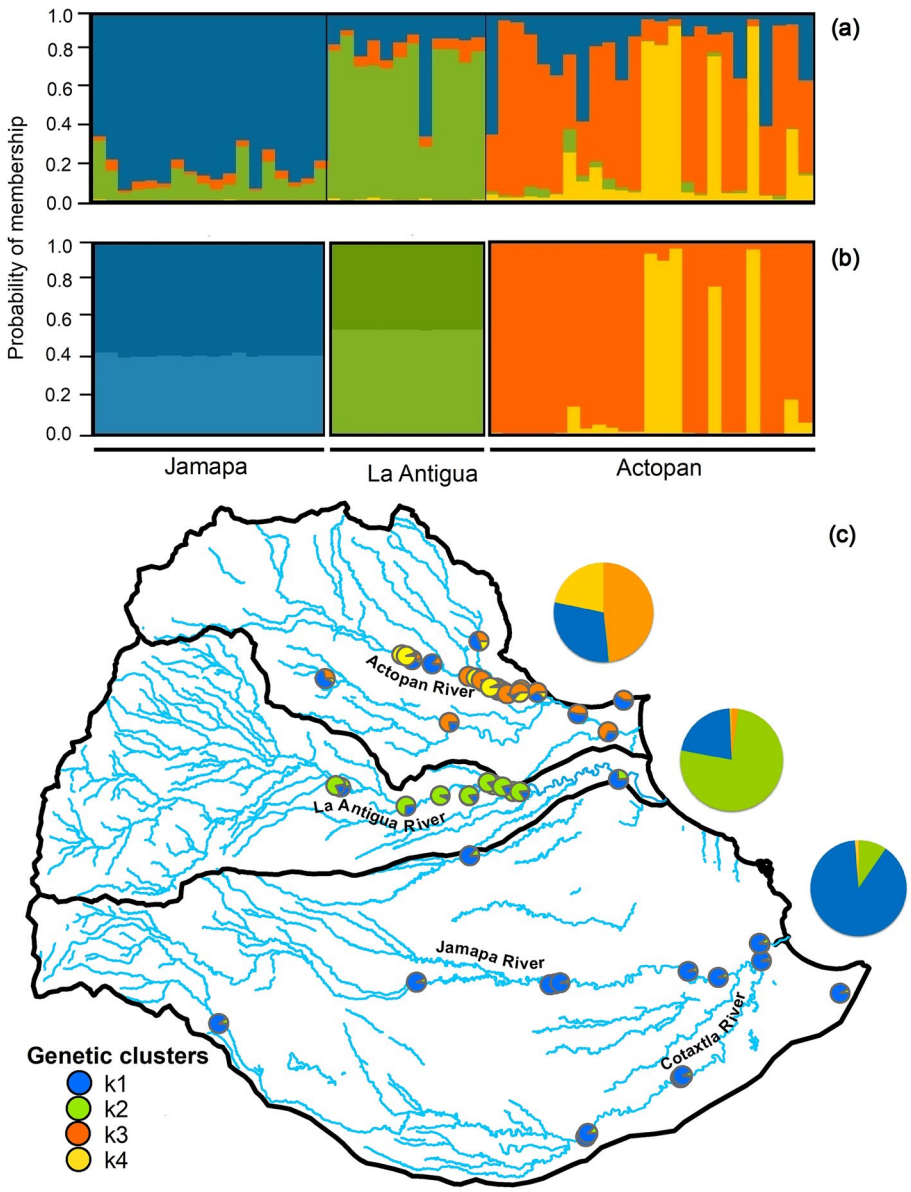

Fig. 3 Assignment of Neotropical otter individuals to genetic groups (K) based on 11 microsatellite loci performed in STRUCTURE. **a** Probability of membership of 55 analyzed individuals to belong to an optimal number of genetic clusters K = 4. **b** Probability of belonging to a group when analyzing 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

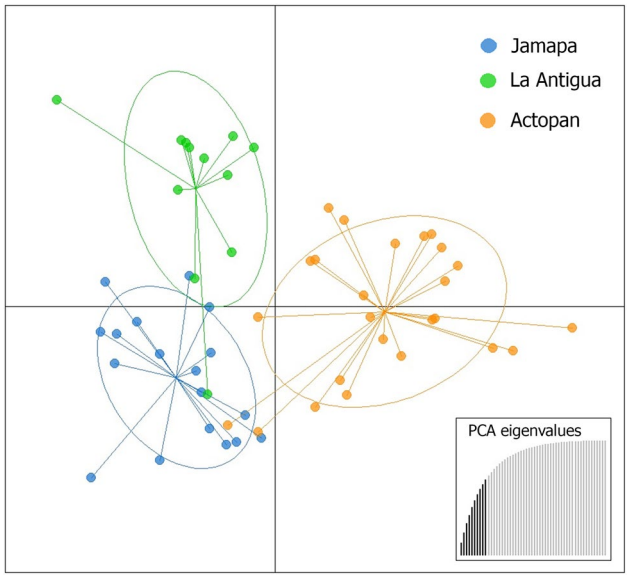

Fig. 4 Scatterplot of discriminant analysis of principal component (DPCA) of the Neotropical otter performed on 11 microsatellites loci. We show genetic clusters in different colors, matching the three basins and the 95% inertia ellipses. Axes correspond to the first two discriminant functions and the circles represent individuals. The lower right inset shows the 10 obtained PCA eigenvalues

Table 2 Pairwise F_{ST}, $F_{ST\text{-}FREENA}$ 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}	$F_{ST\text{-}FREENA}$	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).

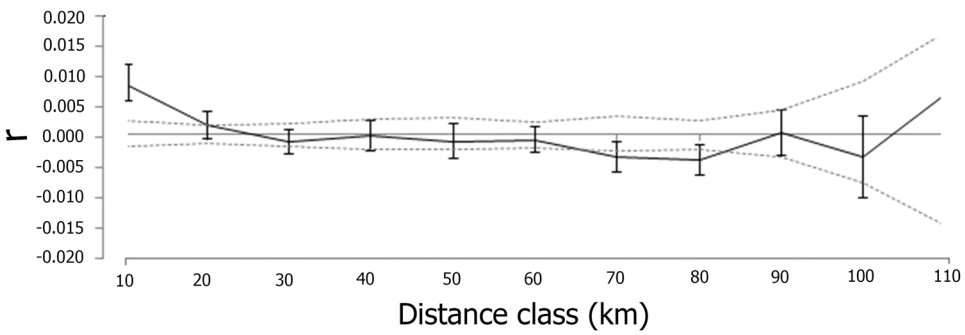

Fig. 5 Plot of the spatial genetic autocorrelation coefficient (*r*) of the Neotropical otter individuals from the three river basins using distance classes of 10 km. The upper and lower 95% confidence intervals (CI) are indicated with dashed lines and represent the null hypothesis of a random geographic 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. STRUCTURE 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. GENECLASS 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.

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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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Electronic supplementary material

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

Conservation genetics

Materials and methods

DNA extraction and PCR amplification conditions

To avoid contamination, before conducting DNA extractions we sterilized all materials (tips, tubes, pipets and double distilled water) and performed extractions under a hood with laminar flow previously sterilized with ultraviolet light. We included a negative control consisting of sterilized doubled distilled water in every batch of DNA extractions to monitor contamination. We always kept DNA extractions separated from amplification products.

To avoid contamination, before performing PCR reactions we sterilized all materials (tips, tubes, pipets and double distilled water) and carried them out in an ultraviolet PCR workstation. PCR amplifications consisted on a final volume of 10 µL containing 1X PCR buffer (Promega), 2.0 mM $MgCl_2$ (in RIO loci reactions) and 1.8 or 2.0 mM $MgCl_2$ (in Lolo loci reactions, see Latorre-Cardenas et al. 2019), 0.25 mM dNTPs, 0.1 µM of a forward primer labeled with a CAG universal tail (CAG tail 5'-CAGTCGGCGTCATCA-3'), 0.15 µM of reverse primer, 0.15 µM of a fluorescently-labeled (with either HEX, FAM or NED) CAG primer, 0.75-1 U GoTaq DNA Polimerasa (Promega) and 2 µL of genomic DNA. We included a negative control, containing all of the above reagents but instead of genomic DNA we added double distilled water. Amplifications were performed in a Veriti™ thermocycler (Thermofisher) using the following conditions: 94°C for 2 min, followed by five cycles with a touchdown (−1 °C per cycle) at 94°C for 45 s, 60–56°C for 45 s and 72°C for 1.5 min; and a second phase of 40 cycles at 94°C for 45 s, 55°C (for RIO loci reactions) and 54°C–57°C (for Lolo loci reactions, see Latorre-Cardenas et al. 2019) for 45 s, 72°C for 1.5 min and a final extension at 72 °C for 5 min.

Table S1. Multiplex panels consisting of three microsatellite loci each one. We indicate the dye of the fluorescently labeled CAG primer.

Panels	Microsatellite array	Dye
A	RIO11	NED
	Lolo18	FAM
	Lolo37	HEX
B	Lolo29	NED
	Lolo48	FAM
	Lolo30	HEX
C	Lolo13	NED
	Lolo41	FAM
	Lolo1	HEX
D	RIO2	FAM
	Lolo39	NED
	Lolo19	HEX

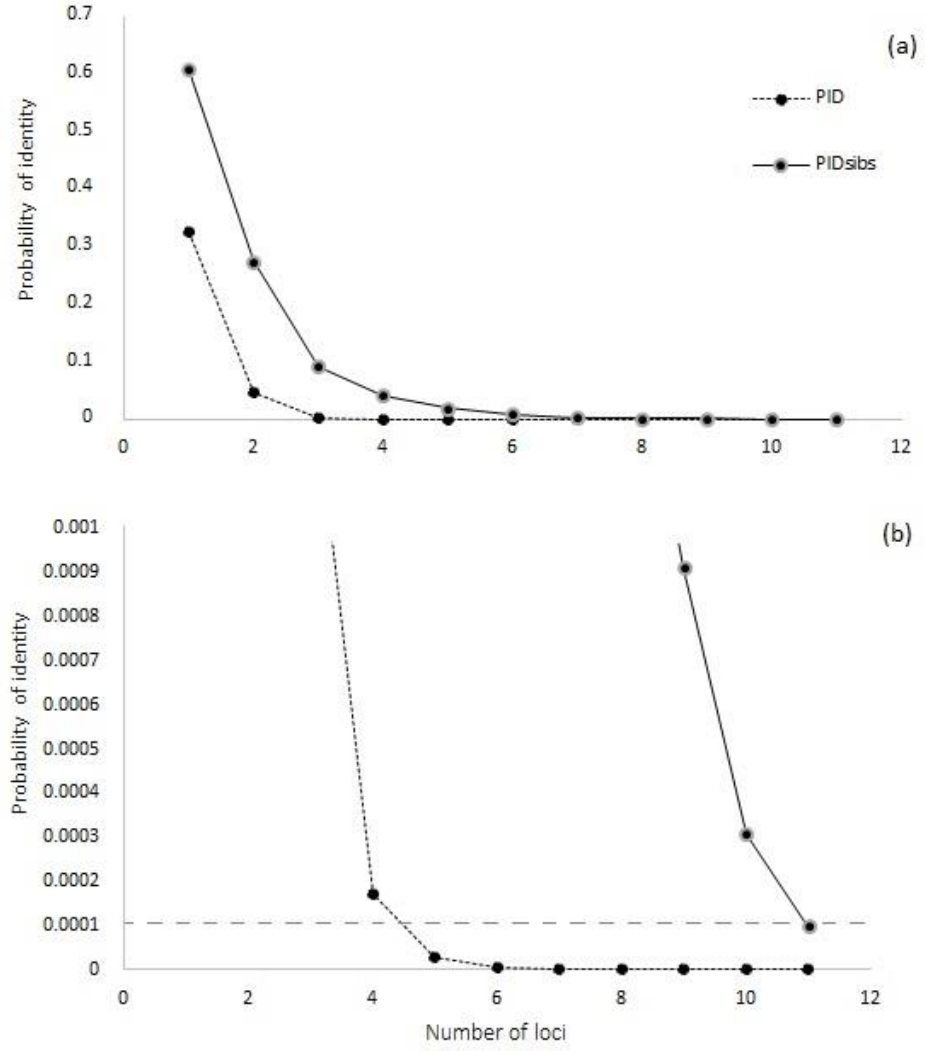

Fig. S1. (a) Probability of identity (P_{ID}) and probability of identity of siblings (P_{IDSIB}) combining different numbers of the 11 loci used in this study (b) P_{ID} and P_{IDSIB} of the number of loci that combined, exhibited probability values ≤ 0.0009. The horizontal dashed line indicates a probability of identity of 0.0001.

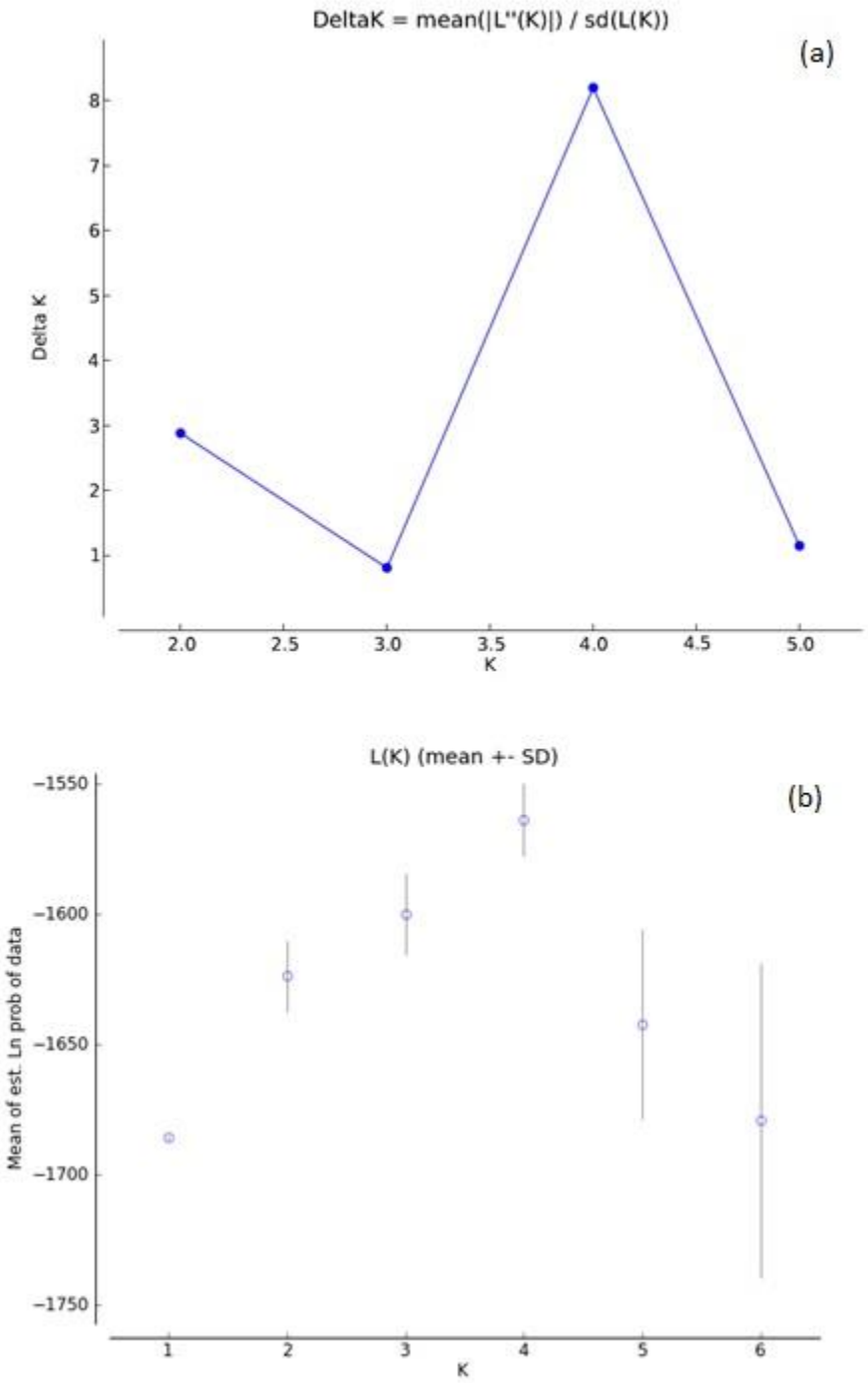

Fig. S2. Plots of (a) delta K calculated according to Evanno *et al*. (2005) and (b) mean log probability of the data (LnP(K) ± SD) over 10 runs as a function of the number of groups (K) for the complete data set.

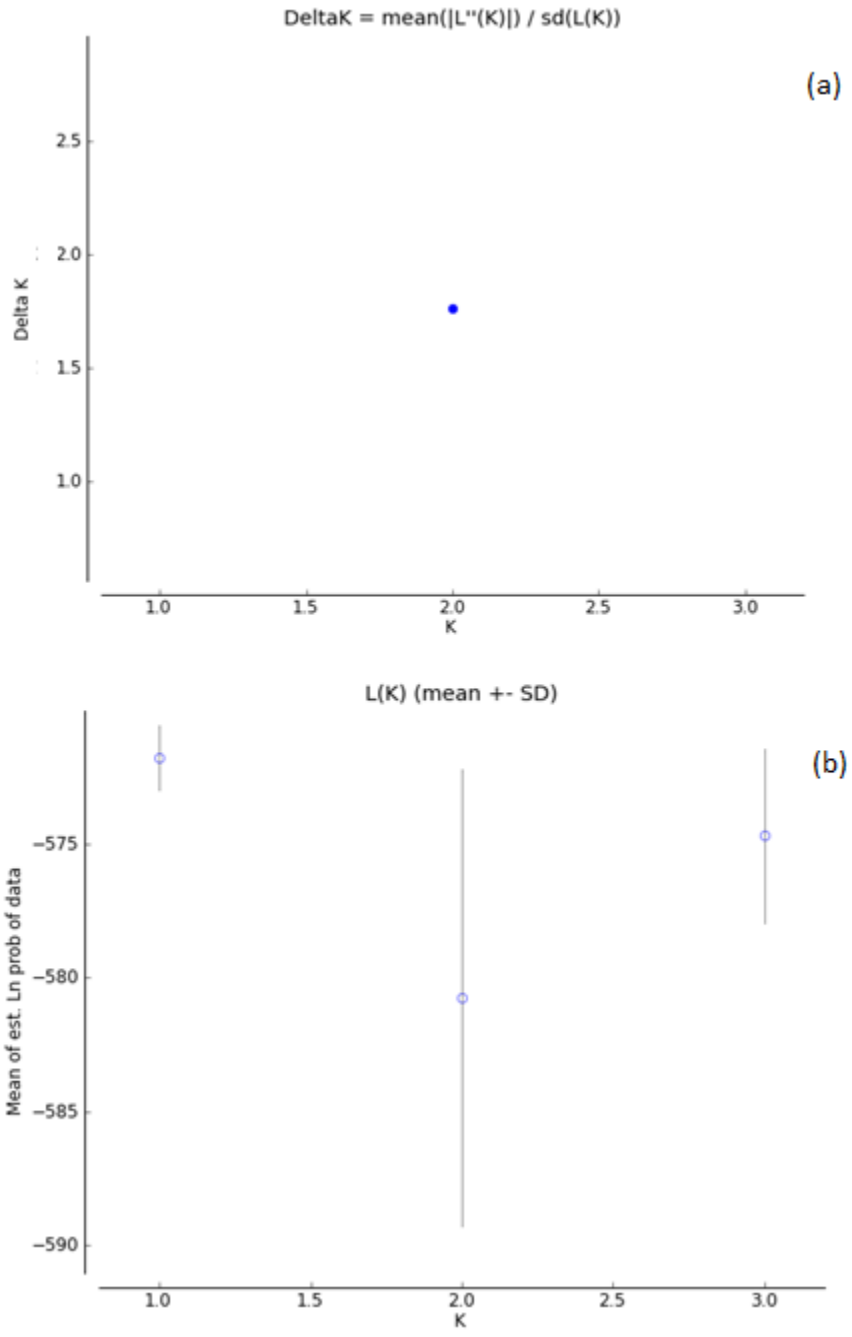

Fig S3. Plots of (a) delta K calculated according to Evanno et al. (2005) and (b) mean log probability of the data (LnP(K) ± SD) over 10 runs as a function of the number of groups (K) for the Jamapa genetic cluster.

