Conservation Genetics of Neotropical otters (*Lontra longicaudis*) in México.

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Abstract

In this thesis I aimed to provide base-line data to inform conservation of neotropical otters (*Lontra longicaudis*) at both the range-wide and local (Mexico) scale. In **Chapter 2**, I compared three commonly used preservation methods for faecal DNA in order to identify the best method for neotropical otter faeces under challenging field conditions and long-term storage: 1) ambient-temperature drying, 2) a two-step protocol involving incubation in 95% ethanol and posterior silica desiccation, and 3) RNAlater. The results of this experiment showed that that RNAlater provides the highest mtDNA amplification success. In **Chapter 3**, I looked into the demographic history, genetic diversity and genetic structure of *L. Longicaudis* in Mexico using mtDNA. I found high genetic structure among North and South regions of the country, potentially due to geographic formations. Analyses of demographic history in Mexico indicated a recent expansion coinciding with the end of the Pleistocene. Given that recent evidence supports the existence of three subspecies of *L. longicaudis* across its range, I combined mtDNA haplotypes identified in this study with available Central and South American haplotypes in order to examine phylogeographic patterns; as a result, a distinct lineage distributed in North and Central America (NCAM) was identified. Due to the monophyly of this lineage, I propose to consider it a distinctive Evolutionary Significant Unit (ESU). In **Chapter 4**, I used landscape genetics to identify landscape features that affect otter geneflow in Mexico by means of microsatellites. I looked into the effect of elevation, slope, river networks and land cover on geneflow at a country-wide scale and two regional scales (North and South Pacific). I used Bayesian clustering to examine country-wide genetic structure. In terms of landscape genetics, elevation and slope were the only variables that explained genetic distance among individuals at the range-wide and North Pacific scale, respectively. The results of Bayesian clustering indicated two population clusters roughly distributed in the North and South of Mexico. The results of this thesis suggest that non-invasive methods can be applied to inform conservation efforts for Neotropical otters. I suggest that the NCAM lineage should be considered a distinct Evolutionary Significant Unit (ESU) throughout the range of *L.longicaudis*. Within Mexico, it is recommended to plan conservation corridors for the species where naturally low elevations and slopes allow genetic connectivity.

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Author’s Declaration

I declare that the work recorded in this thesis is entirely my own and is of my own composition. No part of this thesis has been submitted for another degree.

Chapter 1- General Introduction

Genetics and biodiversity conservation.

Biodiversity can be defined as the existing variability among all living organisms and the ecological complexes of which they are part. As such, there are three recognized levels of biodiversity: ecosystem diversity, species diversity and ultimately, genetic diversity. Genetic diversity has long been recognized as the raw material for evolutionary change given that it confers species the ability to adapt to changing environments and respond to threats such as predators, parasites and disease (Allendorf and Luikart, 2007; Gugerli *et al*., 2008).

The amount of genetic diversity that is present in a given species or population, can be affected by a number of factors. Among them, the effective population size (Ne) is crucial because it determines how fast genetic diversity is lost through genetic drift *i.e*. the smaller the *Ne,* the faster the genetic diversity will be depleted. The rate of loss of genetic diversity is also affected by the degree of isolation of populations, which can be inferred from patterns of genetic differentiation among demes. Thus, by examining the extant genetic diversity and structure in wildlife populations, we can gain insight into the impact of historical and contemporary processes to which they have been subjected, and in this way obtain valuable information for their conservation. This application of genetic methods to the conservation and restoration of biodiversity is the foundation of the research area of conservation genetics, which has the ultimate purpose of dealing with the factors that affect the risk of extinction of species and propose management actions to minimize such risks (Amos & Harwood, 1998; Frankham *et al*., 2007).

Although there are a wide range of research and management problems that are addressed by conservation genetics (Allendorf and Luikart, 2007), this thesis focuses on three major topics: 1) Identifying conservation units, 2) Assesing the effects of Habitat fragmentation 3) Using non-invasive sampling to inform conservation.

Identifying conservation units

In order to achieve biodiversity conservation, it is critical to first identify appropriate taxonomic and population units for management. If the taxonomic status is incorrectly assigned, erroneous management decisions could be made; for instance, failing to recognize species in need of protection could lead to their extinction (Frankham *et al*., 2007). International laws and treaties, such as the Convention on the International Trade of Endangered Species (CITES) and the ´Red List´ by the International Union for the Conservation of Nature (IUCN), focus on the conservation at the taxonomic level of species (Taylor *et al*., 2010). However, there is a general agreement on the need for protecting distinct units below the species level. Among these units, evolutionarily significant units (ESUs) have long been recognized as separate taxonomic entities that are needed to preserve the evolutionary potential of species. In this sense, ESU's can be defined as a population or groups of populations that merit separate management or priority (Moritz, 1994).

There is a lot of controversy on how to recognize an ESU, but so far the most wide spread criterion requires reciprocal monophyly for mtDNA, *i.e.* all lineages within an ESU must share a most recent common ancestor with each other, than with lineages from other Evolutionary Significant Units (Moritz, 2004). In addition, ESUs often display a phylogeographic signal and coincide with recognizable biogeographic provinces (Taylor *et al*., 2010). In terms of the methodology used for defining ESU´s, mitochondrial DNA has been the molecular marker of choice given its particular characteristics. Among the characteristics that make mtDNA extremely suitable for this purpose, is the high number of copies in cells, which makes it easy to amplify in PCR reactions, even from low-quality samples. Additionally, given its smaller effective population size (compared to nuclear DNA) it resolves to monophyly faster after divergence of populations (Moritz, 1994).

Finally, there are alternative proposals on how to define ESUs, for example on the basis of ecological and genetic "exchangeability". Exchangeability implies that individuals, if moved from one population to the other, could occupy the same niche and perform the same ecological role as resident individuals without any reduction in their fitness. Only if the hypothesis of exchangeability between populations is rejected, can ESUs be defined. Although exchangeability can be tested using common garden experiments, this is not so

straight-forward when dealing with endangered species. In such cases, exchangeability can be pondered on the basis of differences in life history traits, morphology and/or habitat (Allendorf and Luikart, 2007; Frankham *et al*., 2007).

Assessing the effects of Habitat Fragmentation

Habitat fragmentation is the transformation of once continuous habitats into smaller isolated patches, separated by an intervening inhospitable matrix (Baguette *et al*., 2013). When habitat connectivity is lost, the expected outcomes are a reduction in population size (Ne), genetic diversity and gene flow among habitat patches; which ultimately results in an increased extinction risk. Habitat fragmentation can occur through a number of ways, one of them being human activities such as agriculture, urbanization, hydroelectric development and forestry, which have been shown to disrupt genetic connectivity in wildlife populations even over short time spans (Forman, 1995; Young and Clarke, 2000; Rabinowitz & Zeller, 2010). These activities have an unquestionable impact on the long-term conservation of species, but they may be even more detrimental for populations located at the limits of a species' range.

At the core of a species´ geographic range, environmental conditions are at their most suitable. Because of this, effective population sizes (Ne) are high and so is genetic diversity. Given that habitat is well-connected within the core of the range, gene flow is facilitated and genetic structure is expected to be low in populations distributed there. However, towards range limits, the habitat starts becoming more and more fragmented. As a result, populations are less likely to receive immigrants from other populations, which results in a reduction of gene flow and consequently an increase in genetic structure. Furthermore, the limited amount of suitable habitat results in lower effective population sizes which ultimately cause a rapid loss of genetic diversity. This puts the evolutionary potential of populations at risk (Fordham *et al*., 2009; Sexton *et al*., 2009). Whether habitat fragmentation is caused by artificial (human activities) or natural (range-limit) processes, in order to achieve long-term survival of species, it is necessary to ensure the connectivity of the landscapes they inhabit.

Landscape connectivity is defined as the interaction between the movement of organisms and the structure of the landscape. In this sense, landscape connectivity has a functional

component and a structural one. The structural component describes the shape, size and location of landscape features; while the functional component refers to dispersal and gene flow among populations habitat patches or populations of interest (Brooks, 2003; Holderegger, 2008). For evaluating landscape connectivity, landscape ecology focuses on the structural component, *i.e.* assessing the quality and characteristics of the landscape found among habitat patches. However, it does not evaluate the functional component of connectivity (i.e. whether the landscape actually results in successful dispersal and reproduction). On the other hand, population genetics can assess functional connectivity using gene flow as a direct measure of it, but the model of isolation by distance explains genetic differentiation only in terms of linear geographic distance, assuming that the landscape between populations is homogeneous. In this case, a better approach to evaluating landscape connectivity is provided by landscape genetics. This novel research area is an amalgamation of molecular population genetics and landscape ecology that can integrate both components of connectivity. Specifically, landscape genetics considers gene flow as a result of functional connectivity and also accounts for structural connectivity by considering landscape features when explaining patterns of genetic differentiation (Manel *et al.*, 2003; Holderegger and Wagner, 2008). In this way landscape genetics can help identify habitat features that are key to allow species movements (Manel *et al.*, 2003; Cushman *et al.*, 2006), which can be extremely useful in the planning of conservation corridors for wildlife.

Non-invasive sampling

Obtaining information on genetic diversity and structure of wildlife populations can be challenging because many of the species in need of conservation or management are also difficult to study using direct methods. In such cases, the use of non-invasive methods is very advantageous. Non-invasive methods rely on DNA recovered from sources such as hair, feathers, and faecal matter and thus do not require to capture organisms.

These methods have allowed scientists to address important issues in conservation genetics (Taberlet *et al*., 1999 ; Marucco *et al*., 2010), such as identification of individuals in studies on population size, wildlife forensics, and delineation of populations (Beja-Pereira *et al*., 2009). However, working with such sources of DNA has complications, for instance,

the low DNA quantity and/or quality usually found in them, favour allelic dropout and detection of false alleles which result in genotyping errors. In this sense, genotyping errors are defined as a discrepancy between the true genotype and the observed genotype of an individual, and may lead to estimation of incorrect allele frequencies, which ultimately results in biased estimates of population parameters such as Fst. In order to minimize genotyping errors when using non invasive sampling, it is necessary to replicate genotyping assays when defining consensus genotypes (Taberlet *et al*., 1999; Pompanon *et al*., 2005).

When using non-invasive methods, it is also crucial to identify the best preservation method for the samples collected, so that subsequent molecular applications are possible (Camacho-Sanchez, *et al.*, 2013). One has to be very aware of climatic conditions expected in the sampling area, as high temperatures and humidity are very detrimental for DNA (Hajkova *et al.*, 2006). This means that working with species that are encountered in tropical areas might be particularly challenging (Soto-Calderón *et al.*, 2009). One of such species are Neotropical otters (*L. longicaudis*).

Study system: Neotropical otters (*Lontra longicaudis*).

There exist 13 species of otters worldwide, six of which can be found in the Americas: sea otter (*Enhydra lutris*), Giant otter (*Pteronura brasiliensis*) and four species that conform the genus *Lontra*: River otter (*Lontra Canadensis*), Southern river otter (*Lontra provocax*), marine otter (*Lontra felina*) and the Neotropical otter (*Lontra longicaudis*). Neotropical otters have the widest distribution range of the four species included in the genus *Lontra*, as they are distributed from northwestern Mexico south to Uruguay, Paraguay, and across the northern part of Argentina (Lariviere, 1999; Kruuk, 2006) (Fig1-1).

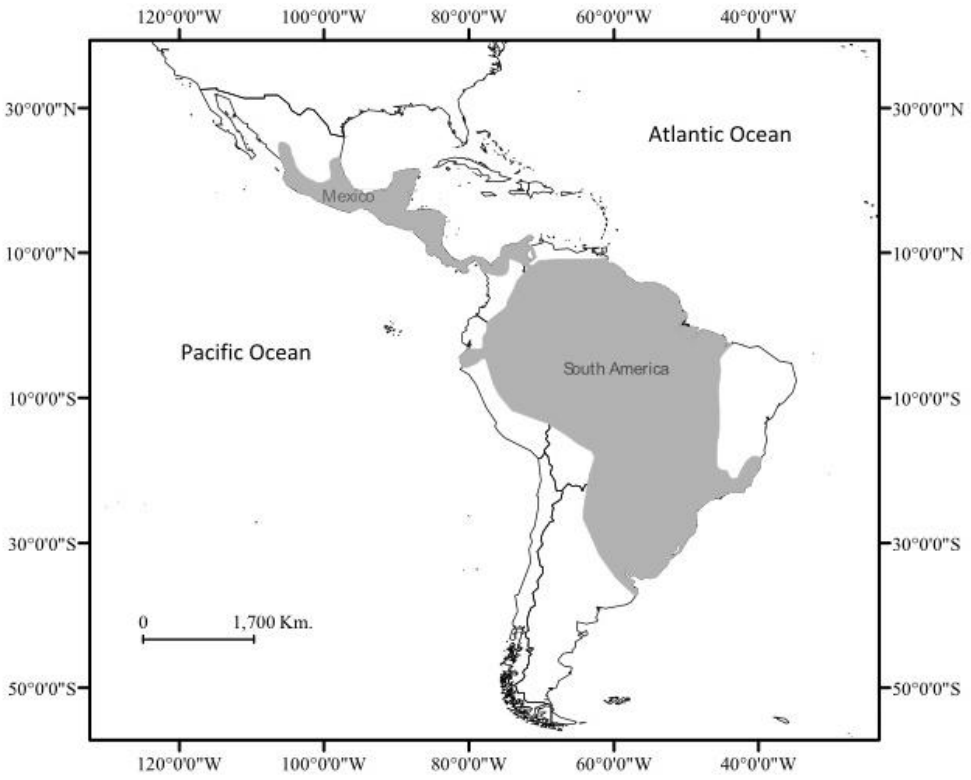

Fig 1-1. Neotropical otter (*Lontra longicaudis*) range.

Modified from: IUCN Red List of Threatened Species. Version 2013.2. <www.iucnredlist.org>

Ecology and Habitat

Throughout its range, the species is found in a variety of habitats which include deciduous and evergreen forests, warm and cool climate rainforests, and coastal savanna swamps. Neotropical otters occur mostly from 300 to 1,500 m of altitude, but there are records of the species below 300m and up to 3,000 m of elevation. Habitat requirements include ample riparian vegetation and abundant potential den sites; the highest abundance of Neotropical otters occurs in areas with extensive aquatic networks, low chemical and organic pollution, and low human density as well as appropriate riparian cover. The species feeds mainly on fish, but crustaceans and mollusks contribute to their diet in some areas. Small mammals, reptiles, birds and insects can be consumed opportunistically (Parera, 1993; Waldemarin and Alvarez, 2008).

Neotropical otters reach sexual maturity at approximately two years old. Breeding occurs mostly in spring, and gestation is 56 days, although delayed implantation can occur (Bertonatti and Parera 1994). Litter size varies from one to five cubs and the male does not provide parental care. The species is mostly solitary, although it may be found in pairs during the breeding season. Communication between Neotropical otters with neighbouring territories occurs through sprainting (Lariviere, 1999).

Conservation status

Currently, the species is categorized as ‘‘Data Deficient’’ by the International Union for the Conservation of Nature (IUCN), and it is suspected that populations are declining due to deforestation, pollution of aquatic systems, agricultural activities, mining and damming. Additionally, there is no information about population size or the cumulative effect of threats across the range of the species (Lariviere, 1999; Kruuk, 2006; Waldemarin and Alvarez 2008). This lack of information on the species might be due to the fact that otters in general are very elusive carnivores, which complicates studying them using direct methods (Kruuk, 2006). In such cases, the use of non-invasive genetic methods can be very advantageous. These methods employ several sources of DNA for which there is no need to capture the organism of interest; faecal samples are among the most used sources of non-invasive DNA. However, DNA in such samples is found at low quantities and quality, and is easily degraded in the presence of high temperatures and humidity (Waits & Paetkau, 2005). The fact that the latter climatic characteristics are prevalent throughout

Neotropical otter range could make it even more challenging to use non-invasive approaches with this species.

Given the sizeable range of this species and the variety of habitats encountered across it, it is not surprising that further taxonomic subdivisions have been proposed for the species across its distribution range. Specifically, based on variation in the rhinarium shape, it has been suggested that there exist three subspecies: *L. longicaudis longicaudis*, distributed throughout most of South America, including Brazil and Uruguay; *L. longicaudis enudris*, distributed through French Guiana, Suriname, Trinidad, and Peru; and *L. longicaudis annectens*, occurring in Nicaragua, Costa Rica, Panama, Colombia, Venezuela, Ecuador and Mexico (Lariviere, 1999; Trinca *et al*., 2012). A recent phylogeographic study conducted in South America, found strong evidence in support of the existence of the subspecies *L. longicaudis longicaudis* and *L.longicaudis enudris*. In addition, based on a single mtDNA haplotype from Colombia, the authors identified a distinctive lineage that could represent the subspecies *Lontra longicaudis annectens*. However, because no samples were available from Central and North America, the authors suggested extending sampling to the entire species range in order to provide support to these preliminary findings and potentially identify further demographic units (Trinca *et al*., 2012).

Neotropical otters in Mexico

In Mexico, the species reaches its northern range limit within the Mexican Transition Zone (MTZ), an area where the Neartic and Neotropical biogeographic regions overlap and is considered one of the 25 biodiversity hotspots (Mittermeier *et al*., 2005; Morrone *et al.,* 2010). As the Neotropical region fades towards the North of the country, the environment becomes drier and water availability decreases, potentially inducing habitat fragmentation for Neotropical otters (Gallo, 1997; Rheingantz *et al*., 2014). Indeed, habitat requirements for the species in Mexico are no different from those known to be key in further parts of its range *i.e*. neotropical otter presence is directly related to abundant riparian vegetation and well-connected waterways (Carrillo-Rubio 2002; Macias-Sanchez, 2003, Diaz-Gallardo 2007).

In support of the above, there have been a few studies on the abundance of the species throughout the country that suggest population size decreases as populations approach the North. For instance, estimates of abundance have ranged from 1.93 individuals/km in

Oaxaca, Southern Mexico (Briones-Salas et al., 2008), 1.22 individuals/km in Veracruz, Gulf of Mexico (Arellano *et al*., 2012) to 0.34 individuals/km in Sonora, Northern Mexico (Gallo-Reynoso, 1996) .

Additionally, within Mexico, it has been proposed that the main threat to otter populations is likely to be habitat fragmentation caused by anthropogenic activities such as ranching, damming and riparian vegetation removal for agricultural purposes (SEMARNAT, 2001). Regardless of which processes are responsible for habitat fragmentation, the consequent decrease in gene flow among populations means that they could be at a higher risk of extinction (Hedrick, 1985; Frankham *et al*., 2003; Stockwell *et al*., 2003). Unfortunately, to this date only one study has addressed the population genetics of neotropical otters; the authors of that study, found evidence for high levels of geneflow within their study area: a single river catchment within a protected area in Chiapas, Southern Mexico (Ortega *et al*., 2012). However, southern Mexico may harbour the highest quality habitats for neotropical otters and thus, studies within them may not necessarily reflect the population structure of otters in further (arguably less favourable) environments in the country. Hence, it remains to be known whether gene flow is on-going or restricted in other areas.

Aims of this thesis

The overall aim of this thesis was to provide base-line data and recommendations for the conservation of Neotropical otters (*L. longicaudis*), by identifying management units at a range-wide scale, as well as investigating which landscape features affect gene flow in Mexico, the northern limit of the species´ range. In particular, there were four main goals for this thesis:

1. To identify the best preservation method for otter faecal DNA under challenging tropical conditions and long-term storage.
2. To contribute to taxonomic clarification of the species and identify potential management units at a range-wide scale.
3. To investigate which landscape features affect gene flow in otter populations in Mexico.

4. Make conservation recommendations for the species at the range-wide and local (Mexico) scale on the basis of the results.

In the next lines, I provide an outline of the topics covered in the present document.

Thesis outline

Chapter 2. *Comparing long-term preservation methods for otter faecal DNA in challenging tropical environments*

Given that the species is distributed in tropical environments (which can be detrimental to non-invasively collected DNA), and that samples would have to be shipped from Mexico to Glasgow University, I expected that obtaining DNA of sufficient quality and quantity for subsequent analyses would be challenging. With this is mind, in this chapter I compared three preservation methods for otter faecal DNA collected in tropical environments and under long-term storage, with the further aim of aiding future studies on the species in challenging environments. Preservation methods were assessed on the mtDNA amplification success they allowed, and theiradvantages/disadvantages are discussed.

Chapter 3. *Genetic Diversity, demographic history and genetic structure of the Neotropical otter (Lontra longicaudis) in Mexico based on mitochondrial DNA.*

Otters in Mexico are distributed in habitats separated by geographic formations that could also act as barriers to gene flow among river basins where the species is distributed. In addition, the species approaches its range limit in increasingly unfavourable environments. I expected this to be reflected in the species ´current patterns of genetic diversity and structure; therefore, in this chapter I looked into said patterns using mtDNA. Furthermore, given the recent findings in terms of the species´ taxonomy and the need for obtaining information from previously unsampled locations throughout the species´ range (Trinca *et al*., 2012), I examined how mtDNA haplotypes retrieved in the present study fit into the recent phylogeographic reconstructions of the species in South America.

Chapter 4. *Using landscape genetics to inform the conservation of Neotropical otters (Lontra longicaudis) in Mexico.*

In this chapter I used microsatellites to estimate genetic diversity and structure in Mexican otter populations. Given that neotropical otters have strong habitat associations and are affected by habitat quality and fragmentation, in this chapter I also examined the effect of landscape features on geneflow in otter populations at two scales: country-wide (Mexico) and two focus regions within the country (North Pacific and South Pacific). I expected that landscape/habitat features known to correlate with presence and/or abundance of the species (specifically: slope, elevation, land cover and water bodies), would influence patterns of geneflow at the scales of study.

Chapter 5. *General discussion.*

In this chapter, I bring the key findings of the present study together and contrast them with recent proposals for the conservation status of the Neotropical otter. I discuss alternatives for the species´ conservation at both the range-wide and local (Mexico) scale. I conclude with proposals for future research on the conservation of the species and suggest specific methodologies for them.

Chapter 2- Comparing long-term preservation methods for otter faecal DNA in challenging tropical environments.

ABSTRACT. Non-invasive genetic techniques have become a popular tool for conservation studies. However, DNA from such sources is extremely vulnerable to high temperatures and humidity, which are common in the tropics. This poses a clear constraint on the number of non-invasive studies that can be accomplished in these biodiversity rich areas. In the present study, I compared mtDNA amplification success from faecal samples of an elusive carnivore: Neotropical otter (*Lontra longicaudis*), after long-term storage (up to 570 days) in three preservation methods: RNAlater, ambient temperature drying and a two-step protocol. The highest amplification success was observed for RNAlater-stored samples (69%), followed by the two-step protocol and ambient temperature drying, respectively. The results show that RNAlater is a highly effective preservation method under challenging field conditions and its use could prove crucial in situations where significant delay between sample collection and molecular analyses is expected.

Introduction

Non-invasive genetic techniques are an effective tool for studying wildlife populations and generating valuable information to support conservation efforts (Bellemain, *et al.*, 2005; Waits & Paetkau, 2005; Schwartz *et al.*, 2007; Mcelwee, 2008; Beja-Pereira *et al.*, 2009). These techniques rely on DNA recovered from sources such as hair (Alberts *et al.*, 2010), feathers (Hogan *et al.*, 2007), and faecal matter (Kovach, *et al.*, 2003; Bhagavatula & Singh, 2006; Piggott *et al.*, 2004; Vianna *et al.*, 2011). Faeces contain DNA from shed cells of the intestinal epithelium and this DNA can be found in sufficient quantities as to allow genetic studies based solely on faecal samples (Waits & Paetkau, 2005). However, there are a number of challenges when working with this source of DNA. First, the quantity and quality of DNA that can be recovered is generally lower than that obtained from tissue. Second, faecal matter contains substances that can inhibit PCR reactions (Kreader, 1996; Marucco, *et al.*, 2010). Finally, the environmental and storage conditions the collected faeces are exposed to, may adversely affect the DNA content and this applies to all stages from the initial faecal deposition by the animal to their collection in the field, their storage by the researcher, and ultimately DNA extraction.

In order to counteract DNA degradation in the field, samples are collected as soon after deposition as possible to avoid increased exposure to high temperatures and environmental humidity, which are known to have adverse effects (Nsubuga *et al.* 2004; Waits & Paetkau, 2005; Beja-Pereira *et al.*, 2009). Given that the method used to preserve samples after collection is crucial for success in subsequent molecular applications (Waits & Paetkau, 2005; Camacho-Sanchez, *et al.*, 2013), the detrimental effect of humidity is counteracted by desiccating samples soon after collection, while the effect of high temperature is minimised by storing samples at low temperatures (Waits & Paetkau, 2005; Hajkova *et al.*, 2006; Lampa *et al.* 2008 ; Beja-Pereira *et al.*, 2009). Additionally, sample collection is usually conducted during the coldest months to further control sample degradation in the field (Hajkova *et al.*, 2006; Marucco *et al.*, 2010). However, in tropical areas, high humidity and temperatures throughout the year, are a major drawback for genetic studies based on faecal samples (Soto-Calderón *et al.*, 2009). Furthermore, if cold storage facilities are located far from collection sites or samples are to be shipped elsewhere for further analyses, it is imperative to find a cost-effective preservation method that keeps the DNA stable when significant delay between collection and extraction is expected.

The use of non-invasive sampling of faeces has particular significance for population genetic studies of elusive species. Otters are elusive carnivores, which makes it difficult to study their populations using direct sampling (Dallas *et al.*, 2003; Hajkova *et al.*, 2006; Ortega *et al.*, 2012). However, otter faeces have been found to be a challenging source of non-invasive DNA and several studies have reported particularly low amplification success rates compared to other carnivores scats (Dallas *et al.*, 1999; Hajkova *et al.*, 2006; Lampa, *et al.*, 2008; Valqui, *et al.*, 2010). Furthermore, many otter species are exclusively found in tropical regions and thus in environmental conditions likely to result in rapid DNA degradation. This is true for the neotropical otter (*Lontra longicaudis*), which is distributed in Latin America (from Mexico to Argentina), making the use of non-invasive techniques with this species particularly challenging. In the present study, I compared three preservation methods in order to identify the most effective approach for long-term storage of otter faeces in tropical environments and to test whether success rates would also depend on local environmental conditions.

