FINAL REPORT

Rufford Small Grants

Project:

METAPOPULATION DYNAMICS AND POPULATION SIZE ESTIMATIVE OF MARINE OTTER (*Lontra felina*) USING MOLECULAR MARKERS




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SUMMARY

Marine otters are an Endangered species by the IUCN, which has patchily distribution along the Pacific from 6ºS to 56ºS, using only rocky seashore and avoiding the sandy beaches. Following the results of the previous RSG project, we collected otter's scats in rocky seashore patches along Chilean and Peruvian seashore In order to test the hypothesis that marine otter population are becoming isolated, and this is a resent historical event. To test the hipótesis, we analyzed mtDNA control region and ND5, and microsatellite loci from blood and feces samples collected along the Chilean coast to study the metapopulation dynamics, which includes the dispersal capacity of the species between rocky parches separated by different distances of sandy beaches and anthropogenic impact. Four study areas were entirely surveyed for faeces, however, one area with higer percentage of sandy beaches no otter sign were detected. Each rocky sea shore fragment, within each study area, had characteristic mtDNA haplotype in higher frequency changing gradually between them. This result indicates population structure between rocky seashore fragment and dispersal is following a steeping stone pattern. The mtDNA showed high population structure (Φ_{st}=0.74) and a clear pattern of isolation by distances but also barriers to gene flow along the species distribution. One barrier and two long areas without any marine otter population separate the species in four management units (MUs). The lack of genetic diversity found in Colcura due to isolation from long areas without marine otter population southern and northern, indicates that females are not capable to disperse long distances and re-colonize new distant areas. Six polymorphic microsatellite loci where selected for marine otter, and they were able to identify unique multilocus genotypes for all individuals (blood samples) from Quintay and Colcura. Using this method we were able to identify for example six individuals from Isla Dama (National Park) from only six faeces samples. Our results indicates that marine otter populations are being fragmented and isolated recently so human activities appear the most probable reason, so this study support Medina-Vogel et al. (2008) results and suggestions: For the conservation of this endangered unique otter species we must implement a network of interconnected large patches of rocky seashore with otter populations, as large marine national parks or reserves several hundred kilometers away between each otters seems not work for the conservation of this top predator based on its population dispersal ecology. Conservation effort should also take attention to the four different management units. Further studies are recommended on Patagonian islands (southern 42^{o} latitude) and channels where little is known about the species.

INTRODUCTION

Among otters, there are only two obligatory marine-living otters, the sea otter *Enhydra lutris*, and the marine otter, *Lontra felina* (Estes 1989). *E. lutris* is highly adapted to the cold coastal waters of the North Pacific Ocean and Bering Sea, with adaptations as the large body size and forages actively (Estes 1989). *L. felina* the most recent extant mammal to have evolved into a marine living condition and is the smallest in its genus, so it might compensate for heat loss imposed by its cold environment by not only increasing heat production but also by spending less time in the water and making trips into the sea mainly to feed (Ostfeld *et al.* 1989, Medina-Vogel *et al*. 2007 Prior Rufford Project). The species is highly dependent of the costal marine habitat, where a radio-tracking study showed that the *L. felina* spend more than 80% in land mostly resting, going to the water mainly to feed (Medina-Vogel et al. 2007 Prior Rufford Project). The marine otter lives in a marine habitat of exposed rocky seashore (Castilla and Bahamondes 1979, Ostfeld *et al*. 1989, Medina-Vogel et al. 2008 Prior Rufford Project) along the Pacific from 6°S near Chimbote (Perú) to 56°S in Cape Horn and Isla de los Estados (Chile and Argentina) (Olrog and Lucero 1981). The marine otter occurrence on the rocky sea shore is dependent of the rocky sea shore size, distance between them and distance to a large rocky sea shore, but also the anthropogenic disturbance (Medina-Vogel et al. 2008 Prior Rufford Project). On this study the species was completed absent in two of eight study areas from approximately 100 km long, but with low percentage of rocky sea shore (14 and 22%). Thus it is important to mention that their might be soon evidences that marine otter populations inhabitant isolated rocky seashore patches might become extinct because of increase of isolation by human activities and direct disturbance (Medina-Vogel et al. 2008 Prior Rufford Project and this study).