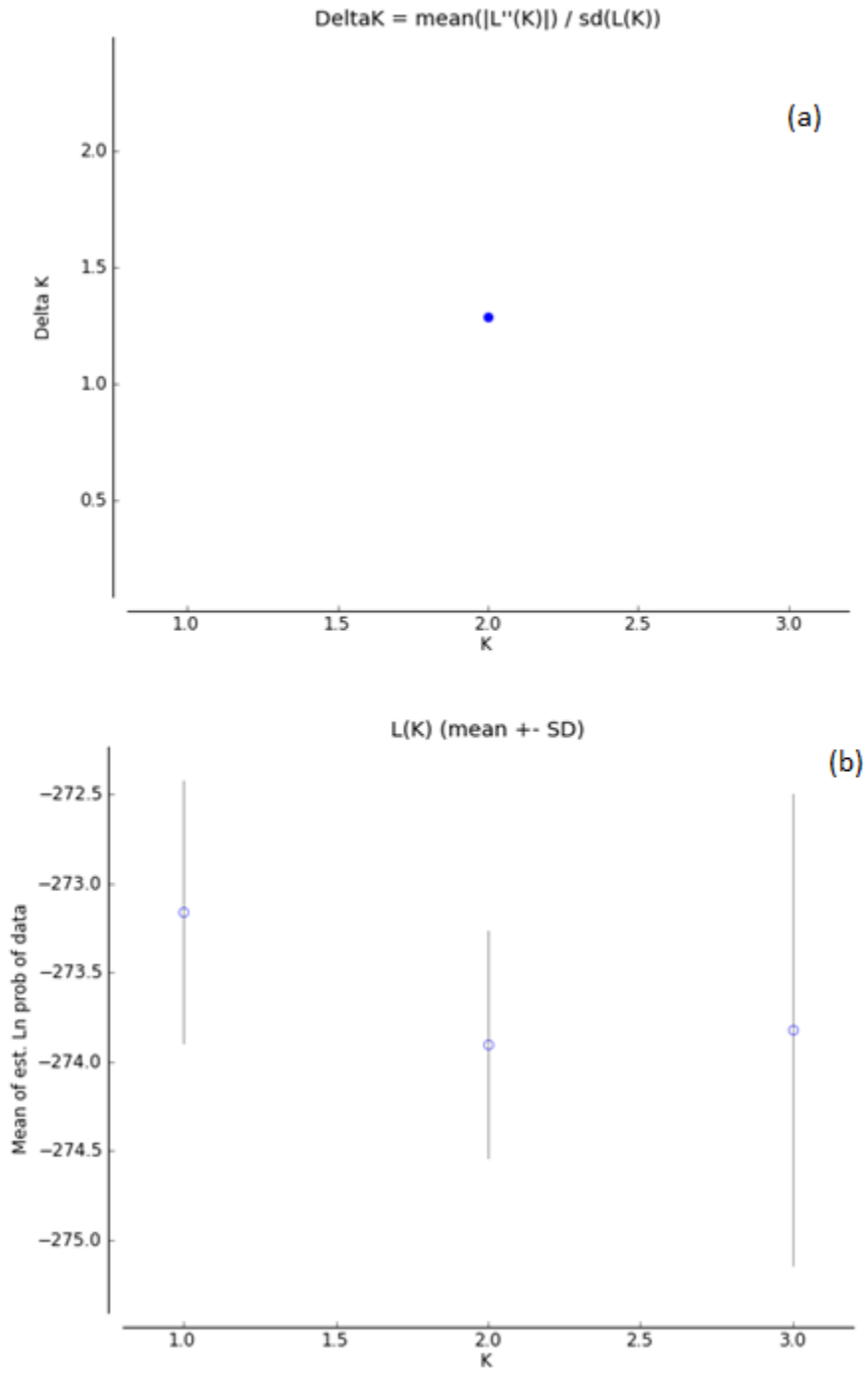

Fig. S4. Plots of (a) delta K calculated according to Evanno *et al*. (2005) and (b) mean log probability of the data (LnP(K) ± SD) over 10 runs as a function of the number of groups (K) for the La Antigua genetic cluster.

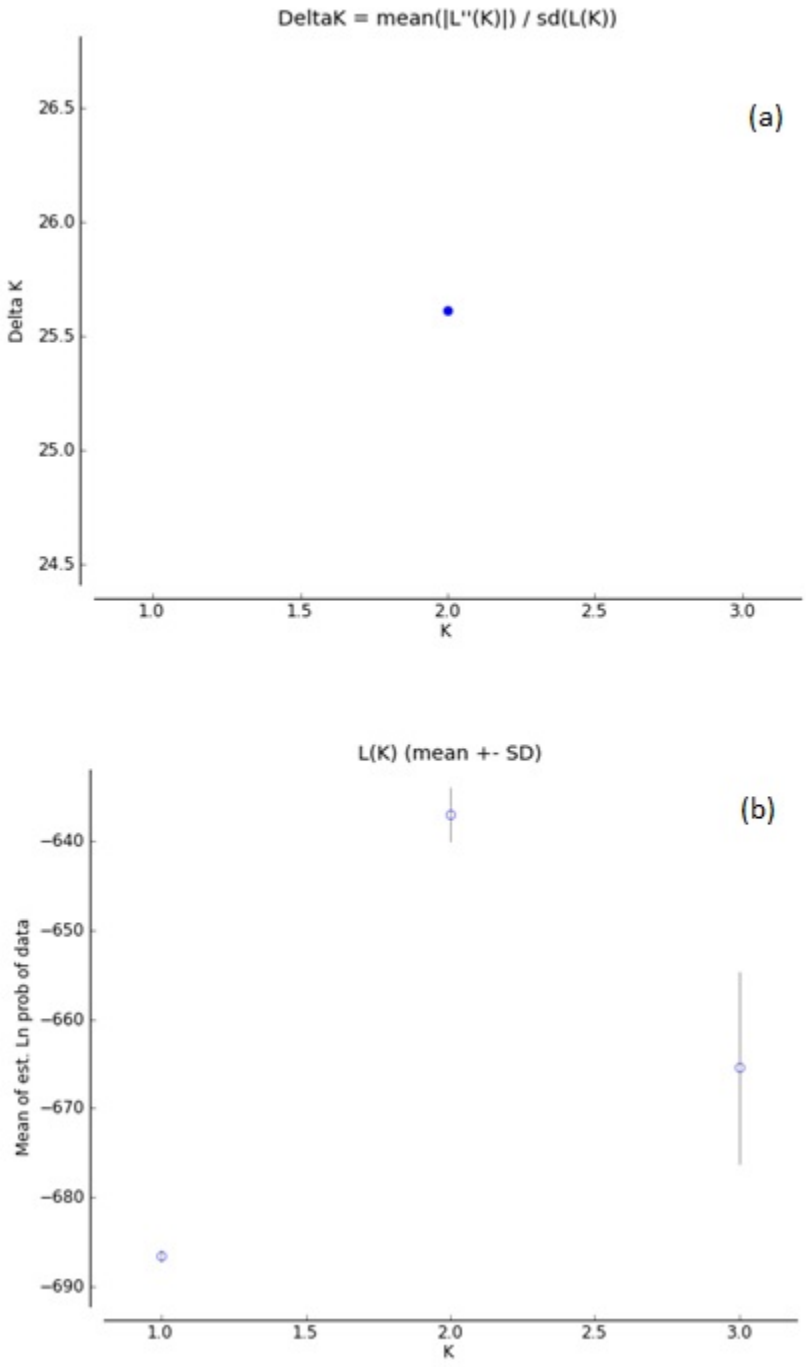

Fig. S5. Plots of (a) delta K calculated according to Evanno *et al*. (2005) and (b) mean log probability of the data (LnP(K) ± SD) over 10 runs as a function of the number of groups (K) for the Actopan genetic clusters.

Table S2. Genetic diversity of the Neotropical otter per basin at each microsatellite locus and combining nine loci, excluding the loci that had high frequency of null alleles. 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* = 0.0019).

	La Antigua Basin (*n*=12)									Actopan Basin (*n* = 25)									Jamapa Basin (*n* = 18)								
Locus	N	Na	AR	NEA	H_O	H_E	F_{IS}	FNA	GE	N	Na	AR	NEA	H_O	H_E	F_{IS}	FNA	GE	N	Na	AR	NEA	H_O	H_E	F_{IS}	FNA	GE
RIO2	11	3	2.82	1.97	0.55	0.49	-0.06	-0.04	0.11	24	4	2.97	1.60	0.29	0.38	0.24	0.06	0.13	13	3	2.99	2.05	0.38	0.51	0.29	0.08	0.15
RIO11	11	3	3.00	1.77	0.36	0.43	0.21	0.05	0.10	25	6	4.68	3.43	0.44	0.71	**0.40**	0.16	0.10	14	5	4.60	3.04	0.64	0.67	0.08	0.02	0.19
Lolo13	11	6	5.40	2.05	0.45	0.51	0.16	0.04	0.17	23	10	7.00	5.29	0.65	0.81	0.22	0.09	0.09	18	11	8.56	6.82	0.83	0.85	0.05	0.01	0.17
Lolo18	11	4	3.79	2.60	0.64	0.62	0.01	-0.01	0.14	25	5	4.05	2.48	0.48	0.60	0.22	0.07	0.03	18	7	5.86	3.31	0.56	0.70	0.23	0.08	0.05
Lolo29	12	6	5.43	2.88	0.75	0.65	-0.11	-0.06	0.09	25	8	4.55	2.44	0.60	0.59	0.00	-0.01	0.04	18	8	6.23	4.73	0.67	0.79	0.18	0.07	0.08
Lolo30	11	4	3.97	3.51	0.55	0.71	0.28	0.10	0.04	21	4	3.82	3.08	0.38	0.68	**0.46**	0.18	0.13	17	4	3.96	3.40	0.41	0.71	0.44	0.17	0.11
Lolo39	10	3	2.90	1.36	0.30	0.27	-0.08	-0.03	0.00	20	6	4.45	2.38	0.50	0.58	0.16	0.05	0.05	18	4	3.84	2.08	0.39	0.52	0.28	0.09	0.00
Lolo41	12	5	4.98	4.36	0.92	0.77	-0.15	-0.08	0.14	25	5	4.64	3.47	0.60	0.71	0.18	0.07	0.08	18	8	6.39	4.32	0.50	0.77	0.37	0.15	0.00
Lolo48	10	4	3.80	2.04	0.60	0.51	-0.13	-0.06	0.04	24	7	5.66	3.81	0.42	0.74	**0.45**	0.18	0.14	17	5	4.57	3.40	0.47	0.71	0.36	0.14	0.13
Mean	11	4.22	4.01	2.50	0.57	0.55	0.02	-	0.09	23.556	6.11	4.65	3.11	0.48	0.64	**0.26**	-	0.09	16.78	6.11	5.22	3.68	0.54	0.69	**0.25**	-	0.10
SE	0.22	0.38	0.33	0.30	0.06	0.05	0.05	-	0.02	0.59	0.62	0.36	0.34	0.04	0.04	0.05	-	0.01	0.60	0.81	0.54	0.46	0.05	0.04	0.04	-	0.02

Table S3. BOTTLENECK summary statistics for the Neotropical otter in the three river basins using the two-phase model (TPM). Sample size (n), observed number of alleles (ko), Hardy-Weinberg heterozygosity (He), expected measured heterozygosity in equilibrium (Heq), standard deviation of mutation-drift equilibrium of heterozygosity (SD), average standardized difference between observed and expected heterozygosity to test for a population bottleneck (DH/sd), and probability of obtaining the measured He in a sample from an equilibrium population (Prob).

		Observed		Under the TPM			
Locus	n	ko	He	Heq	S.D.	DH/sd	Prob
RIO2	48	0.8	0.638	0.555	0.131	-1.298	0.122
RIO11	50	6	0.723	0.693	0.092	0.329	0.46
Lolo13	46	10	0.829	0.835	0.044	-0.137	0.377
Lolo18	50	5	0.609	0.63	0.114	-0.182	0.339
Lolo19	40	6	0.687	0.708	0.086	-0.244	0.314
Lolo29	50	8	0.602	0.778	0.063	-2.808	0.024
Lolo30	42	4	0.692	0.56	0.127	1.04	0.123
Lolo37	48	3	0.526	0.428	0.158	0.62	0.335
Lolo39	40	6	0.595	0.706	0.089	-1.246	0.106
Lolo41	50	5	0.727	0.632	0.113	0.832	0.21
Lolo48	48	7	0.754	0.747	0.075	0.093	0.44

CAPÍTULO II

Do landscape and riverscape shape genetic patterns of the Neotropical otter, *Lontra longicaudis*, in eastern Mexico?

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Enviado a Landscape Ecology

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Abstract

Context Functional connectivity of semiaquatic species is poorly studied despite that freshwater ecosystems are amongst the most threatened worldwide due to habitat deterioration. The Neotropical otter, *Lontra longicaudis*, is a threatened species that represents a good model to evaluate the effect of landscape-riverscape features on genetic structure and gene flow of freshwater species.

Objectives We aimed to assess the spatial genetic structure of *L. longicaudis* and to evaluate the landscape-riverscape attributes that shape its genetic structure and gene flow *at local sites* (habitat patches) and *between sites* (landscape matrix).

Methods We conducted the study in three basins located in Veracruz, Mexico, which have a high degree of ecosystem deterioration. We used a non-invasive genetic sampling and a landscape genetics individual-based approach to test the effect stream hierarchical structure, isolation-by-distance, and isolation-by-resistance on genetic structure and gene flow.

Results We found genetic structure that corresponded to the latitudinal and altitudinal heterogeneity of the landscape and riverscape, as well as to the hierarchical structure of the streams. Open areas and steep slopes were the variables affecting genetic structure *at local sites*, whereas areas with suitable habitat conditions, higher ecosystem integrity and larger streams enhanced gene flow *between sites*.

Conclusions The landscape-riverscape characteristics that maintain functional connectivity of *L. longicaudis* differed between the upper, middle, and lower basins. Our results have important implications for the conservation of the species, including the maintenance of larger suitable areas in Actopan and the necessity to improve connectivity in Jamapa, through the establishment of biological corridors.

Keywords Functional connectivity, freshwater ecosystems, landscape-riverscape genetics, Isolation by resistance, environmental suitability, stream hierarchical structure

Introduction

Understanding the effect of natural and anthropogenic landscape attributes on genetic diversity and dispersal patterns of the species is one of the main objectives of landscape genetics (LG), and has become crucial for preserving evolutionary and ecological processes, such as gene flow, adaptation and functional connectivity (Manel *et al*. 2003; Storfer *et al*. 2007; Manel and Holderegger 2013). Functional connectivity refers to the degree to which the landscape elements influence the movement of individuals, which is essential to maintain gene flow among populations or habitat patches (Fahrig 2005; Cushman *et al*. 2013; Auffret *et al*. 2015). Habitat loss and fragmentation can increase inter-patch distances and reduce habitat patch size, decreasing functional connectivity and leading to genetic differentiation and loss of genetic diversity (Kindlmann and Burel 2008; DiLeo and Wagner 2016). Compared to terrestrial species, few LG studies have focused on semiaquatic species, partly due to the complexity to incorporate not only landscape variables, but also riverscape attributes that influence genetic diversity and gene flow, such as the hierarchical spatial organization of the stream networks, the temporal and spatial variability of stream features (Selkoe et al. 2016; Rico 2019). The assessment of functional connectivity in river systems, that is, the effective dispersal among terrestrial and aquatic habitats (Carranza et al. 2012; Martín-Vélez et al. 2020), is urgently needed, as these ecosystems are amongst the most threatened worldwide due to global warming and habitat deterioration (Poff et al. 2012; Martínez-Meyer et al. 2014).

Riverscapes are mosaics of freshwater river habitats, which are constituted by dendritic networks spatially structured and hierarchically organized across multiple scales. The hierarchical arrangement of streams within and among watersheds, as well as the direction and amount of water flow, have a strong influence on functional connectivity and genetic structure of riverine species (Selkoe et al. 2016; Paz-Vinas y Blanchet 2015; Thomaz et al. 2016). However, the effect of stream network structure is largely dependent on the dispersal capability of the species (Hugues et al. 2009; 2013). For example, the dispersal capability of semi-aquatic species (i.e., amphibians, otters, beavers) is higher than the dispersal of strict aquatic species (i.e., fish and some macroinvertebrates), as they can also use terrestrial paths to move among streams (Hughes et al. 2009). Other riverine features, such as the presence of waterfalls and dams, water pollution and lack of riparian vegetation, can also influence dispersal patterns of riverine species (Davis et al. 2018).

Different theoretical spatial models have been proposed to explain patterns of genetic structure in landscape and riverscapes. The isolation by distance model (IBD) predicts that genetic differentiation increases as a function of geographical distance (Wright 1943). While the isolation by resistance model (IBR) anticipates a positive relationship between genetic differentiation and resistance distance (cumulative cost of movement of an individual across the landscape) imposed by landscape and riverscape attributes (McRae 2006). The Stream Hierarchy Model (SHM) has been used to explain gene flow of riverine species and states that connectivity among populations or individuals should reflect the hierarchical structure of the stream networks (Meffe and Vrijenhoek 1988). SHM predicts lower genetic connectivity among sites or streams belonging to different catchments than among streams within the same catchments (Meffe and Vrijenhoek 1988; Hughes et al. 2009).

The Neotropical otter, *Lontra longicaudis*, is a semi-aquatic species that represents a good model to evaluate the effect of both landscape and riverscape features on genetic patterns of a riverine species. *L. longicaudis* is listed as a Near Threatened species by the International Union for Conservation of Nature (IUCN) due to a decline of its population sizes and to habitat deterioration (Rheingantz and Trinca 2015). Molecular ecology studies have found that the Neotropical otter can move between 3 and 17 km within rivers (Ortega et al. 2012; Trinca et al. 2013), suggesting a moderate dispersal capability. Other studies have documented larger dispersal distances (up to 40 km) for the Eurasian otter, *Lutra lutra*, and the North American river otter, *Lontra canadensis* (Janssens et al. 2008; Spinola et al. 2008). Some landscape-riverscape features have been associated to habitat preferences and recolonization events of otters. For example, conserved and dense riparian vegetation provides resting and breeding dens, as well as protection during terrestrial movements (Mayor-Victoria and Botero-Botero 2010; Loy et al. 2009). Stream features, including the order of the river and water accumulation influence food availability and movement behavior (Ruiz-Olmo 1998; Holland and van der Merwe 2016). Topographic features, such as elevation and slope, have been suggested to constrain the dispersal of the Eurasian otter (Janssens et al. 2008). In addition to natural components of riverine ecosystems, human activities, such as changes in land use, pollution, human settlements, and roads, have detrimental effects on otter populations (Ramos-Rosas et al. 2013; Rheingantz et al. 2014; Trigila et al. 2016). In *L. longicaudis*, the hierarchical organization of river networks and the slope seem to be important landscape components shaping the genetic structure of the species (Latorre-Cardenas et al. 2020b). However, the evaluation of the functional connectivity of *L. longicaudis*, using explicit spatial analyses and LG

analyses to specifically test the effect of these and other landscape attributes on the genetic patterns is lacking. Functional connectivity has traditionally been assessed considering the features of the intervening landscape matrix that can facilitate or impede gene flow *between-sites* (Tischendorf and Fahrig 2000; Ewers & Didham 2006). However, the evaluation of landscape characteristics *at local sites* (habitat patches) that act as attractive or repulsive conditions for migrant otters, is also important to better understand the local genetic structure and genetic connectivity of the species (Murphy et al. 2010, 2014; Dyer et al. 2012). This is particularly important because habitat patches are the landscape elements that hold the resources and conditions required by dispersing individuals for establishing and breeding (Tischendorf and Fahrig 2000; Ewers & Didham 2006; Pflüger and Balkenhol 2014).

The main goal of this study was to identify the landscape-riverscape attributes that shape genetic structure and gene flow of the Neotropical otter. We used a landscape genetics individual-based approach because it does not require *a priori* delimitation of genetically discrete groups, which is challenging to determine when the focal species has a continuous distribution, such as the Neotropical otter. This approach considers each individual as the unit of analysis, which increases the number of observations and the statistical power to detect the effects of the landscape on genetic patterns (Landguth et al. 2010; Kierepka et al. 2015; Shirk et al. 2017). We conducted the study in a hydrological system located in the state of Veracruz in Mexico, comprising three river basins: Actopan, La Antigua and Jamapa. A previous study in this system suggested that genetic diversity and migration patterns of *L. longicaudis* could be associated with habitat quality (Latorre-Cardenas et al. 2020b). Although habitat suitability for the Neotropical otter has not yet been evaluated in these river basins, the human impact in this region is widespread, being the ecological integrity of this area one of the lowest across the country (Equihua et al. 2014; Mora 2019). This implies that for the Neotropical otter, the poor habitat quality of the basins likely reduces gene flow and increases genetic structure. Habitat suitability has previously been associated to the genetic diversity of some populations of *L. longicaudis* throughout its distributional range (Trigila et al. 2016).