Methods

Sample collection

Thirty one otter scats were collected during two sampling periods (January 2011 and March 2013) at six sampling sites within the species' distributional range in Mexico (Fig 2-1) (see APPENDIX A, Table A1 and APPENDIX B, Table B1). Sites ranged in elevation from 0 to 1750 m (above sea level) and were located within three broad climatic types: 1-Semi-arid/steppe (BS); 2-Tropical wet-dry/savannah (Aw) and 3-Tropical monsoon (Am). These regions climates differ from each other in terms of their year-round moisture and insolation (Garcia, 1981; Kottek *et al.*, 2006)

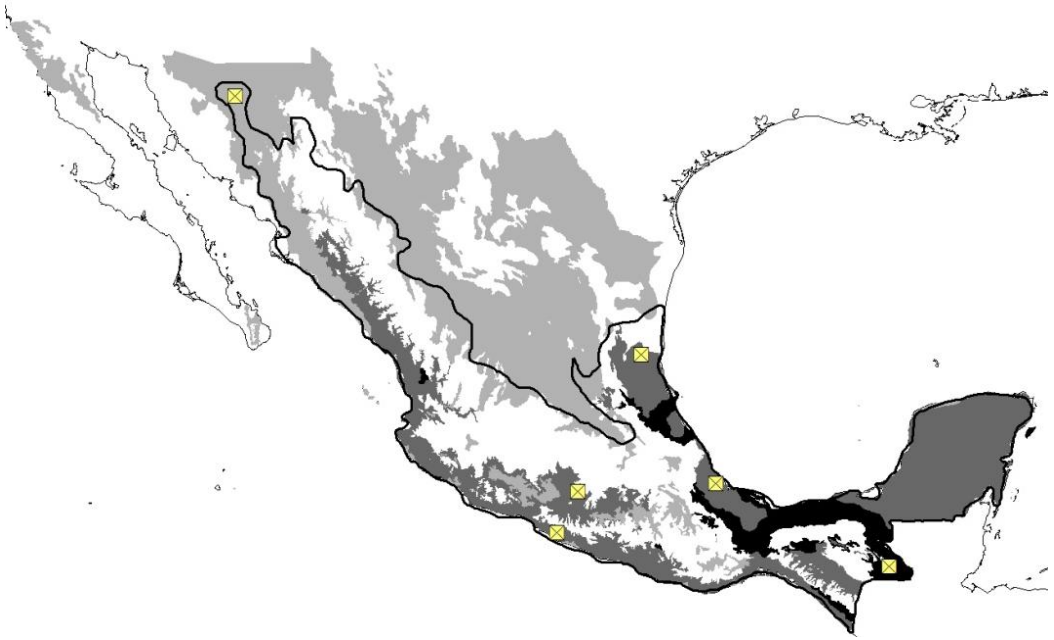

Fig 2-1. Location of the six study sites (squares) at which faecal samples of the Neotropical otter were collected. Sampling was focussed on three climatic regions: tropical monsoon (Am) =black; tropical wet-dry (Aw) =dark grey; semi-arid (BS) =light grey. White area represents various climatic regions from which no samples were collected for the present study. The distribution range of the species is outlined in black.

Sampling was conducted in the early hours of the morning and only scats deemed to be no more than 24 hrs old (based on visual inspection) were collected. Each scat was divided in three equal parts, and each subsample was stored in one of three preservation methods:

1) *Ambient temperature drying*. Samples were placed directly in wax paper envelopes, which were then left open to allow drying. Once dry, they were transferred to plastic bags with 15g of silica beads.
2) *Two-step protocol* (Nsubuga *et al.*, 2004). Samples were submersed in 95% ethanol for 24-36 hrs. Subsequently, ethanol was discarded and the sample was transferred to 15ml micro centrifuge tubes containing 15 g of silica beads.
3) *RNAlater*. Samples were directly placed in 15ml micro centrifuge tubes containing 3ml of RNA*later*® solution (Invitrogen).

Once processed, samples from all three methods were stored at ambient temperature until extraction. Time from collection to extraction ranged from 300 to 570 days.

DNA extraction and PCR Amplification

DNA extraction was performed in a designated area of the lab using a QIAamp® DNA Stool Mini Kit (Qiagen), and following the manufacturer's instructions except for the following modifications: the incubation in ASL buffer was done overnight, the incubation time in AE buffer was increased to 30 minutes and the final elution was done in 100uL of AE buffer. I used primers ProL (5'-CACCACCAACACCCAAAGCT-3') and DLH (5'CCTGAAGTAAGAACCAGATG-3') (Valqui *et al.*, 2010) to amplify a 355bp fragment of *Lontra longicaudis* mtDNA Control Region. The PCR reactions (20µl) contained 2µl DNA, 0.5 µM each primer, 1X reaction buffer, 1.5Mm MgCl, 0.2 mM dNTPmix, 1X BSA and 1U Taq polymerase (Invitrogen) . I used a touchdown PCR protocol with an initial denaturation step of 94°C for 3 min, followed by 94°C for 45s, a touchdown step of annealing from 60°C to 50°C for 45s and extension at 72°C for 90s. Finally, 35 cycles of denaturation at 94°C for 1 min, 50°C annealing for 45 s, and extension at 72°C for 3 min. were added.

In order to confirm amplification, PCR products were run on 2% agarose gels; for each PCR test, the outcome of amplification was recorded as *success* (when a fragment of the expected size was obtained) or *failure* (when no visible fragments were found in the gel). Each sample was tested for amplification twice (in independent PCR reactions). PCR products from successful amplifications were cleaned up by means of Exonuclease I -

Shrimp Alkaline Phosphatase (Affymetrix), and were then sequenced by the DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk). All fragments were sequenced in both directions as a quality control. Lastly, the final sequences were subjected to a BLAST search in Gen Bank to confirm their match to *Lontra longicaudis*.

Statistical Analyses

In order to examine which factors affect PCR amplification success, I used Generalized Linear Mixed Models (GLMM's) with binomial errors as implemented in the 'lme4' package (Bates *et al.*, 2014) in the R programming environment (R Development Core Team 2004). The models assessed the proportion of successful amplifications as a function of the fixed effects 'preservation method' and 'climatic region'. In addition, 'collection site' and 'sample' were included as random effects. Models were fitted by Laplace approximation and were compared based on Akaike's Information Criterion (AIC). To account for the relatively small sample size (n=31), the second order AIC (AICc) was calculated using package 'AICcmodavg' (Mazerolle, 2013).

Results

From sampling across climatic regions, I obtained 25 samples from the tropical wet-dry climate (Aw), three from tropical monsoon (Am) and three from semi-arid steppe (BS). The overall success rate (amplification with any of the methods at least once) was 83%, while 16% of the samples did not amplify in any trial, regardless of preservation method. Additionally, 9 % of samples amplified only when stored with a method different from RNAlater, whilst 74% amplified at least once when they had been preserved in RNAlater. The model with the best fit to the data included both fixed factors: 'climatic region' and 'preservation method' (Table 2-1). Explicitly, 'preservation method' was found to exert an effect on the amplification success. Amplification success was also predicted to be lower in semiarid climates (BS). However, a likelihood ratio test did not allow to reject the simpler model without 'climate' (chi-square=5.12; d.f. =2; P>0.05). Therefore, I present predicted values only for this model, which predicted the highest amplification success for RNAlater preserved samples: 69%, followed by 2step protocol: 32%, and ambient temperature

drying: 11% (Fig 2-2). On the other hand, although keeping ‘climatic region’ in the models did not significantly improve the fit to the data, differing mean success rates were estimated across regions: Am=55%, Aw=38%, BS=11% (Fig 2-3).

Table 2-1. Generalized Linear Mixed Models fitted to dataset.

Models fitted to explain amplification success of mtDNA as a function of preservation method and climatic region of collection.

AICc= Corrected Akaike Information Criterion, LogLik=Log-likelihood of model.

Model	No. of Parameters	AICc	LogLik
Method +Climate	7	168.30	-76.496
Method only	5	168.80	-79.059
Climate only	5	227.16	-108.237
Intercept only	2	226.18	-111.028

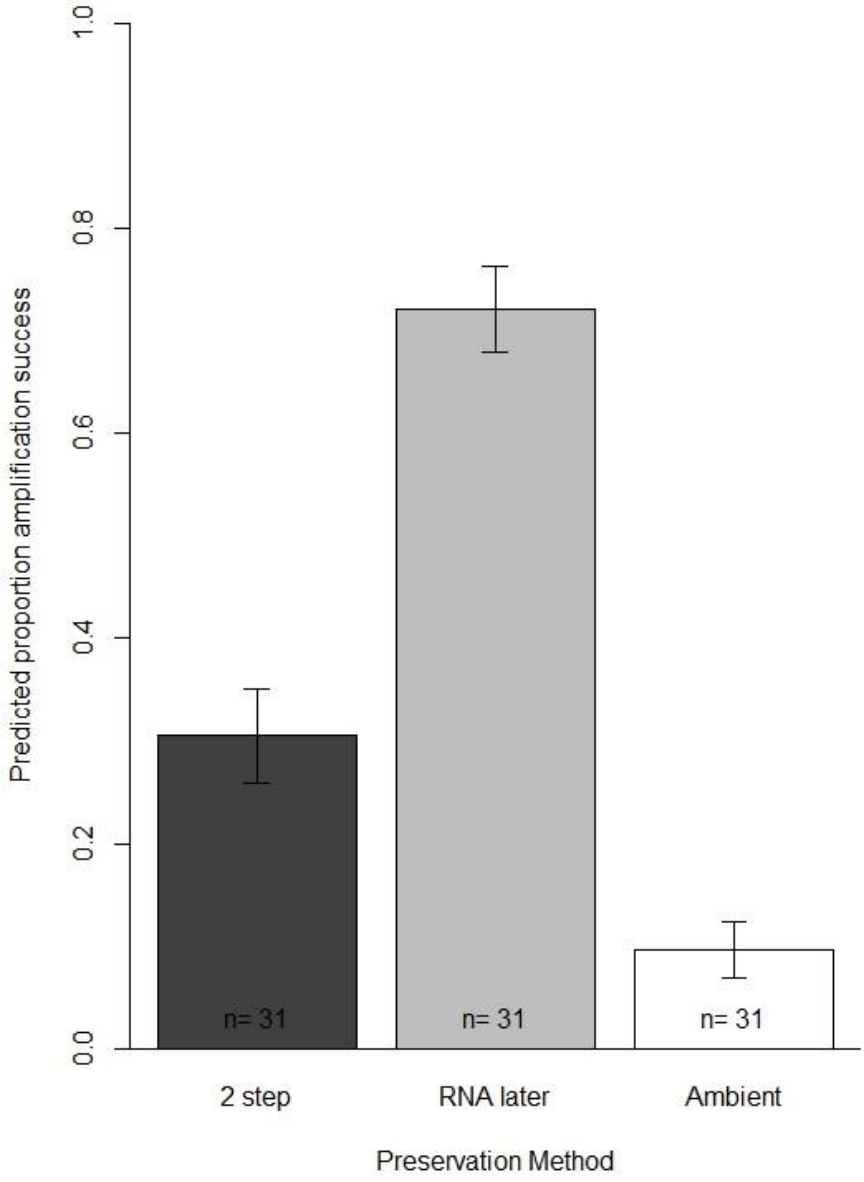

Fig 2-2. Predicted proportion (±SE) of successfully amplified otter faecal DNA samples stored with the three preservation methods. The same 31 faecal samples were tested in duplicate for each method.

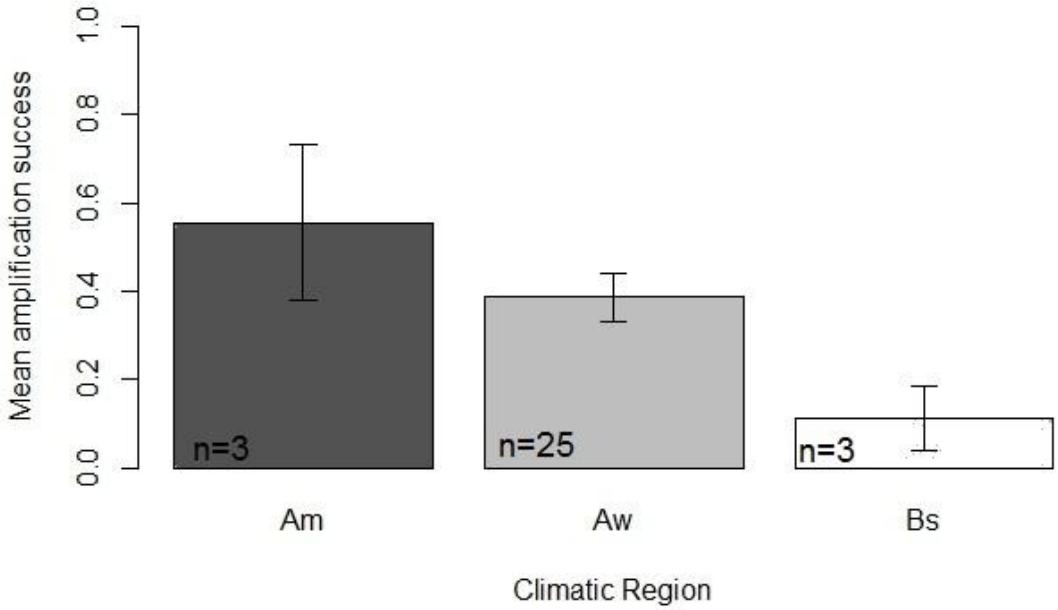

Figure 2-3. Mean amplification success (±SE) of otter faecal DNA from samples collected in three climatic regions.

Am=Tropical monsoon, Aw= Tropical wet-dry and Bs=Semi-arid steppe

Discussion

The mean amplification success for the samples clearly varied across preservation methods. The lowest success rate estimated : 11% (ambient-temperature drying), was close to the lowest estimates for otter species: Southern River otter *(Lontra provocax)*: 26% (Centrón *et al.*, 2008), Neotropical otter (*Lontra longicaudis):* 24% (Ortega et al, 2012); but still low compared to estimates for further vertebrates: Pigmy rabbit (*Brachylagus idahoensis*): 58% (DeMay *et al.*, 2013) and Bengal tiger (*Panthera tigris*): 50% (Sharma *et al.*, 2008). In contrast, the success rate estimated for RNAlater (69%) was superior to the afore-mentioned estimates, and above some of the highest estimates for otter species: Eurasian otter (*Lutra lutra*): 41% (Hajkova *et al.*, 2006); Marine otter (*Lontra felina*) : 43% (Valqui *et al.*, 2010); Eurasian otter (*Lutra lutra*): 46% (Hájková, *et al.*, 2008).
The fact that RNAlater storage was found to predict higher amplification success from fecal samples, agrees with several other studies in which preservation methods were compared, and RNAlater was found to be highly effective in tropical areas (Nsubuga *et al.*, 2004; Rivière-Dobigny., *et al*, 2009; Soto-Calderón *et al.*, 2009). Hence, RNAlater might be the best choice when there is no way to control storage temperature for an extended period. A further advantage of this preservation method is that it involves less handling, which lowers the chances of contamination between samples, compared to the two-step and ambient temperature drying methods. Moreover, despite RNAlater being more expensive (~ £2.50 per sample) than the two alternative preservation methods (under £1 per sample), if relying on any of the latter, it would be necessary to increase sampling effort
(therefore expenses) in order to achieve a success rate comparable to that obtained with RNAlater. This alone compensates for the initial difference in cost and optimizes field logistics.
Numerous studies have found amplification success of mtDNA to be consistently higher than that for nuclear DNA (Waits & Paetkau, 2005; Renan *et al.*, 2012; Vynne, *et al.*, 2012; DeMay *et al.*, 2013). Although looking into this trend was not an objective of the present study, I did test amplification of nuclear DNA (microsatellites) from RNAlater-stored samples that amplified mtDNA in the previous experiments. I found that 30% of these samples allowed obtaining consensus genotypes at four or more microsatellite loci from a panel of 13. This shows it should be plausible to amplify nuclear DNA from RNAlater-stored samples. All in all, the results show that RNAlater is a very reliable

preservation method for non-invasive samples from tropical species, even under challenging environments and after long-term storage. Whenever possible this method should be preferred. Finally, although I did not look directly into the effect of delay on amplification success, it is worth noting that in studies where this has been formally examined, success has been shown to decrease with time and should be avoided as much as possible (Brinkman, *et al.*, 2009; Soto-Calderón *et al.*, 2009; DeMay *et al.*, 2013) .

Chapter 3 - Genetic Diversity, demographic history and genetic structure of the Neotropical otter (Lontra longicaudis) in Mexico based on mitochondrial DNA.

ABSTRACT. Genetic diversity represents the evolutionary potential of a population, and allows insights into its demographic history and genetic structure. Studying these aspects of a population can be extremely useful for conservation, as is the case of the Neotropical otter (*Lontra longicaudis*), distributed from Argentina to Northern Mexico. In this study, I used a 507bp segment of mtDNA control region to examine the genetic diversity, demographic history and genetic structure of *L.longicaudis* in Mexico within three study regions: North Pacific (NP), South Pacific (SP) and Atlantic (AT). Different measures of country-wide genetic diversity in Mexico were lower than recent estimates for South American populations, possibly due to differences in demographic history between these areas. Analyses of demographic history in Mexico indicated an expansion occurring ~8,000BP, coinciding with climatic changes at the end of the Pleistocene. At a regional scale, genetic structure was high among NP and SP/AT, potentially due to the Sierra Madre Oriental and the Transvolcanic Belt acting as barriers to female gene flow among these regions. Additionally, given that recent evidence supports the existence of two subspecies of *L. longicaudis* in South America, the haplotypes identified in Mexico (North America) were combined with Central and South American haplotypes in order to examine phylogeographic patterns. A distinct lineage distributed in North and Central America (NCAM) was identified; this lineage is proposed to represent a distinctive Evolutionary Significant Unit (ESU) and provides evidence in support of the existence of a third subspecies (*L.longicaudis annectens*), in agreement with previous taxonomic proposals (Lariviere, 1999).

Introduction

Genetic diversity is the raw material for evolutionary change, as it confers species the ability to adapt to changing environments and respond to threats such as predators, parasites and disease; thus, it is directly linked to the evolutionary potential of species (Allendorf and Luikart, 2007). Among several processes that can affect the level of genetic diversity, the effective size of a population is crucial because it determines how fast the genetic diversity is lost through genetic drift *i.e*. the smaller the effective population size (*Ne*), the faster the genetic diversity will be depleted (Frankham *et al*., 2007). Most natural populations will experience changes in Ne throughout time, which will leave a signature in their current patterns of genetic diversity (Aspi *et al*., 2006; Pyhäjärvi *et al*., 2007). The rate of loss of genetic diversity is also affected by the degree of isolation of populations, which can be inferred from patterns of genetic differentiation among demes (Amos & Harwood, 1998; Frankham *et al*., 2007). Thus, by examining the current patterns of genetic diversity and genetic structure in the populations of a given species, it is possible to shed light into their demographic histories and genetic connectivity. With this information, conservation units can be defined and management actions can be taken. However, in order to ensure long-term conservation, it is also crucial to have a clear grasp of the taxonomy of the focus species, so as to guarantee that resources are focused at the appropriate level (Frankham *et al.*, 2007).

The Neotropical otter (*Lontra longicaudis*) is distributed in the Americas, from Mexico to Argentina (Fig 1-1). This species is currently classified as data deficient and its populations are suspected to be declining; hence, there is a desperate need for research that sheds light on the status of its populations and the threats they may be facing (Kruuk, 2006; Waldemarin and Alvares, 2008). In Mexico, the species occurs from 30° N to 13°N in the Pacific slopes and from 24°N to 16° N on the Gulf of Mexico slopes (Gallo-Reynoso, 1997). Additionally, in this country, the species' range reaches its northern limit within the Mexican Transition Zone (MTZ); a large biogeographic region that encompasses a complex assemblage of diverse biotas, making it one of Earth's biodiversity hotspots (Mittermeier *et al*., 2005; Morrone *et al.*, 2010). Across the MTZ, geographic formations such as the Sierra Madre Oriental (SMOR), Sierra Madre Occidental (SMOC), the

Transvolcanic Belt (TB) and the Tehuantepec Isthmus (TI) have been implicated in the shaping of large-scale phylogeographic patterns and genetic structure of several terrestrial vertebrate species in Mexico (Mateos, 2005; Arteaga *et al.*, 2012; Bryson *et al*., 2011; González-Porter *et al*., 2013). Furthermore, these formations have also been found to delimitate the distribution of freshwater taxa, including crustaceans, fish and helmynth parasites, as they act as boundaries among hydrological basins and affect directionality of river drainages (Huidobro *et al*., 2006; Quiroz-Martinez *et al.*, 2014). For instance, numerous studies have found biogeographic patterns that divide freshwater taxa into Pacific an Atlantic populations, as the Sierra Madre Occidental and the Sierra Madre Oriental, induce rivers to drain either to the east (toward the Gulf of Mexico), or to the west (toward the Pacific Ocean), thus determining dispersal routes for freshwater biota (Quiroz-Martinez *et al*., 2014). Given that neotropical otters are strongly associated with freshwater habitat, it is likely that the mentioned geographic characteristics have also affected the dispersal and consequently the genetic structure of otter populations in Mexico.

A number of studies have also looked into the demographic histories of taxa within the MTZ and have found patterns that can be attributed to climatic changes occurring throughout the Pleistocene (Eizirik *et al.*, 2001; Milá *et al.*, 2000; Arteaga *et al.*, 2012; Gasca-Pineda, *et al*., 2013; Matte *et al*., 2013). Fossil records establish the presence of Neotropical otters in Mexico in the late Pleistocene (Arroyo-Cabrales, *et al.*, 2013), therefore, the fluctuations in climate throughout this epoch could also have affected the population size of the species in Mexico. Unfortunately, no studies on the demographic history of the species have been attempted and there has only been one study on the genetic diversity and structure of Neotropical otters in Mexico, which was restricted to a small catchment at the Southern end of the country (Ortega *et al*., 2012). In light of this, there is a clear need for studies that investigate the demographic history and genetic structure of the species at a country-wide scale as a baseline for conservation planning.

On the other hand, at a range-wide scale, there have been proposals of taxonomic subdivision of *Lontra longicaudis* based on differences in rhinarium shape (Harris, 1968; Lariviere, 1999). Specifically, three geographically separated subspecies have been proposed: *Lontra longicaudis longicaudis*, distributed through most of South America; *Lontra longicaudis enudris* distributed through French Guiana, Suriname, Trinidad, and

Peru; and *Lontra longicaudis annectens* occurring in Nicaragua, Costa Rica, Panama, Colombia, Venezuela, Ecuador and Mexico (see Appendix C, Figure C1 and C2) (Lariviere, 1999). A recent phylogeographic study of the species (Trinca *et al*., 2012) identified two distinctive mtDNA lineages in South America which were denominated "East South America" (ESA) and "Amazonia" (AMZ). Based on the geographic distribution of these lineages, the authors proposed they could represent the subspecies *Lontra longicaudis longicaudis* and *Lontra longicaudis enudris,* respectively. Additionally, based on a single sample from Colombia, the authors identified a distinct lineage that could represent the subspecies *Lontra longicaudis annectens,* however, no samples from Central and North America were included in their study. Therefore, extending sampling to the entire species range could give stronger support to the proposed taxonomic scheme, as well as resulting in the identification of further demographic units. Especially, since otter populations are faced with numerous geographic barriers and/or environmental gradients across their broad range, which could promote phylogeographic separations.
In brief, given that many aspects of this species´ genetic history and structure remain unexplored at both the range-wide and local (Mexican) scale, my aims for this chapter were:

1) To examine how Mexican and Central American sequences fit into the recent phylogeographic reconstructions of the species in South America, in order to contribute to taxonomic clarification.

2) To investigate the genetic structure of Mexican otter populations and identify potential geographic drivers of the observed patterns.

3) To infer the demographic history of Mexican otter populations from their current patterns of genetic diversity.

4) To identify management units for the species in Mexico/Central America and propose preliminary conservation actions for otter populations in Mexico.

Methods

Sampling

From 2011-2013, sampling was conducted in three focus regions within the range of the species in Mexico: North Pacific (NP), South Pacific (SP) and Atlantic (AT). These regions coincide with proposed biogeographic provinces in Mexico (Morrone, 2005 ; Escalante *et al*., 2013), and were defined on the basis of geographic features that have been shown to delimitate population units in several Mexican taxa (bats: Martins *et al*., 2009; snakes: Bryson *et al*., 2011; mammals: Arteaga *et al*., 2012). Specifically, the Sierra Madre Oriental (SMOR) could form a boundary between Pacific (Western) and Atlantic (Eastern) populations, while the Sierra Madre Occidental (SMOC) and the Transvolcanic belt (TB) would separate North Pacific from South Pacific populations (Fig 3-2).

Within the focus regions, 17 locations were selected for field surveys (Fig 3- 2). During the surveys, otter habitat was visited in the early morning hours in order to collect faecal samples. Habitat was surveyed either by foot or boat, and whenever otter scat were encountered, they were assessed on their appearance *i.e.* colour, smell and moisture content, in order to ensure only those deemed to be no older than 24 hours were collected. The majority of samples were stored in paper envelopes and dried at ambient temperature, but whenever possible they were also stored in *RNAlater*® solution and/or 96% ethanol. Although sampling was based on scats, tissue and hair from specimens found dead in the field were also collected, and donations of hair samples from captive specimens were obtained.