We hypothesized that along the marine otters distribution occurred genetically distinct populations each one related to the rocky sea shore patches with gene flow dependent of distances between them, distance to a large rocky sea shore, and antropogenic factors. Long areas without populations could turn difficult dispersal in a steeping stone pattern working as a barrier to gene flow, in consequence differentiating network of populations in Management Units (MUs) or Evolutionary Significant Units (ESUs).

Furthermore, the marine otter populations distributed along different latitudes could be affected by recent and historical events such as the El Niño Southern Oscillations (ENSO) in the north and glaciations on the south.

2- Material and methods:

2.1- Study Areas and Samples collection:

Samples were collected along the Peruvian and Chilean coast (20°41' to 43°36' latitude) to identify different populations or a metapopulation structure, and also differences between them and historical barriers to gene flow. Four different study areas were selected according to the degree of rocky sea shore length, patchiness and isolation by sandy beaches (Table 1). All rocky seashore fragments from the four study areas were completed surveyed looking for fresh faeces samples (n=233). Currently a total of 389 samples have been collected from blood samples (n=16) from captured animals, tissue (n=46) and bone samples (n=37) from carcasses and scats (faeces) samples (n=327), between 2004 and 2008. All bone samples (20 samples from Perú and 17 from Chile) and few faeces samples have been not analyses yet.

Figure 1- Left picture: collecting blood from captured marine otter; Right picture: Otter being released after capture.

Figure 2- Left picture: marine otter faeces samples; Right picture: collecting marine otter faeces.

Table 1- Four study areas entirely surveyed for faeces samples.

Study Sites	Geographic location (latitud)	Lenght of Study Area (km)	Number of parches	Lenght of Rocky parches (km)		Percentage of rocky seashore within the study sites(%)	Degree of landscape division (*D*)	Number of faeces samples collect
				smaller	bigger			
Area 1 (Pan Azucar)	26°08'-26°18'	33	4	1.6	9.6	57	0.88	40
Area 2 (La Serena)	29°37'-30°17'	150	11	0.4	33.8	65	0.91	96
Area 3 (Palo Colorado)	32°01'-32°30'	79	6	2	18	71	0.88	95
Area 4 (Tirúa)	37°34'-38°40'	201	6	0.3	36	26	0.97	0

2.2- Laboratory sample analysis

Only fresh faeces samples were collected and preserved in absolute ethanol. For DNA extraction from faeces, the absolute ethanol from the interior of faeces vial was transferred to a 2ml vial and centrifuged 13.000 rpm for 10 min and supernatant ethanol was disposed. This process were repeated on the same vial until visualize a pellet. The pellet was extracted with regular DNeasy Tissue Kit (QIAGEN). Tissue and blood samples were extracted using the DNeasy Tissue Kit (QIAGEN) according to manufacture. The primers L15926 (5'-TCAAAGCTTACACCAGTCTTGTAAACC-3') described by Kocher et al. (1989) and CCR-DR1 (5'-CTGTGACCATTGACTGAATAGC-3') (Eizirik pers. comm.) were used to amplify the mitochondrial DNA control region and sequenced. Based on the *Lontra felina* mtDNA control region sequences we designed the specific primers LfCR-F2 (5'-GCACCCAAAGCTGACATTCT-3') and LfCR-R2 (5'-GTTGTGCGATGCGGATAAAT-3'), and the external LfCR-F1 (5'-CTCAAGGAAGAAGCGACAGC-3') and LfCR-R1 (5'-ACCTTATGGTTGTGCGATGC-3'), to facilitate amplification from faeces DNA. ND5_DF1 (5'-TTGGTGCAACTCCAAATAAAAGT-3') and ND5-DR1 (5'-AGGAGTTGGGCCTTCTATGG-3') and primers ND5 were designed using primer 3 (Rozen & Skaletsky 2000).