In this study, we aimed to evaluate: 1) patterns of spatial genetic structure of *L. longicaudis* across three hydrological basins, 2) whether stream network structure or IBD influence the genetic differentiation of *L. longicaudis*. To assess functional connectivity of the Neotropical otter we identified: 3) the landscape-riverscape features that are associated with genetic structure *at-local*

sites (habitat patches), and 4) the landscape-riverscape features of the intervening matrix that better explain gene flow *between-sites*. We expect the Neotropical otter to show a spatial genetic pattern, which will be explained by the SHM rather than by IBD, as riverscapes are spatially and hierarchically structured. In particular, we expect that landscape-riverscape features such as conserved vegetation, higher stream order level, and higher water accumulation constitute suitable habitat conditions for *L. longicaudis*, facilitating dispersal of individuals. On the contrary, high slopes of the rivers and elevation will constrain movement and therefore gene flow of the species.

Materials and Methods

Study area

The study area encompasses Actopan, La Antigua and Jamapa river basins, which are located in the state of Veracruz, Mexico (Fig. 1) and cover an area of 9,372 km^2. Although these basins are considered a priority for biodiversity conservation because they harbor an important number of species, they have been under extreme human perturbation (Cotler-Ávalos et al. 2010). In the last decades, human population growth and changes in land use/cover have altered the functional dynamics of these basins (e.g., hydrological filtration processes, evapotranspiration, sediment movement; Cotler-Ávalos et al. 2010). The main economic activities along the river shores include fishing, livestock, and agriculture (INEGI 2011). Across these basins, *L. longicaudis* is mainly found from 0 to 1,200 m a.s.l. (Macias-Sánchez and Aranda 1999; Hernández-Romero et al. 2018).

Sample collection

We collected fresh spraints and anal glands secretions of *L. longicaudis* throughout the distributional range (from 0 to 1,200 m a.s.l) of the species across the three river basins, restricting sample collection to third-order rivers or higher because they have enough water flow for the otter to inhabit them. We implemented a systematic sampling design to obtain otter samples evenly spread throughout the study area (Oyler-McCance et al. 2013). We divided the study area into 95 blocks of 10 x 10 km, and from May 2017 to February 2018, we surveyed approximately 2 km of the river in the central part of each block by rafting the main rivers, and by foot when sampling points were in smaller tributaries with low water flow (Fig. 1). We collected samples during the mornings using sterile sticks, preserved them in 1.25 ml of RNA later buffer (Sigma Aldrich) and stored them at -20 °C until genetic analyzes were performed. We recorded 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.") that were rescued in the Jamapa basin and a hair sample from an

individual hunted by a fish farmer in the Actopan basin, all obtained for previous studies (Latorre-Cardenas et al. 2020a,b). We recorded the geographic coordinates where these seven individuals were obtained.

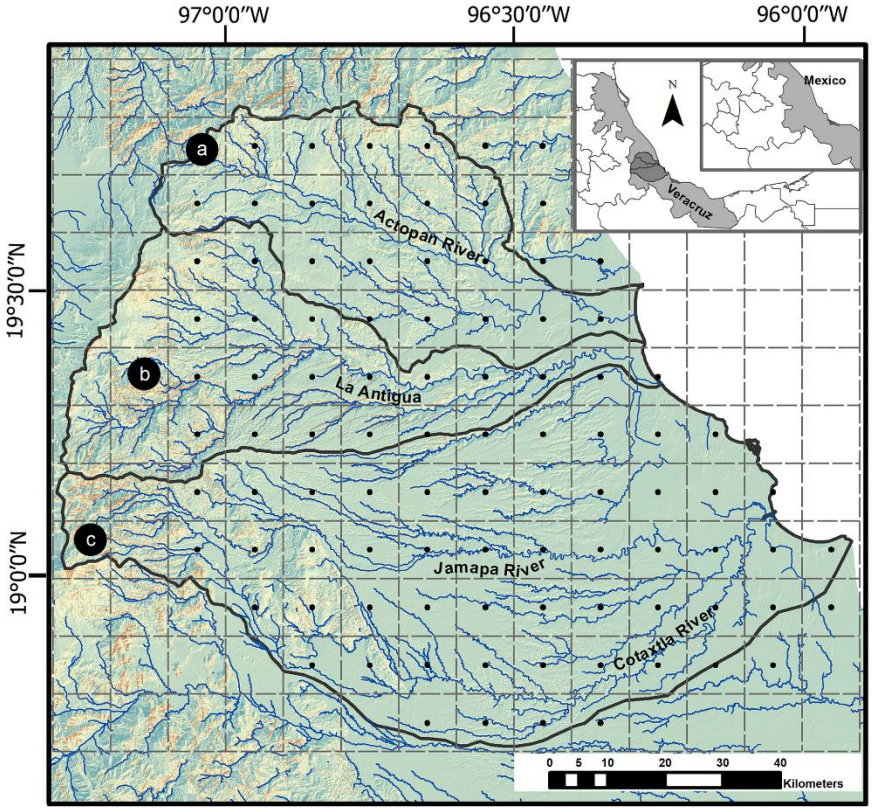

Fig. 1. Map of the study area showing the hydrological network of (a) Actopan, (b) La Antigua and (c) Jamapa basins, where we conducted the systematical sampling of the Neotropical otter by dividing the area in 10 x 10 km plots. Black dots indicate sampled plots throughout the distribution range (from 0 to 1,200 m a.s.l) of the species. The inset on the top right shows the geographic location of the study area within the state of Veracruz in Mexico.

Molecular methods, genotyping error and identification of individuals

Details on DNA extraction, PCR amplification and identification of unique multilocus genotypes (i.e. individuals) are provided in Latorre-Cardenas et al. (2020a, b). Briefly, we extracted genomic DNA from anal secretions and feces using the ZR Fecal DNA MiniPrep kit (Zymo Research), and from blood and hair samples using the DNeasy Blood & Tissue kit (Qiagen), following the manufacturer's instructions of both kits. We used the polymerase chain reaction (PCR) to amplify eleven

microsatellite loci that previously showed a high power to discriminate among individuals (Latorre-Cardenas et al. 2020a). To reduce the genotyping errors that arise when amplifying low-quality DNA from non-invasive samples (i.e. feces and anal secretion), we employed a multi-tube approach (Taberlet et al. 1996) that consists in repeating identical PCR reactions to validate the genotypes assigned to each sample. We assessed genotyping error using the Pompanon et al. (2005) method and determined the presence of large allelic dropout and null alleles in MICROCHECKER (Van Oosterhout et al. 2004). We identified unique multilocus genotypes (i.e., individuals) among the non-invasive samples in ALLELEMATCH (Galpern 2012), and performed all subsequent analyses using the identified unique genotypes.

Spatial genetic structure

To infer spatial genetic structure of *L. longicaudis*, we performed an individual-based spatial analysis of principal components (sPCA) using the ADEGENET R package v.2.1.1 (Jombart et al. 2008). sPCA is a multivariate multilocus method designed to identify cryptic spatial genetic patterns (i.e., additional spatial patterns not necessarily associated to the main genetic variation) at global and local scales, by combining PCA and Moran's I spatial autocorrelation. In sPCA, the product of the variance and the spatial autocorrelation are maximized to separate the effect of global (presence of genetic patches or clines) and local structure (strong differentiation; Jombart et al. 2010). sPCA uses a connection network to define which individuals are neighbors. To reflect the neighborhood of individuals of the Neotropical otter in the river networks, we used the minimum spanning tree algorithm. This network links all individuals using the minimum number of possible connections. We tested for significance of global and local structure with the Monte-Carlo randomization test (999 permutations) using the *spca_randtest* function (Montano and Jombart 2017).

Stream hierarchical and isolation by distance models

To examine whether genetic differentiation of the Neotropical otter is explained by the stream network structure, as the SHM postulates, we constructed a fitted stream tree model in STREAMTREE (Kalinowski et al. 2008). This model reflects genetic distances among individuals as the sum of genetic distances for each stream section that connect them, incorporating explicitly the stream network structure, and excluding the effect of the length of the sections. To construct the model, we used a river layer obtained from INEGI (2019) to determine the structure of the river networks. We estimated pairwise genetic distances (GD) among individuals using D_{PS}, a measure of genetic dissimilarity calculated as 1 - the proportion of shared alleles ($D_{PS} = 1 – Ps$; Bowcock et al.

1994). D_{PS} does not assume Hardy-Weinberg equilibrium and is sensitive to fine-scale structure as allele-frequency-based metrics respond rapidly to recent changes of the landscape (Murphy et al. 2010). We then compared the fitted stream tree model with the observed genetic distance by means of a linear regression in STREAMTREE. Values of the coefficient of determination (R^2) close to 1.0 indicate an excellent fit of the stream tree to genetic distances (Kalinowski et al. 2008).

To test the effect of IBD on genetic differentiation of *L. longicaudis*, we performed a multiple matrix regression with randomization (MMRR), with 999 permutations, using the R function *MMRR* (Wang 2013). We used the D_{PS} genetic distance matrix as our response variable and two different geographic distance matrices as explanatory variables. One of the geographic distance matrices consisted of Euclidian distances (IBD_{geo}) among individuals, calculated in GEOGRAPHIC DISTANCE MATRIX GENERATOR v. 1.2.3. (Ersts 2020). The other distance matrix consisted of pairwise river distances (IBD_{river}), defined as the distance among individuals along the river network, estimated in the RIVERDIST package in R (Tyers 2017).

Environmental variables and environmental suitability model

For the landscape-riverscape analysis and to construct an environmental niche model, we used topographic, climatic, ecological, and anthropogenic variables that are important for the Neotropical otter. All variables were represented as continuous raster layers for the full extent of the study area at a spatial resolution of 30 m. We processed all variables using ArcGIS v.10.2.1. (ESRI 2013).

Topographic features— We obtained elevation from the digital elevation model "Continuo de Elevaciones Mexicano" (https://www.inegi.org.mx/). The slope was derived from this digital model, using the slope tool within the SPATIAL ANALYSIS TOOLS. Stream order and water accumulation of third and higher order streams were derived from the digital elevation model using the HYDROLOGY spatial tool. While stream order indicates the level of branching in a river system, water accumulation reflects the amount of water that the streams accumulate downstream. We used the Strahler method to create the stream order layer. Water accumulation was estimated by counting the number of pixels accumulated downstream along the stream network. We used stream order and stream water accumulation as two separately layers because although they are similar, there are instances where a long section of a river (particularly in the main rivers) maintains its

stream order value but its water accumulation values could change. Ecological features— We calculated the Normalized Difference Vegetation Index (NDVI) using Landsat 7 ETM + multi-spectral images (30 m pixel resolution), from February and March 2015, available in the Global Land Cover Facility Datasets (https://www.usgs.gov/centers/eros/science). NDVI reflects the quantity and quality of vegetation and allowed us to map conserved and dense riparian vegetation that potentially provide cover escape and refuge to the Neotropical otter. NDVI values range from -1 to 1. Negative values correspond to water, including oceans; values close to zero (-0.1 to 0.1) to barren areas of rock or sand; low positive values (0.2 to 0.4) represent shrub, crops and grassland, and higher values (> 0.4) indicate dense vegetation and forest canopy. Climatic features— We used 19 bioclimatic layers updated for Mexico, which express mean, seasonal, and extreme conditions of temperature and precipitation across the country (Cuervo-Robayo et al. 2013). Anthropogenic variables— Human activities and human density affect different aspects of the Neotropical otter (Larivière 1999; Rheingantz et al. 2014; Trigila et al. 2016). To account for these factors, we used the Ecosystem Integrity Index (EII) developed for Mexico (Equihua et al. 2014; Mora 2019; https://www.biodiversidad.gob.mx/sistema_monitoreo/), which expresses the current condition of the ecosystem, including the degree of habitat degradation and transformation by human activities. EII values range from 0 to 1, being the lower values the more degraded areas and the higher the most conserved. The layer extent of the study area included a portion of the ocean, where the Neotropical otter does not inhabit. Although it is common to leave oceans cells as NoData value, the ResistanceGA package (see below) does not allow having NoData values in a raster. Therefore, we assigned arbitrary values to cells falling in the ocean to reflect that these are not used by otters. Because oceans cannot be classified as degraded areas or as potential areas for the occurrence of the otter, we assigned a contrasting value of -1 to ocean cells of the environmental suitability and Ecosystem integrity rasters, whereas for slope, elevation, stream order and stream water accumulation the assigned value was 0.

We estimated the environmental suitability for the Neotropical otter in the study area, following an ecological niche modeling approach (ENM) with the maximum entropy algorithm, implemented in the MaxEnt software (Phillips et al. 2006). Environmental suitability refers to the probability of the occurrence of a species given the state of the local environmental conditions that promote survival and breeding success (Soberón et al. 2017; Drake and Richards 2018). We used 99 occurrence points recorded in the study area between April 2016 and February 2018. To characterize the niche of the otter, we used the layers of the climatic, topographic, ecological, and

anthropogenic variables, excluding the layer of the streams to avoid constraining the niche model to stream courses. In the model construction, we included a bias layer to take into account that otter occurrence was biased to rivers and to terrestrial riparian zones within 500 m distance to the river. Because some variables can be highly correlated, we performed bivariate correlations for the whole set of variables using the NicheToolBox program (Osorio-Olvera et al., 2018) and selected those that were not redundant ($r < 0.7$). The final set of variables that we used for the model were: isothermality (BIO3), seasonal temperature (BIO4), annual temperature range (BIO7), average temperature of the driest quarter (BIO9), annual precipitation (BIO12), precipitation of the hottest quarter (BIO18), elevation, slope, NDVI, and EII. We split our dataset randomly in a 75:25 proportion for calibration and validation, respectively. We tested different values for the regularization multiplier (0.25, 0.5, 0.75, 1, 1.25 and 1.5) and chose the value that obtained the highest area under the curve (AUC) and the lowest omission rate. The final model consisted of 100 bootstrapped replicates with a regularization multiplier of 0.25 and with the clamping option activated. The final product was a raster map representing the average environmental suitability for the species in a continuous scale from 0 to 1. We used this layer as a resistance surface in further landscape-riverscape genetics analyses. In order to describe and compare the environmental suitability within and among river basins, we classified the resulting map into four categories. The unsuitable category corresponds to areas where the presence of the otter was not predicted as the probability of occurrence was lower than the minimum suitability value (0 - 0.12). The other three categories were created by dividing the probability of suitability into terciles above the minimum suitability value, in which all occurrences were included: low (0.12 - 0.40), medium (0.40 - 0.70), and high (0.70 - 1) suitability. We calculated the area of each category per river basin.

Riverscape and landscape genetic analyses

We performed a partial redundancy analysis (RDA) to identify landscape-riverscape features that are associated with genetic structure of *L. longicaudis at-local sites* (habitat patches). RDA is a constrained ordination technique that models multivariate response data to separate the relative contribution of landscape and spatial components on genetic structure (Legendre and Legendre, 1998). This method first uses multivariate linear regression to analyze genetic and landscape data, and then employs the resulting fitted values in a PCA to generate canonical axes. RDA has recently been used in landscape genetic studies because of its high power to detect relationships in

autocorrelated data (Kierepka and Latch 2016; Guerrero et al. 2018). We used genotypic data in the form of spatially lagged scores from the two first principal components retained by the sPCA (see above) as response variables and the set of landscape-riverscape features (slope, elevation, NDVI, EII, stream order, water accumulation, and environmental suitability) as explanatory variables. To obtain a local site measure of these features, we created a buffer of 100 m radius around each sampled individual and calculated the mean of each landscape-riverscape variables within the buffer. We included the geographic coordinates of each sampling location as a conditional variable to control the effect of geographic distance. We performed the RDA analysis in the Vegan R package v 2.5-6 (Oksanen et al. 2019). We first estimated whether the explanatory variables were correlated using the *vif.cca* function, then we performed a stepwise model selection using the *ordistep* function to find the predictor variables that best explained genetic differentiation among individuals. Finally, we ran the *anova.cca* function to test for significance of the best model.

To identify the landscape-riverscape features of the intervening matrix (*between sites*) that explain gene flow of the Neotropical otter, we performed a resistance surface analysis using the R package ResistanceGA (Peterman 2018). ResistanceGA uses a genetic algorithm developed by Scrucca (2013) to optimize resistance surfaces based on pairwise genetic data and effective distances among individuals, without making *a priori* assumptions about the relationship between resistance and the dispersal characteristics of the species. The genetic algorithm relies on an evolutionary (iterative) process of fitness performance, mutation, and selection of populations (set of resistance surfaces) with traits (shape and magnitude parameters to be optimized). By doing this, ResistanceGA allows to determine the resistance surfaces that represent the costs of dispersal inflicted by different landscape features.

Because full optimization in ResistanceGA is more manageable if raster surfaces contain less than 1.5 million cells, we used the set of landscape-riverscape layers with a reduced resolution (500 m) that is still adequate for measuring dispersal movements of *L. longicaudis*. The coarsening of the grid did not reduce the number of samples included in our analysis, and we still had one otter observation per cell, as required by ResistanceGA. The optimization procedure conducted in ResistanceGA consisted of several steps. We first optimized each resistance surface (the set of landscape-riverscape features) separately, using eight transformation functions (monomolecular and Ricker functions; Bolker 2008) and the default parameters (Peterman 2018). We then estimated pairwise effective distances among sampled individuals based on the random-walk commute time approach, using the *conmuteDistance* function of the gdistance package (van Etten 2017). The

random-walk approach considers that individuals can take multiple paths to disperse. We performed linear mixed-effects models with a maximum likelihood population effects parameterization (MLPE; Clarke et al. 2002) to fit genetic distances (D_{PS}) against effective distances, using the AIC (Akaike's information criterion) as the objective function (Nakagawa and Schielzeth 2013). This optimization procedure was repeated until 50-300 generations have passed without improvement of the objective function. Three independent runs for each single surface optimization were enough to verify the convergence of the fitted models and their parameters and transformation functions. After each optimization, we evaluated the level of support for each model selection conducting a bootstrap analysis. This consisted in subsampling 75% of the pairwise effective and D_{PS} distance matrices generated from each optimized surface (1000 times without replacement), refitting the MLPE model, and calculating fit statistics for each surface. We used the frequency at which a model was the top-ranked and the average model weight to assess the level of support. Landscape-riverscape resistance surfaces showing a higher selection percentage than distance alone constituted the set of features important for the dispersal of *L. longicaudis* individuals. We performed a Spearman correlation test of the resistance surfaces to eliminate from further analysis those with a coefficient greater than 0.70. Finally, we performed a multiple surface resistance optimization following the steps of the optimization procedure and selected the fittest models to explain the gene flow of the Neotropical otter.

Results

Genotyping error and identification of individuals

We collected a total of 129 spraints and anal secretion samples along the three river basins. We successfully amplified 55 non-invasive samples (spraints, anal secretions and the hair sample) in at least nine loci, resulting in a successful amplification rate of 42%. Genotyping error and frequency of null allele per locus and river basin are provided in the Online Resource Table S1. From the 55 non-invasive samples that we amplified, we identified 49 unique multilocus genotypes (i.e. individuals), which in addition to the six captive individuals resulted in a total of 55 individuals.

Spatial genetic structure

Permutation tests of the sPCA analysis identified global (P = 0.001) but not local (P = 0.994) genetic structure. The scatter plot of the decomposed eigenvalues (into variance and spatial autocorrelation

Moran's I) showed that the first eigenvalue (λ1) was the largest and exhibited positive autocorrelation (Online Resource Fig. S1). The first and second axes explained 43.5 % of the genetic variation and denoted spatial autocorrelation (I: λ1 = 0.72, λ2 = 0.66, variance: λ1 = 1.46, λ2 = 1.0; Online Resource Fig. S1) among individuals. The first sPCA axis revealed a north-south genetic differentiation, indicating that individuals from Actopan are genetically differentiated from those in La Antigua and Jamapa (Fig. 2a). The second sPCA axis suggested a partial genetic differentiation between the upper and lower zones of the basins, whereas individuals from La Antigua and upper Jamapa were genetically more similar among them, individuals from lower Jamapa were more differentiated. Such altitudinal genetic differentiation was not as clear for Actopan (Fig 2b).

Stream hierarchical and isolation by distance models

The Stream Tree model had a good fit to the observed genetic distances (R^2 = 0.59), suggesting that SHM explains the genetic differentiation of *L. longicaudis* (Online resource Fig. S2). Results of the MMRR indicated that IBD_{geo} and IBD_{river} slightly explained genetic differentiation (R^2 = 0.078, P = 0.002), but only IBD_{geo} contributed significantly to the model (P = 0.01) while IBD_{river} did not (P = 0.603).

Environmental suitability model

The ecological niche model had a good performance (AUC = 0.96; omission rate = 0). The most informative variables for the model were elevation, isothermality (BIO 3) and temperature of the driest quarter (BIO 9; Online Resource Table S2), suggesting that suitable conditions for the presence of *L. longicaudis* are located in areas at mid altitudes with moderate temperatures. The model shows that the Actopan basin has the most extensive areas with high suitability, followed by La Antigua, and by Jamapa with few highly or medium suitable areas (Online Resource Fig. S3, Table S3).