In order to examine large-scale phylogeographic patterns, a hair sample from a single individual from Costa Rica was included, as well as all 29 mtDNA control region haplotypes of South American Neotropical otters available from GenBank (Trinca *et al.*, 2012). The final (large-scale) data set thus comprised North America (Mexico), Central America (Costa Rica) and South America (Bolivia, Brazil, Colombia, French Guyana and Peru)

Fig 3- 1. Study regions, sampling locations and Geographic Features within *L.longicaudis* habitat in Mexico.
The map represents the three study regions and the sampling sites within them; regions/sites are coded by icons: North Pacific (NP) =triangles, South Pacific (SP) =squares, Atlantic (AT) =circles. Numbers and straight lines indicate major geographic features in Mexico: 1=Sierra Madre Oriental (SMOR), 2=Transvolcanic Belt (TB), 3=Sierra Madre Occidental (SMOC), 4=Tehuantepec Isthmus (TI). The Neotropical otter range in Mexico is outlined in black.

DNA Extraction

DNA extraction from faecal samples was performed in a designated area of the lab, using a QIAamp® DNA Stool Mini Kit (QIAGEN) following the manufacturer's instructions, with the following modifications: incubation in ASL buffer was done overnight; incubation time in AE buffer was increased to 30 minutes and the final elution step in AE buffer was repeated, in order to obtain two separate 100uL aliquots of extracted DNA. The DNA extraction from tissue was performed by means of DNeasy Blood & Tissue Kit (QIAGEN), following the manufacturer's instructions. For hair DNA extractions, the same kit and protocol was used with one modification: the incubation in buffer ATL and proteinase K, also included 20uL of 1M DTT.

Amplification of mtDNA and Sequence analyses

I used a combination of primers to amplify two overlapping fragments (~250bp each) of the mtDNA Control Region of *Lontra longicaudis* (Table 3-1). PCR reactions (20µl) contained 2µl DNA, 0.5 µM each primer, 1X reaction buffer, 1.5Mm MgCl, 0.2 mM dNTPmix, 1X BSA and 1U Taq polymerase (Invitrogen). The PCR protocol involved an initial denaturation step of 94°C for 3min, followed by a touchdown scheme of 94°C for 45s, annealing of 60°C to 50°C for 45s, and extension at 72°C for 90s. Finally, 35 cycles of denaturation at 94°C for 1min, 50°C annealing for 45s, and extension at 72°C for 3min. In all PCR reactions, I included one negative control for every 30 samples tested. The PCR products were visualized in 2% agarose gels and products of the expected size were sent out for sequencing at the DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland). All PCR products were sequenced in both directions as a quality control. The retrieved sequences were blasted in Genbank to confirm they matched *Lontra longicaudis* and were aligned and edited using Geneious Pro 5.5.7 (Biomatters Ltd., Auckland, New Zealand).

The estimated home-range of Neotropical otters in Mexico during the dry season (when sampling was conducted) ranges from 2-4km (Gallo, 1989). Therefore, in order to ensure that Mexican samples with identical sequences had originated from different individuals, their respective sampling locations had to be at least 6km apart. The final dataset consisted of 44 sequences deemed to belong to different individuals originating from the three sampling regions in Mexico (Fig 3- 2) (see Appendix B, Table B1).

Table 3- 1. Primers used to amplify mtDNA control region of *Lontra longicaudis*.

[1,2] Indicate primer pairs used together in PCR reactions.

Primer	Sequence	Species	Reference
CENF[1]	5'-GACGTGTACCTCTTCTCG-3'	*Lontra provocax*	Centron *et al*.(2008)
LfCR-R2[1]	5'-GTTGTGCGATGCGGATAAAT-3'	*Lontra felina*	Vianna *et al.* (2010)
ProL[2]	5'-CACCACCAACACCCAAAGCT-3'	*Lontra felina*	Valqui *et al.* (2010)
DLH[2]	5'-CCTGAAGTAAGAACCAGATG-3'	*Lontra felina*	Valqui *et al.* (2010)

Genetic diversity and Genetic structure

I estimated haplotype diversity (*h*), nucleotide diversity (π) and number of unique haplotypes in DnaSP v5 (Librado and Rosas, 2009) for the final set of Mexican sequences, as well as for each of the three study regions separately. Because of uncertainty about the geographic origin of two sequences, these were excluded from within-region estimates. In order to depict relationships between Mexican haplotypes, a median-joining network was built using the software Network 4.6.1.1 and I used an analysis of molecular variance (AMOVA) as implemented in Arlequin v 3.5.1.2 (Excoffier *et al.,* 2005) to estimate overall FST and genetic variance partition among the three study regions. Pair-wise FST values among regions were also estimated with Arlequin, and the significance of statistics was estimated from 1000 permutations.

Selection of nucleotide substitution model.

I used jModelTest (Posada, 2008) to find the best model of nucleotide substitution for two separate data sets: 1-Only Mexican sequences 2- Combined sequences from Mexico, Costa Rica and South America. The model with the best fit according to Akaike information criterion (AIC) was HKY+I for Mexican sequences, and TPM3uf+I+G for the combined dataset. These substitution models were used in subsequent phylogeographic and demographic history analyses.

Demographic history

Fu's Fs test (Fu, 1997) and mismatch distribution (Rogers and Harpending, 1992) analyses were performed with Arlequin to assess whether Mexican otter populations have experienced historical changes in size, and significance values were estimated from coalescent simulations. In order to further explore demographic trends in the population and estimate historical effective population size (*Ne*), a Bayesian skyline plot (BSP) was built using the software BEAST (Drummond *et al*., 2005). The substitution model used was HKY+I and I assumed a strict molecular clock, with a substitution rate of $1.5e^{-7}$ substitutions site/year. This rate was considered suitable because it was estimated in carnivores and has been used in several studies with this taxon (Taberlet & Bouvet 1994,

Savolainen *et al*. 2002, Leonard *et al*. 2005). Three independent MCMC were run for 30´000,000 steps with a burn-in of 10%. Convergence was confirmed using TRACER 1.5 (Rambaut & Drummond 2007), with and effective sample size (ESS) target > 200.

Range-wide Phylogeography

I used MEGA 6.0 (Tamura et al., 2013) to build a Maximum Likelihood phylogenetic tree of haplotypes from the entire species range. Given that the substitution model selected with jModelTest for this dataset, is not available in MEGA, I used the GTR+I+G model for tree building. The nodal support for the tree was estimated from 1000 bootstraps, and I used a sequence of marine otter (*Lontra felina*) as an outgroup (Vianna *et al*., 2010). The divergence dates of phylogeographic lineages observed in the tree were estimated using BEAST 1.8.0 (Drummond *et al.,* 2012) by calibrating with the estimated divergence date of *L. Longicaudis* and *L. Felina*: 0.5–3.2 Ma (Koepfli, *et al.*, 2008). I assumed a normal prior distribution for the node age, an uncorrelated lognormal relaxed molecular clock, and a tree prior based on a Yule process. The MCMC procedure was run for 50 million generations, sampling every 5000 steps. Results were analyzed with the program Tracer v.1.5 (Rambaut and Drummond, 2007) with a burn-in of 10%. Additionally, BEAST was used in combination with TreeAnnotator v.1.5 (Rambaut and Drummond, 2007) to produce a Bayesian phylogenetic tree of haplotypes.

Results

The final alignments for Mexico and range-wide (Mexico +Costa Rica +South America) analyses were 507bp and 491bp long, respectively.

Range-wide Phylogeography

The final data set comprised 41 haplotypes from across the species' range: 11 identified in Mexico in the present study, one from Costa Rica (also identified in this study) and 29 sequences from South America (Trinca *et al*., 2012). Both the Maximum Likelihood and Bayesian trees of these haplotypes, showed similar topologies and strong support (>70%) for a monophyletic group containing all Mexican haplotypes plus the haplotype from Costa Rica and a single haplotype from Colombia (Trinca *et al*.,2012). The trees also showed clades that largely coincide with the lineages East South America (ESA) and Amazonia (AMZ) previously identified by Trinca *et al*. (2012). However, nodal support for these clades was low in the present study (<70%) (Fig 3-3). The mean divergence date between the Mexico/Costa Rica/Colombia lineage from all South American haplotypes was 0.228 Ma (95%HPD: 0.0032-1.138)

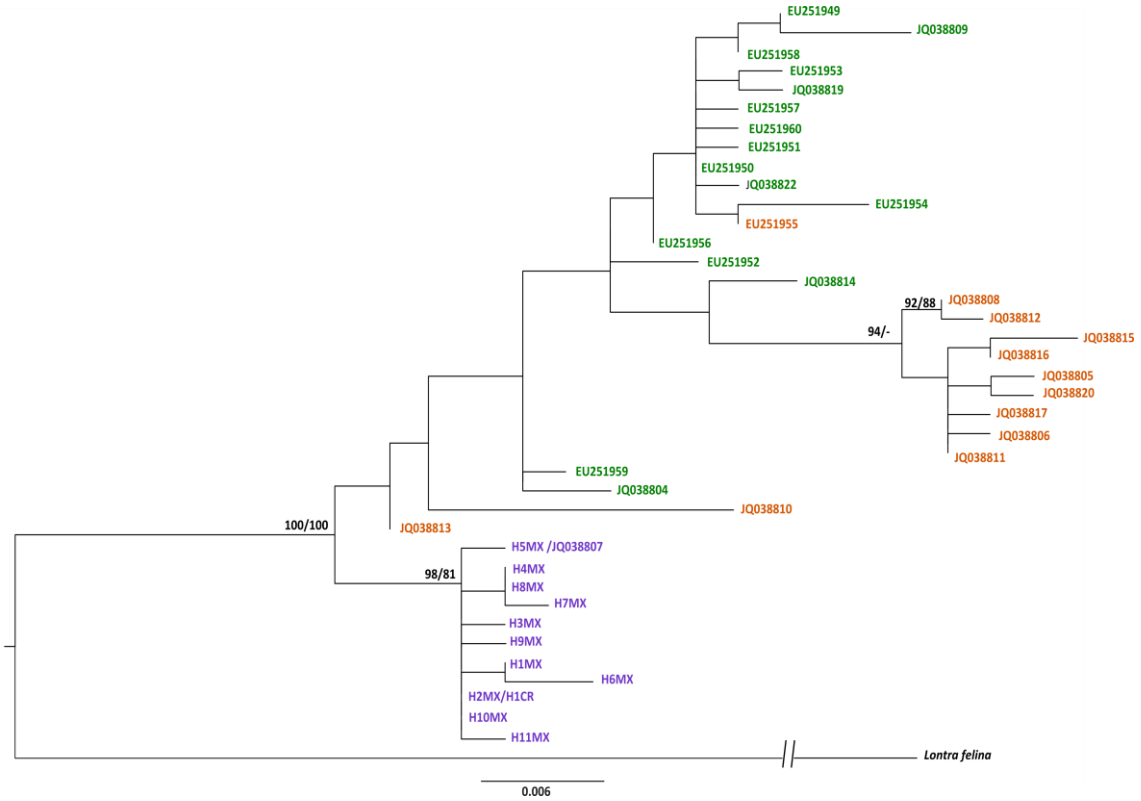

Fig 3-2. Maximum likelihood tree of haplotypes. Scale bar = substitutions per site. Left to right, values on branches represent support for the adjacent node based on Bayesian (BEAST) and Maximum Likelihood (MEGA) analyses. Only support >70% is shown. The clade of Haplotypes from Mexico (HAP1MX to HAP11MX); Costa Rica (H1CR) and Colombia (JQ038807) is displayed in blue. South American haplotypes (EU2511949-EU2511960 and JQ038804-JQ038822) are colour-coded according to the lineages identified by Trinca *et al.* (2012): East South America = green, Amazonia = orange.

Genetic diversity

The country-wide genetic diversity in Mexico was found to be lower than previous estimates for Neotropical otters in South America, as well as for the closely related species *Lontra felina*. Within Mexico, haplotype and nucleotide diversity varied among sampling regions; the highest diversity was estimated in the South Pacific (SP) followed by Atlantic (AT) and North Pacific (NP) respectively (Table 3-2).

Table 3- 2. Genetic diversity estimates in South American and Mexican populations of Neotropical otter (*L.longicaudis*) and the related species, Marine otter (*Lontra felina*).

Species/Study Area	N	Haplotype diversity (SE)	Nucleotide Diversity (SE)
L. felina/Peru and Chile[1]	168	0.9315 (0.0055)	0.0047 (± 0.0028)
L.longicaudis/South America[2]	36 (ESA)	0.8187(± 0.0084)	0.0062 (±0.0018)
	14 (AMZ)	0.9487(± 0.0059)	0.0121 (± 0.0025)
L. longicaudis/ Mexico[3]	44 (Country)	0.7620 (±0.0085)	0.0021 (±0.0000)
	17 (SP)	0.8160(± 0.0147)	0.0023 (± 0.0000)
	17 (AT)	0.4260 (± 0.0356)	0.0016 (± 0.0000)
	8 (NP)	0.2500 (± 0.0636)	0.0004 (± 0.0000)

[1] Vianna *et al.* (2010); [2] Trinca *et al*. (2012): ESA=East South America, AMZ=Amazonia; [3] This study: SP=South Pacific, AT=Atlantic, NP=North Pacific.

Genetic structure

The Haplotype network of Mexican haplotypes showed a star-like pattern; that is, a single high-frequency haplotype (the only one shared by the three sampling regions), as well as several low-frequency haplotypes with few mutational steps (maximum of two) between them (Fig 3-4).

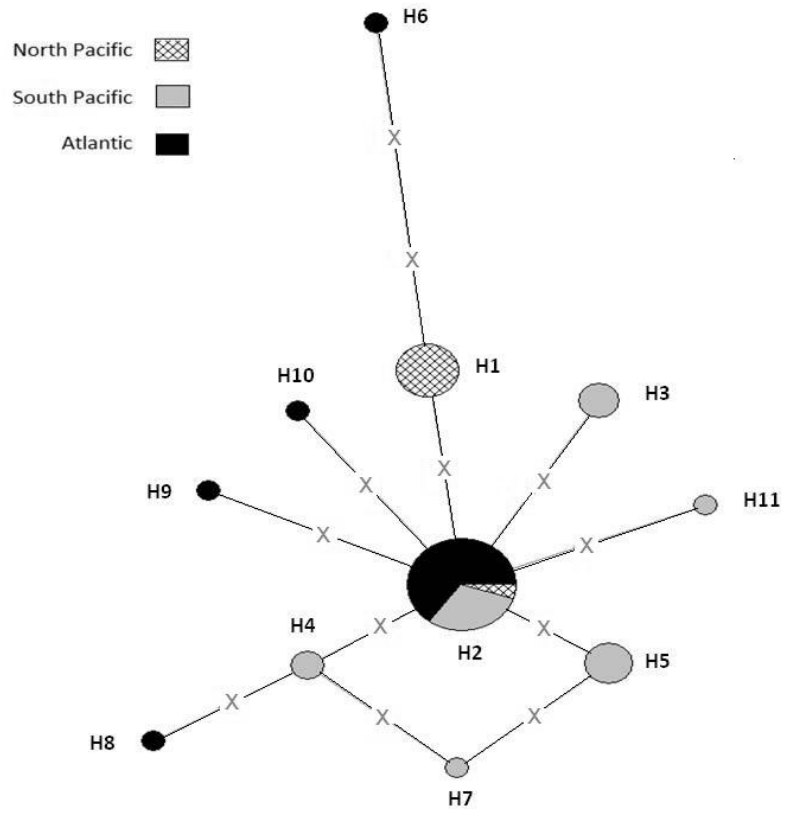

Fig 3-3. Median-Joining Network of Mexican haplotypes.

The circles represent individual haplotypes (H1 to H11) coloured by region, and their sizes vary according to their frequencies in the sample. The marks represent mutations between the haplotypes.

The AMOVA showed evidence of significant genetic structure (FST=0.31, P<0.05) across the three sampling regions (Table 3-3). The estimated pair-wise FST values among regions were all significant (P<0.05) and ranged from 0.08 to 0.49, suggesting moderate to high differentiation. The highest values were estimated among North Pacific and the two other regions, while the value was low among Atlantic and South Pacific (Fig 3- 5).

Table 3-3. AMOVA among three regions (North Pacific, South Pacific and Atlantic) within the distribution range of Neotropical otters (*Lontra longicaudis*) in Mexico.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
Among regions	2	6.289	0.20292 Va	31.85
Within regions	39	16.937	0.43428 Vb	68.15
Total	41	23.226	0.63719	

Fixation Index F_{ST} : 0.31 (P<0.05)

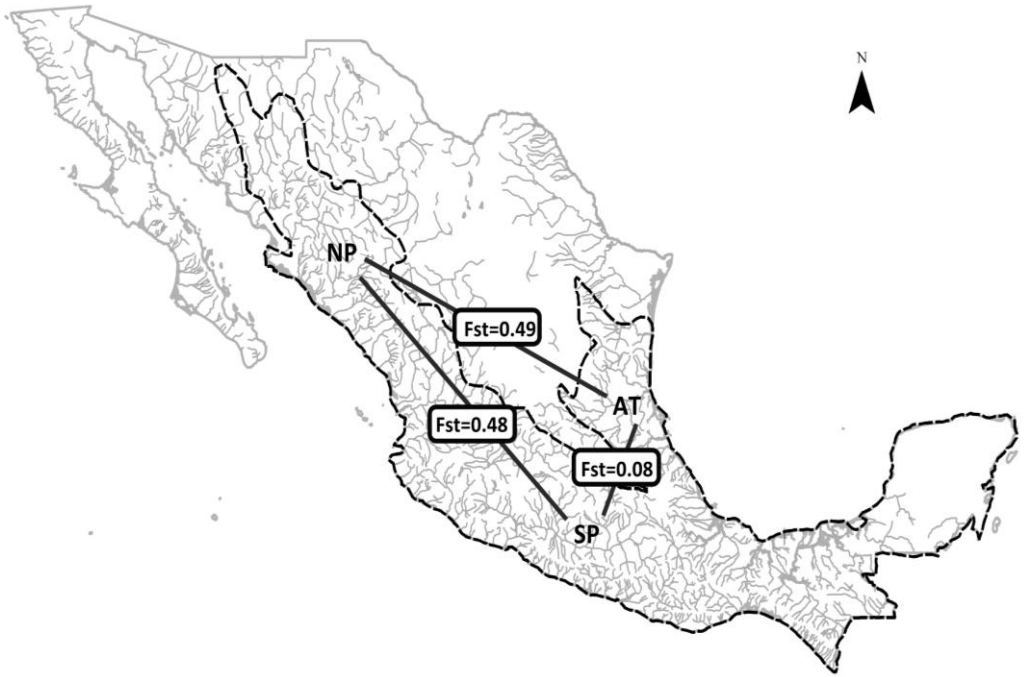

Fig 3-4. Pairwise estimates of genetic differentiation (F_{ST}) among Neotropical otter populations in three biogeographic regions in Mexico. Population pairs from which FST values were estimated are indicated by straight lines. NP=North Pacific, SP=South Pacific and AT=Atlantic. The Neotropical otter (*L.longicaudis*) range is outlined in black.

Demographic History

Fu's Fs statistic was negative and significant: -6.20 ($P<0.05$), which suggests an excess of rare haplotypes (Valqui *et al.*, 2010). Additionally, Harpending's raggedness index turned out to be low and non significant: 0.12 ($P=0.33$). These combined results can be taken as evidence of a recent demographic expansion. Moreover, the Bayesian skyline plot (Fig 3-6) also hinted to a recent demographic expansion (starting roughly 8,000 BP) and showed an increase in effective population size (*Ne*) of approximately one order of magnitude. The time to the most recent common ancestor (T_{MRCA}) of all Mexican sequences was estimated to be 11,819 BP (95% HPD: 3,138-24,127 BP).

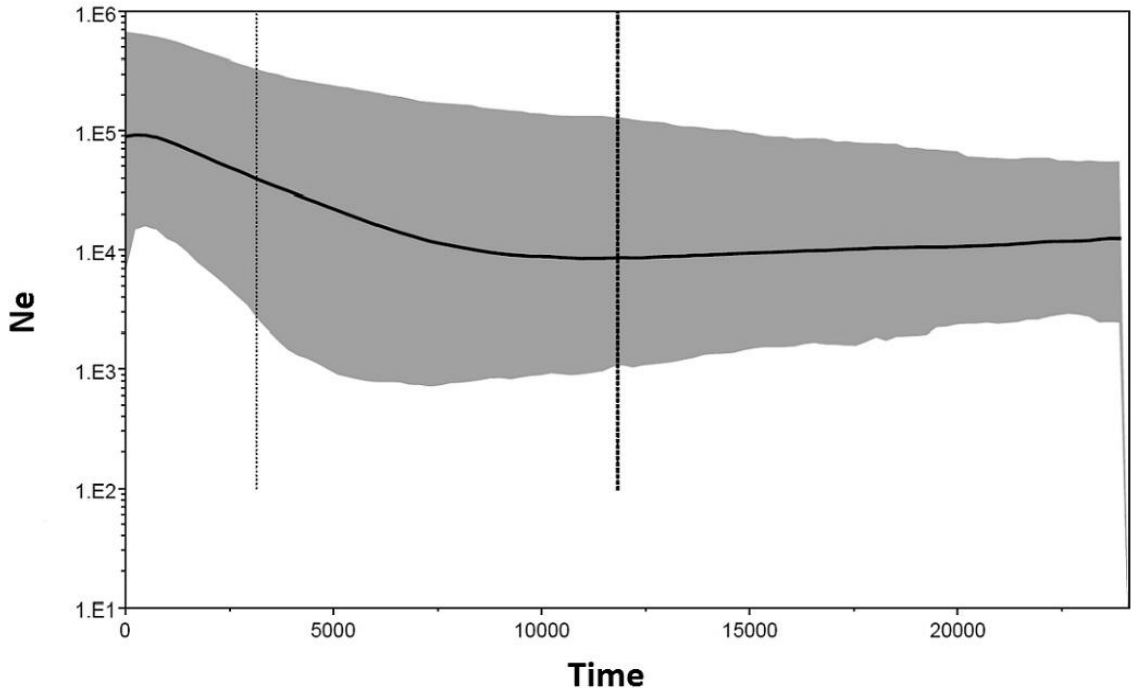

Fig 3-5. Bayesian Skyline Plot of Neotropical otter (*L.longicaudis*) populations in Mexico.

The *x* axis shows time in years before the present and the *y* axis depicts the estimated effective population size (*Ne*). The thick solid line represents the median *Ne* and the shaded area shows the 95%HPD. The vertical dashed lines refer to the time to the most recent common ancestor (T_{MRCA}): the thick dashed line represents the median and the thin dashed line represents its lower 95%HPD.

Discussion

Range-wide Phylogeography

The study by Trinca *et al*. (2012) identified two major mtDNA lineages of neotropical otters: East South America (ESA) and Amazonia (AMZ), that coincided with the proposed geographic distribution of the subspecies *L. longicaudis longicaudis* and *L. longicaudis enudris*, respectively. Additionally, a third lineage was identified, represented by a single sequence from Colombia. This lineage was estimated to have diverged from the rest of South American haplotypes 0.58 Ma and was suggested to represent the separation of the subspecies *Lontra longicaudis annectens*. The results of the present study, support this subdivision, given that haplotypes from Costa Rica, Mexico and the Colombian outlier sequence were found to be monophyletic, with strong nodal support for the lineage (NCAM). The estimated divergence date of this lineage from the South American haplotypes was 0.22 Ma, which implies a more recent divergence than previously estimated (Trinca *et al*., 2012). This difference in estimates could be due to the fact that they were derived from different mtDNA markers, *i.e.* the estimate in the present study was based on mtDNA control region, while the estimate by Trinca *et al.* (2012) was obtained using the coding mtDNA genes: ATP8, ATP6 and ND5.

In the present study, identification of a lineage distributed (with the exception of Colombia) in North America and Central America (NCAM), provides further phylogeographic evidence for the previously proposed subspecies scheme (Lariviere, 1999). This grouping of North American and Central American lineages separately from South American ones, is consistent with phylogeographic patterns frequently observed in large-scale studies of Neotropical taxa (Matte *et al*., 2013; Bagley & Johnson, 2014). The findings of the present study also suggest limited geneflow between NCAM populations and those in South America. This restricted connectivity could be related to the presence of the Cordillera Central and Cordillera Oriental located in Northern Colombia (Trinca *et al.*, 2012). However, because of the limited sample size from Central America (only one individual from Costa Rica) future studies are needed with increased sampling effort in this area as well as in the virtually unstudied insular populations such as those in Trinidad (Lariviere, 1999); this could reveal further lineages of the species and help clarify the present taxonomic uncertainties. It is also recommended to include nuclear markers in future studies to increase taxonomic resolution. Furthermore, closer examination of

morphological differences among proposed subspecies, as well as differences in life history traits and habitat, could strengthen the case for the proposed taxonomic scheme (Crandall *et al*., 2000; Padial *et al*., 2010).

Genetic diversity and genetic Structure in Mexico.

The lower genetic diversity estimated for Mexican otter populations compared to South American populations and closely related species, could imply historically lower effective population size. Additionally, there were differences in regional genetic diversity within Mexico, with the lowest estimate obtained in the NP (*h*=0.25). This estimate is comparable to two of the lowest estimates of haplotype diversity that have been obtained in other otter species (*Enhydra lutris*: h = 0.412; Larson *et al*., 2002; *Lutra lutra:* h = 0.16 ± 0.06; Ferrando *et al*., 2004). Low genetic diversity found in the NP, could be related to the isolation of this region from SP and AT populations, as evidenced from the results of genetic structure analyses. Although all pair-wise population comparisons yielded evidence of significant genetic structure, the highest estimates were among the NP and both the SP and AT regions; while the lowest genetic structure was estimated between SP and AT.