PCR reactions were carried out in a 50µL volume containing 4µL of DNA, 1x reaction buffer, 1.5mM of MgCl2, 200µM of each dNTP, 0.4µM of each primer, and 1unit of Taq DNA polymerase (Invitrogen ®). The PCR amplification profile was as follows: 10min at 95^{o}C, a touchdown of 95^{o}C for 15s, 60–50^{o}C for 30 sec, 72^{o}C for 45 sec, with 2 cycles at each annealing temperature, and 35 amplification cycles of 95^{o}C for 15 s, 50^{o}C for 30 sec, 72^{o}C for 45 sec, followed by a final extension period of 30 min at 72^{o}C. PCR products were purified using QIAquick PCR purification kit [Qiagen ®], and were sequenced by Macrogen Inc., Seoul, Korea.

Nine microsatellite loci were selected to test polymorphism in *Lontra felina*, developed by Dallas and Piertney (1998) and Huang et al. (2005) for *Lutra lutra* and Beheler et al. 2004, 2005 developed for *Lontra canadensis*. A M13 tail (5'CAC GAC GTT GTA AAA CGA3') was added to forward primer to allow incorporation during PCR of a M13 tail sequenced labelled with NED, FAM or VIC fluorescent dyes. Sizes of alleles were scored using ABI GeneScan Version 3.1 and genotyper Version 2.1 software (Applied Biosystems, Foster City, CA,USA). Among the nine loci, six tetranucleotide microsatellite loci were selected according to high polymorphism and easy amplification to *Lontra felina*.

PCR products amplification was confirmed on an acrylamid 8% gel electrophoresis died by silver solution. The PCR products were screened on an ABI 377XL automated DNA sequencer (Applied Biosystems) to assess polymorphisms.

2.3- Genetic analysis

2.3.1- mtDNA sequences

The sequences were aligned and the bases were confirm according to the quality using Proseq v. 2.91 (Filatov, 2002). All sequenced were aligned to detect haplotypes ClustalX v. 1.83 (Thompson et al., 1997).

The geographical differentiation of haplotypes was quantified using a hierarchical analysis of variance by AMOVA (Excoffier et al. 1992). We assigned portions of total genetic variation to divergence either among groups (North, Central, South), or within the 23 populations. The significance of variance components and Φ-statistics, was tested by multiple (1000) random permutations. All estimations were performed using Arlequin, version 2.000 (Schneider et al. 2000). We have also used Arlequin software to calculate pairwise Φ_{ST} and to perform a Mantel test with 10 000 permutations to assess the correlation significance using geographic distances.

We calculated several standard and molecular diversity indexes with the dnasp version 4.0 (Rozas et al. 2003) and arlequin version 2.0 (Schneider et al. 2000). Effective population sizes (Ne) were calculated using the indexes NS, h and π according to the Wright–Fisher model.

Relationships among control region haplotypes were inferred by a median-joining network (MJN) analysis using the program network version 4 (Bandelt et al. 1999). Phylogenetic analyses were performed with mega version 3 (Kumar et al. 2004) to generate trees by different methods with 1000 bootstrap replicates.

2.3.2-Microsatellite Loci

Observed heterozygosity (H_O), expected heterozygosity (H_E), genetic diversity, F_{IS} values to detect inbreeding for individual populations were calculated by Arlequin version 2.0 (Schneider et al. 2000). The program was also used to calculate AMOVA, including differences between genotypes among populations and among groups (F_{IT}), genotypes among individuals within populations (F_{IS}), individual genotypes among populations but within groups (F_{SC}), populations among groups (F_{CT}). Further analysis will be complete and the publications will be sent to Rufford Small Grant for Conservation.

2.4- Research team:

The study was part of one PhD tesis and one Master's degree dissertation. Three undergraduate students participated in the field work surveys:

PhD student: Juliana A. Vianna .

Dr. Gonzalo Medina Vogel: Principal Investigator

Master degree student: Paula Ayerdi

From left to right: René Monsalve (field assistant), Paula Ayerdi (Master degree student), Nicole Salabberry (undergraduate student), Marcelo Fuentes (undergraduate student).