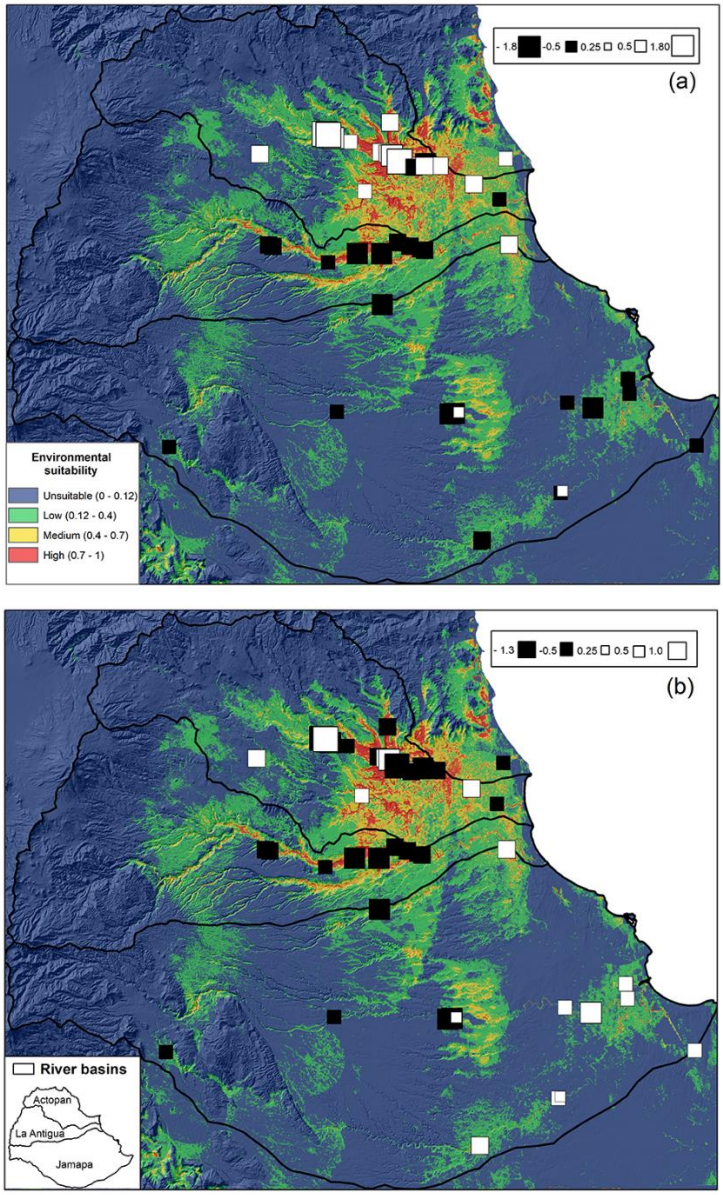

Figure 2. Plots of the spatial principal components analyses (sPCA) for the first (a) and second (b) axes depicting spatial genetic structure of Neotropical otter individuals across the basins. Each square represents the score of an individual's genotype located by their spatial coordinate. Black squares represent positive values and genetic similarity, and white squares indicate negative values and genetic differentiation. The size of the squares corresponds to the magnitude of the score. The colors behind the graph of the sPCA analysis correspond to the environmental suitability of the Neotropical otter based on MaxEnt models. Categories of environmental suitability are indicated by different colors.

Landscape-riverscape genetics

We included in the landscape-riverscape analysis some of the variables (slope, elevation, NDVI, and ecosystem integrity index) that we also used to create the environmental suitability model because we wanted to analyze their individual contribution to the genetic structure of the otters. These variables had a low percentage of contribution to the suitability model (except for elevation, see Online Resource Table S2), thus we consider that the ecological information that each variable represent by itself for the otter was not duplicated in these analyses.

Results of the RDA analysis to identify features associated with genetic structure *at-local sites* (habitat patches), indicated that slope, stream water accumulation, and NDVI explained 19.2 % of the genetic variation (P = 0.002; Table 2) after accounting for the effect of geographical distance, while distance alone accounted for 20.1 % of the variance. RDA 1 and RDA 2 explained 87.8 % and 12.2 % of the constrained genetic variation, respectively (Fig. 3). NDVI and stream order were the variables with the strongest effect on the genetic structure of the Neotropical otter *at-local sites* (Fig. 3). Stream order and slope correlated positively with the genetic structure of *L. longicaudis* contained in the first axis of the sPCA. This indicates that *at local sites*, genetic similarity between individuals is higher in habitat patches with steepest slopes and streams with higher order or branching levels. NDVI correlated negatively with the genetic structure retained by the second sPCA axis, denoting that genetic similarity between individuals is higher *at local sites* with low NDVI (open areas such as grassland and crops, Fig. 3).

Table 1. Partial redundancy analysis (RDA) model selection showing landscape-riverscape variables that explained the genetic variance contained in Axis 1 and Axis 2 of the sPCA. We include values of the Akaike Information Criterion (AIC) and F statistics. *P-values* (<0.05) depicting significant differences are shown in bold.

RDA model	AIC	F	*P-value*
Slope	**-34.88**	**4.36**	**0.02**
Stream order	**-34.69**	**4.53**	**0.04**
NDVI	**-36.09**	**3.27**	**0.05**
Stream water accumulation	-39.13	0.63	0.56
Ecosystem integrity index	-63.84	0.35	0.65
Environmental suitability	-39.54	0.29	0.70
Elevation	-39.63	0.21	0.80

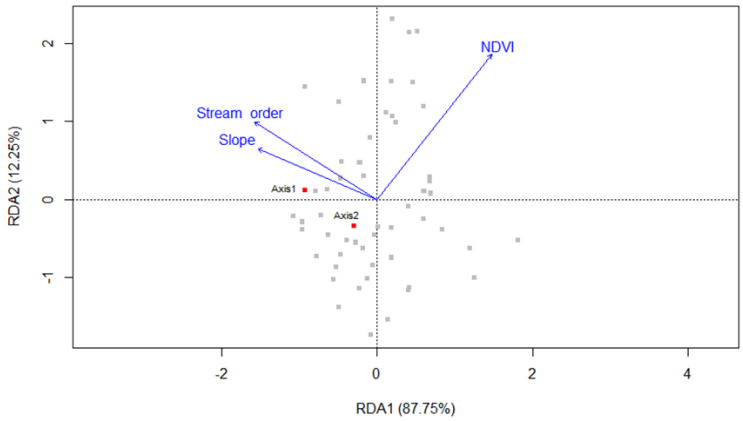

Figure 3. Triplot showing the landscape-riverscape features that *at local sites* explained the genetic structure of the Neotropical otter, using redundancy analysis (RDA) conditioned by geographic distance. Red squares represent the scores of the first and second axes of the sPCA that were used as the genetic response variables. Blue arrows depict the variables that were significant for the model (P = <0.05). The length of the arrows represents the magnitude of their contribution and the angle between each arrow is the correlation among variables. Grey squares represent sampled individuals and are graphed according to their scores of the RDA.

ResistanceGA optimization and model selection indicated that environmental suitability, ecosystem integrity, NDVI, slope, and stream water accumulation explained gene flow *between sites* better than geographical distance, while no significant relationships were found for elevation and stream order (Online resource Table S4). Because all resistance surfaces, except for stream water accumulation, were highly correlated (r > 0.7), we simultaneously optimized the environmental suitability and stream water accumulation to generate a combined resistance surface. The bootstrap analysis that included all surfaces and the combined (environmental suitability + stream water accumulation) resistance surface, showed that environmental suitability (38.4 %), ecosystem integrity index (38.1 %) and the combined resistance surface (13.9%,) had the best model support based on the top ranked percentage (see Table 2 for other statistical criteria). The rest of the resistance surfaces (NDVI, slope, stream water accumulation) showed a lower contribution to gene flow (Table 2). The response curves of optimized resistance surfaces showed that resistance cost decreases as environmental suitability and ecosystem integrity increases; resistance cost also decreased in streams with larger water accumulation (Fig 4). In areas with higher NDVI (i.e., areas with denser vegetation) and soft slopes, the resistance costs of *L. longicaudis* across landscape increased (Fig. 4). Maps showing the resistance cost for each landscape and riverscape feature across the study area are shown in Figure 4. Environmental suitability represents lower resistance

costs at the middle and in some patches at the upper and lower parts of Actopan and La Antigua basins. Higher resistance is imposed in the lower parts of Jamapa. The resistance cost that imposes ecosystem integrity is lower in mid and upper basins, increasing downstream in the three basins. Stream water accumulation inflicts a resistance cost that increases gradually from downstream to upstream in the three basins. The resistance imposed by the slope is higher in the low basins, decreasing in the mid and upper zones of the basins. Although the resistance cost imposed by NDVI has not a clear spatial pattern, it was lower in the mid basins.

Table 2. Model selection results for the generalized linear mixed-effects models testing the effect of landscape-riverscape features on gene flow *between-sites* of the Neotropical otter. We show the best fitted transformation equation and statistics for each resistance surface and for the combined resistance surface. Higher top-ranked percentage and lower average weight indicate the best supported models.

Resistance surface	Transformation equation	avg.AICc	avg.weight	avg.rank	Top-ranked %
Environmental suitability	Ricker	-1222.61	0.23	2.34	38.4
Ecosystem integrity	Ricker	-1220.74	0.25	2.76	38.08
NDVI	Inverse Ricker	-1219.68	0.13	3.69	7.75
Environmental suitability + stream water accumulation	NA	-1219.70	0.143	3.75	13.9
Slope	Reverse Ricker	-1215.69	0.10	3.94	0.08
Stream water accumulation	Inverse monomolecular	-1215.69	0.03	6.05	1.24
Geographic distance	NA	-1215.07	0.02	6.66	0.54

Abbreviations: avg.AICc: average of the Akaike information criterion for each model, corrected for sample size; avg. weight: average model weight representing the probability that a model is the best in the model set, averaged over 1000 bootstrap replicates; avg.rank: average model ranking from 1000 bootstrap replicates; top-ranked %: proportion of times during 1000 bootstrap iterations that each model was ranked as the top model.

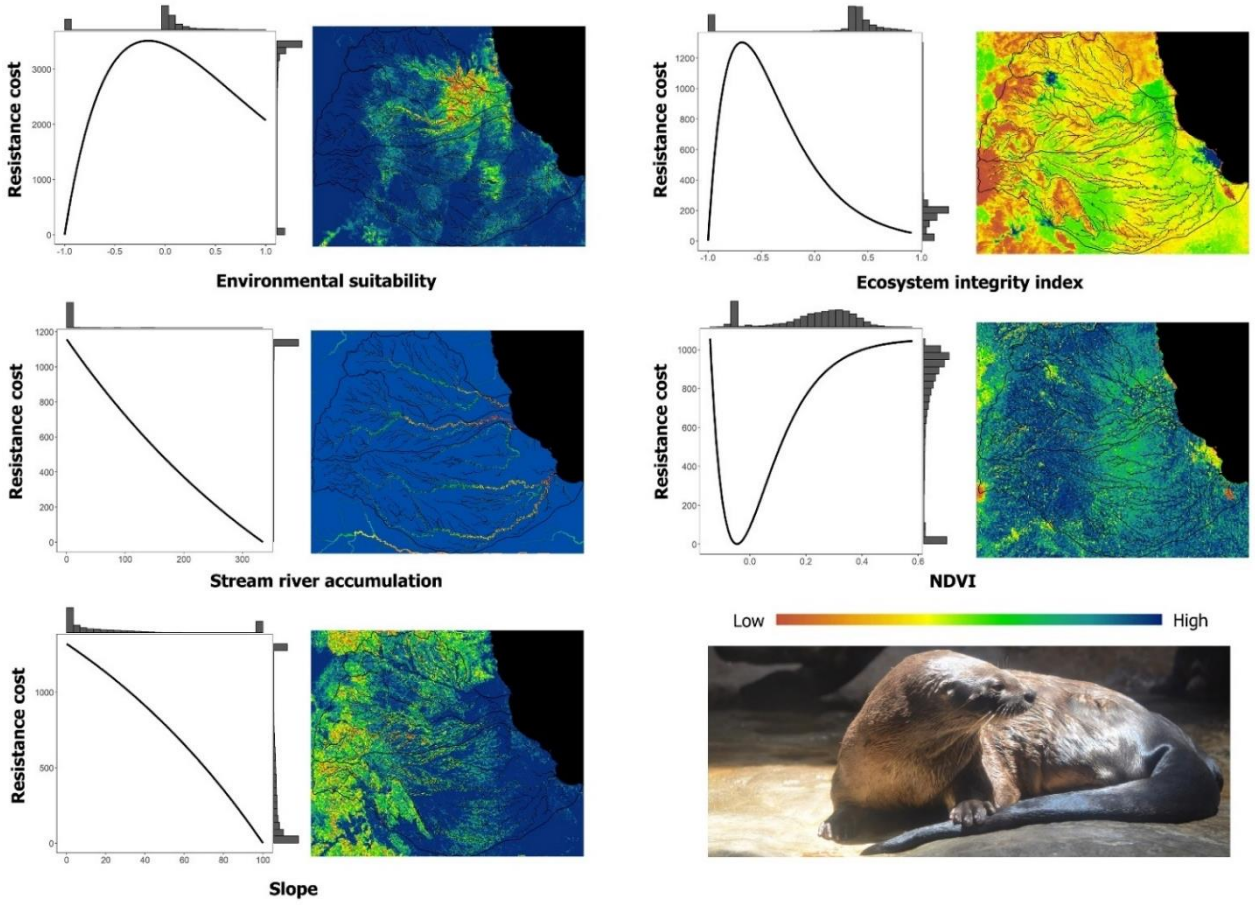

Figure 4. ResistanceGA single surface optimization response curves for landscape-riverscape features that explained the genetic distance among individuals of the Neotropical otter. Each plot shows the resistance cost values obtained after the optimization procedure. Histogram bars indicate the frequency of each resistance value in the landscape-riverscape. Maps show the assigned resistance cost for each landscape or riverscape feature across the study area, which is indicated by the color of the bar. Environmental suitability and EII response curves had values starting at -1 in the X axis because we assigned this arbitrary value to cell falling in the ocean.

Discussion

Spatial genetic structure and habitat suitability

As we expected, the Neotropical otter exhibited a discontinuous genetic pattern, characterized mainly by a latitudinal genetic differentiation across the landscape, with individuals of the northern basin (Actopan) being differentiated from those in the southern basins (La Antigua and Jamapa). Our results agree with the genetic structure previously reported for the species in the study area, which found that each river basin represents a different genetic group of *L. longicaudis*, with individuals from La Antigua and Jamapa being more similar among them than to individuals from Actopan (Latorre-Cárdenas et al. 2020b). The ecological niche model is to some extent concordant with the genetic structure of *L. longicaudis*, as it revealed that environmental suitability also varied latitudinally across the study area. More extensive areas of high suitability were detected in Actopan, and these areas were progressively smaller in La Antigua and Jamapa, possibly restricting the movement of individuals. Interestingly, although the environmental conditions between La Antigua and Actopan are similar, individuals from the two basins are genetically differentiated. Recently, Latorre-Cardenas et al. (2020b) found that Actopan receives more first-generation migrants and expels fewer individuals than La Antigua and Jamapa. Individuals in suboptimal habitats tend to disperse more frequently to high-quality habitats (Rémy et al. 2011; Day et al. 2019). However, the mortality risk of dispersing individuals may increase in hostile landscape matrices (Atkins et al. 2019). For example, one of the main threats for young dispersers of the Eurasian otter is being killed on roads (Quaglietta et al. 2013). Therefore, our findings suggest that the higher habitat quality in Actopan is critical for the persistence of *L. longicaudis* in the region. In addition to the latitudinal differentiation, the sPCA identified a cryptic altitudinal genetic differentiation among individuals from lower and upper zones of the basins. Genetic structure between areas with no evident physical barriers to dispersal may reveal interesting ecological differences, such as food availability and social behavior. Such is the case in the Artic fox populations, in which genetic differentiation matched the distribution of its main prey (Lai et al. 2017). Similarly, in the North America river otter, differences on coastal and inland habitat use, food availability along the year and social group composition are factors that could modify the breeding seasonality of populations, leading to genetic differentiation among them (Latch et al. 2008). There is no available information on the reproductive periods or prey availability of *L. longicaudis* in the study region, therefore future studies evaluating these and other ecological differences among

basins will be important. It is likely that differences of the environmental suitability between the upper and lower basins (particularly in Jamapa), such as the availability of water along the year and the degree of human perturbation, may be reducing gene flow of *L. longicaudis*.

Stream hierarchy and isolation by distance models

Genetic differentiation among individuals of the Neotropical otter was for the most part explained by the structure of the stream network, supporting the stream hierarchy model. In contrast, geographical distance had little (Euclidean distances) or no (river distances) effect on the genetic distances of the species. These findings suggest that the structure of the streams play an important role on the dispersal patterns of *L. longicaudis* and that individuals not only use rivers but also land for dispersing. The SHM has explained patterns of genetic structure of several riverine species restricted to water channels, such as fish (Hughes et al. 2013; Osborne et al. 2014; Cole et al. 2016; Brauer et al. 2016) and medium-size semiaquatic mammals (Crawford et al. 2009; Byrne et al. 2015). However, the SHM has not always explained connectivity patterns of semiaquatic species, particularly in those that are habitat generalists and perturbation tolerant species. For example, the genetic structure of the muskrat *Ondatra zibethicus*, the American mink, *Neovison vison* and the water vole, *Arvicola sapidus*, did not reflect the watershed network structure because individuals of these species used anthropogenic landscape features, such as roads, agriculture channels and urban areas to disperse (Zalewski et al. 2009; Centeno-Cuadros et al. 2011; Laurence et al. 2013).

The effect of the hierarchical structure of streams has not been tested in other otter species, but IBD is known to shape the genetic structure of *L. lutra*, *L. canadensis* and *L. longicaudis* at fine spatial scales (Quaglietta et al. 2013; Trinca et al. 2013; Pagacz 2016). These studies reported spatial genetic autocorrelation with distances up to 20-25 km, suggesting that the effect of IBD decreases at longer distances. The dispersal patterns of most mammals are sex-biased, with males dispersing longer distances from natal sites than females, and females being more philopatric (Greenwood 1980; Mabry et al. 2013). It would be important to assess whether *L. longicaudis* sexes are influenced differently by the hierarchical structure of the streams and by IBD. For example, for the fisher, *Pekania pennanti*, the effect of rivers and roads on genetic structure differed between sexes, while females were affected by these features, males were not (Tucker et al. 2017). In the study region, the recorded killed otters were mainly males (*personal observation*). This may impact the population size, sex ratio and the effective dispersal of young individuals, resulting in gene flow

reduction and genetic structure of *L. longicaudis*, as it has been observed in other carnivores, such as the black bear and the American mink (Coster and Kovach 2012; Zalewski et al. 2016).

Landscape and riverscape genetics and isolation by resistance

As we expected, several landscape and riverscape attributes influenced the genetic structure and gene flow of *L. longicaudis*, supporting our hypothesis that IBR explains better the genetic patterns of the species than IBD. Interestingly, the landscape features influencing gene flow *at* local sites (within habitat patches) were different from those affecting dispersal *between* sites across the intervening matrix. Within habitat patches, slope, stream order, and NDVI explained local genetic structure in the Neotropical otter. However, it is important to point out that the proportion of constrained and unconstrained variation of the RDA was very similar, which suggests that only a small amount of the genetic variation is explained by these landscape-riverscape features. On the other hand, environmental suitability, ecosystem integrity, and stream water accumulation were the landscape-riverscape features that mostly influenced gene flow *between* sites, while slope and NDVI had a lower effect on the dispersal of the Neotropical otter. Other LG studies have also shown that ecological factors have different effects on genetic patterns of medium and large size mammals, depending on the spatial and temporal scales, highlighting the importance of including different scales in the same study (Galpern et al. 2012; Thatte et al. 2019).

As we predicted, areas with suitable habitat conditions, higher ecosystem integrity, and streams with higher water accumulation enhanced gene flow *between* sites of *L. longicaudis* as they represent less resistance to dispersal. Environmental suitability was the feature contributing the most to the dispersal of the Neotropical otter throughout the study area. Environmentally suitable conditions were characterized by medium to higher altitudes and lower temperatures during the driest season of the year. Areas with cooler temperatures guarantee that the streams have enough water throughout the year, particularly during the driest season. Streams with cooler temperatures contain higher levels of dissolved oxygen, which could be supporting larger bodied fish for otters to feed (Casariego-Mandorell et al. 2008). Deeper and wider streams imply less resistance to movement of otters, as they are more agile in water than in land (Kruuk 2006), and also represent greater availability of fish and aquatic crustaceans, the main preys of *L. longicaudis* (Carrillo-Rubio and Lafón 2004; Smith et al. 2020). This is concordant with the positive contribution of the stream order to explain the genetic similarity among individuals *at-local sites*.