The Sierra Madre Oriental (Fig. 3-2) has been consistently identified as being associated with high genetic structure between Pacific and Atlantic populations of diverse taxa in Mexico (Sullivan *et al*. 2000; Leon-Paniagua *et al* 2007; McCormack *et al.* 2008; Arteaga *et al.*, 2012). Nevertheless, in this study I found a only moderate genetic structure (FST=0.08) between the Atlantic (AT) and Pacific (SP) regions, consistent with recent or ongoing gene flow between these regions. A potential explanation for this pattern is the presence of the Tehuantepec Isthmus (TI) between the geographical East and West (Fig 3-2). This low-altitude, narrow isthmus is almost entirely transversed by a fast-flowing river: the Coatzacoalcos river, and is unique as it appears to be the single region in Mexico where numerous riparian species have spread between the Atlantic and Pacific drainages (Garcia, 1981; Mateos *et al*., 2002; CONABIO, 2010). Low-elevation mountains, as well as high surface water availability, are environmental characteristics that have been shown to facilitate otter dispersal and may promote genetic exchange (Latch *et al.*,2008; Loy *et al.*,2009; Santiago-Plata, 2013).. Recent studies have proposed that the TI might also act as a geneflow corridor for other mammals in Mexico (Arteaga *et al.*, 2011; Arteaga *et al*., 2012). In contrast, genetic structure was high among the NP and the SP/AT; which could

be related to the presence of the Sierra Madre Occidental and the Transvolcanic Belt (Fig. 3-2). Specifically, these geographic formations could represent a barrier between NP and SP/AT populations, as has been suggested for several other species in Mexico (Bryson *et al*., 2011) . Moreover, surface water availability decreases towards the North of the country (Conabio, 2010), which could further interfere with genetic connectivity among NP and SP/AT given that permanent, well connected waterways are key to allow geneflow among otter populations (Hobbs *et al*., 2006; Janssens *et al*., 2007).

Given that genetic structure estimates were obtained by means of mtDNA, the use of nuclear genetic markers will be necessary to examine whether the observed structure is related to sex-biased dispersal. Female philopatry is widespread in mammals and has been observed to occur in both Eurasian and Neotropical otters (Quaglietta, *et al.,* 2013; Trinca *et al.,* 2013); this pattern of dispersal typically results in higher estimates of genetic structure from maternally inherited markers than estimates obtained from bi-parentally inherited makers (Allendorf and Luikart, 2007).

Demographic history

The results of the mismatch distribution and Fu´s Fs analyses, as well as the ´star-like´ configuration of the Haplotype network, consistently suggested a recent demographic expansion for otters in Mexico (presumably the entire NCAM clade). Furthermore, although Heller *et al* (2013) have shown that Bayesian Skyline Plots can give false signals of demographic decline when there is population structure (as was found in the present study), the Bayesian skyline plot of Mexican sequences showed a clear signal of demographic expansion. Given that fossil records of Neotropical otters in Mexico date to the late Pleistocene (Arroyo-Cabrales, *et al.*, 2013), it is important to consider how the climatic changes through this epoch could have impacted otter populations in the country. During the Pleistocene, Mexico was not covered by the Laurentide glacier, and there is no clear consensus on whether this glacier's advance-retreat dynamics could have had a direct effect on glacier patterns in Mexican territory (Ferrusquía-Villafranca *et al.*, 2010). Studies on Pleistocene climate in Mexico have provided strong evidence of the presence of glaciers in the territory; however, these were most likely constrained to the top of high altitude mountains (Metcalfe *et al*., 2000). During the Last Glacial Maximum, colder and dryer conditions than at present prevailed throughout Mexico; specifically, there are records of a

dramatic decrease in rainfall (~50% compared to present conditions) which caused depletion of major water bodies, particularly in the south and centre of the country (Metcalfe *et al*., 2000; Ferrusquía-Villafranca *et al*., 2010). Hence, even if glaciers did not directly cover Neotropical otter habitat, the climatic conditions brought about by their presence could have impacted otter populations by limiting the areas of suitable habitat, as well as decreasing the availability of fish, their main prey (Gallo, 1997; Kruuk, 2006). As mountain glaciers began retreating ca.14,000 BP, climatic conditions started shifting toward overall higher humidity and temperature (Arroyo-Cabrales *et al*., 2008; Arroyo-Cabrales, *et al*., 2010), which could have favoured otter population growth. This postglacial expansion scenario coincides with results of the BSP, which showed an estimated expansion starting ca. 8,000 BP, which roughly coincides with the end of the Pleistocene epoch (Gibbard and van Kolfschoten, 2004). Furthermore, there are records of mass extinction of large mammals (>100 kg) in Mexico during the late Pleistocene (Ferrusquía-Villafranca *et al.*, 2010). Therefore, it is possible that otter demographic expansion was also related to the elimination of larger, potentially competing species. A recent study on cougar (*Puma concolor*) populations throughout the Neotropics, estimated a similar date for a demographic expansion of this species and proposed akin mechanisms underlying it (Matte *et al*., 2013) .

Differences in demographic histories between Mexican and South American otter populations, should account for the lower genetic diversity found in Mexico. The estimated time of the most recent common ancestor (T_{MRCA}) of Mexican populations is very recent (11,819 BP) compared to estimates of coalescence times obtained for the South American lineages ESA and Amazonia: 280,000BP and 190,000BP, respectively (Trinca *et al.*, 2012). A more recent coalescence would also imply there has been less time for mutation to increase genetic diversity in Mexican otter populations (Amos & Harwood, 1998). Furthermore, differences in historical effective population size (*Ne*) could also explain dissimilarities in genetic diversity. Specifically, the estimated median *Ne* for Mexican populations before the expansion, is about one order of magnitude lower than historical estimates for South American populations (Trinca *et al*., 2012). However, this comparison should be taken with caution given overlap in the highest posterior density intervals (HPD) of these estimates.

Very little is known in terms of more recent demographic history of the species in Mexico as opposed to further otter species throughout the world. For instance, in Europe, organochlorine pesticides (PCBs) caused a massive decline in Eurasian otter populations (*Lutra lutra*) starting in the 1950s. During this period, the species was restricted to refugia and subsequently recovered in response to the regulations imposed on the use of PCBs in the 70s (Kruuk, 2006). However, in Mexico it wasn't until the year 2000 that specific regulations on the use of PCBs were introduced (SEMARNAT, 2000). Furthermore, throughout the country there continue to be cases of inadequate disposal of these compounds into the environment, including water bodies (Sanchez *et al*., 2007). In addition, a recent study provided evidence for accumulation of heavy metals in otter faeces; according to the authors, the levels that were estimated in Mexico are at least an order of magnitude higher than what has been found in studies on European otter populations (Ramos-Rosas *et al*., 2013).

Although no recent surveys on the status of otter populations in Mexico have been conducted, several studies have included interviews to locals in which they were asked whether neotropical otters were as commonly observed in the present, as compared to decades ago; in all cases, interviewees stated that sightings had become less common, and this trend has been observed throughout the country (Macias-Sanchez, 2003; Sanchez *et al*., 2007). This implies that Mexican otter populations might continue to be at risk and it is suggested to start systematic surveys of otter populations as soon as possible, in order to estimate and monitor changes in population size.

Implications for range-wide and local conservation.

For South American Neotropical otter populations (Trinca *et al*., 2012), two distinctive lineages were identified (Amazonia and ESA) which the authors proposed represent separate Evolutionary Significant units (ESUs) (Moritz, 1994). Given the evidence for the almost complete monophyly of the North/Central America (NCAM) clade identified here, as well as its clear separation from South American lineages, I propose to consider this lineage as a third ESU for the species. Such a proposal is also consistent with recognized biogeographic subdivisions in the Neotropics (Escalante *et al*., 2013; Morrone, 2006), and implies that NCAM should be managed as a distinctive unit for conservation (Frankham *et al*., 2007). Nevertheless, it is important to note that identification of conservation units

solely on the basis of genetic data has been controversial and it is proposed to include both genetic and ecological data, as well as testing for exchangeability when defining conservation units (Crandall et al., 2000). Furthermore, a new type of conservation unit: the Elemental Conservation Unit (ECU) has recently been proposed. An ECU is defined as a demographically isolated population whose probability of extinction over a given time scale is imminent, unless there is human action to prevent it. Under the ECU framework, scientists would assist in identifying conservation units at risk, but decision makers and the public would also participate in selecting the ECUs that are prioritary on the basis not only of their ecological/genetic uniqueness, but also taking into account the ecosystem services they provide (Wood and Gross, 2008)

Mexican otter populations were found to be overall less genetically diverse than South American populations. A lower genetic diversity translates into a diminished evolutionary potential and therefore an increased risk of extinction (Frankham *et al.*, 2007). Based on the results of this study, it follows that conservation efforts might have to be tailored to specific regions in Mexico. In principle, maintaining connectivity between SP and AT populations should be prioritary. Given that evidence points to the Tehuantepec isthmus as a potential geneflow corridor between both areas, maintaining habitat connectivity within this region could be a good starting point. On the other hand, the North Pacific region poses different conservation challenges given that populations there appear genetically depauperate and potentially isolated from other regions. Furthermore, the NP populations exist in arguably the most extreme environments found throughout the species range i.e. arid habitats at the limit between the Neotropical and the Neartic regions. In this sense, otter populations in the NP might be showing local adaptation, as is frequently encountered in populations at range limits (Gaston, 2009; Sexton *et al.*, 2009). Given the genetic distinctiveness of NP populations, as well as their low genetic diversity, immediate conservation actions seem necessary.

A reasonable course of action for conservation of NP populations would be to first look closely into the reasons for the low genetic diversity encountered in this area. For instance, it is crucial to investigate whether declines in population size have taken place within the region; and if so, whether they are historical or recent. Conservation efforts should also be directed to maintain (or improve where possible) the extant habitat of the species in the North Pacific; one way of achieving this would be to designate new protected areas. Although there already exist natural reserves throughout the country (CONANP, 2002),

recent studies have suggested that current national protected areas might not adequately represent mammalian biodiversity and habitat in Mexico (Vasquez and Valenzuela-galván, 2009). In light of this, it is recommended to re-evaluate whether current protected areas in North Pacific Mexico are indeed serving the purpose of conserving Neotropical otter habitat. For instance, there is a single protected area within the NP that includes potential otter habitat: The "Sierra de Alamos-Rio Cuchujaqui" reserve in the state of Sonora (CONANP, 2002). However, this reserve only includes one river basin (out of several throughout the NP) where the presence of the species has been confirmed (Gallo, 1997). Thus, this protected area is unlikely to be sufficient to guarantee long-term conservation of otter populations in the NP, as gene flow with populations outside the reserve is not being contemplated, and this would be key to avoid inbreeding in the long term.

In the context of long-term conservation, future studies should focus on identifying specific landscape/habitat features that promote or hinder geneflow among otter populations, as well as the impact that human activities have had on the genetic connectivity of populations throughout Mexico. In this sense, nuclear markers will be necessary to address these questions, as they could reveal genetic patterns at a finer temporal and spatial scale.

Finally, although there are questions that remain to be addressed, to my knowledge, the present study is the first large-scale investigation on population genetics and demographic history of the species in Mexico. Furthermore, the findings of the present study should be relevant not only for the conservation of this species, but given that the observed phylogeographic patterns coincide with clearly identifiable bioregions, it is likely that research on other taxa will yield similar patterns and contribute to establish further units and/or large scale areas for conservation.

Chapter 4 -Using landscape genetics to inform the conservation of Neotropical otters (*Lontra longicaudis*) in Mexico

ABSTRACT. Landscape genetics is a multidisciplinary area of research that is well-suited to address questions across a number of disciplines, including conservation biology. In this sense, this discipline can aid in identifying specific landscape features that affect gene flow among wildlife populations and thus provide invaluable information for their conservation. The Neotropical otter (*Lontra longicaudis*) is catalogued as data deficient by the International Union for the Conservation of Nature (IUCN) and hence, it is imperative to conduct research that helps to identify the challenges the species faces in its natural habitat in order to inform conservation strategies. In this study, landscape genetics was used to investigate how selected landscape characteristics affect gene flow of otter populations in Mexico, with the ultimate aim of proposing conservation actions for the species and its habitat. Non-invasive sampling and 13 microsatellites were used to obtain genotypes of 54 individuals at a country wide scale and 38 at two regional scales: North Pacific and South Pacific. Mantel tests and Multiple regression on distance matrices (MRM) were used to investigate how elevation, slope, aquatic networks and land cover are related to gene flow at a country-wide and regional scale in Mexico. Additionally, Bayesian clustering algorithms were used to examine country-wide genetic structure. The results of this study show that elevation (country-wide) and slope (North Pacific) can indeed hinder otter gene flow. Therefore, it is suggested that conservation corridors for the species are planned in areas with low elevations and slopes, so as to allow connectivity among basins inhabited by the species. Finally, the results of Bayesian clustering suggest the existence of two distinct population clusters in Mexico which show moderate genetic differentiation among them (FST=0.19). However, given that the identified clusters appear to have migrant exchange and isolation by distance was observed in the study area, further studies will be needed in order to assess whether these clusters can be considered distinctive management units.

Introduction

Landscape genetics (LG) is an exciting area of research that combines landscape ecology, population genetics and spatial statistics, with the aim of assessing the interaction between landscape features and micro evolutionary processes (Manel *et al.*, 2003). Being an interdisciplinary area of research, LG is well-suited for addressing questions across several disciplines, such as evolutionary biology, ecology and conservation biology (Manel *et al.*, 2003; Holderegger and Wagner, 2008).

There are two core steps in the landscape genetics workflow; the first step consists of using molecular markers to obtain a genetic dataset from which patterns such as gradients or discontinuities can be identified. Given that in LG the individual is the preferred operational unit of study, sampling is usually conducted in a continuous way throughout the study area without defining populations *a priori*. Once genetic patterns are identified, the second step consists of correlating them with landscape features (Manel *et al.*, 2003). For the second step, there exist a variety of statistical tools such as simple and Partial Mantel tests, which have been extensively used in LG studies to this day (Spear *et al*., 2005; Lind *et al.*, 2010; Zalewski *et al.*, 2009; Moore *et al.*, 2011). However, in simulation studies, Mantel tests have been found to suffer from high type-1 error rates (i.e. they suggested statistically significant landscape-genetic relationships when no such relationships had been simulated). Hence, it is proposed to use Mantel tests in the first steps of landscape genetics studies (for preliminary analyses), but also use other methods in conjunction with them. For this purpose, there exist alternative and arguably more powerful statistical approaches which are currently gaining importance in landscape genetic studies (Balkenhol *et al.*, 2009).

In terms of incorporating landscape effects into a LG study, one option is to use Least-Cost-Path (LCP) analysis, which generally involves overlaying the landscape with a grid and assigning "cost" values to each grid cell according to the habitat needs of the focus species. Next, the one path (between individuals or populations) that represents the least cost to movement, is used as a distance metric which is then correlated to genetic distances (Adriaensen *et al*., 2003; Vignieri, 2005; McRae, 2006). However, this approach could be improved if it could take into account that dispersal most likely occurs through numerous different paths, not just a single optimal one (McRae, 2007). In this sense, an alternative to LCP analysis is the use of the isolation by resistance (IBR) model (McRae, 2006). This model builds

up from electric circuit theory and is used to incorporate landscape heterogeneity into studies dealing with genetic differentiation. Specifically, IBR predicts a positive relationship between genetic differentiation and the so-called "resistance distance"; a distance metric that considers all possible pathways as well as landscape heterogeneity between sampled populations (McRae, 2007). In order to obtain resistance distances for landscape genetic studies, geographic information systems are used to create "resistance surfaces", which are spatial layers that assign a value to each landscape feature representing the degree to which it facilitates or hinders dispersal for the study species. In this way, resistance surfaces are a way of representing hypothesized relationships between landscape variables and dispersal or gene flow. The values assigned to landscape features are usually based on prior knowledge about the ecology of the focal species (McRae, 2006; Wang *et al.* 2008; Spear *et al.,* 2010)

Regardless of the particular approach, one of the most important applications of LG is in conservation biology (Manel *et al*., 2003). Specifically, the discipline can help identify habitat features that facilitate species movements (useful for wildlife corridor planning), aid in locating barriers to gene flow across a landscape and thereby assess how anthropogenic alterations contribute to habitat fragmentation. In this way, landscape genetics provides an effective means to evaluate habitat connectivity; a central issue when attempting the long-term conservation of species (Manel *et al*., 2003; Coulon *et al*., 2004; Pearse and Crandall, 2004; Cushman *et al*., 2006 Holderegger and Wagner, 2008).

Thus far, landscape genetics has been used for conservation purposes in numerous vertebrate species (fish: Castric, *et al*., 2002; salamander: Dudaniec, *et al*., 2012; giraffe: Thomassen *et al*., 2013), including carnivorous mammals (marten: Broquet, *et al*.2006; mink: Zalewski *et al*., 2009; marten: Koen *et al*., 2012). Carnivores are considered key in the conservation of biodiversity as their habitat requirements comprise many other species' habitats and it has been shown there is a direct link between top predator conservation and ecosystem-level Conservation (Noss *et al*., 1996; Fabrizio, 2006). Thus, research on the association between carnivores and the landscapes in which they prevail is most important; particularly for those species with strong habitat associations. One example of such species are otters, which are intimately associated with riparian habitats and are considered bio-indicators of ecosystem health (Kruuk, 2006; Waldemarin and Alvares, 2008).

Otters are very charismatic carnivores, but have been extremely affected by human activities throughout history. Hunting, pollution and habitat fragmentation have caused otter populations

to decline drastically and even disappear from large areas around the world (Kruuk, 2006). Research on arguably the most studied species, the Eurasian otter (*Lutra lutra*), has found that habitat features such as riparian vegetation, human density and water availability/quality are strongly correlated with the species distribution in Europe (Mason, 1995; Kruuk, 2006). This illustrates how vulnerable otters may be to human activities and alterations in their habitat.

In the Americas, the Neotropical otter (*Lontra longicaudis*) is distributed from Argentina to Mexico (Chapter three) (Larivière, 1999; Kruuk, 2006). The basic habitat requirements of the Neotropical otter (*L. longicaudis*) include ample riparian vegetation, well-connected aquatic networks and low human density (Larivière, 1999; Pardini, & Trajano, 1999; Kruuk, 2006; Waldemarin and Alvares, 2008). Recent studies have also found a strong association between the presence of the species and land cover, stream order and riverbank slope (Cirelli, 2005; Santiago-Plata, 2013). Given that the species is currently classified as data deficient by the International Union for Conservation of Nature (IUCN), there is a desperate need for research that sheds light on the status of its populations and the threats they may be facing. Specifically, identifying key habitats and areas where large populations subsist is considered a priority (Kruuk, 2006; Waldemarin and Alvares, 2008).

In Mexico, it has been proposed that the main threat to otter populations is likely to be habitat fragmentation (Gallo, 1990; SEMARNAT, 2001), which restricts gene flow among populations and thus increases their risk of extinction (Hedrick, 1985; Frankham *et al*., 2003; Stockwell *et al*., 2003). Habitat fragmentation relevant to otter populations may occur through anthropogenic activities such as river desiccation and damming, urbanization, removal of riparian vegetation and water pollution (Mason, 1995). Within Mexico, all these activities are common place; for instance, riparian vegetation is often cleared for agricultural purposes, while the demand for water to fulfill the growing population's needs has caused extensive construction of dams throughout the country (Sanchez *et al*., 2007). In spite of the clear threats that these activities pose on otters, very little is known about the status or recent changes in otter population size in Mexico. For instance, it is widely accepted that the release of organochlorine pesticides and heavy metals into the rivers caused a dramatic decline in Eurasian otter (*Lutra lutra*) populations in Europe from the 1950s to the mid 1970s (Kruuk, 2006). However, to my knowledge there are no studies that have directly evaluated changes in otter population size (contemporary or historical) in Mexico, as a consequence of riparian pollution. The only

information available in this regard, consists of anecdotal reports from inhabitants of areas where neotropical otters are distributed; most of these reports indicate that otter sightings have become much less frequent in recent years (Macias-Sanchez, 2003; Sanchez *et al.*, 2007; Guerrero-Flores *et al*., 2013). Given that appropriate regulations on the use of organochlorine pesticides were not in place in Mexico before the year 2000, and the fact that discharge of heavy metals into the rivers is not unusual (SEMARNAT 2001, Sanchez *et al.,* 2007) it is possible that the perceived decline in otter numbers is related to water pollution. In support of this, a recent study found evidence for accumulation of heavy metals (mercury, lead and cadmium) in otter faeces collected within two protected areas (Ramos-Rosas *et al*., 2013).

Moreover, Mexico represents the northern limit of the species' range, which could further compromise the genetic connectivity of otter populations as habitats in range limits tend to be more fragmented and of marginal quality (Eckert, *et al*, 2008; Gaston, 2009; Sexton *et al.*, 2009). Additionally, natural landscape features such as mountain chains could further hinder dispersal of otters, as it has been suggested that the species is less abundant at high elevations (Lariviere, 1999). Therefore, investigating how habitat features affect gene flow in Neotropical otter populations could provide invaluable information to conserve the species. Given this scenario, in this chapter I make use of the landscape genetics approach with the following aims:

1- To assess genetic diversity and to test for evidence of population structure of the species in Mexico based on microsatellites

2- To investigate the effect of elevation, slope, aquatic networks and land cover on otter gene flow at a country-wide (Mexico) and regional (focal regions within Mexico) level.

3- To propose conservation actions for the species and its habitat in Mexico based on the results.

Methods

Study Area and sampling.

From 2011 to 2013, sampling was conducted along the distribution range of the species in Mexico in 21 sampling locations, in order to examine the effect of landscape features on gene flow at a country-wide scale. Additionally, two distinct focal areas were defined in the North Pacific (NP) and South Pacific (SP) in order to conduct analyses at a regional scale (Fig.4-1)

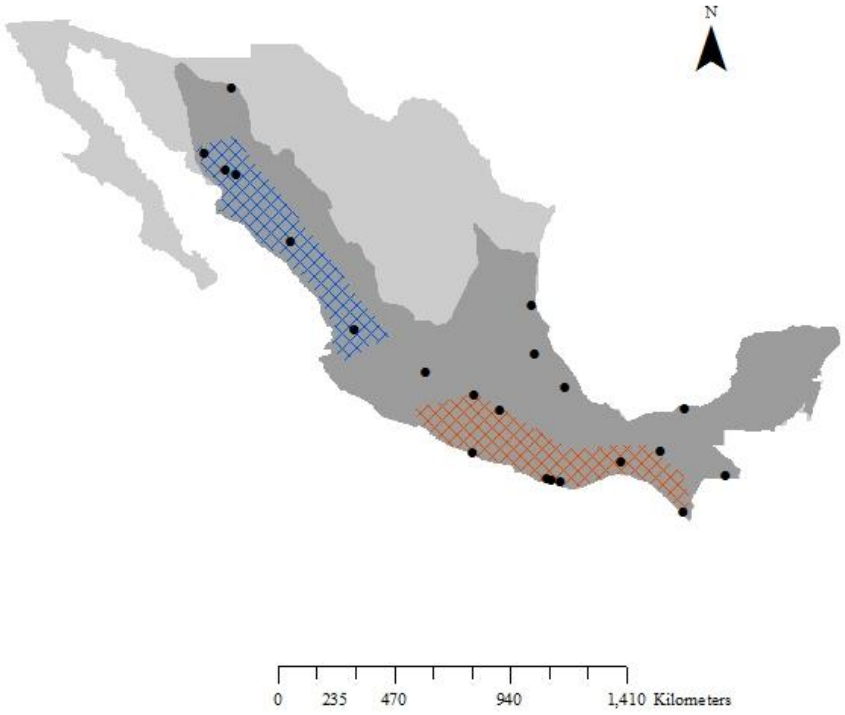

Fig 4-1. Sampling sites and focus regions within the range of the Neotropical otter (*Lontra longicaudis*) in Mexico.
The map shows the range of the species (dark grey) and sampling sites (circles); focus regions within the range are color-coded: North Pacific (blue) and South Pacific (orange).

During the sampling surveys, otter habitat was visited in the early morning hours in order to collect fresh faecal samples. The majority of samples were stored in paper envelopes and dried at ambient temperature, but whenever possible they were also stored in *RNAlater*® solution and 96% ethanol. Although sampling was based on scats, tissue and hair from specimens found dead in the field were also collected, and donations of hair samples from captive specimens were obtained. All samples were geo referenced in the field. From this sampling, 159 fecal samples were collected from the North Pacific, 151 from the South Pacific and 93 from the Atlantic (total=403). The rest of the samples (10) included hair/tissue from dead specimens, as well as hair donations from zoos.

DNA Extraction and PCR amplification

DNA extraction from faecal samples was performed in a designated area of the lab, using a QIAamp® DNA Stool Mini Kit (QIAGEN) following the manufacturer's instructions, with the following modifications: incubation in ASL buffer was done overnight; incubation time in AE buffer was increased to 30 minutes and the final elution step in AE buffer was repeated, in order to obtain two separate 100uL aliquots of extracted DNA. The DNA extraction from tissue was performed by means of DNeasy Blood & Tissue Kit (QIAGEN), following the manufacturer's instructions. For hair DNA extractions, the same kit and protocol was used with one modification: the incubation in buffer ATL and proteinase K, also included 20uL of 1M DTT.

As a first step, I tested 20 microsatellite primers originally developed for *Lontra canadensis* (Beheler *et al.*, 2004, 2005) for amplification in *L. longicaudis* DNA (extracted from muscle tissue) in single-locus PCR reactions. These reactions (20 μl) contained 2μl DNA, 0.5 μM each primer, 1X reaction buffer, 1.5Mm MgCl, 0.2 mM dNTPmix, 1X BSA and 1U Taq polymerase (Invitrogen). The PCR conditions consisted of 35 cycles of denaturation at 94°C for 60s, annealing at 55°C for 60s and extension at 72°C for 60s. Out of this initial panel of microsatellites, 15 could be amplified from Neotropical otter DNA and were subsequently used in Multiplex reactions with DNA extracted from all sources (hair, tissue and faecal). These reactions (14 μl) contained 7 μl of Qiagen Type-it microsatellite PCR mix, 0.2 μm primer mix and 4 μl DNA. The PCR conditions consisted of an initial step of 95°C for 5 m, 45 cycles of denaturation at 95°C for 30 s, 58°C annealing for 1.30 m and 72°C for 30 s. The last step was an extension of 60°C for 30 m.

In all PCR reactions, I included one negative control for every 30 samples tested. The PCR products were visualized in 2% agarose gels and products of the expected size were sent out for fragment analysis at the DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland).