Claudio Soto: Veterinary surgeon in charge of marine otter capture.

3- RESULTS

MtDNA

A total of 112 samples were sequenced the mtDNA control. Analysis from 570-bp mtDNA control region sequences revealed 15 haplotypes and 12 polymorphic sites, including two indels (Figure 1).

From all four studied area entirely surveyed (Table 1) one did not show any otter record due the long sandy beaches isolating the rocky fragments. Within each of the other three study areas each rocky sea shore showed characteristics haplotype in higher frequency when compared to the next rocky fragment.

Grouping all samples from different locations in four different groups or considering only the samples from the defined Area 1 to 3, the haplotype and nucleotide diversity within populations decreased from north to south (Table 2,3). The increase of genetic diversity on the areas 1 to 3 (Table 3) are also correspondent to the increase of percentage of rocky seashore within the study area (Table 1). Isolated populations such as Quintay (33º11'S) and Colcura (37º08'S) showed no genetic diversity. No genetic difference was found between the Chiloe's Island and the continent, due to the only one haplotype (C) found on the island and the continent (Mehuin).

Table 2- Genetic Diversity from all analyzed samples divided in four different groups.

	Sample size	Number of Haplotype	Diversity Haplotype	Nucleotide
North 1	12	5	0.7879 +/- 0.0898	0.003100 +/- 0.002174
North 2	42	8	0.7607 +/- 0.0411	0.003546 +/- 0.002263
Center	46	5	0.6860 +/- 0.0427	0.001551 +/- 0.001229
South	12	2	0.5303 +/- 0.0764	0.000935 +/- 0.000940
Total	112	16	0.8626 +/- 0.0112	0.004171 +/- 0.002536

Table 3 – Three study areas with different rocky sea shore sizes and distances between them, entirely surveyed for marine otter faeces.

	Sample size	Number of Haplotype	Diversity Haplotype	Nucleotide
Area 1	9	4	0.6944 +/- 0.1470	0.002743 +/- 0.002044
Area 2	34	7	0.6809 +/- 0.0446	0.003688 +/- 0.002348
Area 3	35	4	0.5109 +/- 0.0249	0.001802 +/- 0.001374
Total	78	13	0.8345 +/- 0.0164	0.004290 +/- 0.002605

High population structure was found for the 23 populations from each rocky sea shore patches (Φ_{st}=0.74). Only one mtDNA control region haplotype (E) was highly distributed,

found in Peru (18º15'S) as well as Colcura in Chile (37º08'S). However this haplotype was differentiated by the mtDNA ND5 sequences. The 700-bp from mtDNA ND5 sequences revealed 4 haplotypes and 3 polymorphic sites. The haplotype E was found in different locations, however is differentiated in three haplotypes (north-E1, central-E2, south-E3) by ND5 sequences, as well as the control region haplotype F became F1 and F2 (both from north) in combination with ND5 haplotypes. A clear pattern of population structure and isolation by distance can be seen on the map of haplotype distribution (Figure 1).

The mtDNA control region and ND5 haplotypes relationship was reconstructed by Median Joining Network (MJN) not showing clusters highly differentiated. However, two barriers seem to prevent the animal's dispersal homogenizing the haplotypes along the distribution whole distribution. The haplotype widely spread (E) was a central haplotype on the MJN (Figure 2).

Calculating the pairwise F_{st} for the four different groups (table 1) and correlating with geographic distance it was found a pattern of isolation by distance (Figure 3).