The Neotropical otter can live in habitats with some degree of human perturbation, including urban areas (Rheingtanz et al. 2014; Aceituno et al. 2015). However, our results indicated that costs of dispersal were lower through areas with higher ecosystem integrity, suggesting that dispersing otters avoid areas impacted by human activities. The negative impact of the human footprint (i.e., human population density, settlements, land cover use changes, and presence of roads) on patterns of gene flow has been reported even for large and highly mobile mammals (Wultsch et al. 2016; Draheim et al. 2018). However, the magnitude of the effect of human presence depends on the degree of habitat specialization of the species and on the sensitivity to habitat loss (Thatte et al. 2019). Transformation of natural vegetation into human-dominated landscapes reduces the habitat quality of *L. longicaudis* (Mayagoitia-González et al. 2013; Navarro-Picado et al. 2017). Nevertheless, to a lesser extent than other variables, we found that open areas (low NDVI), such as croplands and grasslands, as well as steep slopes enhance gene flow *between sites* and are the variables with the strongest effect on genetic structure *at local sites*. At the local scale, landscape features constitute attractive or repulsive conditions that are likely to influence the selection of habitat-patches by dispersing individuals, which likely influence fine-scale genetic structure (Murphy et al. 2010; Zero et al. 2017). Our study area is dominated by croplands and grasslands, which are mainly established on the river buffer zones. It is possible that otters may be forced to use these transformed landscapes, even if they represent suboptimal features. However, some crops, like sugar cane, have irrigation channels, which may represent attractive conditions as they facilitate the movement of otters and provide escape cover from predators and humans. Steepest slopes (>30°) facilitated the dispersal of individuals, suggesting that *L. longicaudis* disperses through the middle and upper zones of the basins. These findings are contrary to our predictions and to previous studies on semiaquatic species, which found that steep mountain slopes reduced the dispersal of the Eurasian otter (Janssen et al. 2008) and the American mink (Zalewski et al. 2009). Dispersal of the Neotropical otter could be occurring in areas with steepest slopes because dispersing individuals tend to settle in areas with high-quality habitat (Romanowski et al. 2013; Pagacz 2016), which in our study area are mostly located in the middle and in some upper areas of the basins. Therefore, in concert, our results suggest that the elements favoring functional connectivity of the otter differ along the basins. In the lower basins, large rivers with large amount of water accumulation seem to be the main characteristic maintaining the connectivity of the Neotropical otter, reducing the negative effect of human disturbance in these zones. In the middle and upper basins, the rivers are smaller, which could lead to less food availability, but they are

relatively less impacted by human activities and have sufficient areas with environmental suitable conditions, favoring connectivity for the otter. It is possible that upper and middle areas of the basins act as inter-basin dispersal areas, particularly between La Antigua and Actopan given their narrow basin configuration, which could lead to shorter land-travel distances. This could also explain that higher altitudes and steep slopes enhance the dispersal of the otter in the region. Inter-basin dispersal promoted by irrigation channels has been documented in fish (Muñoz-Ramírez et al. 2015) and could have a similar effect in the Neotropical otter. Recently, a human-otter conflict due to competition for food resources was reported in the upper areas of La Antigua basin (Hernández-Romero et al. 2018). This conflict induces the hunting of *L. longicaudis* and represents a potential threat for the survival of individuals dispersing in the upper basins (Andrade-Ponce and Angarita-Sierra 2017; Hernández-Romero et al. 2018). Therefore, it would be important to monitor and evaluate the effect of this conflict on the dispersal patterns of the Neotropical otter.

In conclusion, we found genetic structure on the Neotropical otter that mostly corresponded to the latitudinal and altitudinal heterogeneity of the landscape and riverscape throughout the study area. Even though this area has a considerable degree of environmental deterioration, the functional connectivity of the species is maintained by different elements of the riverscape and the landscape. In general, middle zones of Actopan and La Antigua harbor larger suitable areas, favoring the dispersal of the otter. On the contrary, functional connectivity throughout Jamapa basin was low, which can potentially affect its populations in the short and medium term. It would be important to extend our study to nearby basins to have a better understanding on regional dispersal patterns and genetic structure of the Neotropical otter. We recommend that future studies focusing on the design and establishment of biological corridors consider the contribution of both streams and terrestrial elements on the dispersal of the Neotropical otter within and among basins.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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Electronic Supplementary Material

Do landscape and riverscape shape genetic patterns of the Neotropical otter, *Lontra longicaudis,* **in eastern Mexico?**

Landscape Ecology

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Table S1. Quality of the microsatellites of the Neotropical otter showing the frequency of null alleles (FNA) and genotyping error (GE) at each microsatellite locus and combining all 11 loci. We include the number of total individuals detected in each basin (n) and number of individuals that successfully amplified and that were used for the analyses (N).

	Actopan Basin (n = 25)			La Antigua Basin (n=12)			Jamapa Basin (n = 18)		
Locus	N	FNA	GE	N	FNA	GE	N	FNA	GE
RIO2	24	0.061	0.127	11	-0.036	0.111	13	0.084	0.150
RIO11	25	0.157	0.096	11	0.049	0.102	14	0.016	0.188
Lolo13	23	0.087	0.094	11	0.038	0.173	18	0.010	0.167
Lolo18	25	0.073	0.033	11	-0.012	0.139	18	0.083	0.050
Lolo19	20	0.131	0.167	9	0.011	0.083	17	0.216	0.063
Lolo29	25	-0.006	0.040	12	-0.058	0.091	18	0.068	0.083
Lolo30	21	0.175	0.132	11	0.098	0.036	17	0.172	0.107
Lolo37	24	0.092	0.029	12	0	0.025	16	0.276	0.031
Lolo39	20	0.050	0.047	10	-0.027	0.000	18	0.085	0.000
Lolo41	25	0.065	0.076	12	-0.082	0.139	18	0.151	0.000
Lolo48	24	0.184	0.141	10	-0.059	0.036	17	0.137	0.125
Mean	23	-	0.089	11	-	0.085	17	-	0.088
SE	0.604	-	0.010	0.285	-	0.017	0.524	-	0.016

Table S2. Estimation of the relative contribution of the environmental and landscape variables to the Maxent model for the Neotropical otter. The percent contribution indicates the variables that are contributing to fitting the model, while the permutation importance shows the contribution of each variable by randomly permuting the values of that variable among the training points and measuring the resulting decrease in the training AUC.

Variable	Percent contribution	Permutation importance
Elevation	27	40.1
BIO3	24.6	4.9
BIO9	13.3	23.6
NDVI	8	2.5
BIO18	7.9	4.4
Ecosystem integrity	7.1	7.5
Slope	6.6	1.7
BIO12	1.7	4
BIO7	0.5	5.5
BIO4	0.4	3.6

Table S3. Environmental suitability of the Neotropical otter in the three studied basins. We show the total area and the percentage of the area of each basin falling in the different suitability categories.

Basin	Total area (km^2)	Unsuitable	Low	Medium	High
Actopan	2009.1	57.8	23.8	12.3	6.2
La Antigua	2208.6	66.5	25.1	6.2	2.2
Jamapa	5154.4	77.1	20.7	2.1	0.2

Table S4. Model selection of the generalized linear mixed-effects models testing the effect of each landscape-riverscape feature on gene flow *between sites* of the Neotropical otter. Resistance surfaces showing a greater selection percentage than geographic distance alone constituted the set of features that are important for dispersal of *L. longicaudis* individuals. Higher top-ranked percentage and lower average weight and rank indicates the best supported models.

Resistance surface	avg.AICc	avg.weight	avg.rank	Top-ranked %
Environemntal suitability	-1220.821	0.911	1.006	99.41
Geographic distance	-1214.877	0.089	1.994	0.59
Ecosystem integrity	-1220.597	0.835	1.108	89.22
Geographic distance	-1214.93	0.165	1.892	10.78
NDVI	-1220.224	0.804	1.125	87.48
Geographic distance	-1215.644	0.196	1.875	12.52
Slope	-1218.868	0.805	1.117	88.3
Geographic distance	-1214.371	0.195	1.883	11.7
Stream water accumulation	-1215.478	0.573	1.297	70.35
Geographic distance	-1214.844	0.427	1.704	29.65
Stream order	-1169.985	0	2	0
Geographic distance	-1215.063	1	1	100
Elevation	-1214.501	0.441	1.685	31.49
Geographic distance	-1215.01	0.559	1.315	68.51

Abbreviations: average of the Akaike information criterion for each model, corrected for sample size; avg. weight: average model weight representing the probability that a model is the best in the model set, averaged over 1000 bootstrap replicates; avg.rank: average model ranking from 1000 bootstrap replicates; top-ranked %: proportion of times during 1000 bootstrap iterations that each model was ranked as the top model.

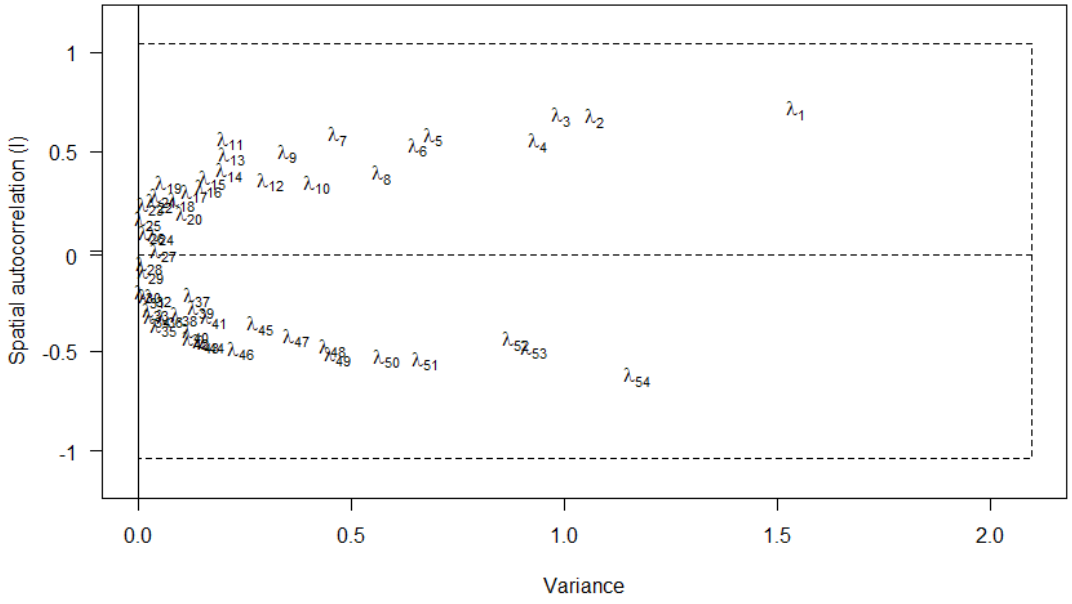

Fig. S1. Eigenvalues of the spatial principal component analysis plotted as variance and spatial autocorrelation (Moran's I) components. The two horizontal dashed lines indicate the range of variation of Moran's I, given the spatial weighting matrix that was used. The vertical dashed line indicates the maximum attainable variance. This figure is useful to assess whether a given score of entities contains relatively enough variability and spatial structuring to be interpreted. For instance, λ1 is the largest eigenvalue in terms of variance and spatial autocorrelation, it can therefore be distinguished from all the other eigenvalues.

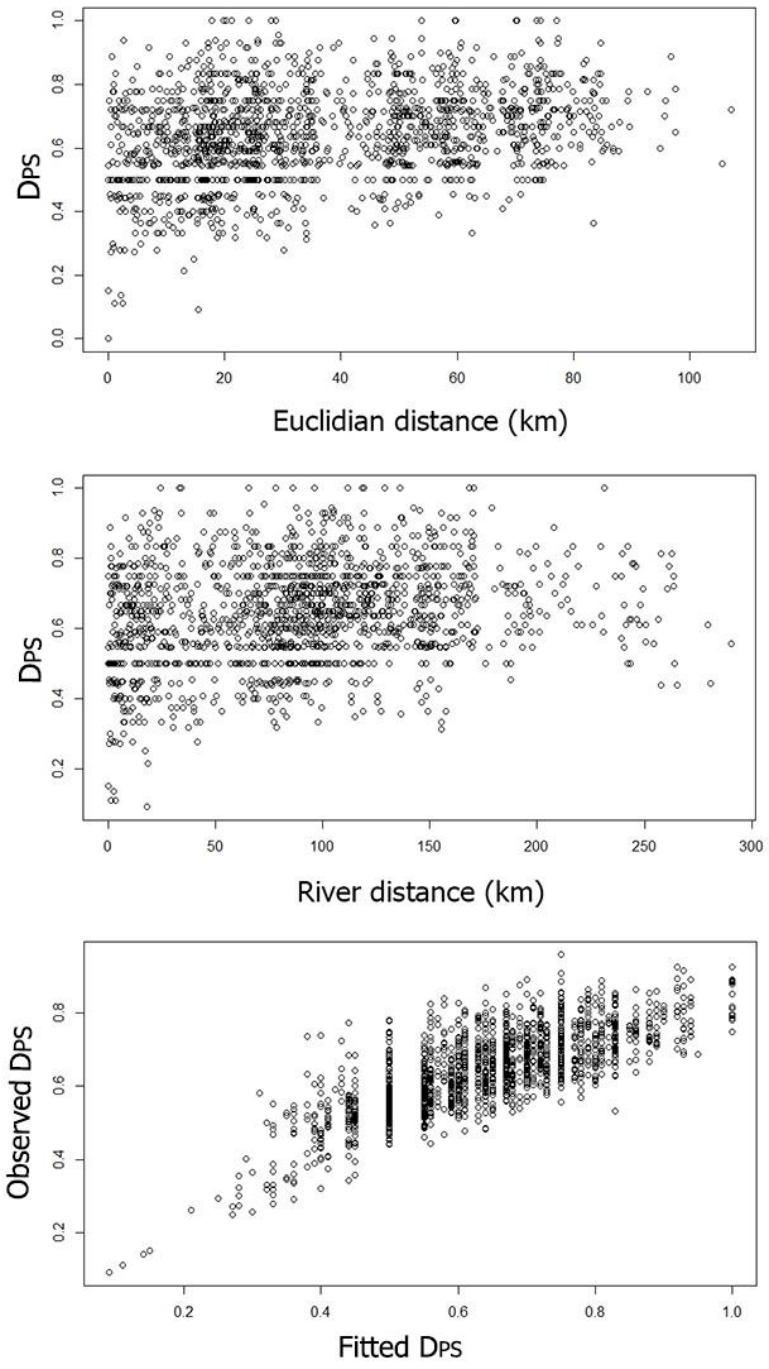

Fig. S2. Plots showing the results of the isolation by distance (IBD) using (a) Euclidian and (b) river distances and the (c) StreamTree analyses for the Neotropical otter. The StreamTree plot depicts the relationship between the fitted genetic distance (genetic distance among individuals in the stream network) and the observed pairwise genetic distance among individuals.

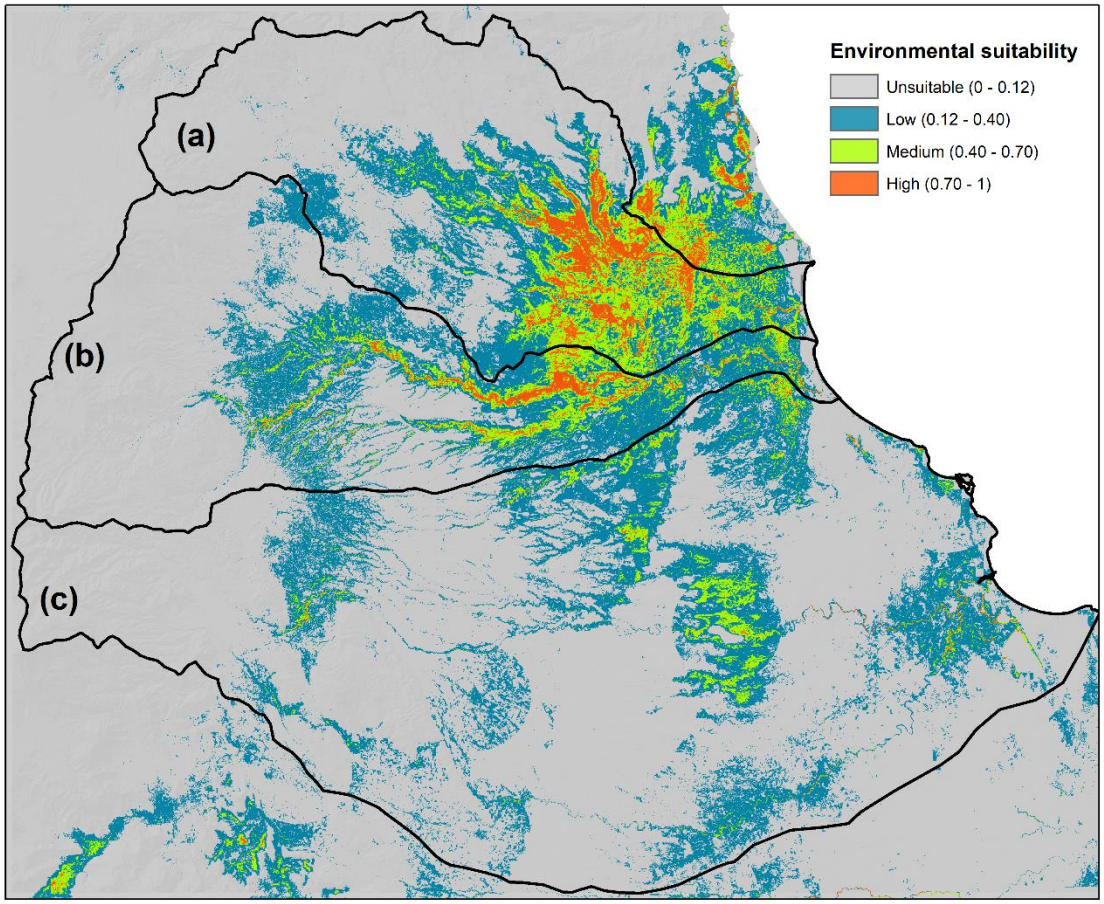

Fig. S3. Map of the environmental suitability of the Neotropical otter in (a) Actopan, (b) La Antigua, (c) Jamapa based on environmental niche modelling performed in MaxEnt. Unsuitable areas and three categories of environmental suitability are indicated by different colors in the map.

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SHORT COMMUNICATION

Isolation and characterization of 13 microsatellite loci for the Neotropical otter, *Lontra longicaudis*, by next generation sequencing

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Abstract
The Neotropical otter, *Lontra longicaudis*, is an ecologically important species for freshwater ecosystems that is threatened due to habitat destruction and hunting. However, there is limited information regarding the population sizes, genetic diversity, genetic structure and gene flow of the species, which is crucial for the elaboration of conservation plans. The aim of this study was to isolate and characterize microsatellites for *L. longicaudis*, using Illumina paired-end-sequencing. Initial amplification tests were performed in 48 loci, out of which, 13 yielded high-quality PCR products and thus were further evaluated. Genetic diversity and discrimination power of the 13 microsatellite loci was assessed using 19 non-invasive samples collected in the Jamapa basin in Veracruz, Mexico and blood samples from six captive individuals. All loci were polymorphic, the number of alleles per locus ranged from 4 to 10, the observed heterozygosity from 0.21 to 0.69, and the expected heterozygosity from 0.55 to 0.82. The combined set of 13 microsatellites showed a high power for discriminating among individuals (probability of identity $P_{ID} = 1.551 \times 10^{-16}$) and among siblings (probability of identity of siblings $P_{IDSIB} = 3.349 \times 10^{-06}$). A combination of nine loci are sufficient to discriminate among siblings with high confidence ($P_{IDSIB} < 0.0001$). The new set of microsatellites for the Neotropical otter reported here will provide a useful genetic tool to assess population genetic patterns and ecological parameters of the species.

Keywords Discrimination power · Genetic diversity · *Lontra longicaudis* · Microsatellites · Freshwater ecosystems

Introduction

The Neotropical otter, *Lontra longicaudis*, is an ecologically important species of freshwater ecosystems. As a top predator, the otter regulates its prey populations and it is an important biomonitor of the quality of the habitat [1]. Because of the reduction of its population sizes, mainly due to habitat destruction and hunting, the Red List of the International Union for Conservation of Nature (IUCN) has classified the Neotropical otter as an "almost threatened" species [2]. Therefore, actions for the conservation of *L. longicaudis* are crucial, including performing studies that estimate population sizes, genetic diversity, dispersal patterns and connectivity.