Generation of data set

Genotyping was done in Genemapper 3.7 (Applied Biosystems); all 15 microsatellites that could be amplified in *Lontra longicaudis* were polymorphic. However, I discarded two (RIO 10 and RIO 20) that showed consistent amplification artifacts (stutter bands) and hence were prone to inaccurate scoring. Thus, the final microsatellite panel consisted of 13 useable loci (Table 4-1). However, because of varying quality of samples originating from different areas in Mexico, for the country-wide analyses only data for 7 loci were available for individual genotyping. On the other hand, for regional analyses (NP and SP), the entire panel of microsatellites was used (Table 4-1). In order to determine the ability of these two sets of microsatellites to distinguish between individuals, the probability of identity (P_{ID}) and P_{ID} between siblings (Waits *et al*., 2001) were estimated using 6 otter muscle tissue samples, and the software GenAlEx 6.5. (Peakall and Smouse, 2012). Given the non-invasive source of DNA, samples were genotyped repeatedly in order to obtain and validate multilocus genotypes. This was achieved by following the protocol developed by Frantz *et al.* (2003), which is a modification of the multitube approach (Taberlet *et al.*, 1996). The protocol involves accepting a genotype as heterozygote after each allele has been observed twice (in independent PCRs), while a homozygote is accepted after being observed in three independent PCRs. This approach has the advantage of requiring less replicates than the multitube approach (Taberlet *et al*., 1996) and has been used in several studies dealing with non-invasive sources of DNA (Quéméré *et al.*, 2009; Quéméré *et al.*, 2010; Morán-Luis *et al.*, 2014). Next, GIMLET 1.3.3 (Valière, 2002) was used to identify individuals within the pool of genotypes. In this sense, multilocus genotypes that were identical or differed in a single allele, were assumed to belong to the same individual (Bellemain *et al.*, 2005). As a result, 54 individuals (typed at 7 loci) were identified for country-wide analyses, while 25 individuals from the NP and 13 individuals from the SP (all typed at 13 loci) were identified (see APPENDIX B, Table B1 and APPENDIX D, Table D1). Taking into account the number of faecal samples originally collected in each region, and the number of these for which consensus genotypes could be obtained, I

estimated a genotyping success of 16% from the North Pacific, 7% from the South Pacific and 10% from the Atlantic. These percentages are lower than have been found in recent studies with otters: 33% (Trinca *et al*., 2013) and further mammal species such as ungulates: 58.3 to 83.3 % (Soto-Calderon *et al*., 2009) and elephants: 98.6% (Okello *et al*., 2005). The low genotyping success of the present study could be explained by significant delay between collection and DNA extraction. After obtaining consensus genotypes, from a subset of 12 samples, I estimated a mean allelic dropout rate (ADO) across loci as the number of times in which an allele from a heterozygote genotype was not observed, divided by number of replicates. In adittion, I estimated a false allele rate (FA) as the number of times a spurious allele appeared, divided by number of successful replicates for a given locus (Broquet and Petit, 2004).

I used GenePop 4.0 (Raymond and Rousett, 1995) to test for linkage disequilibrium among loci (applying a Bonferroni correction). By means of the software R (R Development Core Team, 2008) and the package PopGenReport (Adamack & Gruber, 2014) , I tested for Hardy Weinberg equilibrium (applying a Bonferroni correction), estimated null allele frequencies, number of alleles per locus, and obtained matrices of genetic distances among individuals (Smouse and Peakall, 1999) to be used for successive landscape genetics analyses. Genetic distances were estimated separately for the country-wide and each of the regional scales.

Table 4 - 1. Characteristics of Microsatellite loci used to amplify *Lontra longicaudis* DNA in the present study (Beheler 2004; 2005).

Locus	Forward sequence	Reverse sequence	Repeat Units	Size range	Fluorescent dye
RIO01	AAGGGCACCTCGAGACAAT	CATGCTTGACCTTGAGCAAC	$(GATA)_9(GAT)_4$	264-280	5'FAM
RIO02	GTAGAGTGGGGCGCCTAAG	TGTCCTTGGAAGAGACATGC	$(TC)_9(AC)_{12}(GA)_4$	184-198	5'HEX
RIO03*	ATCAGCCTGAGTCCCTGAAC	ACAGCCAGAACCAAAAGACA	$(CT)_{26}(CA)_7$	194-218	5'FAM
RIO06	GCCAAGATGGCAACTACTCC	GAAGCACATTCTCTCTCCATCA	$(TCTA)_2(TCTA)_8$	252-264	5'AT550
RIO07*	AAGCACTTCCAGATATCAGTTGC	CCCAACTTGAGTGGGACTTT	$(AC)_{21}$	167-177	5'FAM
RIO08	TTTCCAGAGCCAATTTGTCA	CTTGCCTGCTGACATTGAAG	$(TG)_{15}$	204-214	5'AT550
RIO09*	GCTCTATTATTAGGAGCAAACCA	AGCTGGCTTGGAATTCTCTC	$(AG)_{11}$	252-256	5'FAM
RIO11*	TCTTCCACTTTTCAATTTAGGTA	GCCCAAGGTTCACTATCAAG	$(AC)_{14}$	156-168	5'HEX
RIO12*	GTATCGTCCAGGCTGCTCTC	CCACAGCCAGCTCTGAATAA	$(AG)_{12}$	207-213	5'FAM
RIO13*	GCTCAGCTGTGCAGAATGAT	GCACACGTGGTAAGATGAGC	$(GT)_{20}$	254-274	5'FAM
RIO15	AGTGCACAGTGGTGGTCTTG	TCCTGATTCTGCTTGGTTCA	$(TC)_8(CA)_{10}$	253-261	5'FAM
RIO16	GGTGCTTCTTAAGGAACTGAGC	ATTTATTGGGCATGGAAGCA	$(GT)_{14}$	266-280	5'HEX
RIO19*	GGTCCCAGGTGCAAATCTTA	GATTTGGGTCTTCCAATGGTT	$(CT)_{12}(ATCT)_9$	273-285	5'AT550

* Loci used for country-wide analyses

Genetic diversity and structure

I used PopGenReport (Adamack & Gruber, 2014) to obtain genetic diversity estimates of the North Pacific and South Pacific separately. Specifically, average Observed (*Ho*) and Expected (*He*) heterozygosity as well as the mean number of alleles per locus were estimated. To determine the genetic structure at a country-wide scale, I used STRUCTURE 2.3.4 (Pritchard *et al.*, 2000). Analyses were performed for values of *K* from 1 to 10, with 10 independent runs per *K*, using the admixture model and correlated allele frequencies with 50,000 iterations and a burn-in of 5,000. The output was analyzed in STRUCTURE HARVESTER (Earl and von Holdt, 2011), in order to examine Δ K (Evanno *et al.*, 2005) and mean log-likelihood of data. Next, I used the software CLUMPP to calculate the means of all estimated cluster membership coefficients (Jakobsson & Rosenberg, 2007) and the software ArcGIS 10 to visualize the clustering pattern. In addition, I used GenePop 4.0 (Raymond and Rousett, 1995) to estimate FST among the clusters identified by STRUCTURE.

Landscape genetics

In order to assess the interaction between gene flow and landscape features in the study area, I used a landscape resistance approach based on circuit theory (McRae & Beier 2007). This approach requires generating resistance surfaces, by assigning cost or "resistance" values to cells in habitat rasters; these surfaces are then used to estimate "resistance" distances among individuals (Spear *et al.*, 2010). Thus, as a first step I used the software ArcGIS 10 to generate and parameterise resistance surfaces based on the landscape features: *elevation, slope, land cover* and *aquatic networks* as they are known to affect abundance, dispersal and/or occupancy of otters (Table 4-2). For this purpose, I obtained land cover data and a digital elevation model (DEM) from the Mexican Instituto Nacional de Estadística y Geografía (INEGI): http://www.inegi.org.mx . Data on slope was estimated from the DEM; in order to generate aquatic networks rasters, data on river networks was obtained from HydroSHEDS (http://hydrosheds.cr.usgs.gov/index.php) and combined with a raster of lentic water bodies (lakes, lagoons and wetlands) obtained from INEGI.

Resistance surfaces for the country-wide scale were generated with a cell size of 500m, while for the regional scales (NP and SP) the surfaces had 300m cell size. All surfaces were univariate *i.e.* generated separately for each landscape feature. When landscape variables were divided in categories, different cost ratios (1:10, 1:100, 1:1000, 1: 10000) were tested. In addition to these variable-specific resistance surfaces, for both scales I generated "flat" resistance surfaces in which all cells were assigned the same cost of one. This approach is akin to using Euclidean distance (to test for isolation by distance as a null model), but accounts for the finite size of the landscape (Moore *et al.*, 2011; Dudaniec *et al.*, 2012).

Landscape resistance surfaces for both *elevation* and *slope* were parameterised in two ways: 1) keeping the original values per cell (elevation in meters and slope in degrees) assuming increasing resistance with increasing values of these features, and 2) assuming a "threshold" effect (With & Crist, 1995) in which gene flow would only be disrupted beyond critical values of the landscape feature. The use of thresholds in landscape ecology and landscape genetics is a wide-spread approach to model non-linear responses among landscape features and geneflow/dispersal (Short Bull *et al*., 2011). In the case of this study, this meant assigning the highest resistances to values beyond predefined thresholds in landscape features of interest. Specifically, in the case of slope, I used a threshold of 10° as it has been shown that Neotropical otter presence is associated with gentle slopes across riparian habitats (Cirelli, 2005; Santiago-Plata, 2013). In the case of elevation, I tested a threshold of 1500m, which is the average elevation at which Neotropical otters are found throughout their world-wide range (Waldemarin and Alvares, 2008). For the NP and SP, I also tested a threshold of 300m because within these regions there were few elevations over 1500m.

In the case of *land cover*, resistance surfaces were generated by grouping the original categories of the raster into three broader categories: *vegetation, agriculture*, and *urban land cover*. The category '*vegetation*' included all types of native vegetation in an area; while the category "*agriculture*" included rain-fed and irrigation agriculture, as well as all types of cultivated grasslands. In these resistance surfaces, *vegetation* was assigned the lowest resistance, *agriculture* was assigned intermediate resistance and *urban land cover* was assigned the highest resistance. In addition, I generated two- category surfaces by

grouping *urban land cover* and *agriculture* together, while keeping *vegetation* as a separate category. In this instance, *vegetation* was assigned the lowest resistance, while the category *urban+agriculture* was assigned the highest.

For *aquatic network* surfaces, I assigned higher resistance to cells with land, and lower resistance to cells where rivers (or other waterbodies) were present. Additionally, because the presence of Neotropical otters has been associated with the permanence of rivers (Cirelli, 2005; Plata, 2013), I parameterised
additional surfaces by assigning resistance values according to stream order; a measure of stream branching in which higher order streams have more tributaries and hence tend to be more permanent (Strahler, 1952). Specifically, while cells with land were still assigned the highest resistance, cells with streams of a lower order were assigned higher resistance than cells with higher stream order.

Next, I used all generated resistance surfaces and coordinates of sampling locations of individuals, as input for the software Circuitscape 4.0 (Shah and McRae, 2008). This software uses circuit theory to generate surfaces that model the "resistance" that landscape features pose to movement of organisms, while accounting for all possible pathways among individuals simultaneously. I chose this approach because it is an improvement over least cost path analysis, which assumes that there is a single optimal path and that organisms know *a priori* which one it is (McRae and Beier, 2007). Circuitscape software was run using the pair-wise mode; the nodes (locations of individuals) were connected to their 8 nearest neighbours and connections between neighbours were calculated as average resistances. In this way, I generated matrices of resistance distances based on the landscape variables of interest. However, an unknown issue prevented Circuitscape 4.0 from generating country-wide slope matrices, so for this scale only elevation matrices were obtained.

Table 4-2. Landscape features tested for their effect on otter *(L.longicaudis*) gene flow

Landscape feature	Ecological relevance	Prediction	References
Elevation	High elevations create barriers between basins, Lower fish biomass in higher elevations	High elevation will cause decreased gene flow among otter populations.	Lariviere, 1999; Janssens *et al.*, 2007.
Slope	Steep slopes create barriers between basins, easier accessibility to prey with gentle slopes at river margins	Steep slopes will cause decreased gene flow among otter populations.	Janssens *et al.*, 2007; Santiago-Plata, 2013.
Land cover	Agriculture and urban land cover require removal of native vegetation, which provides shelter and safe pathways for dispersal.	Agricultural and urban land cover will cause decreased gene flow among otter populations compared to natural vegetation.	SEMARNAT, 2001; Kruuk, 2006
Aquatic networks	Well-connected aquatic networks (i.e. rivers, lakes and lagoons) provide suitable habitat for the species and are involved in dispersal.	Well-connected aquatic networks will facilitate gene flow among otter populations.	Waldemarin and Alvares, 2008

Statistical analyses

For both regional and country-wide scales, I used the package Ecodist (Goslee and Urban, 2007) in the software R (R Development Core Team, 2008), to conduct all statistical analyses. As a first step, I performed simple Mantel tests between genetic distance matrices and the "flat" distance matrices in order to examine whether there was evidence of isolation by distance (IBD) in any of the study areas. Next, I conducted partial Mantel tests on genetic and landscape resistance matrices, while controlling for "flat" distance. For both simple and partial Mantel tests, the significance of Spearman correlations was determined from 10,000 permutations. From the partial Mantel tests, I identified the resistance matrices (of each landscape variable) that showed the highest significant correlation ($p<0.05$) with genetic distance matrices. Only these matrices were used for subsequent analyses using 'multiple regression on distance matrices' (MRM).

MRM is a powerful statistical approach for landscape genetic studies, allowing to examine the contribution of each explanatory variable to the overall fit of models. In addition, it has been shown to provide a good balance between type-1 error and statistical power (Lichstein, 2006; Balkenhol, *et al.*, 2009; Blair *et al.*, 2013). In the MRM models examined in the present study, the selected landscape resistance matrices and the flat matrices were used as predictors of genetic distance (the response variable). However, prior to model selection, I checked for correlation between predictors and whenever a high correlation was detected ($r >0.6$), only one of the pair was included in the candidate models. Specifically, I kept the one predictor that had shown the highest correlation with genetic distance in partial Mantel tests. Finally, model selection was done using a backward elimination approach, starting with a maximal model that included all candidate predictors and removing them one by one (starting with the one with largest p value) until only the predictors with a P-value under a pre-defined threshold (in this case $p <0.05$) were kept in the model. Finally, the significance of regression coefficients and R^2 values was assessed from 10,000 permutations of the response variable (genetic distance).

Results

There was no evidence for linkage disequilibrium among the loci used in the present study after Bonferroni correction; the estimated ADO was 0.21 and FA was 0.02; these values are within the ranges estimated in previous studies relying on Neotropical otter faecal DNA (Ortega *et al*., 2012; Trinca *et al*., 2013). The estimates of P_{ID} were 1.6^{-9} (for 13 loci) and 6.2^{-6} (for 7 loci); P_{ID} between siblings was 1.5^{-4} and 5.4^{-3}, respectively. At the country-wide scale and the NP, there was an initial pattern of heterozygote deficiency across loci, suggesting Wahlund effect. The Wahlund effect is a result of unknowingly pooling separate panmictic populations together *i.e.* even if when separate, the populations are in Hardy Weinberg equilibrium, when pooled together they produce a pattern of heterozygote deficiency (Dakin & Avise, 2004; Pompanon *et al.*, 2005; Hartl and Clarke, 2006). Indeed, when pooling individuals according to the clusters inferred with STRUCTURE, The only locus that was observed to be consistently out of Hardy Weinberg across country-wide clusters and the NP, was RIO7; potentially indicating deviation due to null alleles (Allendorf and Luikart, 2007). Although its estimated null allele frequency was ~0.2 across clusters and such null allele frequencies have been shown not to significantly bias estimates of population differentiation (Dakin & Avise, 2004); in order to be conservative, I repeated landscape genetic analyses with and without RIO7. The results did not change. In the NPAC only two more loci remained out of Hardy Weinberg (RIO1 and RIO13), while in SPAC only one locus (RIO 9) was out of Hardy Weinberg. It is worth noting that heterozygote deficiency can also be caused by allelic dropout (which was detected in the present study), and inbreeding (Castric *et al*., 2002). In the case of the North Pacific, most samples were obtained within two dams in the state of Sonora. If within these dams, matings among relatives occur frequently, this could also explain the general pattern of heterozygote deficiency across loci. Results of Linkage Disequilibrium, Hardy-Weinberg tests and No. of alleles per locus are shown in the Appendix D, Tables D2-D7)

Genetic diversity and Genetic structure

In terms of genetic diversity, estimates of *He* ranged from 0.51 (NP) to 0.55 (SP), while *Ho* estimates were 0.25 and 0.47, respectively. The mean number of alleles per locus was 4.6 in the North and 4.5 in the South. Overall, these estimates are very similar to the ones obtained by Ortega *et al* (2012) in a small catchment in Chiapas (Southernmost Mexico).

However, Trinca *et al.* (2013) recorded higher genetic diversity estimates in Brazilian populations of the species (Table 4- 3).

Table 4- 3. Genetic diversity estimates in Mexican and Brazilian populations of Neotropical otter (*L. longicaudis*).The table shows expected heterozygosity *(He)* , observed heterozygosity *(Ho)* and mean number of alleles per locus estimated in the present study (for NP and SP) as well as two previous ones in Mexico and Brazil.

Species/Study Area	n	*He*	*Ho*	Mean number of alleles/locus
L. longicaudis/ Mexico[1]	38	*0.51/0.55	*0.25/0.47	*4.6/4.5
L. longicaudis/ Mexico[2]	34	0.59-0.67	0.48-0.59	4.7
L.longicaudis/Brazil[3]	28	0.73	0.83	6.5

[1] This study * NP/SP; [2] Ortega *et al*. (2012); 3 Trinca *et al.*, 2013.

Genetic structure

From STRUCTURE analyses, the optimal K-value based on Δ K (= 41.87) was for K=2; posterior probability = - 873.6 (Fig 4-2). The mean likelihood plot showed a peak at K=2. Although variance increased after this value, there was another peak at K=4, therefore, a plot of estimated membership coefficients at K=4 is available in Appendix D, Figure although variance increased after (Fig 4-3). The two identified clusters roughly correspond to the north and south of the species' range, although several individuals were located away from the 'core' of their assigned cluster (Fig 4-4). The estimated FST among clusters was 0.19 ($p<0.05$).

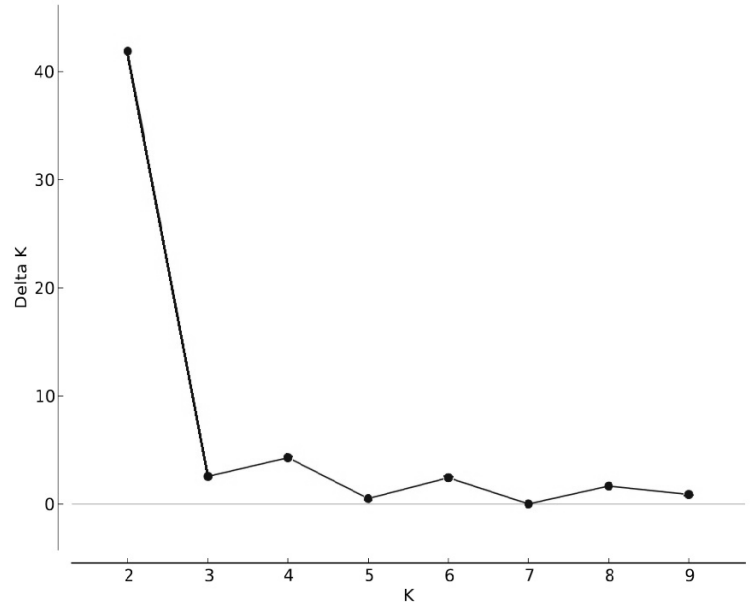

Fig 4-2. Plot representing the second order rate of change (Δ*K*) as a function of the number of populations *K*. Following Evanno *et al.* (2005).

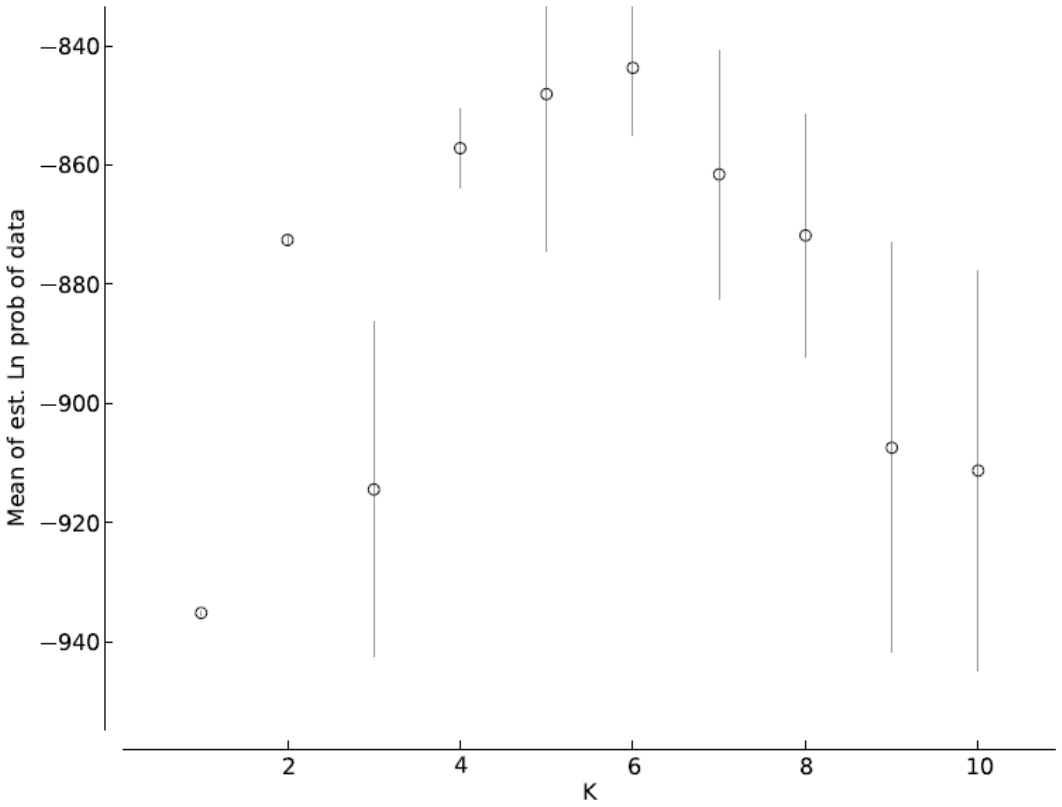

Fig 4-3. Plot of mean likelihood L(K) and variance per *K* . The plot shows a peak at *K*=2, with variance (vertical bars) increasing with larger values.

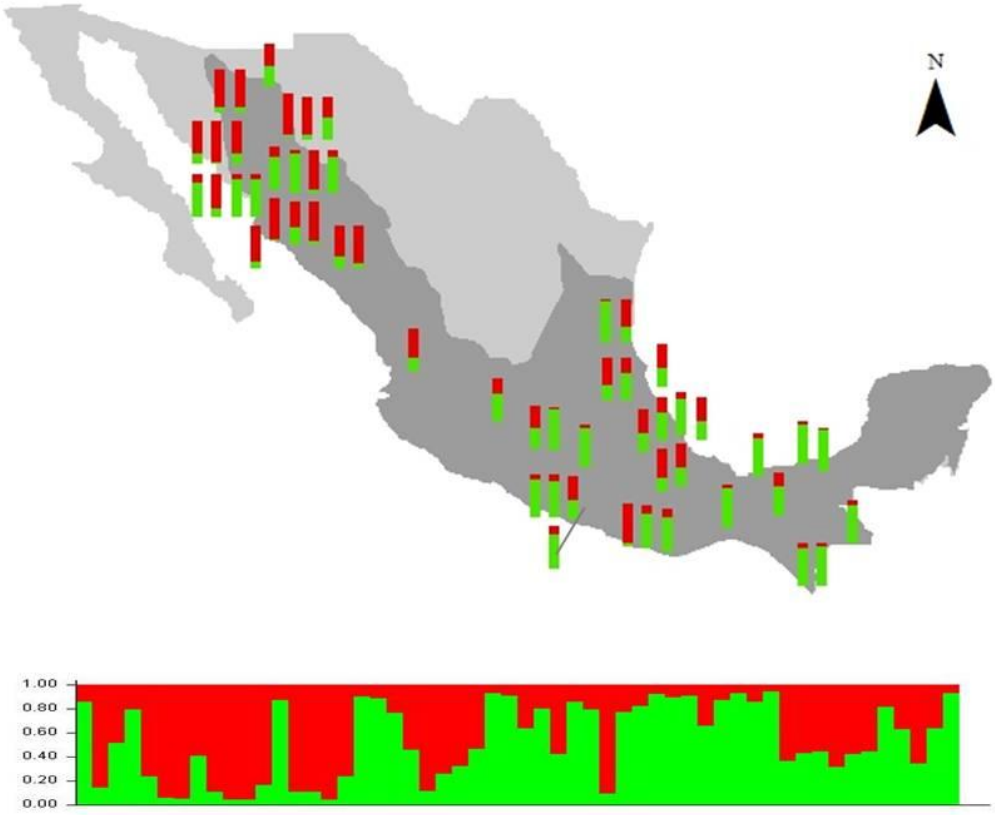

Fig 4- 4. Summary plot (*K*=2) of estimated membership coefficients and sampling locations for each individual. Each bar in the plot represents an individual and the proportion of its genome assigned to the inferred clusters. The bars (from left to right), represent individuals sorted from North to South. For illustration purposes (due to the large scale of sampling), the map displays approximate locations of individuals.

Landscape genetics

At the country-wide scale, the simple Mantel test showed a significant, positive correlation between genetic distance and the flat resistance surface (r=0.12; $p<0.05$), showing an effect of IBD. When controlling for the flat resistance distance in partial Mantel tests, genetic distance showed significant, positive correlations with elevation categorized using a threshold of 1500m (r=0.20; $p<0.05$) and land cover, when natural vegetation had low resistance, and high resistance was assigned to urban and agricultural cover grouped (r=0.16, $p<0.05$), suggesting an increase in genetic distance with increasing (resistance) values of these landscape features. Although land cover was correlated to genetic distance, it was also highly correlated (>0.60) to elevation. Given that in partial Mantel tests

elevation had a higher correlation with genetic distance, land cover was not included in MRM models.