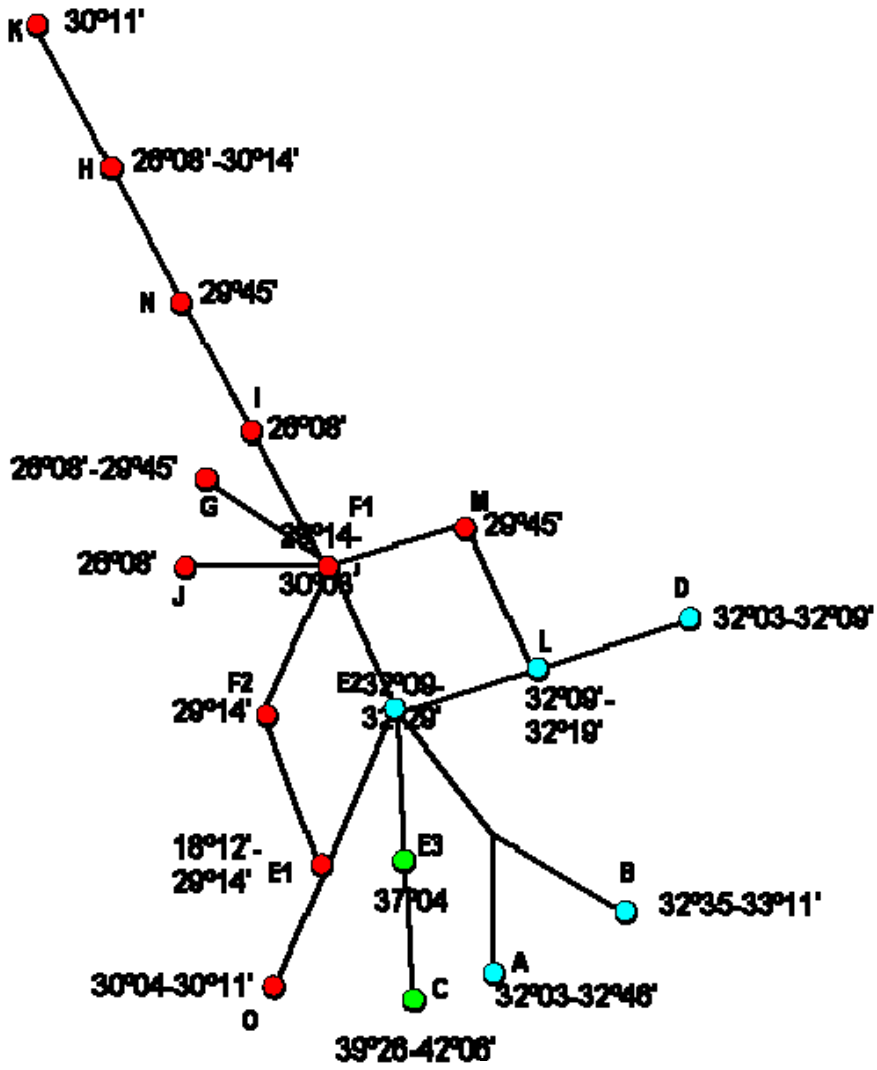

Figure 2- Median Joning Network of mtDNA control region and ND5 sequences. Each circle is a haplotype represented by the letters. The red circles are samples from north, blue circle from central area and green from south.