Because the Neotropical otter is a rare species that has elusive behaviors, the identification of individuals through direct observations is problematic. Consequently, most otter studies have used non-invasive sampling (e.g. collection of feces, anal secretions and hairs) in combination with molecular markers to estimate different population parameters and movement patterns [3]. Such combination of techniques has proven to yield accurate estimations of density, abundance and population sizes [4]. It is also more efficient than other approaches, such as photo-trap and the direct counting of

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signs (e.g. feces and tracks), since with less sampling effort similar or better results can be obtained [5–7].

Microsatellites are one of the molecular markers that in combination with non-invasive methods have been used to estimate population parameters. Microsatellites are short DNA fragments (100–500 bp) consisting of tandem repeats of simple sequence motifs that are relatively easy to amplify even from small or partially degraded samples. These markers are codominant, allowing the distinction between heterozygotes and homozygotes; and have high mutation rates (10^{-6} to 10^{-2} mutations per site per generation), making them very polymorphic [8]. Although the use of heterologous loci is a good initial approximation in species for which microsatellites are not available, they have the disadvantage of not being as polymorphic as the microsatellites developed in the focal species, and null alleles could be common [9, 10]. When using heterologous microsatellites, it is a common practice to choose the most polymorphic loci, which could bias the results as a consequence of using the markers with the greatest number of alleles. Therefore, for estimating genetic diversity and other population parameters, it is recommended to use a set of randomly selected polymorphic microsatellite loci developed in the target species to avoid biases resulting from using the most variable markers [11, 12].

The aim of this study was to isolate and characterize microsatellite loci for *L. longicaudis*, using Illumina paired-end-sequencing. These markers will be useful for estimating genetic diversity, population structure and gene flow, as well as for assessing different ecological parameters such as population size and dispersal patterns.

Materials and methods

Sample collection and DNA extraction

A total of 20 non-invasive samples that included seven anal secretions and 13 feces of *L. longicaudis* were collected along the main river and tributaries of the Jamapa basin (from 19° 13′ 39.49″ N, 97° 0′ 30.25″ W to 18° 50′ 52.62″ N, 95° 53′ 58.38″ W) in Veracruz, Mexico. Samples were preserved in RNA later at − 20 °C until DNA extractions were conducted. Blood samples from six captive individuals in the Veracruz aquarium ("Acuario de Veracruz, A.C."), which were originally from the Jamapa basin were also included. Blood samples were taken by veterinarians of the aquarium, following "NOM-135-SEMARNAT-2004" and the "Code of ethics and welfare animal of the World Association of Zoos and Aquariums (WAZA)". Samples were preserved in EDTA at − 20 °C until DNA extractions were performed. Genomic DNA from anal secretions and feces was extracted using the ZR Fecal DNA MiniPrep kit (Zymo Research), and from blood samples using the DNeasy Blood & Tissue kit (Qiagen), following the manufacturer instructions. To avoid contamination, before conducting the extractions all materials (tips, tubes, pipets and double distilled water) were sterilized and extractions were performed under a hood with laminar flow previously sterilized with ultraviolet light. A negative control consisting of sterilized doubled distilled water was included in every batch of DNA extractions to monitor contamination. DNA extractions were always kept in a different area than amplification products.

Isolation of microsatellite loci and primer design

An Illumina paired-end shotgun library was prepared using otter DNA extracted from blood samples following the methods described in O'Bryhim et al. [13]. Following the protocol of the Illumina TruSeq DNA Library Kit, and using a multiplex identifier adaptor index, the Illumina paired-end shotgun library was constructed by shearing 1 µg of DNA with a Covaris S220. Illumina sequencing was performed on a NextSeq 500 with 100 bp paired-end reads. Strands of nucleotides at the ends of the reads with scores lower than Q15 were removed. A total of five million reads were obtained and analyzed in the program PAL_FINDER_v0.02.03 [14] to identify reads containing di-, tri-, tetra-, penta-, and hexanucleotide microsatellites. The sequences that PAL_FINDER detected to have repeats, were batched to a local installation of the program Primer3 v2.0.0 [15] for primer design. Loci with primer sequences that occurred only one or two times in the five million reads were selected, in order to avoid issues with copy number of the primer sequences in the genome. A total of 15,202 loci conformed to this criterion, out of which, 48 tetra and penta-nucleotides loci were selected for initial screening. The forward primer of each pair designed to amplify the 48 loci was modified at the 5′ end with an engineered sequence (CAG tag 5′-CAG TCGGGCGTCATCA-3′). This allowed the use an extra primer (identical to the CAG tag) in the PCR reactions that was fluorescently labeled for detection with either 6-FAM or HEX fluorescent dyes. The 48 designed primer pairs were tested for amplification using DNA from both non-invasive and blood samples. Thirteen (Table 1, Electronic Supplementary Material) of the 48 primer pairs amplified high quality PCR products consistently and were used for further analyses.

Microsatellite genotyping and genotyping error

In order to avoid contamination, before performing the polymerase chain reactions (PCRs) all materials (tips, tubes, pipets and double distilled water) were sterilized and PCRs were carried out in an ultraviolet PCR workstation. PCRs consisted on a final volume of 10 µL containing

Table 1 Characteristics and amplification conditions of the 13 microsatellite loci developed for *Lontra longicaudis*

Locus	Primer sequence 5′–3′	Repeat motif	Size (bp)	Ta (°C)	$MgCl_2$ [mM]
Lolo1	F:*TCTTATTCAGGGCCTGTGGG	TTTC(15)	327–359	56	2.0
	R: TTTCCTGCTGTTCACAGTTCC				
Lolo13	F: *CAGCCAGGCACACTATCCC	AAAG(15)	178–206	55	2.0
	R: AGGCAAAGCACAGGATTTGG				
Lolo18	F:*CATCCCAAACTCAAAAGACTGC	GGAAA(14)	214–264	55	2.0
	R: TGTGTAATGATTGTTCACTAATCGG				
Lolo19	F:*CCATTTTCTCCCTCCTTTCC	TTCC(14)	291–303	56	2.0
	R: ACTGCAATGCACTCTGAGCC				
Lolo23	F:*AGGATTCTCACTCACTCTCTGCC	GAAA(15)	371–417	56	2.0
	R: AATGGTGAAAGATTTTGGAAGC				
Lolo29	F:*TCATAACTGACTTGCCTCAAGC	TTTC(17)	207–231	55	2.0
	R: AAATAGGGTTGTAAGATCTCAAGGC				
Lolo30	F:*ATAATTGCCGCTGTATCCCC	TTCC(15)	328–344	57	2.0
	R: TGACTTCTAAAGAAATACAAGCACTCC				
Lolo36	F:*TTGCGTTTGAGAGATGGTGG	AAAG(14)	332–368	54	2.0
	R: GGGAGGGGTCTTTCACTCG				
Lolo37	F:*TTCCCTCTATTCATTCCTCCC	TTCC(15)	295–311	56	1.8
	R: CCAAACATTTAAAGAACATTTATGCC				
Lolo38	F:*TCATGTCACACCAATTACAACAGC	TTTC(14)	228–288	56	1.8
	R: TCAGGTAAATACCCAGCAGTGG				
Lolo39	F:*CTTGCAGGGACTTTACTGCC	TTTC(14)	240–252	56	2.0
	R: CGGAAAGAGCCTTTTCTAGGG				
Lolo41	F:*AGGTCCAAGTTCCTGTTGGG	TTTC(15)	251–275	56	2.0
	R: CCTCAAGAAAGTGTGACAATAAGACG				
Lolo48	F:*GCCTACAGAAATGTGGCTGG	TTCC(15)	233–269	56	2.0
	R: TTCTGCTCAAGTCACGGTCC				

The size indicates the range of observed alleles in base pairs (bp) and includes the length of the CAG tag sequence, Ta (°C) is the annealing temperature of the second phase of the PCR cycling, $MgCl_2$ [mM] is the final concentration used in the PCR

*Indicates that the CAG tag sequence (5′-CAGTCGGGCGTCATCA-3′) was added

1 × PCR buffer, 1.8–2.0 mM of $MgCl_2$ (depending on the locus, see Table 1), 0.25 mM of dNTPs, 0.1 µM of the forward primer, 0.15 µM of the reverse primer, 0.15 µM of the fluorescently-labeled CAG primer, 0.5 U of GoTaq DNA polymerase (Promega) and 1–2 µL of genomic DNA. To monitor contamination, in all PCR reactions a negative control was included, which contained all of the above reagents but instead of genomic DNA double distilled water was added. Amplifications were performed in a Veriti™ thermocycler (Thermofisher) using the following conditions: 94 °C for 2 min, followed by five cycles with a touchdown (−1 °C per cycle) at 94 °C for 45 s, 60–56 °C for 45 s and 72 °C for 1.5 min; and a second phase of 40 cycles at 94 °C for 45 s, 54–57 °C (depending on the locus, see Table 1) for 45 s, 72 °C for 1.5 min and a final extension at 72 °C for 5 min. Amplicons were read in an ABI 310 DNA sequencer (Thermofisher) and the size was determined using the size standard GeneScan™ 600 LIZ and the software GeneMapper 4.0 (Thermofisher).

Because the low amount and quality of the DNA contained in anal secretions and feces could increase genotyping errors (i.e. allelic drop out, null alleles, false alleles), a multi-tube approach [16] was employed to verify the genotypes assigned to each of the non-invasive samples. A sample was considered to be either a homozygote or a heterozygote when the same alleles were obtained in three and in two separate and identical PCR reactions, respectively. Using the genotypes from the PCR repetitions, the consensus genotypes of each sample at each locus were determined in GIMLET v. 1.3.3 [17]. The genotyping error per locus was assessed based on the number of mismatches between resulting genotypes of the PCR replicates as suggested by [18]. MICROCHECKER [19] was used to determine whether allelic dropout and/or null alleles were present in all samples.

Identification of individuals and characterization of microsatellites loci

When employing a non-invasive sampling approach, several samples of the same individual could be collected during the same or in different sampling sessions. Therefore, *L. longicaudis* individuals were identified among the collected samples using the obtained genotypes and the R package ALLELEMATCH [20]. This program identifies unique multilocus genotypes in data sets where the number of individuals is unknown, taking into account the genotyping error and the missing data.

The analyses described below were conducted using the genotypes of the individuals identified by ALLELEMATCH from the non-invasive samples and the genotypes of the six captive individuals. The software FSTAT 2.9.3.2 [21] was used to calculate linkage disequilibrium between all pairs of loci, observed (H_O) and expected (H_E) heterozygosity, as well as deviations from Hardy–Weinberg equilibrium (HWE) by means of the inbreeding coefficient (F_{IS}) and 10,000 permutations. To assesses the discriminating power of the microsatellites in identifying individuals, the probability of identity (P_{ID}) and the probability of identity of siblings (P_{IDSIBS}) [22] were computed for each locus and combining all loci in CERVUS 3.0. [23].

Results and discussion

Out of the 20 non-invasive samples, 13 yielded amplification products for more than eight loci, resulting in a successful amplification rate of 65%. Genotyping error per locus ranged from 0 to 0.17 (Table 2) and no allelic dropout was detected for any of the loci. Using the genotypes of the 19 non-invasive samples that amplified for at least eight loci, ALLEMATCH identified 18 unique multilocus genotypes (i.e., individuals).

Hereafter, the results are based on the genotypes of 24 individuals, 18 identified from the non-invasive samples and six from the aquarium. All loci were polymorphic and between 21 and 24 individuals successfully amplified at each of the microsatellite loci (Table 2). Combining all 13 loci, a total of 84 alleles were found, ranging between 4 and 10 alleles per locus (mean = 6.5, SD = 1.80, Table 2). The observed heterozygosity ranged from 0.21 to 0.79 (mean $H_O = 0.44$), and the expected heterozygosity from 0.55 to 0.82 (mean $H_E = 0.72$, Table 2). These values of genetic diversity are similar to those reported for other populations of the Neotropical otter in Mexico, Argentina and Brazil (number of alleles: 4.7–6.5, H_E: 0.52–0.73, H_O: 0.56–0.83) [24–26]. The moderate to low values of genetic diversity found in this study could be the result of reductions in population sizes of the species as a consequence of habitat destruction. Alternatively, the levels of genetic diversity detected in the Neotropical otter could have been maintained through time. Further studies that evaluate these alternative scenarios are needed.

Table 2 Genetic diversity, frequency of null alleles and probability of identity of the 13 microsatellite loci developed for *Lontra longicaudis*

Locus	N	N_A	H_O	H_E	F_{IS}	FNA	P_{ID}	P_{IDSIB}	GE
Lolo1	23	6	0.47	0.74	0.38	0.15	0.08	0.41	0.16
Lolo13	24	7	0.79	0.82	0.07	0.00	0.04	0.36	0.17
Lolo18	24	7	0.68	0.76	0.12	0.04	0.05	0.39	0.05
Lolo19	23	5	0.21	0.67	**0.67**	0.27	0.14	0.46	0.06
Lolo23	21	8	0.28	0.81	**0.61**	0.26	0.00	0.13	0.07
Lolo29	24	7	0.58	0.81	0.27	0.11	0.04	0.33	0.08
Lolo30	23	4	0.42	0.74	**0.46**	0.18	0.08	0.40	0.11
Lolo36	22	10	0.37	0.8	**0.46**	0.19	0.03	0.36	0.00
Lolo37	21	4	0.21	0.55	**0.56**	0.21	0.21	0.54	0.03
Lolo38	22	8	0.37	0.7	0.40	0.14	0.11	0.44	0.15
Lolo39	24	4	0.42	0.56	0.28	0.06	0.18	0.53	0.00
Lolo41	24	7	0.47	0.74	0.37	0.14	0.07	0.40	0.00
Lolo48	24	7	0.47	0.72	**0.41**	0.16	0.09	0.42	0.13

Calculations were performed using the 24 genotyped individuals, except for the genotyping error, which was calculated for the 18 individuals identified from non-invasive samples

N number of individuals identified, N_A number of alleles per locus, H_O observed, and expected H_E heterozygosity, F_{IS} inbreeding coefficient, *FNA* frequency of null alleles, P_{ID} probability of identity, P_{IDSIB} probability identity of siblings, *GE* genotyping error. F_{IS} values in bold indicate significant deviations from HWE after Bonferroni corrections (Adjusted *P* value = 0.00385)

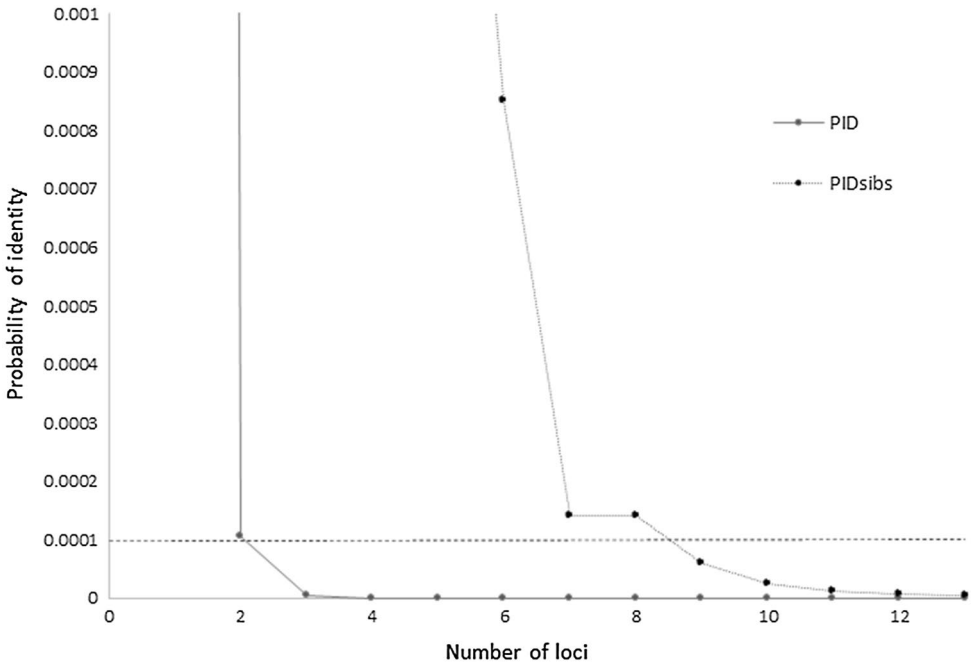

Fig. 1 Probability of identity (P_{ID}) and probability of identity of siblings (P_{IDSIB}) combining different numbers of the loci developed for *Lontra longicaudis*. The horizontal dashed line indicates a probability of identity of 0.0001

For each locus, P_{ID} values ranged between 0 and 0.21, and P_{IDSIB} from 0.13 to 0.54 (Table 2). Combining all 13 loci P_{ID} and P_{IDSIB} values were 1.55×10^{-16} and 3.35×10^{-06}, respectively. These results indicate that the microsatellite markers reported here have a high power to discriminate individuals. Three loci are enough to distinguish between unrelated individuals and nine to differentiate among siblings, with a probability of identity lower than 0.0001 (Fig. 1). Because of the philopatric behavior previously reported for the Neotropical otter [22], it would be advisable to use at least nine loci to accurately discriminate among related individuals.

There was no evidence of linkage disequilibrium for any of the 78 paired loci comparisons after the Bonferroni correction ($P < 0.0064$). Six loci (Lolo19, Lolo23, Lolo30, Lolo36, Lolo37, Lolo48) had positive and significant F_{IS} values, suggesting the presence of null alleles and/or inbreeding (Table 2). These six loci were among those with the higher frequencies of null alleles. However, only for three of these loci (Lolo 19, Lolo23 and Lolo 37), the frequency of null alleles was higher than 0.20, while for the other three the frequency ranged between 0.16 and 0.19. The presence of null alleles translates into a higher proportion of homozygotes and thus in reduced estimates of genetic diversity; it can also lead to an overestimation of genetic differentiation. However, frequencies of null alleles lower than 0.2 are considered not to introduce significant biases in the results [27]. Alternatively, the positive and significant F_{IS} values could be the result of inbreeding. It has been suggested that males of the Neotropical otter disperse more than females, presumably to reduce inbreeding [22] but dispersal could be limited by low quality habitat. Further investigations in different *L. longicaudis* populations are required to assess dispersal patterns in the species and the possible effect of habitat reduction.

The 13 microsatellites described here are the first set developed for the Neotropical otter, *L. longicaudis*. The characteristics of these markers make them suitable to assess population genetic patterns of the Neotropical otter as well as different ecological parameters including population size and dispersal patterns. These kind of studies are valuable as they can contribute to the management and conservation of the species.