In the NP region, the simple Mantel showed that genetic distance had a positive, but non-significant correlation with the flat resistance surface ($r=0.22$, $p>0.05$). On the other hand, genetic distance had positive and significant correlations with slope coded using a threshold of 10° ($r=0.50$, $p<0.05$) and elevation with a threshold of 300m ($r=0.42$, $p<0.05$). Nevertheless, elevation and slope were highly correlated (>0.6) and were therefore not used together in the MRM models (Table 4- 4). For illustration purposes, plots of the highest partial Mantel correlations for the country-wide scale and the NP can be found in Fig 4-5. and Fig 4-6, respectively**.** On the other hand, in the SP region, no correlation was found between genetic distance and the flat resistance surface or any landscape resistance matrix. Therefore, no further analyses were conducted for this region and plots are not shown.

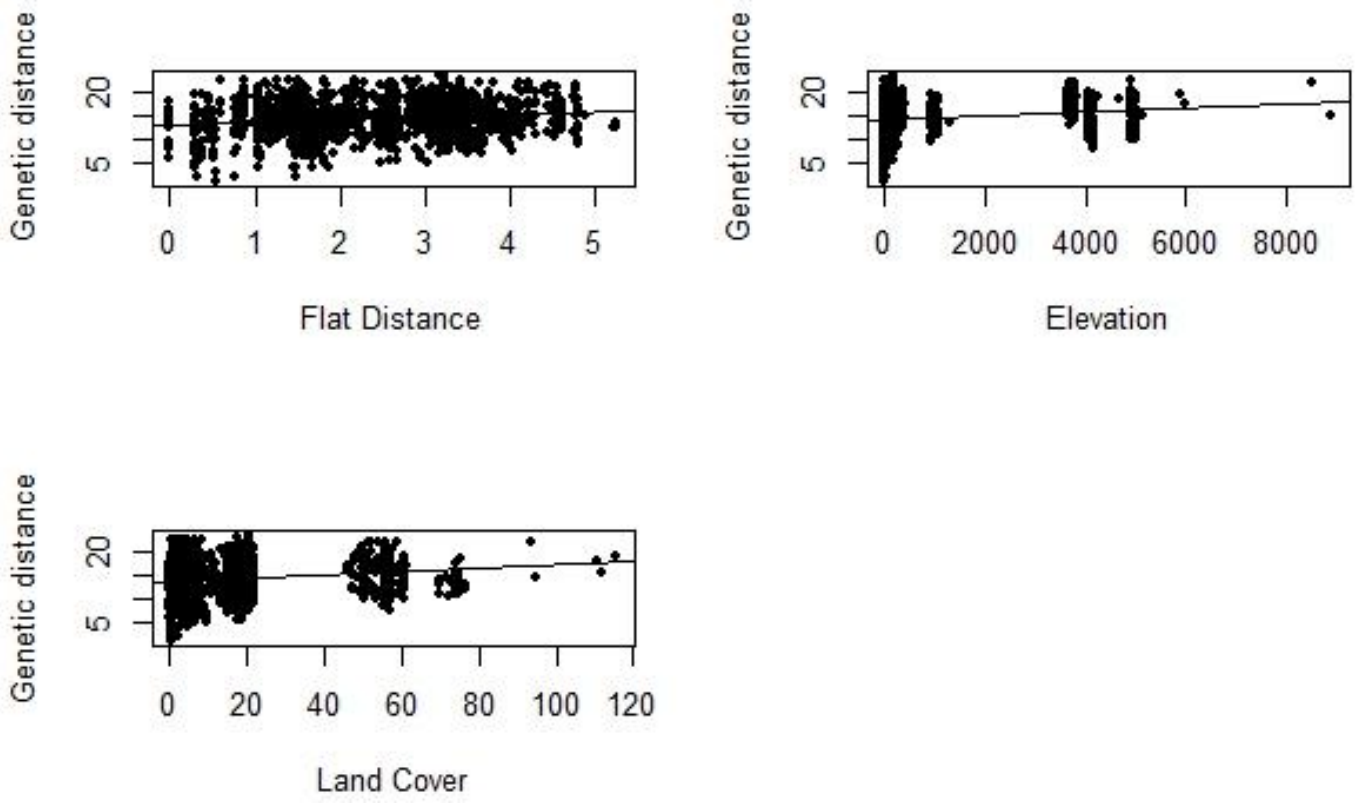

Fig 4-5. Country-wide plots of genetic distance (Smouse and Paekall, 1999) against raw resistance estimates for "flat" distance, elevation and slope.

The X-axis shows resistance distances (unitless) obtained from Circuitscape.

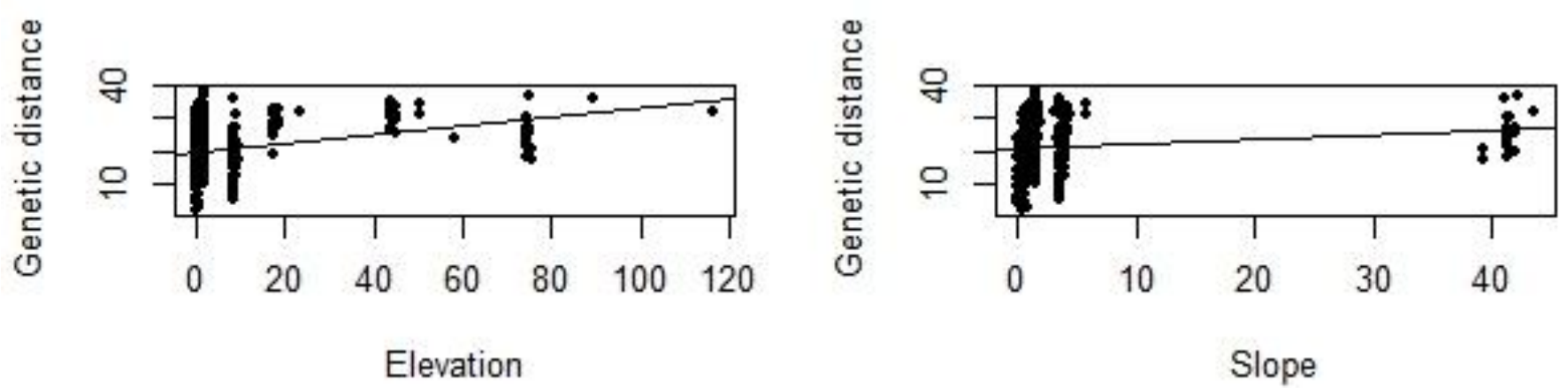

Fig 4- 6. North-Pacific plots of genetic distance (Smouse and Paekall, 1999) against raw resistance estimates for elevation and slope. The X-axis shows resistance distances (unitless) obtained from Circuitscape.

Table 4-4. Simple Mantel and partial Mantel (Spearman) correlations between genetic distance and resistance distances derived from landscape resistance surfaces.

Simple Mantel tests (*italic*) show correlation between genetic distance and flat resistance distance (akin to Euclidean distance). Partial Mantel tests were performed controlling for flat distance. Significant ($p<0.05$) correlations are shown in **bold*.** ID=name assigned to resistance surface.

Scale	Landscape feature	Mantel *r*	p-value	ID	Categories	Cost-ratio
Country-wide	*Flat distance*	***0.125****	***0.001***	flat1	all cells resistance =1	-
	Elevation	**0.204***	**0.009**	rsurfbx	elv<1500:elv>1500	1:100
	Aquatic	-0.097	0.883	rivlndxx	aquatic:land	1:1000
	Landcover	**0.169***	**0.023**	lu2x	vegetation: urban/agriculture	1:100
North Pacific	*Flat distance*	*0.221*	*0.070*	nflat	all cells resistance =1	-
	Elevation	**0.422***	**0.009**	ndmcat	elv<300m : elv>300m	1:100
	Slope	**0.505***	**0.000**	nsl3	slp <10°: slp>10°	1:100
	Aquatic	-0.106	0.241	rilnd2	aquatic: land	1:100
	Land cover	-0.052	0.458	nlu2b	vegetation: agriculture:urban	1:100:10000
South Pacific	*Flat distance*	*0.158*	*0.165*	sflat	all cells resistance =1	-
	Elevation	-0.247	0.115	sdmcat	elv<300m: elv>300m	1:100
	Slope	-0.238	0.142	ssl3	slp <10° : slp>10°	1:100
	Aquatic	0.129	0.231	str1	stream order	lower order=higher resistance
	Land cover	0.111	0.281	slu1	vegetation: urban+agriculture	1:10

ID=unique identifiers for resistance surfaces, Aquatic=Aquatic networks, elv=elevation, slp=slope.

For the MRM models, at the country-wide scale, the maximal model included both elevation and (flat) distance. However, following model selection, the final model included only elevation and explained 5.6 % of the variation in genetic distance. In the case of the NP, the final model included only slope and explained 15% of variation in genetic distance (Table 4-5).

Table 4-5. Multiple regression on distance matrices (MRM).

The models examine the relationship between pairwise genetic distance (Smouse and Peakall, 1999) and resistance distances estimated from landscape features at the country-wide scale and the North Pacific region. The final models are indicated in **bold***

Scale	Model	Variable	B	Pval	R^2	Pval
Country-wide	A	Flat distance	1.329e-03	0.9838	0.0568	0.003
		Elevation	2.377e-01	0.0108		
	B*	**Elevation**	**0.238**	**0.0002**	**0.0568**	**0.000**
North Pacific	**C***	**Slope**	**0.3890**	**0.0062**	**0.1513**	**0.006**

Discussion

Levels of genetic diversity of neotropical otters found in the present study can be considered moderate, consistent with previous estimates for a local study in southern Mexico (Ortega *et al*., 2013). Higher estimates have recently been obtained for the same species in Brazil (Trinca *et al*., 2013). This result corroborates findings of chapter three (based on mtDNA) and could reflect a lower effective population size of Mexican otters compared to Brazilian populations and/or different population history (Frankham *et al.*, 2007). In the STRUCTURE analysis support was highest for two clusters at the country-wide scale and the location of these roughly corresponded to the North and South of the species range in Mexico, separated by the Mexican Transvolcanic belt (MTBV). This mountain range has been observed to contribute in varying degrees to the genetic structure of Mexican fauna and flora (Bryson *et al.*, 2011; Ruiz-Sanchez & Specht, 2013). However, in the present study, a few individuals were observed at locations incongruent with their assigned cluster (i.e. *potential migrants)*, which suggests that limited gene flow has been maintained between regions. Therefore, it appears that the MTBV is not an entirely impermeable barrier to otter dispersal. Furthermore, in chapter three (using mtDNA), I estimated high genetic differentiation between the North Pacific region and two sampling regions in the south of Mexico (FST=0.49). In contrast, using microsatellites, the differentiation between the clusters identified by STRUCTURE (which roughly correspond to the regions defined in chapter three) was lower (FST=0.19). This could indicate male-biased dispersal, which has already been documented in different otter species (Blundell *et al*., 2002; Janssens *et al*., 2007), including Neotropical otters (Trinca *et al*., 2013) .

One of the main goals of this chapter was to investigate which landscape features affected gene flow in otter populations. Across different scales of analysis, *elevation* and *slope* were found to have a positive relationship with genetic distance, implying a reduction in gene flow with increasing values of these landscape features. These results are in agreement with previous findings combining landscape and genetic data to examine gene flow in other otter species. For instance, Janssen *et al*. (2008) used a landscape genetic approach to examine how the ridges that delineate basins affected the gene flow in European otters (*Lutra lutra*) in France. The authors found a positive correlation between the genetic distance of individuals in different basins, and the mean slope separating the catchments, showing that in their study area, steep and dry lands could actually hinder the dispersal of

otters. Furthermore, studies on habitat suitability of *Lutra lutra* in Italy, have also found a negative effect of slope and elevation on otter dispersal (Loy *et al*., 2009; Carranza *et al*., 2011).

Although not included in the final MRM model at the country-wide scale, flat (geographic) distance had a positive relationship with genetic distance, suggesting the presence of IBD over the scale examined here. IBD occurs when gene flow between individuals closer in space is higher than among those separated by greater distances, reflecting restricted dispersal by individuals within a population (Wright, 1943). This result is in agreement with the findings of several studies on *Lutra lutra* populations across Europe that have found evidence of limited dispersal of otters at different spatial scales (Cassens *et al*. 2000; Dallas *et al.,* 2002; Mucci *et al*., 2010) as well as river otter (*Lontra canadensis*) in North America (Latch *et al*., 2008). At the scale of NP, flat distance was not significantly correlated to genetic distance. A potential reason for this could be the smaller size of the study area. Specifically, with the exception of two individuals, all samples were taken <140 km of each other. Although estimates of average dispersal distance in otters vary, dispersal movements of 20 to 50 km in a single day are not uncommon in *Lutra lutra* (Jenkins *et al*., 1980; Chanin *et al*., 2003) and there is evidence for long distance dispersal also in other otter species. As an example, a study on river otters (*Lontra Canadensis*) in Alaska, that combined radio tracking and population genetic approaches, estimated dispersal distances of up to 96km (Blundell *et al.*, 2002) . Thus, it is possible that the scale of sampling in the NP region was too small relative to the dispersal capability of the species. In the case of SP, no correlation was found between genetic distance and geographic distance either. This observation could be related to the small sample size (n=13) available for this region. Additionally, locations of three samples were approximations provided by the collectors who donated them to this project, which could have obscured the true relationship between genetic and resistance distance in those cases.

Given that it is generally acknowledged that well-connected waterways and low levels of human disturbance (*i.e*. agriculture, urban settlements etc.) are key to Neotropical otter occurrence and survival (Kruuk, 2006; Santiago-Plata, 2013), I hypothesized that urban land cover and agricultural lands would have a detrimental effect on otter geneflow. However, I didn´t find any correlation between genetic distance and land cover at any scale

of study. There are several potential explanations for this: for example, otters have been observed to tolerate human disturbance as long as there is a sufficient amount of prey available (Lariviere, 1999; Kruuk, 2006; Reid *et al.*, 2013). In support of this, there are several accounts of Eurasian otters (*Lutra lutra*) obtaining a large amount of their prey from fish farms in agricultural landscapes, to the degree of causing significant losses of fish stock in certain areas (Sales-Luís, *et al*., 2009; Václavíková *et al.*, 2011). In this study, I did not incorporate any measure of prey availability as a predictor of genetic distance in otters; if indeed this is an important driver of gene flow, it could explain why no correlations were found with land cover. Additionally, my results could also be related to the scale and/or parameterization employed for resistance surfaces. The smallest scale at which landscape features could be quantified in this study was at 300m resolution, but there could be landscape features affecting dispersal which weren't detectable at this scale. For example, in habitat suitability studies of both Eurasian and Neotropical otters, riparian vegetation density and river bank slope have been shown to be important for otter habitat connectivity and to predict the presence of the species within river catchments (Jeffress *et al*., 1982; Pardini and Trajano, 1999; Loy *et al*., 2009; Santiago-Plata, 2013). In these studies, said habitat characteristics were measured in the field or from rasters at high resolution. While these features may play a determinant role in connectivity *along* rivers (i.e. longitudinal connectivity), they couldn´t have been properly examined with the available raster resolution for this study. In terms of the parameterisation of resistance surfaces, it has been suggested that the choice of data classes used to model landscape features (i.e. thematic resolution) can also affect the results of landscape genetic studies (Cushman & Landguth, 2010). For example, for land cover rasters, I decided to group the original categories into three simplified classes: vegetation, agriculture and urban land cover; assuming that they would accurately represent the overall effect that each of these features have on otter geneflow. However, it is possible that different types of vegetation, agricultural practices or human settlements affect otter gene flow in different ways. For instance, rain-fed agriculture might not have as much of a negative impact on otter dispersal as irrigation agriculture, given that the former usually does not require extreme alterations to the habitat or continuous human presence.

Furthermore, there might be cases in which relationships between genetic distance and certain values of landscape variables are non-linear. In such cases, the use of threshold values can be useful (Spear *et al.*, 2010). In this study I did test threshold values for both

elevation and slope. However, it must be acknowledged that the applicability to conservation of critical thresholds may not be straight-forward, as they may be of limited use in a predictive sense (Lindenmayer *et al*., 2005).

In addition, in this study I used circuit theory for all landscape genetic analyses. This approach is wide-spread, but is not the only one available for landscape genetic approaches. In this sense, an alternative could have been to use least cost path analyses. Although analyses based on circuit theory and least cost path have been found to yield similar results in a number of studies (Moore *et al*., 2011; Munshi-South, 2012; Blair *et al*., 2013), these approaches can also be used in conjunction, as they can complement each other. This might be specially the case when identifying conservation corridors, because least cost paths would identify *single* optimal routes among patches/individuals, which may be difficult to identify from circuit theory graphical output (*i.e.* current maps). In turn, circuit theory can help identify suitable *alternative* corridors that might be overlooked by least cost path approaches (McRae *et al.,* 2008). Thus, using these approaches together can, in some cases, strengthen inferences made from landscape genetics studies.

Conservation implications

Otter populations in Mexico were found to be genetically less diverse than South American (Brazilian) populations studied to date, coinciding with the findings of chapter three on mtDNA. This could translate into a diminished evolutionary potential and therefore an increased risk of extinction (Frankham *et al.,* 2007). Although in the previous chapter, lower genetic diversity in the North was estimated, this didn´t seem to be the case using microsatellites. The estimated expected heterozygosities in SPAC and NPAC were similar to each other and also similar to what has been estimated before in Mexico (Ortega *et al*., 2012). In terms of population structure, the results of the present study suggest the existence of at least two potential management units located in the North and South of the species range. However, it is recommended to increase sampling effort, particularly in areas where homogenous sampling was not possible in this study, as it has been shown that non-homogeneous sampling can bias the results of Bayesian clustering algorithms, especially in the presence of isolation by distance (Frantz *et al.,* 2009; Anderson *et al.,* 2010), of which there was some evidence in the present study. Although the genetic structure between the North and South clusters was found to be lower than expected from

the results of chapter three, it is also possible that estimates obtained from microsatellites still reflect historical processes and are not an accurate reflection of more recent changes in the connectivity of otter populations; for example, those changes brought about by human activities that have an impact on the landscape.

In terms of the landscape features that affect dispersal of the species, high elevations and steep slopes were found to restrict gene flow in the present study and thus their presence within otter habitat will likely interfere with the connectivity among otter populations. In the case of semiaquatic species, such as the otters, habitat connectivity is usually evaluated making a distinction between longitudinal and lateral connectivity. In this regard, longitudinal connectivity would refer to otters being able to move *within* a river catchment, while lateral connectivity would refer to dispersal movements *across* catchments, thus contributing to gene flow among otter populations living in adjacent basins (Carranza *et al.*, 2011; Van Looy *et al.,* 2014) . Elevation and slope could affect lateral connectivity. For instance, in terms of lateral connectivity, the natural boundaries separating watersheds (i.e. ridges) could obstruct dispersal across certain basins. Moreover, the effects of anthropogenic disturbance to otter habitat in terms of both lateral and longitudinal connectivity should also be considered. For instance, it has been proposed that damming can disrupt connectivity of otter populations (Gallo, 1997; Palmeirim *et al.*, 2014). Although it was my intention to test for the effect of dams on genetic connectivity in the present study, and sampling in certain areas of the North Pacific was specifically designed for this purpose (*i.e.* sampling was done in areas upstream and downstream from dams), the low amplification success of microsatellites prevented me from achieving this goal. However, the construction of dams within a river could change the natural configuration of the landscape and impose a further barrier to otter dispersal (Mason, 1995; Pedroso and Santos-Reis, 2009). Under this scenario, it is proposed that conservation corridors for the species are established in hydrological regions in which adjacent river basins are delimited by low elevations and slopes and whenever possible, avoid heavy human infrastructure within the catchments. Nevertheless, this might prove challenging, given that in addition to the natural topology of Mexico (numerous mountain ranges), there already exist over 600 high capacity dams (i.e. higher than 15 m) throughout the country, distributed all along the Neotropical otter range (Fig 4- 7.); and there are plans to increase hydrologic infrastructure within the next four years (CONAGUA: http://www.conagua.gob.mx/). In addition, there is very little actualized information on the current status of the species in Mexico, as the

only country-wide survey that has been conducted to date, was conducted approximately 20 years ago (Gallo, 1997). Since then, several studies have been conducted on the species´ ecology, distribution, habitat requirements and population genetics, providing much needed insight into the current status of the species in the country (Maldonado and Lopez, 2003; Carrillo-Rubio and Lafon, 2004; Charre-Medellin *et al*., 2011; Ortega *et al*., 2012). However, given the increasing anthropogenic pressure on the species habitat, there is a clear need for establishing long-term monitoring programmes of otter populations. To this date, there has only been one nation-wide survey (Gallo, 1999) which focused on recording presence/absence of the species throughout Mexico. However, no direct estimates of abundance/density were obtained from the survey, which means that at the moment we do not have a reliable reference to determine whether otter populations have declined in recent years. This is most important, because pollutants (organochlorines) that caused a dramatic decline in Eurasian otter populations in the 50´s (Kruuk, 2006) were only formally regulated (not entirely banned) in the year 2000 in Mexico (SEMARNAT, 2000).

Furthermore, it crucial to identify prioritary hydrological regions based on their potential for harbouring healthy populations of the species. In this sense, there have been proposals for hydrological regions that might be used for otter protection (based on recent records of the species). Among these, key locations are: the river Yaqui in the North Pacific, the lower Balsas River and Lacantun river in the South Pacific, as well as the rivers Tamesi and Conchos in the Atlantic coast (Sanchez *et al*., 2007). However, it is necessary to determine whether these basins are sufficiently conserved on their own, so as to allow long term survival of otter populations. Furthermore, based on the results of the present study, it is important to also consider the natural topology to ensure that geneflow occurs among river basins. Specifically, it is recommended to select low land conservation corridors, as elevation and steep slopes were found to interfere with gene flow. Indeed, this recommendation might be relevant not only for conservation planning within Mexico, but potentially throughout the species´ range.

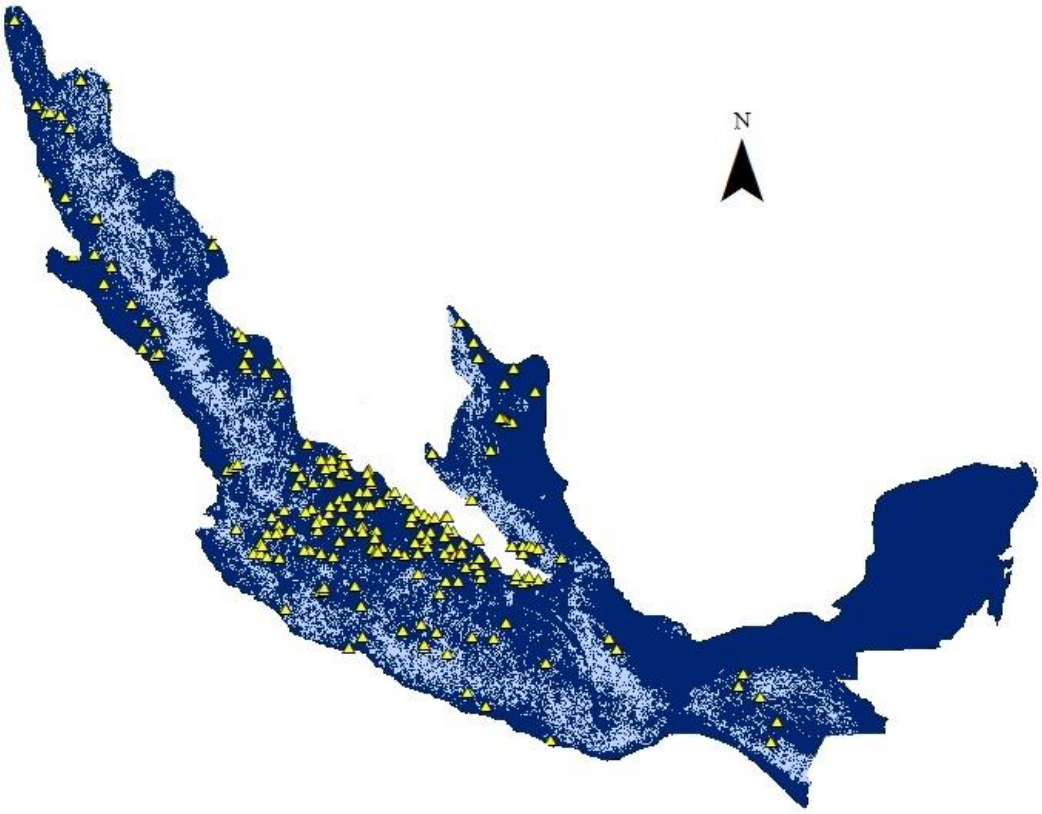

Fig 4- 7. Distribution of high capacity dams throughout Neotropical otter (*Lontra longicaudis*) habitat. The dark areas in the map are characterized by gentle slopes, while the light areas represent slopes >10° (found to hider otter gene flow in this study). The yellow marks show the location of high capacity dams across Mexico (INEGI: http://www.inegi.org.mx/).

Chapter 5- General Discussion

Throughout this final chapter, I will revisit the key findings of this thesis, starting on the challenges of working with elusive species in tropical environments. Next, I will discuss recent proposals for the conservation management of the Neotropical otters throughout their range, and re assess them in light of the findings of my study. Following this discussion, I identify the challenges that Neotropical otters face in Mexico and make conservation recommendations for Mexican otter populations on the basis of my results. Finally, I propose future studies that need to be conducted at both the range-wide and local (Mexico) scale and potential methodologies for them.

Non-invasive methods for studying wildlife under challenging conditions.