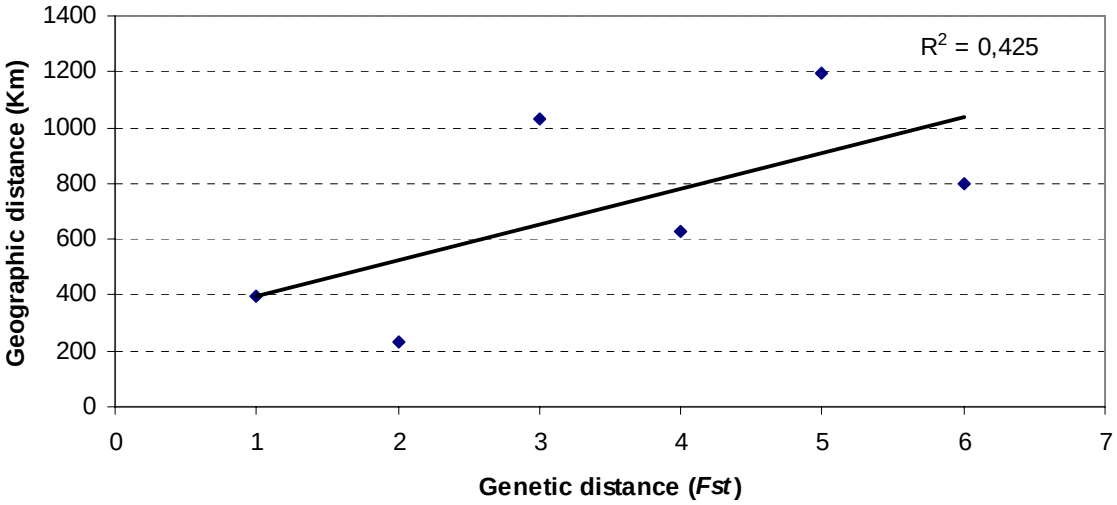

Figure 3- Pattern of isolation by distance from the four groups, correlating genetic distance measured by F_{st} and geographic distance (Km) from each group pair.

Microsatellite loci

All samples from Quintay (n=12) and Colcura (n=7) are from different individuals due to are all blood samples from alive captured animals. Although Quintay and Colcura had no mtDNA diversity within each population, using these six microsatellite loci, we were able to identify unique multilocus genotypes for all individuals examined. The combination of mtDNA, microsatellite loci and genetic sex identification can be even more powerful to detect individuals. The small island of Isla Damas, a National Park, and all six faeces samples showed a unique genotype or different individuals.

Table 4 – Three study areas with different rocky sea shore sizes and distances between them, entirely surveyed for marine otter faeces.

Populations	*N*	*Fis*	*P*	*H obs*	*Hesp*	Gene Diversity
Pan Azucar	9	0.52834	0	0.36111	0.74183	0.593464 +/- 0.363951
Isla Dama	4	0.56627	0	0.30000	0.63571	0.635714 +/- 0.418052
Arrayan	5	0.94118	0.001955	0.04000	0.60889	0.608889 +/- 0.391223
Totoralillo	3	0.80000	0.062561	0.16667	0.70000	0.560000 +/- 0.395980
Tongoy	2	0.42857	0.309873	0.40000	0.60000	0.600000 +/- 0.469042
Palo Colorado	8	0.43003	0	0.40000	0.68167	0.681667 +/- 0.412095
Puquen	8	0.46292	0	0.37500	0.67667	0.676667 +/- 0.409530
Papudo	8	0.59809	0	0.30000	0.71667	0.716667 +/- 0.430033
Quintay	10	-0.03041	0.672532	0.64000	0.62211	0.622105 +/- 0.376264
Colcura	7	0.02439	0.507331	0.55556	0.56863	0.454902 +/- 0.292816

Differences between genotypes among populations and among groups (F_{IT} = 0.54557) were higher, followed by genotypes among individuals within populations (F_{IS} =0.47934),

individual genotypes among populations but within groups (F_{SC} = 0.08702), populations among groups (F_{CT} = 0.04403). However, Quintay and Colcura are the only two populations which did not show high values of F_{IS}, and significant differences between heterozygosity expected and observed. This can be due to null allele o Hardy-Weinberg disequilibrium, due to Quintay and Colcura are all blood samples from captured animals and the other 8 populations we use faeces samples. Further analyses are being complete and all will be sent to Rufford Small Grant for Conservation with a copy of all scientific publications.

4- DISCUSSION

High genetic diversity on the north compared to the south corresponds to larger populations in the north compared to south (Medina-Vogel et al. 2008, prior Rufford Project). High population structure was found for the 23 populations from each rocky sea shore patches (Φ_{st}=0.74).

In a broad scale, populations are isolated by long areas without the species presence and in a finer scale by the distance of rocky sea shores and human populations.

Haplotypes are shared gradually between north (1 and 2) populations in a pattern of isolation by distance, with the haplotype H widely distributed from 26°12' to 32°26'. However no haplotype (mtDNA control region and ND5) are shared between North, Central and South. Although the Median Joining Network didn't show clusters highly differentiated, this distribution of haplotypes within each geographic area can be observed. Apparently, three barriers seem to prevent the animal's dispersal homogenizing the haplotypes along the whole distribution: I- the Punta Lengua de Vaca (30°14'S) dividing north and central areas and II- the lack of species' populations in wide range between the central and south areas (Medina-Vogel et al. 2008; personal observations) III- the lack of species' populations between the two southern populations (Medina-Vogel et al. 2008; personal observations).