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Compliance with ethical standards

Conflict of interest All authors declares 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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Electronic Supplementary Material

Isolation and characterization of 13 microsatellite loci for the Neotropical otter, *Lontra longicaudis*, by next generation sequencing

Molecular Biology Reports

María Camila Latorre-Cardenas[1,2], Carla Gutiérrez-Rodríguez[1], and Stacey Lance[3]

Microsatellite sequences

>Lolo1

TGAACAATGATCCTGTTGCCTTCTTATTCAGGGCCTGTGGGGGATGAGGCTTCAGGGTAGGGAGTGAGCAG
TTAGTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTTCTTTCTTCCTTTCT
GCAGTTTTCCCAAATAGGCTGATCCCCAGTCCCCCCTGTCTGGGCTCTCTGGAAGGGACAGAAAGAGGAAC
TGTGAACAGCAGGAAAGATATTCAAATCAGGTCCATTTTAAATTCTAATTCTCCTGCCTTTTAGTAATTTTGA
TTTCCCT

>Lolo13

TGGGGCTTGAACTCACAACCCTAAGATCAAGAGTTGCATGCTCTACTGACTGAGCCAGCCAGGCACACTAT
CCCCATTAAAAAAGAAAGAAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGA
AAGAAAGAAAGA

AATTCACGAGCTAGTACATTGCAAAGCAGATGGTCTCCAAATCCTGTGCTTTGCCTACTGTGCAATACTGAC
TAAAGAGTGTGAACACCAACAGAATATTTAAAATGAGAATACTATGTACTACATATAATTGCATTGTTTGTG
TGACTCA

>Lolo18

AAACCCTCCCCTAGTACTGAATACTAAGTCATGCAACATCCCAAACTCAAAAGACTGCAGTCTTTAGCTCTAA
AATAAAAATACTGTGAACCAGGTTAGTGTTTAGAGAGATGAAGTAAAGAAGGAAAAAAGGAAGGAAGGA
AGGAAAGAGGGAAGGAAAGGAAAGGAAAGGAAAGGAAAGGAAAGGAAAGGAAAGGAAAGGAAAGGA
AAGGAAAGGAAAGGAAGGGAGGAATTAAATTAAAAAACACCGATTAGTGAACAATCATTACACATTTTCAA
AATCTGTTTCATGATCCTCTGTT

>Lolo19

AGTGCCATTTTCTCCCTCCTTTCCTCCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCT
TCCTTCCCTTCTTTTGTGGGGGAGATAGGGTGCCAGCAGGGCAGGGGGGGGGGTTGCTGGGCGGAGGCA
GAGGGGAGATAACCTTAAGCCGGCTCCACACCCAGCTCGATCTCAGGACCCTGAGATCAAGACCTGAGTCG
GAACCAAGAGTCCTGCAGAGCCCCCCGGGTGCGGGGTGCTGACTTCCACAGCCTGGGCTCAGAGTGCATT
GCAGTGTTAAACTTT

>Lolo23

TAGAGCCTGCTTAGGATTCTCACTCACTCTCTGCCCAAAACACTCTCTCTTTAAAAAAGAAAGAAAGAAAGA
AAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGGAAAAGAAAGAAAGAAATGGAAGTG
ATTTTAGTATAACTGATAGAAGTGGTTGAGGGTGGACATCCTTTCTTGTTCCCAATCTTGAGGGAAAGCTTC
CAAAATCTTTCACCATTAAGTATGCTGTTAACTGCAGGTTTTTCACAGATGCCCTTTAACAGACTAACAAAGT
TCCCTTCTATTCCCAAT

>Lolo29

AATAGTTCCTGGGAATTGAGTCTGACATCATCATAACTGACTTGCCTCAAGCACAAGAGACTCAATTTTCTTT
CTTTCTCTCTTTCTTTTCCTTCCTTCTTTCTTTTCTTTCTCTCTCCCTCACCCTGTCTTCCTCCCTCCTTCTGCTCCC
CTTTCTCTCTCCCTCACCCTGTCTCCCTCCCTCCTTCTGCTCACTCTTTCTTTCTTTCTTTCTTTCTTTCTTTC
TTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTCCTGCCTTGAGATCTTACAACCCTATTTATAGTTCAGAT

>Lolo30

ACTTGTCTCTCCAGGTACTGATCTCTGGACAAGGCTCCAGATAACCTGGAAATGGGTATAATTGCCGCTGTA
TCCCCTCCCCTGCACCCTGCTGGGATGGTGTGCTCTCAGTGGGGCTGCCATCAGCGGCTCTTCAGACTTGAA
GACCAGGTCCTTCCTTCCCTCTTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTC
CTTCCTTCTCATCTGACATTTTTTTATGGAGTGCTTGTATTTCTTTAGAAGTCAAGTGTTTGATTTAACGAACA
AGGAATA

>Lolo36

CTCTTTAGGTAGGTGGCTAGAGGCATTTGCGTTTGAGAGATGGTGGTATGTGTGTGTGTTTTCCCCTTCCCT
CTCCCTACTATGTGAACGTCTCGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAA
AGAAAGAAAGGATCTTTCAAAAAGGTGAGCACCTGAGTTTGAAAAAAGACTTCTGGGCATGGGTATTGGTT
CGGTTTTCACCATCTGCTCTCCATCTCCATTTATTTCCATCATACATTTGCTTAAAGTGTTAGCGAGTGAAAGA
CCCCTCCCATCCAAC

>Lolo37

TCCTTCCTTCCCTCTATTCATTCCTCCCTTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCT
TCCTTCCTTCCCTTTCTTTCTTTCTTTCCTTCCCCTTCCTTCCTCTCTCTTTTCCTCCTTTCTTTCTTTCCTTCTTCCT
TCCTTCTTCTTTCTTTCTTAGTGTCTTTATCTGGTTTTGGTATCAGAATAATGCTGACTTTGAAGAACTAGTTT
GGAAGCATTTCTTTCAATTTTTTGGAATAGTTAGAGAAAGATAGGCATAAATGTTCTTTAAATGTTTGGTA

>Lolo38

ATATCATGTCACACCAATTACAACAGCTAATATCAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAG
AAAGAAAGAAAGAAAGAAAGAAAAAAGGAAGAAACCAAGATATAACAAGTGTTAGCAAGGATGTGGAGA
AATGACGACAGTACCCTCATGCACTCCTGGTAGAAATGTCGACTGATGTGACCATAATGGAAACCGGTATG
GAAATTTCTCAAGAAATTAAAAAATAAAAAGCCATAAAATTCAGCATTCCACTGCTGGGTATTTACCTGAAG
AAAACAAGAAACACTAATTT

>Lolo39

CCTATCATCTCTCTTGACTGACAGTCGTGAGTTTGCAGACTTTTTCCCTTGCTTGCAGGGACTTTACTGCCTTG
TGCCCATCTAGAAAAGAGTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTC
CTTATTTACAGAATCTCTACCTCCGACATGGGGCTCCATCTCACGACCCCACGATTAAGAGTCTCATGCTGTT

CCTGACTGAGTCAGCACCCCTAGAAAAGGCTCTTTCCGAACCATATTGACCATGTCATTTTCTTGCTTAAGAA
ACCC

>Lolo41

TGCAAGGTTAGATCCATTTGTCTGAGGTCCAAGTTCCTGTTGGGTCTGCAGTGGGCCAGGACTGGTAGTGT
CAGTGAGCCCTCATGCTCAAACTAGAGACCCTAACAATTAAAACCACAAGTAGACAAGACCACTTAACTCAC
GCTCTAACNCAANACCACTTAACTCANGCTCTAACTGCAACCCATTGTTCGTTCGTTTTCTTTCTTTCTTTCTTT
CTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTCTTATTCTTCTCGTCTTATTGTCACACTTTCTTGAG
GCATT

>Lolo48

CTGCCTACAGAAATGTGGCTGGAATTTTGATAGGAATTGTGTTAAACCTGTATGTCAGTTTCCCTCCCTCCCT
TCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTATCTTGGTTTGTTTTTTT
CCTTCCTTCCTTCCTTCCTTCCTATCTAGGTTTGGTTATTCTAGGGCTGGGGCATGAGGGGCAGAGGGTGAA
GGAGAGAAGCAGACTCCCCGCTGAGCACAGAGCCCAACGTGGGGCTTGATCTCAGGACCGTGACTTGAGC
AGAACTCAG

CAPÍTULO IV

Non-invasive sex determination of the Neotropical otter using *ZFX/ZFY* sequence

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Conservation genetics resources

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Abstract

The development of molecular sexing techniques is relevant for conservation and managing purposes of endangered species, particularly when working with elusive and non-abundant species. Here, we describe a new protocol that amplifies a short fragment (180 pb) of the zinc finger gene, ZFY/ZFX, using specific primers (ZFMC-F2 and ZFMC-R2) that we designed for the Neotropical otter, *Lontra longicaudis*. The primers were designed to amplify a fragment that contains three polymorphic sites that differ between X and Y chromosomes, which allowed us to determine the sex of non-invasive genetic samples. The new set of sex-specific primers for the Neotropical otter reported here will provide a useful molecular tool to determine differences on dispersal patterns and population parameters between the sexes.

Keywords molecular sexing; amplification of homologous fragments; sex-linked marker; *Lontra longicaudis*

Technical note

Molecular sexing of wildlife individuals is relevant for conservation and managing purposes, particularly when working with elusive and low-abundant species that are difficult to observe and capture (Hrovatin and Kunej 2018). Sexing individuals provides information that is relevant for the demography (sex ratio), population dynamics, reproductive behavior and dispersal of the species (Tucker et al. 2017; Mengüllüoğlu et al. 2019). Molecular sexing methods in mammals have been based on the amplification of fragments on male-specific genes or of homologous fragments present in both sex chromosomes. The determination of sex based on the amplification of a male Y-specific fragment (i.e., chromosome Y-linked SRY gene) and the non-amplification of such fragment in females could result on the misidentification of the sex. The lack of amplification of the target fragment could not necessarily indicate that it is a female as PCR failures could be for other reasons (Statham et al. 2007). One way to control for errors that result from the lack of amplification of the samples is to simultaneous amplify, by multiplex PCR, the Y-linked marker along with another fragment (i.e. mitochondrial or microsatellite locus) that function as a positive control (Hrovatin and Kunej 2018). However, using such positive control does not guarantee the correct identification of the sex, as the target fragment might not amplify for several reasons. This is particularly true when working with low quality DNA samples, such as scats and hair, as the risk of the PCR amplifications failing and having genotyping errors increases (Taberlet 1996; Waits and Paetkau 2005; Pompanon 2005). The use of homologous fragments presented in both sex chromosomes (e.g., zink-finger protein genes *ZFX* and *ZFY*) has been proved to be more reliable (Ortega et al. 2004; Fontanesi et al. 2008; Hrovatin and Kunej 2018). This method is based on the identification of differences on sex chromosomes sequences of males and females. Such differences could consist on nucleotide polymorphisms (Mucci et al. 2007), or length differences of X and Y chromosomes homologue fragments (Statham et al 2007; Brinkman and Hundertmark 2009).

The Neotropical otter, *Lontra longicaudis*, is an ecological important species of freshwater ecosystems (Rheingantz et al. 2014) that is classified as “Near Threatened” species by the Red List of the International Union for Conservation of Nature (IUCN; Trinca and Rheingantz 2015). Therefore, actions for the conservation of *L. longicaudis* that include the assessment of population demography, proportion of sexes and dispersal patterns of individuals are imperative. Because the Neotropical otter is an elusive species, sex identification through non-invasive sampling and the use of molecular markers is crucial. Trinca and Ezirik (2012) described a protocol that uses the Lut-SYR

marker (Dallas et al. 2000) to sex samples of *L. longicaudis*. Although the method involves the co-amplification of a microsatellite locus developed for *L. lutra* (Huang et al. 2005) as a positive control, the lack of amplification of the SYR marker could lead to the incorrect identification of females. Here, we described a new protocol to amplify and sequence a short fragment of the zinc-finger protein gene, ZFY/ZFX, using specific primers that we designed for the Neotropical otter, which allows to determine the sex of the Neotropical otter. We used the zink-finger protein gene ZFX/ZFY, because it is highly conserved in mammals and it is present in both X and Y chromosomes (Luo and Page 1994).

To designed primers to amplify a fragment of ZFY/ZFX specific for *L. longicaudis*, we used blood samples of nine sex-known captive individuals (four males and five females) from the Veracruz aquarium ("Acuario de Veracruz, A.C."), which were rescued from the Jamapa River in the last five years. Veterinarians of the aquarium took blood samples following the ethical protocol "NOM-135-SEMARNAT-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^{o}C. We extracted genomic DNA using the DNeasy Blood & Tissue kit (Qiagen), following the manufacturer instructions. We amplified a fragment of approximately 380 bp using primers P1-5EZ and P2-3EZ, previously employed in other mammals (Ansen and Medrano 1990) and the polymerase chain reaction (PCR). PCRs consisted on a final volume of 15 µL containing 1X PCR buffer (Promega), 2.0 mM $MgCl_2$, 0.3 mM dNTPs, 0.13 µM of each primer, 0.75 U GoTaq DNA Polimerase (Promega) and 1 µL of genomic DNA. We included a negative control, containing all of the above reagents but instead of genomic DNA we added double distilled water. Amplifications were performed in a Veriti™ thermocycler (Thermofisher) using the following conditions: 94°C for 2 min, followed by a phase of 40 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 45 s, and a final extension at 72 °C for 10 min.

We purified PCR products using the Exo Nuclease I and FastAP enzymes (Thermofisher) and sequenced them at Macrogen, South Korea. We assembled the sequences in SEQUENCHER v 4.1.4. (Gene Codes Corp.) and aligned them in PhyDE v 0.9971 (http://www.phyde.de). The final alignment consisted of 348 bp, containing three polymorphic sites at positions, 35, 56 and 98 that differed between sexes (Fig. 1). The rest of the sequences were identical among individuals. We used PRIMER3 v. 0.4.0 (Untergasser et al. 2012) to design internal primers ZFMC-F2 (5'- GAAAGGAGCCAACAAAATGC- 3') and ZFMC-R2 (5'- GCAGTACTGGCACTGGTACG- 3'), which amplify a shorter fragment (180 bp) that included the three polymorphic sites. The small size of the fragment

is ideal for amplifying partially degraded samples or containing smalls amount of DNA, such as scats and hair.

We used the newly designed primers to determine the sex of a total of 40 non-invasive samples (17 anal secretions, 22 scats and 1 hair sample) that were collected for a previous study in Jamapa, La Antigua and Actopan rivers, located in the state of Veracruz, Mexico (Latorre-Cardenas et al. 2020). When employing a non-invasive sampling approach, several samples of the same individual could be collected during the same or in different sampling sessions. Therefore, we used previously extracted DNA that was identified as a unique individual in Latorre-Cardenas et al. (2020). PCRs were conducted as described above, except that we used the ZFMC-F2 and ZFMC-R2 primers, 2-3 ul of genomic DNA and an annealing temperature of 54 °C. We conducted purification and sequencing of PCR products, and sequence analysis as described above. We determined the sex based on the three polymorphic sites at positions 35, 56 and 98 of the sequences (Fig. 1).

We obtained a total of 34 sequences from the non-invasive samples, as six samples did not amplify, resulting in a success amplification of 85%. Based on the three polymorphic sites we identified a total of 12 males and 22 females. All female and male sequences were identical with the exception of the three polymorphic sites, which were identical within sexes. The use of homologous sex-specific sequences for sexing mammals have shown to be reliable for assigning sex (Ortega et al. 2004; Lynch and Brown 2006) but PCR failures and allelic dropout are frequent in non-invasive genotyping. Therefore, PCR replicates should be carried out to achieve reliable sex identification, with two replications generally being enough to corroborate sex determination (Lynch and Brown 2006). In our study, we did not perform PCR replicates because we used samples that had been previously amplified at 11 microsatellites loci, which yielded PCR products ranging between 180 and 400 bp, and for which a multi-tube approach was carried out (Latorre-Cardenas et al. 2020). In addition, the error in determining the sex with the method we proposed here, is minimized because the differences between the two sexes consist on three polymorphic sites. These primers will be useful to assess behavioral, demographical and ecological aspects that are lacking for the Neotropical otter (Rheingantz and Trinca 2015). For example, assessing the effect of sex-biased dispersal on genetic patterns and the sex ratio of breeding individuals, is essential information for supporting conservation efforts of the species (Latorre-Cardenas et al. 2020).

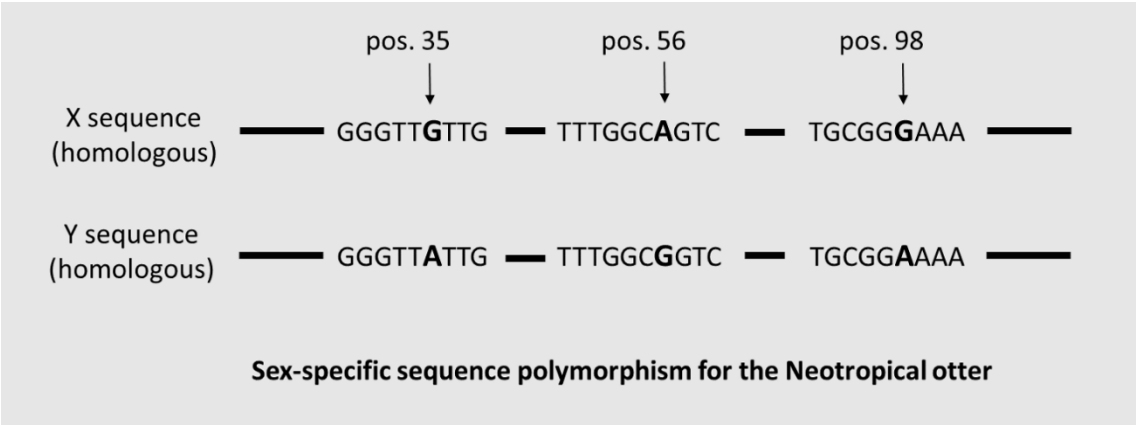

Fig. 1. Sex-specific sequence polymorphisms used for sexing the Neotropical otter. We show the three polymorphic sites (nucleotides in bold) of the homologous sequences Zinc Finger (ZFY/ZFX) from both sex chromosomes. We obtained the sequences by first amplifying a fragment using primers ZFMC-F2 and ZFMC-R2.

COMPLIANCE WITH ETHICAL STANDARDS

Ethical approval

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

CONFLICTS OF INTEREST AND ETHICAL APPROVAL

The authors declare that they have no conflict of interest.

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DISCUSIÓN GENERAL

En este estudio se investigaron los tamaños poblacionales, patrones genéticos contemporáneos y elementos del paisaje que mantienen la conectividad funcional de la nutria neotropical a una escala local (parches de hábitat) y del paisaje (configuración de la matriz). Esta información es relevante para conocer el estado de conservación de la especie en la región y para efectuar estrategias que mantengan la conectividad del hábitat de la nutria, sobre todo si se considera que en el área de estudio la modificación del paisaje y la pérdida de hábitat es rápida. La diversidad genética de la nutria fue moderada, aunque es posible que ésta se vea reducida en el corto plazo debido a que el tamaño efectivo de las poblacionales fue bajo y la calidad del hábitat en la región es pobre. La idoneidad del hábitat, la organización de los ríos y la perturbación humana fueron los elementos del paisaje que mejor explicaron la estructura genética y el flujo génico de *L. longicaudis*. Las partes altas y medias de las cuencas parecer ser las zonas que facilitan la conectividad de la nutria en la región.

Diversidad genética y tamaños poblacionales

La diversidad genética de *L. longicaudis* fue moderada (promedio: H_O = 0.50, H_E = 0.62, AR = 4.56) y varió significativamente entre las cuencas, siendo más alta en Jamapa (H_E = 0.69), seguida de Actopan (H_E = 0.63) y La Antigua (H_E = 0.54). Se esperaba que la diversidad genética de *L. longicaudis* fuera más baja, debido al alto deterioro ambiental reportado en el área de estudio (Cotler et al. 2010, Equihua et al. 2014). Sin embargo, los niveles de diversidad encontrados fueron similares a los reportados para la especie tanto en una zona conservada de México (río Lacantún; Ortega et al. 2012) como en zonas perturbadas de Suramérica (río Paraná en Argentina; Trigila et al. 2016). De forma similar, se esperaba que la diversidad genética variara entre cuencas y reflejara el diferente grado de deterioro ambiental (Actopan < La Antigua < Jamapa) al que están sujetas las cuencas. Esto no se cumplió ya que Jamapa tuvo la diversidad genética más alta a pesar de tener la idoneidad ambiental más baja para la nutria (de acuerdo con el modelo de nicho ecológico) y un deterioro ambiental extremo (Cotler-Ávalos et al. 2010).

A pesar de que se ha reportado que la pérdida y la fragmentación del hábitat tienen efectos negativos sobre la diversidad genética, esto no fue evidente en este estudio (Keyghobadi 2007). Esto pudo deberse a que se requieren entre 5 a 10 generaciones para que el efecto negativo del deterioro

del hábitat se refleje en la diversidad genética de las poblaciones (Goossens et al. 2004, Wozney et al. 2011, Epps y Keyghobadi 2015). La nutria neotropical tiene una longevidad de 10 a 15 años, y aunque no se conoce con certeza la edad de su madurez sexual, se ha reportado que otras especies de nutrias la alcanzan entre los dos a cinco años, si las condiciones ambientales son óptimas (Redd-Smith 2012, Hutchinson et al. 2015, Hung et al. 2016). Esto sugiere que se necesitan entre 12 a 50 años para alcanzar ese número de generaciones, lo cual puede ser tiempo suficiente para enmascarar los efectos negativos de la pérdida del hábitat sobre la diversidad genética. En la región de estudio, el deterioro ambiental por la agricultura y ganadería data desde la década de los años 30 del siglo XX y ha incrementado en un 20-30% desde 1970, principalmente a causa del crecimiento urbano (Cuevas et al. 2010). Otra posibilidad es que en el área de estudio no se haya alcanzado el valor umbral en el cual la pérdida de hábitat genera cambios drásticos en la diversidad genética, la cual se ha calculado que ocurre cerca del 50% de pérdida del hábitat (Pflüger et al. 2019).

Las estimaciones de N_e para *L. longicaudis* en cada una de las cuencas (Jamapa = 37.3, Actopan = 19.6, La Antigua = 6.4) fueron considerablemente más bajas que el tamaño mínimo (N_e = <100) para evitar la depresión por endogamia y asegurar que las poblaciones mantengan la diversidad genética (Frankham et al. 2014). Esto evidencia la vulnerabilidad de las poblaciones de la nutria frente a cambios estocásticos demográficos, ambientales y genéticos. Además, en poblaciones considerablemente pequeñas, se puede acumular un número de mutaciones deletéreas suficiente para que se tenga una acelerada pérdida de diversidad genética y disminución del tamaño poblacional (Higgins y Lynch 2001).