Non invasive genetic sampling allows biologists to effectively study many individuals and populations without disturbing, or even seeing the organisms of interest. Thus, non-invasive genetic techniques are especially useful when it comes to studying elusive species (Beja-Pereira, *et al*., 2009). A common source of DNA in non-invasive studies are faecal samples, which have the advantage of being generally easy to obtain, but the disadvantage of containing very low quantity and quality of DNA. In addition, conducting non-invasive studies with species that are distributed in tropical environments, might be particularly challenging because climatic conditions will degrade DNA at a faster rate (Michalski et al., 2011). Furthermore, there are situations in which a significant delay between collection of samples and DNA extraction is expected, and maintaining samples at low temperatures through this time might not be possible in certain isolated areas. With this in mind, in chapter two I compared three preservation methods for otter faecal DNA, in order to identify the best method for working with such samples in tropical environments and after long-term storage. My results showed that RNA later is the most efficient preservation method under such challenging conditions. However, in my project it was not always possible to keep samples in RNAlater, especially for the first sampling season, which had to be started before the preservation methods comparison had been done. In addition, it is still important to consider the somewhat elevated price of RNAlater in relation to other

methods. In this sense, it would be interesting to know if providers of RNAlater would consider offering discounts to institutions or researchers in developing countries. In order to address this topic, I recently contacted (e-mail) two major providers of the reagent: Invitrogen and Qiagen, in order to know their position on the matter. At the moment I am awaiting for their response. Nevertheless, I expect that the results obtained from my study will be very useful for future studies not only with *Lontra longicaudis* but also with other species distributed in tropical environments, especially when long-term storage is expected to occur.

New perspectives on the conservation status of the Neotropical otter.

According to the International Union for Conservation of Nature (IUCN), there is currently not enough data to determine the range-wide conservation status of the Neotropical otter. Because of this, to this day the species' status has remained 'data-deficient' and it has been recommended that research on Neotropical otters, should focus on defining its current distribution, population status, and habitat requirements (Waldemarin and Alvarez, 2008). However, this year, a study used updated records of Neotropical otters throughout their distribution range, with the aim to re-evaluate the species' distribution and identify environmental, climatic and population variables that influenced the suitability of *L. longicaudis* habitat (Rheingantz, *et al.*, 2014). The study provided evidence that annual temperature is the one environmental variable that most contributes to otter habitat suitability and generated an updated map of both current distribution and habitat suitability across the range (Fig 5-1). Given that the size of the species´ range estimated in the study is beyond 20,000 km^2 (larger than previously estimated), the species cannot be included in any of the 'Threatened' categories (*i.e.* ´Critically Endangered´, ´Endangered´ or ´Vulnerable´) according to IUCN criteria (IUCN, 2014). Hence, Rheingantz *et al.* (2014) have proposed that the species should be moved to either 'Near threatened' or 'Least concern' status.

According to IUCN, 'Least concern' status is given to taxa that are wide spread and have no immediate threats, while ´Near threatened´ is given to species that cannot currently be ascribed to a ´Threatened´ category, but in the near future could be endangered (IUCN,

2014). In this sense, there are good reasons to support the ´Near threatened´ status for *L. longicaudis* as opposed to 'Least concern'. First and foremost, although the current distribution range of Neotropical otters is considerable, all the activities that have historically endangered otter populations across the globe, *i.e.* deforestation, water pollution, agriculture, mining and damming, will most likely only increase in the years to come (SEMARNAT,2001; Waldemarin and Alvarez, 2008; Ortega *et al*., 2012; Trinca *et al*., 2012). In addition, the criteria in the IUCN list are designed to assess the status of species at global levels, but there are cases in which species have a global status that is clearly not concordant with the particular challenges their populations face at regional levels (IUCN, 2014). This is extremely relevant, because some countries straightforwardly adopt the IUCN red list criteria into their local legislation without conducting any regional assessments; this can result in overlooking populations in serious need of protection (Mace, 2004). This might be particularly true for the Neotropical otter, given the recent identification of distinctive ESU´s across its range. Specifically, North+Central America (NCAM) was identified as a potential ESU in the present study, in addition to two ESU's (Amazonia and ESA) recently identified in South America (Trinca *et al.*, 2012). These findings combined, provide strong evidence for the need of management actions to be tailored to specific regions. If, on the other hand, the 'Least concern' status were assigned, and local governments decided to follow the global criteria as opposed to conducting their own regional assessments, we could risk dismissing real threats that specific populations of the species face. For this reason, I strongly advocate for assigning the species the status of ´Near threatened´, which is a realistic depiction of the overall standing of the species across its range, but at the same time it is not so relaxed a criterion, that it could result in underestimating the endangerment that particular populations of the species are experiencing at the moment. This might be key for the conservation of populations located in the northern limit of the species range, where habitat suitability begins to decrease and anthropogenic activities represent an added pressure to their long-term survival.

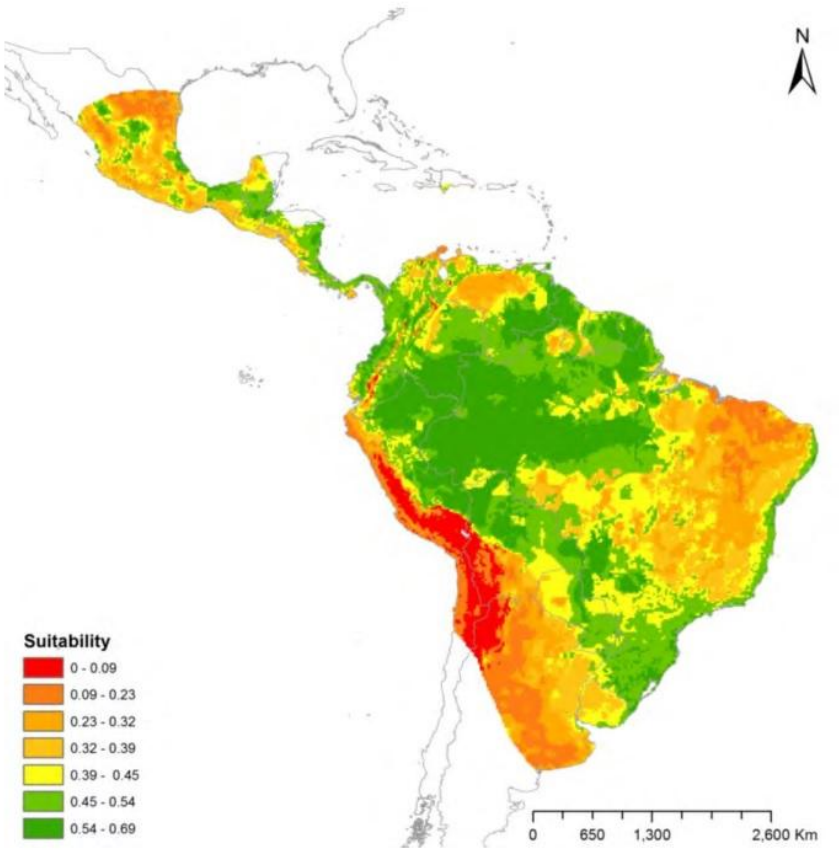

Fig 5-1. Map of habitat suitability for *L. longicaudis* across its entire range. From Rheingantz *et al*.(2014)**.**

The challenge of populations at the range-limit: Neotropical otters in Mexico.

As species approach the limit of their range, habitat becomes more and more fragmented, causing populations to become isolated from each other. As a result, there is a stark reduction of gene flow and an increase in genetic structure. Furthermore, the limited amount of suitable habitat, results in lower effective population sizes (Ne) and a rapid loss of genetic diversity; putting the evolutionary potential of populations at risk (Allendorf and Luikart, 2007; Gaston, 2009) . Because of this, range limits have been termed "the land of the living dead" (Channel and Lomolino, 2010) and are often overlooked when attempting to conserve species. However, there are good reasons for conserving range-limit populations. For instance, such populations may harbour unique adaptations to extreme environments and thus could provide insight into how species will react to climate change. Moreover, it has been proven that under extreme anthropogenic pressure, it is often the range-limit populations the ones that ultimately persist (Channell and Lomolino, 2000; Fordham *et al*., 2009; Sexton *et al*., 2009).

Neotropical otters reach their northern range-limit within Mexico; where the Neotropical and Neartic regions merge into what is known as the Mexican Transition Zone, a large bio geographic region considered one of the 25 biodiversity hot spots of the world (Myers *et al.*, 2000). As the Neotropical region fades towards the Neartic, otter populations inhabit increasingly more fragmented and dry habitats (Gallo, 1997). This can be observed in Fig 5-1, where habitat suitability for otters decreases towards the North of Mexico.Consistent with range-limit expectations, my results showed lower mtDNA and microsatellite diversity than that found in otter populations at the core of their range in South America (Trinca *et al*., 2012; Trinca *et al*., 2013). These results clearly show that Mexican otter populations are at an added risk of extinction, and potentially more vulnerable to anthropogenic habitat fragmentation.

In order to counteract habitat fragmentation, conservation corridors can be established to prevent the loss of genetic diversity and maintain the evolutionary potential of isolated populations (Frankham *et al*., 2007). In chapter three, the results from mtDNA analyses

demonstrated the existence of genetic connectivity within southern Mexico; specifically, between the South Pacific and Atlantic regions. In the same chapter, I postulated that given its geographic location, as well as low elevations that characterize it, the Tehuantepec Isthmus (TI) appeared to be an appropriate low-land corridor connecting Atlantic and Pacific coastal populations in Southern Mexico (Fig 5-2). This area has also been found to promote gene flow and species diversity in other mammals such as armadillos, rodents and bats (Arteaga *et al*., 2011; Barragán *et al*., 2010). Moreover, south to the TI lays the Mesoamerican Biological Corridor (MBC). This conservation corridor was designated in 1997, and begins in the southern states of Campeche and Chiapas (Mexico), and continues south to Panama. The corridor is an ambitious conservation project that aims to provide connectivity for a wide range of taxa across participating countries (Mendoza *et al*., 2013). Given that Neotropical otters are distributed throughout the MBC; this corridor along with the TI, could serve the purpose of maintaining connectivity between otter populations in the south of Mexico and into Central America. Nevertheless, it is worth noting that even within these areas in southern Mexico, habitat fragmentation (mainly due to deforestation) continues to be a problem which needs to be urgently addressed (Mendoza *et al*., 2013) .

On the other hand, the question remains whether the remainder of Neotropical otter range in Mexico is currently well connected? In chapter four I found that elevation and steep slopes hinder geneflow among otter populations. Based on these characteristics, it is evident that the better connected areas (with gentle slopes and low elevations) are found in the South Atlantic coast of Mexico; while the opposite pattern is found in the Pacific coast, where the terrain is largely mountainous due to the Sierra Madre del Sur (SMS) (Fig 5-2). This could imply that connectivity on the Pacific side is limited to a narrow strip of coast-line running from the south Pacific to the North Pacific. Although there are not many studies on connectivity corridors across the Pacific coast in Mexico, a recent study that modelled range-wide conservation corridors for jaguar (*Panthera onca*), also identified the South Pacific coast as a corridor of concern given its width and the potential for it to become severed by human activities (Rabinowitz & Zeller, 2010).

Following the Pacific coast towards the North of Mexico, in the area of the Sierra Madre Occidental (SMO), the climatic conditions become drier and generally more extreme. Within this North Pacific (NP) region, otter populations exist in possibly the most extreme and fragmented environments found throughout the species range, which could difficult

genetic connectivity with southern otter populations. In support of this, the results of mtDNA and microsatellites (chapters three and four) both showed evidence for genetic structure between NP and populations in the South of Mexico, implying limited connectivity among these areas. This partial isolation of otter populations in the NP could make them especially vulnerable to human activities. As an example, given the limited amount of surface water in the NP, natural water courses are constantly altered in order to maintain agricultural lands in this area. Furthermore, there is extensive mining going on in this region and leakage of mining waste into rivers is unfortunately not uncommon; this causes irreparable damage to river ecosystems, and has a direct impact on fish availability (Gallo, 1997; SEMARNAT, 2001). In addition, the current protected areas in the North of Mexico have been found to be insufficient to protect the extant biodiversity in the region (Grigione *et al*., 2009; Valenzuela-galván, 2009). Neotropical otter populations are no exception to this, as only one protected area within the NP includes otter habitat, and is limited to only one of several river basins, where otters are present in the region (chapter three). In summary, although there exist designated (MBC) as well as potential (TI, SMS) conservation corridors within Neotropical otter range, it is evident that more corridors and protected areas are needed, especially towards the North of the country.

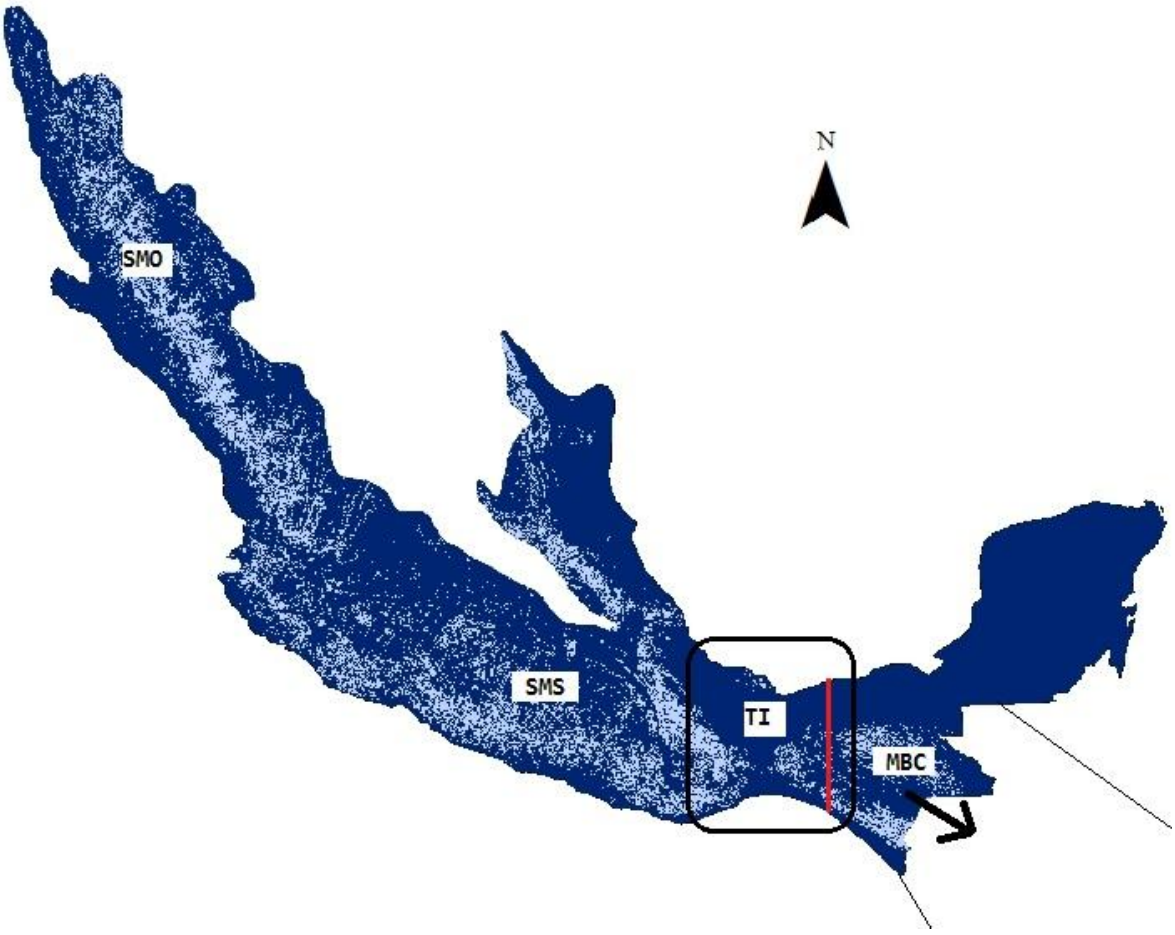

Fig 5-2. Existing and potential conservation corridors for *L. longicaudis* in Mexico. From North to South: SMO=Sierra Madre Occidental, SMS=Sierra Madre del Sur, TI=Tehuantepec Isthmus, MBC=Mesoamerican Biological Corridor; the red line marks the beginning of the MBC in Mexico and proceeds south to Panama.

Future directions in Neotropical otter conservation.

Regardless of which global conservation status is ultimately assigned to *L. longicaudis,* I propose that regional assessments should still be conducted, as they will be crucial for the long-term conservation of the species. These assessments could in principle be done at the level of the different ESU's that have been identified so far, and should follow the

guidelines for regional Red List assessments established by the International Union for the Conservation of Nature (IUCN, 2014).

In terms of local (within Mexico) conservation actions, it is necessary to increase sampling across the country in order to identify appropriate population units for management. Given that landscape characteristics that form natural divisions among watersheds (i.e. steep slopes and high elevations) were found to hinder otter gene flow, it is likely that distinct river basins also harbour separate otter populations. In this sense, a good starting point for identifying management units, would be to select basins or (at a coarse scale) hydrologic regions as sampling units, and use non-invasive methods to genetically characterize otter populations.

Achieving successful conservation of elusive species, also requires reliable estimates of population size (Hájková *et al*., 2008). Although acquiring such data can prove very challenging, a way of estimating population sizes within Mexico could be to make use of capture–recapture analyses based on non-invasive genetic data. These type of analyses have become an important tool to estimate population size of elusive species (Marucco *et al*., 2010) and have recently been applied with *L. longicaudis* in Brazil (Trinca *et al*., 2013). In the present study, the sampling design did not allow to conduct such a study because some sampling sites were only visited once. However, in Mexico one capture-recapture study was conducted recently, within a small river catchment in Southern Mexico; in this area, the authors identified 34 different individuals within an area of approximately 80 km (Ortega, *et al*., 2012). However, the tropical environments found in Southern Mexico are likely the most favourable for the species and hence may harbour large population sizes compared to those present in other parts of the country. In this sense, it would be advisable to conduct capture-recapture studies in different climatic regions across Mexico, in order to make regional comparisons of population size and thus identify areas of conservation concern. These studies could be repeated periodically in order to monitor Neotropical otter populations.

Ultimately, the landscape genetics approach proved extremely useful in identifying features that interfere with gene flow among otter populations and thus allowed to recognize potential conservation corridors and areas of concern for *L. longicaudis*. However, the effects of finer-scale landscape features on gene flow could not be

thoroughly examined in the present study. Thus, it is recommended that future studies applying the landscape genetics approach to study otter populations, aim to examine landscape features at a finer scale. This could yield valuable insight into the species ecology, as well as providing further support to conservation corridor planning.

Lessons learnt

Throughout my study, several challenges arose that should be taken into account for future studies. The main challenge was the difficulties faced when importing samples from Mexico. Although paper work was always thoroughly completed for each sampling season, we were continuously faced with unexpected bureaucratic situations that severely delayed the onset of lab work, and in the end limited the number of samples that were usable by the time they were received in Glasgow university facilities. This was most evident for the first sampling season, for which the majority of samples were preserved using ambient temperature drying or the two-step protocol, which were found to be inferior to RNAlater. With the delay in getting samples to Glasgow, we could not implement the RNAlater preservation method at the start of the second field season. I would recommend that researchers contemplate the possibility of such unexpected situations arising when doing research in collaboration with foreign institutions. On the other hand, there were a couple of areas that had been previously identified as potential sampling sites which were no longer accessible by the time sampling was conducted. In such cases, it is always desirable to have alternatives for sampling locations, as long as they still serve the purpose of the study.

Although the present study did not benefit as much as possible from its own findings in terms of preservation methods, the findings remain for future studies. In this sense, there is a growing interest to conduct non-invasive studies on Neotropical otters in Mexico. As an example, I have recently been contacted by a Mexican NGO: Biodiverso A.C. (http://bio-diverso.org/sitio/) about the present project. This NGO recently collaborated in the designation of a new RAMSAR site: " Presa La Vega", and has been attracting public attention to the site using Neotropical otters as a flagship species (M. Meiners, personal communication). The NGO is interested in conducting non-invasive monitoring programmes for the species, and I believe this can be a unique opportunity to apply the knowledge gained in this project. All in all, this project has laid a foundation for the use of conservation genetics on Neotropical otters in Mexico and provides novel information that should be considered when it comes to establish the conservation status of the species throughout its range. By identifying an efficient long-term preservation method for faecal samples in tropical environments, it could also enable those researchers in the tropics with

no access to storage/lab facilities, to collaborate with institutions based overseas. This is exciting as there might be many species that remain under-studied in the tropics partly because of the lack of facilities available. In these cases, international collaborations could be key to conservation, precisely in the regions where it might be most needed.

APPENDIX A: Supplementary information for chapter two.

Supplementary Table A1. Information of *L. longicaudis* faecal samples used in this study.
Climatic regions (Kottek *et al*., 2006): Am=Tropical monsoon (Short dry season, high moisture); Aw=Tropical wet/dry (Winter dry season, Intermediate moisture); BS=Semi-arid (Low moisture year round).

Sample Id.	Locality	Climate	Year of Collection
1X	Estado de Mexico	Aw	2011
2X	Estado de Mexico	Aw	2011
3X	Veracruz	Aw	2011
4X	Veracruz	Aw	2011
5X	Tamaulipas	Aw	2011
6X	Tamaulipas	Aw	2011
7X	Tamaulipas	Aw	2011
8X	Guerrero	Aw	2011
9X	Guerrero	Aw	2011
10X	Estado de Mexico	Aw	2011
11X	Guerrero	Aw	2011
12X	Guerrero	Aw	2011
13X	Guerrero	Aw	2011
14X	Chiapas	Am	2011
15X	Veracruz	Aw	2011
16X	Chiapas	Am	2011
17X	Sonora	BS	2011
18X	Chiapas	Am	2011
19X	Sonora	BS	2011
20X	Sonora	BS	2011
21X	Estado de Mexico	Aw	2011
22X	Guerrero	Aw	2011
23X	Guerrero	Aw	2011
24X	Guerrero	Aw	2011
263	Guerrero	Aw	2012
265	Estado de Mexico	Aw	2012
266	Estado de Mexico	Aw	2012
267	Estado de Mexico	Aw	2012
268	Estado de Mexico	Aw	2012
270	Veracruz	Aw	2012
271	Veracruz	Aw	2012

APPENDIX B: Supplementary information for chapters two, three and four.

Table B1.Geographic origin of *Lontra longicaudis* samples used in the present study.
All samples included in the table were successfully genotyped at mtDNA and/or microsatellites, and used in subsequent genetic analyses. Coordinates are in decimal latitude/longitude degrees, and the table provides reference to the specific chapters in which individual samples were used.