Punta Lingua de Vaca is a biogeographic limit (30°14'S), for many costal marine species (Camus, 2001). No haplotype were share among north and south from this limit. Southern from this biogeographic limit, different haplotypes are found from 32°01' (Southern Los

Vilos) all the way to 33°11'S latitude (Quintay). Southern Quintay, Algarrobo (33º21'S) was a limit from Central area, followed by long sandy beaches without any register of species occurrence all the way to Península de Tumbes (36º36'S). Another area of discontinuity is from Sur Golfo Arauco (37º10') to Quele, 39º23'. Those two areas without register of species occurrence due two long sandy beaches could work as barrier to dispersal of *L. felina*. Supporting this observation is the marine otter population at Colcura (37º08'), a population isolated by north and south, and so shows a lack genetic diversity due to the absence of gene flow and the genetic drift effect.

Chiloé Island is characterized by endemic species and populations due to its isolation from the coast by strong currents of cold waters of the Chacao channel. However, no genetic difference was found between Chiloé Island and the continent, sharing the same unique haplotype (C). Chiloé Island is another biogeographic limit marked by different currents and limit of glaciations. This lack of diversity is probably due to a founder effect as recolonization process after the recent glacial period (about 30 000–12 000 bp). No sample was analyzed southern from Chiloé Island, but low genetic diversity is also expected. It is interested to mention that otters form Chiloé island separtad from the continent by a several kilometers wide sea channel (Chacao), a channel with stron sea currents due sea tides has no genetic differences, but otters from the continent separted by long sandy beaches showed differences.

Management Units (MU) is a term used to differentiate any population witch exchange few migrants with other to be genetically different (Moritz, 1994a). In the case of marine otter, none mtDNA haplotype was share between Management Units. MUs can be identified by significantly divergence in the allele frequency of neutral loci (Moritz, 1994a). MUs are demographically autonomic, however in the case o overexploited o extirpated, is improbable to be re-established via natural recruitment of migrants individuals during a time ecological scale relevant for management interest (Avise, 1995).

Four management units (MUs) could be defined for marine otter along its distribution from Perú to Chiloé Island, marked by biogeographic limits and areas without marine otter populations. One management unit is in high risk of extinction considering its isolation, the lack of genetic diversity found in Colcura, and the proximity to the second biggest city in Chile, Concepción city (Figure 1).

The lack of genetic diversity found in Colcura due to isolation from long areas without marine otter population southern and northern, indicates that females are not capable to disperse long distances. Consequently new populations from long distances from the source can not be reestablished without intermediated rocky sea shore parches occupied by the species. Furthermore, population extirpations caused by human activities can increase population isolation and even extinction.

Our results indicates that marine otter populations are being fragmented and isolated by the human activities supporting Medina-Vogel et al. (2008) results and suggestions of conservation efforts should be made in a network of patches, and that this isolation are associated to those areas were high anthropogenic activities that together occur with long sandy beaches and small rocky seashore patches. This study highlight the suggestion that if soon there are no direct actions against the factors that are producing the isolation between otter populations we will observing in no to much time evidences that marine otter populations will become extinct from large regions because the increase of isolation by human and domestic animals disturbance (Medina-Vogel et al. 2008 Prior Rufford Project).

Further south from Chiloé Island, along the southern limit of the species distribution, there are many islands with different sizes and distances, where the populations are separated by cold Ocean on the Patagonia channels. On this area little is known about the species distribution, ecology, population genetics and conservation. Furthermore, the species has sympatric distribution on this area with the endangered southern river otter (*Lontra provocax*) and the alien North American mink (*Mustela vison*) (Medina 1997). Studying these species along their distribution would give us much information about its biology, ecology, species interactions and diseases transmission, all very important aspect to have a full idea of the conservation situation of these endangered otter species and promote conservation actions. However, this area is very rainy, intensively harvested by salmon farms, there is no road, and the transport is only by boat what turns researches very costly.

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