La estimación del N_c para toda el área de estudio fue de 259 individuos, lo cual equivale a una densidad de 1.21 individuos km^{-1}. Este valor de densidad es similar al reportado por estudios moleculares para la especie en poblaciones de Brasil (1 individuos km^{-1}; Trinca et al. 2013), pero es menor al obtenido en el río Lacantún, ubicado en la cuenca del Usumacinta-Grijalva en Chiapas, México (1.95 individuos km^{-1}; Ortega et al. 2012). Esta diferencia puede deberse a la alta conectividad hídrica y la mayor integridad ecosistémica en dicha cuenca, en comparación con las estudiadas, lo cual implica una mejor calidad de hábitat para mantener poblaciones más grandes de la nutria (Morrison et al. 2012, Pflüger et al. 2019). También la densidad de nutrias en la cuenca de Actopan fue ligeramente mayor (1.66 individuos km^{-1}) que las estimadas en La Antigua (1.44 individuals km^{-1}) y Jamapa (0.88 individuals km^{-1}), lo cual puede asociarse con la mejor calidad de hábitat para la nutria en dicha cuenca. A pesar de que varios estudios han reportado que existe una

proporción constante entre el tamaño efectivo y censal de las poblaciones (N_e/N_c = 0.1 - 0.3; Kalinowski y Waples 2002, Willoughby et al. 2015), en este estudio los tamaños poblacionales no variaron de forma similar para cada cuenca, puesto que Jamapa presentó el mayor N_e, pero el menor N_c y densidad. Esto puede deberse a que la propoción N_e/N_c varía en el tiempo dependiendo de la variación en el éxito reproductivo de las poblaciones y la migración entre poblaciones. N_e puede mantenerse a lo largo del tiempo si la migración es constante, aunque el tamaño censal disminuya por cuestiones demográficas o ambientales (Luikart et al. 2010). También debe considerarse que la estimación de N_c deben tomarse con precaución, ya que sus amplios intervalos de confianza sugieren que las poblaciones pueden ser más grandes (Miller et al. 2005).

Aunque no se encontró evidencia de cuellos de botella recientes en ninguna de las cuencas, los tamaños poblacionales (N_e y N_c) de la nutria fueron bajos. Los N_e pequeños podrían ser resultado de la historia evolutiva de las poblaciones; sin embargo, se ha reportado que las poblaciones de las cuencas de La Antigua y Jamapa han estado en un proceso de expansión desde el último periodo glacial (Hernández-Romero et al. 2018a), por lo que es probable que los pequeños tamaños se deban a reducciones recientes. Por ejemplo, se ha determinado que los principales factores que han llevado a la extinción local de poblaciones de la nutria euroasiática y la nutria marina son la contaminación de los ríos, la reducción del alimento y la caza (Larson et al. 2002, 2012; Tison et al. 2015; Pigneur et al. 2019).

Estructura genética y detección de migrantes

Como se esperaba, *L. longicaudis* presentó una estructura genética que se correspondió con la organización espacial de las cuencas; es decir, se identificaron tres grupos genéticos (K = 3) que correspondieron a cada cuenca. Este resultado sugiere que el arreglo jerárquico (anidamiento) de los ríos entre cuencas promueve la diferenciación genética de *L. longicaudis*, como lo predice el modelo de jerarquización de ríos (SHM), el cual será discutido más adelante. Adicionalmente, se encontró estructura genética espacial críptica que sugiere la presencia de clinas genéticas a lo largo de un gradiente latitudinal y altitudinal. La estructura genética latitudinal diferenció a los individuos de Actopan, la cuenca situada más al norte, de aquellos de La Antigua y Jamapa, mientras que la estructura altitudinal, indica que los individuos de las cuencas bajas están genéticamente diferenciados de los individuos de las cuencas medias y altas. Estos patrones genéticos

correspondieron con la variación espacial que tuvo la calidad (idoneidad) del hábitat de la nutria a lo largo del área de estudio. Las zonas con una mejor idoneidad de hábitat se localizaron en la cuenca de Actopan, y el tamaño de estas áreas disminuyó latitudinalmente hacia Jamapa.

Se identificaron migrantes de primera generación (10 individuos), lo cual indica que, a pesar de existir estructuración genética, se mantiene el flujo génico entre las poblaciones. Este resultado es importante, ya que el flujo génico entre poblaciones aisladas y con N_e pequeños, como el caso de la nutria en este estudio, ayuda a contrarrestar los efectos de la pérdida de diversidad genética y de la diferenciación genética (Bouzat 2010, Frankham et al. 2017). La mayoría de los emigrantes identificados provenían de La Antigua, y el mayor número de inmigrantes se encontraron en Actopan. Varios estudios han reportado que la baja calidad de hábitat promueve la dispersión de los individuos hacia hábitats con condiciones óptimas (Bowler y Benton 2005, Rémy et al. 2011; Honorato et al. 2015, Day et al. 2019), lo cual concuerda con los resultados de este estudio que indican que Actopan tiene las condiciones de hábitat más favorables de las tres cuencas. Todos los resultados de estructura genética sugieren que la Antigua y Jamapa son genéticamente más similares, mientras que Actopan es la cuenca más diferenciada. Parte de esta diferenciación podría deberse a que Actopan recibe individuos migrantes que provienen de cuencas ubicadas al norte del área de estudio, por lo que sería importante incluir dichas cuencas en futuros estudios para tener un mejor entendimiento de los patrones de dispersión de *L. longicaudis* en la región.

Efecto de modelos de aislamiento por distancia y de jerarquización de ríos

La diferenciación genética de la nutria neotropical fue explicada principalmente por la estructura de las redes de ríos y, en menor medida por la distancia geográfica (euclidiana y distancia de río) entre los individuos. Esto apoya nuevamente que el modelo de jerarquización de ríos (SHM) se ajusta a los patrones de estructura genética de la nutria, así como se ha reportado para varias especies estrictamente acuáticas (Osborne et al. 2014; Byrne et al. 2015, Cole et al. 2016, Brauer et al. 2016), y para algunas semiacuáticas que tienen una dispersión moderada, como el castor, *Castor canadensis* (Crawford et al. 2009) y el capibara, *Hydrochoerus hydrochaeris* (Byrne et al. 2015). Sin embargo, el modelo de jerarquización de ríos no se ajusta a todas las especies semiacuáticas, especialmente si son generalistas y usan de forma oportuna elementos del paisaje que han sido

introducidos por el humano, como caminos, canales agrícolas y zonas urbanas (Zalewski et al. 2009, Centeno-Cuadros et al. 2011, Laurence et al. 2013).

El efecto de la estructura jerárquica de los ríos no se ha evaluado en otras especies de nutrias, pero si se ha reportado que el modelo de aislamiento por distancia (IBD) explica la diferenciación genética de *L. lutra*, *L. canadensis*, y *L. longicaudis*. Estos estudios, incluyendo el presente, han documentado una autocorrelación espacial genética, la cual sugiere que el efecto de la distancia geográfica se reduce a partir de los 18 a 25 km (Quaglietta et al. 2013, Trinca et al. 2013; Pagacz 2016). La dispersión diferencial entre sexos es común en mamíferos, siendo los machos los que tienen una mayor dispersión desde sus sitios natales hacia nuevas zonas de reproducción; mientras que las hembras son filopátricas y se dispersan distancias más cortas (Greenwood 1980; Mabry et al. 2013). Por lo tanto, se esperaría una mayor estructuración genética en las hembras, y que el efecto de SHM, IBD, y de otras variables del paisaje sea diferente entre sexos. Por ejemplo, los ríos y caminos afectan la dispersión de las hembras del pescador, *Pekania pennanti*, pero no la de los machos (Tucker et al. 2017).

Genética del paisaje y el efecto del aislamiento por resistencia

De acuerdo con lo esperado, diferentes elementos del paisaje influyeron sobre la estructura genética y la dispersión de *L. longicaudis*, apoyando la hipótesis de que el aislamiento impuesto por la resistencia (IBR) del paisaje explica mejor los patrones de diferenciación genética que la distancia geográfica (IBD). Es interesante que fueron diferentes los elementos del paisaje que a una escala local y del paisaje explicaron la diferenciación genética de la nutria. En los parches de hábitat, la pendiente, el NDVI y el orden del río se asociaron con la estructura genética de la nutria, mientras que la idoneidad ambiental, integridad ecológica y acumulación de agua en ríos fueron los elementos de la matriz del paisaje que facilitaron o limitaron la dispersión de la especie. Este efecto diferencial de los elementos del paisaje, dependiente de la escala espacial, ha sido reportado para otras especies de mamíferos (Galpern et al. 2012, Thatte et al. 2019).

Como se esperaba, las áreas con mejor idoneidad de hábitat para la nutria, alta integridad ecológica y ríos con mayor cantidad de agua, facilitaron la dispersión de *L. longicaudis* a través de la matriz del paisaje. La idoneidad del hábitat fue la variable que más contribuyó a la dispersión de la nutria, y se caracterizó por incluir zonas con altitudes medias a altas, y temperaturas moderadas

durante la época más seca del año, lo que podría relacionarse con la permanencia de cuerpos de agua durante todo el año. Los ríos con mayor cantidad de agua impusieron menor resistencia a la dispersión de la nutria, lo cual era de esperarse porque ésta es más ágil en agua que en tierra (Kruuk 2006, Holland and van der Merwe 2016). Además, los ríos profundos y anchos pueden albergar una mayor disponibilidad de presas (Carrillo-Rubio y Lafón 2004, Smith et al. 2020), lo cual facilita el proceso de dispersión. A pesar de que la nutria es una especie tolerante a la perturbación humana y puede observarse cerca de zonas urbanas (Rheingtanz et al. 2014, Aceituno et al. 2015), los resultados de este estudio demuestran que la nutria prefiere dispersarse por áreas con alta integridad ecológica, es decir, zonas con menor impacto humano. Esto también se ha reportado para mamíferos grandes que tienen una alta capacidad de dispersión (Wultsch et al. 2016; Draheim et al. 2018).

Los elementos que componen los parches de hábitat actúan como condiciones atractivas o repulsivas para los individuos migrantes, por lo tanto, la selección de estos elementos tiene una influencia final sobre la estructura genética a una escala espacial fina (Murphy et al. 2010, Zero et al. 2017). Como se esperaba, el orden del río fue una de las características atractivas de los parches de hábitat para la nutria, y se asoció con la estructura genética de la especie a una escala local. Contrario a lo esperado, las áreas abiertas (NDVI bajo), como pastizales y cultivos, así como las pendientes pronunciadas (> 30°), fueron elementos de los parches de hábitat que explicaron la estructura genética de la nutria a una escala local, y que favorecieron la dispersión de la nutria a través de la matriz del paisaje. El área de estudio está dominada por pastizales y cultivos, los cuales se encuentran cerca de la orilla de los ríos. Es probable que las nutrias se vean forzadas a usar este tipo de elementos del paisaje, a pesar de que son condiciones sub-óptimas del hábitat (Mayagoitia-González et al. 2013, Navarro-Picado et al. 2017). No obstante, varios cultivos, incluyendo los de caña de azúcar, tienen canales de irrigación que pueden facilitar el movimiento de la nutria, y ser elementos atractivos porque proveen cobertura de escape frente a depredadores. Por otra parte, las zonas con pendientes pronunciadas, las cuales se ubican en las zonas medias y altas de las cuencas, facilitaron la dispersión de la nutria. Este resultado es contrario a lo esperado, y a lo reportado para otras especies de mustélidos semiacuáticos, como la nutria euroasiática (Janssen et al. 2008) y el visón americano (Zalewski et al. 2009). Se ha documentado que cuando la nutria euroasiática se dispersa, tiende a asentarse en zonas con una alta calidad de hábitat (Romanowski et al. 2013; Pagacz 2016). Es factible que *L. longicaudis* esté usando áreas con pendientes pronunciadas debido a que los sitios con mejor idoneidad de hábitat se encuentran localizados

principalmente en la zona media de las cuencas, y en algunos parches de las zonas altas. Recientemente se ha reportado un conflicto entre la nutria y el ser humano en las partes altas de la cuenca La Antigua, como consecuencia de la competencia por los mismos recursos pesqueros (Hernández-Romero et al. 2018b). Esta situación puede afectar la dispersión de la nutria en estas zonas, ya que incrementa la caza de la nutria y el riesgo de mortalidad de los individuos dispersores (Andrade-Ponce y Angarita-Sierra 2017; Hernández-Romero et al. 2018b). De forma similar, se ha encontrado que elementos antropogénicos, como la densidad de caminos, limitan la dispersión de la nutria euroasiática, y es una de las principales causas de mortalidad de los dispersores sub-adultos (Quaglietta et al. 2013).

Implicaciones para la conservación de *L. longicaudis*

Los niveles de diversidad genética reportados en este estudio fueron moderados y similares a los estimados para especies de nutrias que están en peligro de extinción, como la nutria marina, *Enhydra lutris* (H_O = 0.49, H_E = 0.47; Gagne *et al*. 2018) y la nutria gigante, *Pteronura brasiliensis* (H_O = 0.56, H_E = 0.57; Pickles et al. 2011). Aunque *L. longicaudis* no se encuentra en un grado de amenaza extremo, se predice que las áreas núcleo de su distribución serán impactadas de forma negativa por la caza ilegal, la sobrepesca, la contaminación del agua, la construcción de presas y el turismo (Cianfrani et al. 2018). Además, los cambios de la temperatura y precipitación afectarán la disponibilidad de agua de los ríos (Pletterbauer et al. 2018), lo que resultaría en la reducción de la conectividad funcional de la nutria. Por esta razón, es importante monitorear a corto y mediano plazo si los cambios en la configuración del paisaje y la calidad del hábitat reducen gradualmente la diversidad genética y el tamaño de las poblaciones de *L. longicaudis* al punto de que se enfrenten al riesgo de extinción.

Los efectos negativos de la pérdida de la diversidad genética y del aislamiento genético de las poblaciones puede contrarrestarse si existe flujo génico entre las poblaciones. En este estudio, se encontró que la conectividad funcional de la nutria neotropical está asociada con diferentes elementos del paisaje y que la importancia de estos atributos varía entre las zonas de las cuencas. En las cuencas bajas, los ríos al ser más grandes y acumular más agua, facilitan la dispersión de la nutria, proveen mayor cantidad de alimento, probablemente contrarrestan el efecto negativo que ejercen la presencia humana y la baja idoneidad del hábitat. En las zonas medias y altas de las

cuencas, los ríos son más pequeños y la disponibilidad de alimento es menor, pero en estas áreas se encuentran las zonas menos perturbadas por el humano y con una mejor idoneidad de hábitat para la nutria. Estos hallazgos son importantes para sustentar estrategias de manejo y conservación de la nutria neotropical en la región, como por ejemplo el diseño y establecimiento de corredores biológicos, la restauración del hábitat e incluso la reintroducción de poblaciones. Estas estrategias han permitido mantener la conectividad funcional y la diversidad genética de poblaciones amenazadas de especies de carnívoros, incluyendo los de especies de nutria (Yumnan et al. 2014, Mowry et al. 2015, Gantchoff et al. 2020).

En este estudio no se encontró una relación clara entre la diversidad genética de la nutria y la calidad del hábitat, pero si entre la idoneidad del hábitat y la diferenciación genética de las poblaciones e individuos. Se ha documentado en varios estudios que la escala temporal y espacial en la que responden el tamaño poblacional, la diversidad genética y la diferenciación genética a la pérdida de hábitat y a la alteración del paisaje son diferentes (Anderson et al. 2010, DiLeo y Wagner 2016, Pflüger et al. 2019). Por ello, cuando se cuantifica el efecto del paisaje sobre la variación genética es importante considerar este desfase de tiempo (lag time), y la posibilidad de que las poblaciones comienzan a diferenciarse más rápido que lo que la diversidad genética se pierde dentro de las poblaciones (DiLeo y Wagner 2016). Por ejemplo, se ha visto que la calidad y configuración del paisaje contemporáneo explica mejor la diferenciación genética de las poblaciones, mientras que paisajes históricos explican mejor la diversidad genética (Keyghobadi et al. 2005, Flavenot et al. 2015, Balkenhol et al. 2013). Esto puede explicar el porqué, a pesar de que el área de estudio es un paisaje altamente transformado, la diversidad genética de la nutria aún es moderada. De forma similar, esto puedo explicar el hecho de que la diversidad genética de la nutria sea mayor en Jamapa, a pesar de que actualmente es la cuenca más perturbada y tiene poca conectividad funcional. Posiblemente en el pasado, la mejor calidad del hábitat, aunado al gran tamaño de la cuenca, permitió que las poblaciones fueran más grandes y diversas genéticamente, las cuales han podido mantenerse a pesar de la presión humana actual.

CONCLUSIONES GENERALES

La diversidad genética de la nutria neotropical fue moderada y similar a la reportada en ríos de México y Suramérica. No obstante, también fue similar a la diversidad genética que presentan otras especies de nutrias que se encuentran en peligro de extinción. Esto señala la importancia de monitorear posibles reducciones de su diversidad genética que impliquen un riesgo para la supervivencia y adaptación de sus poblaciones en el corto y mediano plazo. El tamaño efectivo de las poblaciones estudiadas fue menor al valor mínimo necesario (N_e = 100) para evitar la pérdida de la diversidad genética en el corto plazo. Esto sugiere que las poblaciones de la nutria son vulnerables a la extinción local, sobre todo si se considera que en el área de estudio la modificación del paisaje y la pérdida de hábitat es rápida.

La nutria neotropical presentó un patrón de estructura genética que refleja la organización jerárquica de los ríos entre cuencas. Adicionalmente, se encontró estructura genética espacial que diferenció genéticamente a los individuos en un gradiente latitudinal y altitudinal. Esta diferenciación genética se correspondió con la variación de la idoneidad ambiental a lo largo del paisaje. A pesar de que las poblaciones se encuentran estructuradas genéticamente, existe flujo génico contemporáneo, el cual fue evidenciado por la identificación de migrantes de primera generación. El modelo de aislamiento por resistencia (IBR) tuvo mejor soporte que el de aislamiento por distancia (IBD) para explicar la diferenciación genética de la nutria. Asimismo, el modelo de jerarquización de ríos (SHM) tuvo mejor soporte que el IBD. Esto indica que los elementos del paisaje terrestre y acuático de la nutria tienen un papel importante sobre sus patrones genéticos.

Varios elementos del paisaje influyeron sobre la estructura genética y flujo génico de *L. longicaudis*. A una escala local (parches de hábitat), la pendiente, el NDVI y el orden del río explicaron la diferenciación genética de la nutria. A una escala del paisaje, la idoneidad ambiental, la integridad ecológica y la acumulación de agua en los ríos fueron elementos de la matriz del paisaje que facilitaron el flujo génico de la nutria. La conectividad funcional de la nutria parece ser mayor entre las zonas medias de las cuencas, puesto que en estas áreas se localizan los elementos del paisaje que representan menor resistencia para la dispersión de la especie. En las partes altas de las cuencas, la dispersión de la nutria es favorecida por la presencia de zonas conservadas (alta idoneidad de hábitat y baja perturbación humana). Sin embargo, es importante incluir en futuros estudios el efecto negativo que podría tener el conflicto de la nutria con el humano sobre la

dispersión de la nutria en estas zonas. Pese a que las partes bajas de las cuencas parecen generar una mayor resistencia al movimiento de la nutria, la conectividad de los ríos en estas zonas es clave para mantener el flujo génico.

Al relacionar los resultados obtenidos sobre la diversidad genética, tamaño poblacional y la calidad del hábitat, se puede concluir que, las poblaciones de la nutria en las tres cuencas tuvieron una diversidad moderada que podría verse reducida en las próximas generaciones. Aunque la cuenca de Jamapa tiene el tamaño efectivo y los valores de diversidad genética más altos, la calidad de su hábitat es pobre, lo que anticipa un riesgo para el mantenimiento de su población. La Antigua, al tener un tamaño efectivo muy bajo, es la más vulnerable frente a la extinción local. Por el contrario, la cuenca de Actopan tiene la mejor calidad de hábitat, y al parecer mantiene el flujo génico entre las poblaciones, por lo cual es un elemento clave para la conservación de la conectividad funcional de la nutria en la región. La calidad del hábitat explicó los patrones de diferenciación genética de la nutria neotropical, pero no de diversidad genética. No obstante, los efectos negativos de la pérdida del hábitat sobre la diversidad genética pueden tardar más generaciones en reflejarse. Por esto, es necesario seguir monitoreando la diversidad genética de la nutria en la región.

La combinación de un muestreo genético no invasivo y el diseño de un conjunto de microsatélites para *L. longicaudis* permitió conocer aspectos demográficos y ecológicos que son de gran importancia para la conservación de la especie. El diseño de marcadores específicos para el sexado de individuos de la especie permitirá evaluar el efecto diferencial del paisaje sobre los patrones genéticos y demográficos de machos y hembras de la nutria neotropical, y otros aspectos ecológicos y comportamentales que sean importantes para su conservación.

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