Sample	*Region*	*Locality*	*Coordinates*	*Chapter*
1x	South Pacific	Temascaltepec,Estado de Mexico	19.056,-100.050	2,3,4
2x	South Pacific	Temascaltepec,Estado de Mexico	19.012,-100.097	2,3
3x	Atlantic	Jalcomulco,Veracruz	19.328, -96.773	2
4x	Atlantic	Jalcomulco,Veracruz	19.331,-96.773	2,3,4
5x	Atlantic	La Vega Escondida, Tamaulipas	22.335,-97.905	2,3,4
6x	Atlantic	La Vega Escondida, Tamaulipas	22.329,-97.947	2
7x	Atlantic	La Vega Escondida, Tamaulipas	22.279,-97.904	2
8x	South Pacific	Coyuca, Guerrero	16.960 ,-100.111	2
9x	South Pacific	Coyuca, Guerrero	16.960,-100.110	2,4

10x	South Pacific	Temascaltepec, Estado de Mexico	19.015 ,-100.100	2
11x	South Pacific	Coyuca, Guerrero	16.960,-100.111	2,4
12x	South Pacific	Coyuca, Guerrero	16.955,-100.111	2
13x	South Pacific	Coyuca, Guerrero	16.960,-100.110	2,4
14x	South Pacific	Miranda, Chiapas	16.138 , -90.921	2
15x	Atlantic	Actopan,Veracruz	19.502,-96.619	2,3,4
16x	South Pacific	Lacantun, Chiapas	16.128,-90.925	2,3,4
17x	North Pacific	Bavispe, Sonora	30.217,-108.901	2
18x	South Pacific	Lacantun, Chiapas	16.104, -90.995	2
19x	North Pacific	Bavispe, Sonora	30.209 ,-108.894	2,4
20x	North Pacific	Mayo, Sonora	27.220,-97.111	2
21x	South Pacific	Temascaltepec, Estado de Mexico	19.015, -100.106	2,4
22x	South Pacific	Coyuca, Guerrero	16.961, -100.111	2
23x	South Pacific	Coyuca, Guerrero	16.961,-100.111	2
24x	South Pacific	Coyuca, Guerrero	16.993,-100.110	2,3

10	South Pacific	Rio Verde, Oaxaca	16.162,-97.738	3
14	South Pacific	Rio Grande, Oaxaca	16.036,-97.429	3,4
18	South Pacific	Manialtepec, Oaxaca	15.980,-97.240	4
21	South Pacific	Manialtepec, Oaxaca	15.985,-97.239	3
22	South Pacific	Manialtepec, Oaxaca	15.986,-97.238	4
23	South Pacific	Colotepec, Oaxaca	15.909,-96.897	3
37	South Pacific	Rio Grande, Estado de Mexico	19.017,-100.088	3
58	North Pacific	San Lorenzo, Sinaloa	24.603, -112.734	4
64	Atlantic	Laguna de Terminos, Campeche	18.597,-92.411	3
70	Atlantic	Laguna de Terminos, Campeche	18.600,-92.410	4
99	Atlantic	La Vega Escondida, Tamaulipas	22.320,-97.960	4
101	Atlantic	Tehuacan/Cuicatlan	17.663,-96.918	3
104	Atlantic	Tehuacan/Cuicatlan	17.894,-96.994	3
109	Atlantic	Tehuacan/Cuicatlan	17.777,-96.958	3
136	North Pacific	Bavispe, Sonora	30.210,-108.898	3

177	Atlantic	Jalcomulco,Veracruz	19.330,-96.770	4
191	South Pacific	Lacantun, Chiapas	16.104, -90.984	3
204	Atlantic	Jalcomulco, Veracruz	19.330,-96.772	3,4
205	Atlantic	Jalcomulco, Veracruz	19.330,-96.770	4
208	Atlantic	La Vega Escondida, Tamaulipas	22.2453,-97.892	3
235	South Pacific	Rio Grande, Estado de Mexico	19.029,-100.143	3
263	South Pacific	Rio Grande, Estado de Mexico	16.939,-100.052	2,3
265	South Pacific	Rio Grande, Estado de Mexico	19.015,-100.106	2
266	South Pacific	Rio Grande, Estado de Mexico	19.018,-100.113	2
267	South Pacific	Rio Grande, Estado de Mexico	19.014, -100.098	2
268	South Pacific	Rio Grande, Estado de Mexico	19.012,-100.094	2

270	Atlantic	Pescados,Veracruz	19.330,-96.770	2,4
271	Atlantic	Actopan,Veracruz	19.500,-96.620	2,4
320	South Pacific	Coyuca,Guerrero	16.960,-100.110	4
322	South Pacific	Espiritu Santo, Oaxaca	16.620, -94.753	3,4
335	North Pacific	Mocuzarit, Sonora	27.220,-109.090	4
336	North Pacific	Mocuzarit, Sonora	27.220,-109.090	4
337	North Pacific	Mocuzarit, Sonora	27.220,-109.090	4
338	North Pacific	Mayo, Sonora	27.223,-109.109	4
339	North Pacific	Mocuzarit, Sonora	27.230,-109.110	4
341	North Pacific	Mocuzarit, Sonora	27.220,-109.090	4
343	North Pacific	Mocuzarit, Sonora	27.220,-109.090	4
344	North Pacific	Mocuzarit, Sonora	27.220,-109.090	4
346	North Pacific	Mocuzarit, Sonora	27.227,-109.095	3,4
347	North Pacific	Mocuzarit, Sonora	27.210,-109.060	4
349	North Pacific	Mocuzarit, Sonora	27.220,-109.090	4

350	North Pacific	Mocuzarit, Sonora	27.220,-109.090	4
351	North Pacific	Mocuzarit, Sonora	27.210,-109.060	4
353	North Pacific	Mocuzarit, Sonora	27.220,-109.090	4
354	North Pacific	Mocuzarit, Sonora	27.255,-108.982	3,4
358	North Pacific	Yaqui, Sonora	27.719,-109.908	3,4
359	North Pacific	Oviachic, Sonora	27.820,-109.900	4
361	North Pacific	Oviachic, Sonora	27.820,-109.900	4
362	North Pacific	Oviachic, Sonora	28.022,-109.789	3,4
363	North Pacific	Yaqui, Sonora	27.810,-109.890	4
364	North Pacific	Yaqui, Sonora	27.896,-109.806	3,4
371	North Pacific	Cuchujaqui, Sonora	26.950,-108.870	4
375	North Pacific	Cuchujaqui, Sonora	27.040,-108.710	4
385	North Pacific	Cuchujaqui, Sonora	27.040,-108.710	4
3qro	South Pacific	Hondo, Quintana Roo	18.492,-88.473	3

4s	South Pacific	Estado de Mexico	19.016,-100.090	3
colo	South Pacific	Colotepec, Oaxaca	15.909,-96.897	3,4
hch	Uncertain Location	Campeche/Tabasco*	18.257,-92.647	3,4
hg	Uncertain Location	Campeche/Tabasco*	18.232,-93.798	3,4
lv1	Atlantic	La Vega Escondida, Tamaulipas	22.318,-98.042	3
lv5	Atlantic	La Vega Escondida, Tamaulipas	22.328,-97.947	3
mor	South Pacific	Amacuzac, Morelos	18.154,-99.135	3,4
nay	North Pacific	Santiago, Nayarit	21.163,-104.192	3,4
pue1	Atlantic	Mecapalapa,Puebla	20.529,-97.881	3,4
pue2	Atlantic	Mecapalapa,Puebla	20.776,-97.877	3,4
s2	North Pacific	San Lorenzo,Sinaloa	24.603,-106.734	3,4
zm1	South Pacific	Mazatan, Chiapas	15.057,-92.350	3,4
zm4	South Pacific	Mal Paso, Chiapas	17.171,-93.641	3,4
zm6	South Pacific	Mazatan, Chiapas	14.862,-92.484	3,4
zmor	South Pacific	Zacapu, Michoacan	19.363,-102.058	3,4

APPENDIX C: Supplementary information for chapter three.

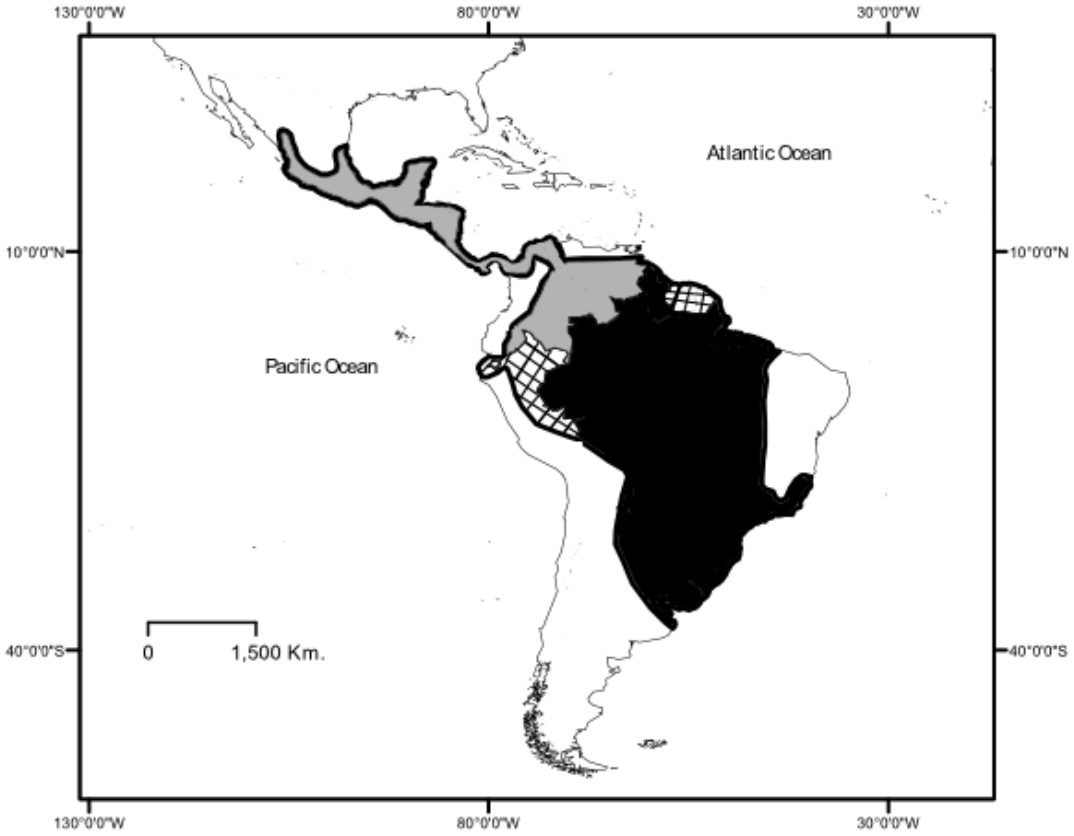

Fig C1. Distribution of three proposed subspecies of *Lontra longicaudis*.

The distribution of *L. longicaudis* in the Americas is outlined in black, and shaded areas represent approximate boundaries for the distribution of subspecies *L. longicaudis annectens* (grey), *L.longicaudis enudris* (hatched) and *L.longicaudis longicaudis* (black) (Van Zyll de Jong, 1972; Lariviere, 1999).

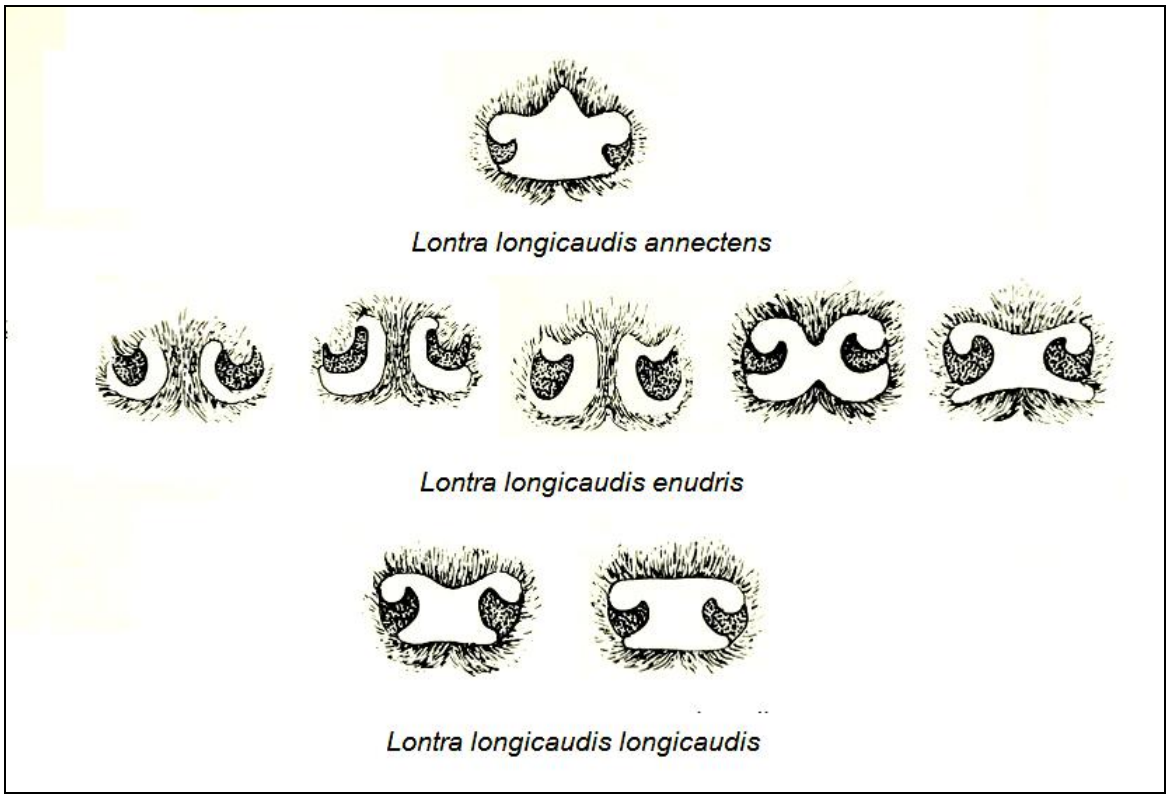

Fig C2. Differences in rhinarium shapes described in three proposed subspecies of *L. longicaudis*. Modified from Van Zyll de Jong (1972).

APPENDIX D: Supplementary information for chapter four.

Table D1.Genotypes of samples used in the present study and number of repeats done to achieve consensus (dashes = non-amplified loci)

ID	RIO13	RIO19	RIO3	RIO1	RIO2	RIO8	RIO6	RIO11	RIO7	RIO9	RIO12	RIO15	RIO16	Repeats
335	252/272	-	190/190	-	-	-	-	142/142	161/163	250/250	208/210	255/255	-	11
336	250/266	276/276	200/200	266/266	184/184	209/209	266/266	140/140	163/163	250/252	208/210	-	267/267	13
337	252/252	-	198/198	-	-	-	-	140/142	161/161	252/256	-	-	-	13
338	250/250	280/280	200/200	262/276	184/190	209/209	260/260	142/142	161/161	252/252	208/208	255/255	265/267	17
339	-	-	190/190	256/256	182/182	-	-	142/142	163/163	252/260	210/210	251/257	267/267	14
341	266/266	276/276	200/200	-	-	-	-	142/142	163/163	252/258	206/208	255/255	267/267	13
343	266/266	276/280	200/200	262/276	184/184	209/209	260/266	142/142	161/161	252/252	208/208	255/255	267/267	12
344	-	280/280	200/200	262/276	184/184	209/209	260/260	142/142	161/161	252/252	208/208	255/255	267/267	13
346	250/266	276/276	200/200	262/276	184/184	209/209	260/260	140/142	161/163	252/252	208/208	255/255	267/267	12
347	266/266	-	-	-	184/184	209/209	-	142/142	161/161	250/260	208/214	255/255	-	16
349	266/266	280/280	-	-	184/198	-	266/266	-	161/161	252/252	208/210	251/255	-	11
350	266/266	280/280	196/200	262/262	184/184	209/209	256/266	142/142	161/161	252/252	208/210	255/255	265/267	17
351	266/266	276/276	200/200	262/262	184/184	209/209	260/260	142/142	161/161	252/252	208/208	255/255	267/267	12
353	266/266	276/280	200/200	262/276	184/184	209/209	250/260	142/142	161/163	252/252	208/208	255/255	267/267	14
354	266/266	276/276	200/200	276/276	184/184	209/209	250/260	140/142	161/161	252/252	208/208	255/255	267/267	12
358	266/266	276/280	198/198	-	184/184	207/207	250/266	140/140	161/177	260/260	208/208	255/255	273/273	13
359	254/254	272/282	-	270/270	190/190	211/211	-	-	161/165	252/252	210/210	255/255	273/273	13
361	250/266	276/276	198/200	-	184/184	209/209	266/266	140/162	161/161	250/252	208/208	255/261	267/273	12
362	250/250	280/280	198/200	276/276	184/184	209/209	256/266	140/146	161/161	252/252	208/208	255/255	265/265	13

ID	RIO13	RIO19	RIO3	RIO1	RIO2	RIO8	RIO6	RIO11	RIO7	RIO9	RIO12	RIO15	RIO16	Repeats
363	250/266	280/280	198/200	276/276	184/184	209/209	256/256	140/140	161/161	252/252	208/208	255/255	265/265	14
364	266/266	280/280	198/200	276/276	184/184	209/209	256/266	140/140	165/165	252/256	208/210	251/255	265/265	14
371	252/258	-	202/202	-	186/186	207/207	264/264	-	161/161	250/250	208/210	255/255	-	13
375	250/250	-	218/218	-	-	-	-	-	163/163	-	210/210	-	-	15
385	246/246	-	198/198	-	186/186	207/207	250/250	146/160	161/161	-	208/210	257/257	-	14
s2	266/266	272/276	200/200	262/262	184/184	209/209	260/260	140/162	161/161	252/252	208/208	255/255	265/267	15
58	268/268	276/276	190/200	-	182/184	209/209	260/260	140/140	161/163	252/252	-	-	-	11
NAY	272/272	276/276	198/200	262/262	182/184	209/209	-	140/146	177/177	252/252	208/208	251/251	-	12
14	250/250	276/276	-	-	-	-	-	-	161/161	252/252	-	-	-	9
18	266/268	268/280	196/196	-	-	-	-	-	161/161	250/250	-	-	-	9
70	276/276	-	218/218	-	-	-	-	-	163/163	250/250	-	-	-	12
99	252/252	272/272	220/220	-	-	-	-	-	177/177	256/256	-	-	-	9
177	250/268	280/280	200/200	-	-	-	-	142/142	163/165	250/250	-	-	-	8
204	250/250	268/268	200/200	-	-	-	-	-	163/165	252/252	-	-	-	9
205	-	280/280	200/200	-	-	-	-	166/166	161/161	-	-	-	-	9
270	268/268	272/276	200/204	-	-	-	-	-	161/163	252/252	208/208	-	-	13
271	250/250	-	200/200	-	-	-	-	-	161/163	250/250	-	-	-	12
1x	252/276	-	200/200	-	-	-	-	140/140	163/167	252/252	-	-	-	7
16x	268/268	268/268	200/204	-	-	-	-	-	161/161	250/250	206/206	-	-	12
19x	-	-	-	-	-	-	-	140/140	161/161	250/250	208/210	-	-	9
21x	246/250	-	-	254/254	184/184	209/211	-	-	157/157	258/258	210/210	-	-	12
mor	266/270	272/272	196/196	262/284	184/184	211/211	256/260	146/146	161/163	250/250	206/208	255/255	265/273	12
ID	**RIO13**	**RIO19**	**RIO3**	**RIO1**	**RIO2**	**RIO8**	**RIO6**	**RIO11**	**RIO7**	**RIO9**	**RIO12**	**RIO15**	**RIO16**	**Repeats**
zmor	266/266	268/272	202/202	254/262	184/184	209/209	256/256	142/164	163/163	252/252	208/208	255/255	265/267	12

320	250/250	268/272	196/202	254/276	184/184	211/211	256/260	160/162	161/161	252/252	208/210	255/255	265/265	14
322	-	-	202/202	-	-	-	-	-	161/163	250/250	202/202	-	-	13
4x	268/272	272/272	200/200	-	-	-	-	-	161/161	-	-	-	-	10
5x	266/266	282/282	196/200	-	-	-	-	140/162	161/161	252/252	-	-	-	9
11x	246/250	268/268	198/198	254/256	182/182	211/209	-	140/140	161/161	252/252	208/210	255/255	-	12
13x	250/254	272/272	196/202	254/276	184/184	211/211	-	-	161/167	252/252	208/210	255/255	265/265	13
15x	252/266	-	-	-	-	-	-	150/166	161/161	250/250	-	-	-	9
colo	250/250	268/272	196/202	254/254	184/184	209/211	256/260	140/146	161/167	252/252	206/208	252/252	265/265	12
22	250/250	268/272	196/202	254/276	182/184	211/211	256/260	-	-	-	-	-	-	10
9x	250/262	268/272	202/202	254/276	184/184	209/211	256/256	-	161/161	252/252	208/210	255/255	265/265	13
322	-	-	202/202	256/270	184/184	209/209	256/256	-	161/163	250/250	202/202	255/261	-	13
zm1	250/250	272/284	196/202	276/276	184/184	207/209	256/260	146/154	157/161	250/252	208/210	255/255	265/267	12
zm4	250/250	268/280	200/200	254/284	184/186	209/209	-	166/170	161/161	250/250	206/208	255/255	265/267	12
zm6	244/268	276/280	202/204	254/262	184/184	209/209	-	-	161/163	250/250	206/206	255/255	267/267	11
HCH	250/268	268/284	202/202	-	-	-	-	146/160	161/161	250/250	202/208	-	-	12
HG	266/268	272/274	200/204	-	-	-	-	150/160	161/163	250/252	210/210	-	-	12
PUE1	250/250	276/276	196/196	254/254	182/184	209/209	256/256	-	161/163	252/252	-	-	-	12
PUE2	250/250	272/280	196/204	-	-	-	-	-	161/163	252/252	-	-	-	8

Table D2.Test of Linkage Disequilibrium for 13 microsatellite loci used in *Lontra longicaudis*

Loc1	Loc2	p-val	Loc1	Loc2	p-val	Loc1	Loc2	p-val
rio13	rio19	0.239050	rio9	rio2	0.440490	rio2	rio12	0.025710
rio13	rio3	0.092920	rio1	rio2	0.154110	rio8	rio12	0.060030
rio19	rio3	0.474500	rio13	rio8	0.056610	rio6	rio12	0.093360
rio13	rio11	0.161500	rio19	rio8	0.040580	rio13	rio15	0.166690
rio19	rio11	0.737960	rio3	rio8	0.004630	rio19	rio15	0.667680
rio3	rio11	0.063840	rio11	rio8	0.083160	rio3	rio15	0.264990
rio13	rio7	0.446900	rio7	rio8	0.166830	rio11	rio15	0.120250
rio19	rio7	0.113060	rio9	rio8	0.095760	rio7	rio15	0.206720
rio3	rio7	0.329800	rio1	rio8	0.130180	rio9	rio15	0.132760
rio11	rio7	0.812980	rio2	rio8	0.007920	rio1	rio15	0.152400
rio13	rio9	0.470510	rio13	rio6	0.587160	rio2	rio15	0.054690
rio19	rio9	0.599920	rio19	rio6	0.173860	rio8	rio15	0.630610
rio3	rio9	0.069540	rio3	rio6	0.011210	rio6	rio15	0.168680
rio11	rio9	0.941040	rio11	rio6	0.792930	rio12	rio15	0.320440
rio7	rio9	0.047370	rio7	rio6	0.643060	rio13	rio16	0.371820
rio13	rio1	0.593310	rio9	rio6	0.031670	rio19	rio16	0.011250
rio19	rio1	0.373190	rio1	rio6	0.194000	rio3	rio16	0.000680
rio3	rio1	0.024340	rio2	rio6	0.502350	rio11	rio16	0.029800
rio11	rio1	0.139170	rio8	rio6	0.026760	rio7	rio16	0.109760
rio7	rio1	0.018460	rio13	rio12	0.488030	rio9	rio16	0.380590
rio9	rio1	0.041460	rio19	rio12	0.279810	rio1	rio16	0.049070
rio13	rio2	0.000690	rio3	rio12	0.149520	rio2	rio16	0.478940
rio19	rio2	0.177960	rio11	rio12	0.814040	rio8	rio16	0.026360
rio3	rio2	0.052270	rio7	rio12	0.186370	rio6	rio16	0.061680
rio11	rio2	0.390490	rio9	rio12	0.011210	rio12	rio16	0.999990
rio7	rio2	0.232240	rio1	rio12	0.017350	rio15	rio16	0.163140

Table D3. Tests of Hardy Weinberg for seven loci in two regions (North and South) in Mexico.

Hardy Weinberg test "North Cluster" (country-wide)
Departures from Hardy-Weinberg (after Bonferroni correction) are shown in **bold.**

locus	Expected He	Observed He	p-val	Null allele freq
Loci11	0.565	0.384	0.405	0.10
Loci12	0.269	0.307	0.934	0.00
Loci13	0.500	0.285	**0.001**	0.14
Loci19	0.504	0.133	0.011	0.29
Loci3	0.411	0.384	0.914	0.00
Loci7	0.464	0.200	**0.002**	0.18
Loci9	0.348	0.266	0.117	0.06

Hardy Weinberg test "South Cluster" (country-wide)

locus	Expected He	Observed He	p-val	Null allele freq
Loci11	0.810	0.700	0.432	0.00
Loci12	0.665	0.555	**0.005**	0.29
Loci13	0.845	0.550	0.122	0.19
Loci19	0.698	0.692	0.756	0.00
Loci3	0.801	0.368	**0.000**	0.27
Loci7	0.605	0.409	**0.002**	0.18
Loci9	0.586	0.150	**0.000**	0.25

Table D4. Test for Hardy Weinberg NP (North Pacific)
Departures from Hardy-Weinberg (after Bonferroni correction) are shown in **bold.**

Locus	Expected He	Observed He	p-val	Null allele freq.
loci1	0.564	0.357	**0.002**	0.12
loci11	0.593	0.316	0.036	0.18
loci12	0.278	0.316	0.995	0.00
loci13	0.482	0.211	**0.000**	0.20
loci15	0.245	0.167	0.320	0.06
loci16	0.521	0.253	0.014	0.20
loci19	0.522	0.211	0.030	0.22
loci2	0.195	0.211	1.000	0.00
loci3	0.398	0.389	0.993	0.00
loci6	0.671	0.412	0.075	0.16
loci7	0.471	0.200	**0.000**	0.20
loci8	0.105	0.000	0.046	0.09
loci9	0.348	0.253	0.234	0.06

Table D5. Test for Hardy Weinberg SP (South Pacific)
Departures from Hardy-Weinberg are shown in **bold.**

Locus	Expected He	Observed He	p-val	Null allele freq.
loci1	0.707	0.769	0.259	0.00
loci11	0.836	0.714	0.155	0.00
loci12	0.690	0.666	0.012	0.11
loci13	0.628	0.550	0.092	0.03
loci15	0.243	0.090	0.011	0.05
loci16	0.475	0.444	0.887	0.00
loci19	0.648	0.727	0.229	0.00
loci2	0.269	0.153	0.164	0.03
loci3	0.649	0.550	0.005	0.04
loci6	0.429	0.625	0.643	0.00
loci7	0.593	0.550	0.395	0.00
loci8	0.535	0.384	0.499	0.30
loci9	0.559	0.083	**0.000**	0.30

Table D6. Number of alleles per locus observed Country-wide.							
Locus	RIO13	RIO19	RIO3	RIO11	RIO7	RIO9	RIO12
No.alleles	12	7	8	10	6	5	5

Table D7. Number of alleles per locus observed in both focal regions: North Pacific (NP) and South Pacific (SP).													
Locus	RIO13	RIO19	RIO3	RIO11	RIO7	RIO9	RIO1	RIO2	RIO8	RIO6	RIO12	RIO15	RIO16
NP	8	4	5	5	4	5	5	5	3	5	4	4	3
SP	8	5	5	9	4	3	6	3	3	2	4	3	3

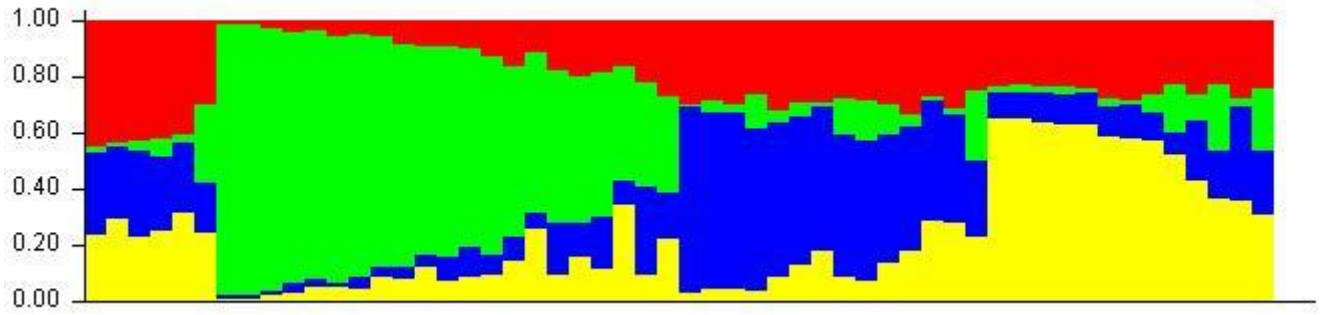

Fig D1. Summary plot of estimated membership coefficients at *K*=4.Each bar in the plot represents an individual and the proportion of its genome assigned to the inferred clusters. The bars (from left to right), represent individuals sorted from North to South.

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