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Population Structure and Genetic Diversity of the Endangered South American Giant Otter (*Pteronura brasiliensis*) from the Orinoco Basin in Colombia: Management Implications and Application to Current Conservation Programs

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Abstract

Endangered giant otters, *Pteronura brasiliensis,* are found along the Amazon and Orinoco rivers and most of their tributaries. Hunting in the mid-1970s pushed giant otter populations to the brink of extinction. We studied population structure and genetic diversity of giant otters from Colombia's Orinoco basin using analyses of partial mitochondrial DNA control region sequences obtained from scat material. We collected and analyzed 54 scat samples from 22 latrines, 2 tissue samples primarily from captive giant otters and 2 from hunted animals near Puerto Carreño and Puerto Inírida (Colombian Orinoco), as well as one tissue sample from Puerto Leguizamo (Colombian Amazon). Thirty-nine partial control region sequences were obtained (258 bp), corresponding to 15 unique haplotypes. Most of these haplotypes, found in samples collected around Puerto Carreño, defined one phylogeographic group (phylogroup) not previously described. Higher genetic diversity in the Colombian Orinoco populations than in other South American populations suggests that this newly described phylogroup, as well as a second phylogroup defined from a few Colombian Orinoco and Amazon samples, should be considered distinct genetic management units. National conservation programs, particularly those aiming to establish protected areas, should manage these independently. Current Colombian confiscated animal reintroduction and captive reproduction programs should also consider such differentiation when determining reintroduction locations or improving husbandry practices.

Resumen

Las nutrias gigantes, *Pteronura brasiliensis,* se distribuyen en la región Amazónica y en la Orinoquía. La cacería intensiva y el comercio de pieles las llevó al borde de la extinción a mediados de los años 70. En este estudio investigamos la estructura poblacional y la diversidad genética de las nutrias gigantes en la Orinoquía Colombiana analizando secuencias parciales de la región control del ADN mitocondrial obtenidas a partir de heces. Colectamos y analizamos 54 muestras

de heces de 22 letrinas, dos muestras de tejido obtenidas cerca a Puerto Carreño y Puerto Inírida (Orinoco Colombiano) y una muestra de tejido colectada en cercanías de Puerto Leguízamo (Amazonas Colombiano). Obtuvimos treinta y nueve secuencias parciales de región control (258 pb), correspondiendo a 15 haplotipos únicos. La mayoría de estos haplotipos, identificados en muestras colectadas alrededor de Puerto Carreño, definieron un grupo filogeográfico (filogrupo-phylogroup) que no había sido previamente descrito para esta especie. Se encontró alta diversidad genética en las poblaciones de la Orinoquía Colombiana, mayor que la encontrada en otras poblaciones alrededor de Suramérica, sugiriendo que este filogrupo, así como un segundo filogrupo definido por unas pocas muestras de la Amazonía y Orinoquía Colombiana, deben ser considerados como unidades de manejo para la especie. Los programas de conservación a nivel nacional, particularmente aquellos cuyo objetivo es establecer áreas protegidas requieren manejar la especie según este criterio de unidades de manejo independientes. Los programas de reintroducción y reproducción en cautiverio deben también considerar esta diferenciación genética al definir zonas para llevar a cabo reintroducciones o para mejoramiento de protocolos de cría en cautiverio.

Subject areas: Conservation genetics and biodiversity, Population structure and phylogeography
Key words: conservation genetics, Colombia, mtDNA, Orinoco, reintroduction

The giant otter (*Pteronura brasiliensis*) is a mustelid endemic to South America, where it is found in the Amazon, Orinoco, and Paraná-Paraguay basins (Carter and Rosas 1997). The main threats to giant otters in Colombia and throughout most of their range include loss of habitat (Weber-Rosas and De-Mattos 2003), hunting, and competition from human fisheries, illegal trade of pelts and pups, and water and soil pollution from gold mining. Between the 1940s and 1970s, populations were also diminished due to export of hides and sale of pups as pets (Trujillo-González *et al.* 2006). As a result of these threats and their reduced populations, the International Union for Conservation of Nature (IUCN) has classified this species as Endangered (*EN*) (IUCN 2011). The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) has included this species in Appendix I (CITES 2011). This species is also protected by Colombian law (number 17 of 1981) (Ministerio-de-Ambiente-y-Desarrollo-Sostenible 2012).

The giant otter is a top predator in aquatic ecosystems and maintains food web balance (Trujillo *et al.* 2013). River otters are also considered to be bioindicators of aquatic integrity (Utreras and Jorgenson 2003). For these reasons, this species has been included in the National Action Plan for Conservation of Aquatic Mammals in Colombia, an initiative started in 2009 by the Ministry of Environment (Trujillo *et al.* 2013). One of the short-term goals of this initiative is to understand the status, genetic diversity, and structure of giant otter populations in Colombia (Trujillo *et al.* 2013). The resultant understanding of the degree of genetic exchange (or isolation) occurring between locations will highlight key regions in which to focus conservation efforts for management and recovery of this species.

In several provinces of the Colombian Amazon and Orinoco (Putumayo, Amazonas, Meta) giant otters have disappeared or human activities have fragmented their populations (Trujillo-González *et al.* 2006). Additionally, it has been suggested that in the River Apaporis (Colombian Amazon), topographic features such as rapids limit the distribution of giant otters and other aquatic species such as dolphins (*Inia geoffrensis* and *Sotalia fluviatilis*). Such biogeographical barriers may contribute to natural isolation among giant otter populations (Botello-Castillo 2000).

Recent studies have examined the population structure and evolutionary history of giant otters. Evidence of phylogeographic structuring between the Amazon and Paraná-Paraguay basins in Brazil was detected using 30 partial mitochondrial control region and cytochrome *b* sequences (García *et al.* 2007). Pickles *et al.* (2011) described four phylogeographic groups (phylogroups) from analyses of partial mitochondrial control region and cytochrome *b* sequence data: 1) Amazon-Orinoco-Guianas, 2) Madre de Dios-Madeira, 3) Iténez, and 4) Pantanal. These authors proposed that such defined phylogroups should be considered conservation units for giant otters. Pickles *et al.* (2012) reported initial evidence of a reduction in effective population size and a recent genetic bottleneck in the Amazonian population from analyses of microsatellite loci. They suggested that this reduction could have been the result of overharvesting due to hunting for pelts about 40 years ago.

These prior studies only included a few samples (n = 4) from the Colombian and Venezuelan Orinoco and from the Colombian Amazon. As a result, discrimination of the phylogeographic patterns for the Upper Amazon and Orinoco regions as described by Pickles *et al.* (2011) is not entirely clear.

In this study, our objectives were to 1) describe the population structure of giant otters in Colombia, 2) determine levels of genetic diversity in the Colombian Orinoco and Amazon, and 3) provide initial information for genetic management of Colombian giant otter populations. This knowledge can be used in current management conservation strategies in Colombia, specifically to underpin the designation of conservation areas and management units. It might also help define areas of origin for animals confiscated from illegal trading activity and guide efforts for their reintroduction, and can be used in captive breeding programs to help optimize mating pairs and improve husbandry practices.

Methods

Sample Collection and Storage

Due to the difficulty of obtaining and handling wild giant otters for blood or tissue sampling, we collected scat samples; these have

proven to be a useful source of DNA in a number of mustelid species, including giant otters (García *et al.* 2007), the southern river otter (*Lontra provocax*) (Centrón *et al.* 2008), and the neotropical river otter (*Lontra longicaudis*) (Trinca *et al.* 2007). Samples were collected in the Colombian Orinoco, in the vicinity of the townships of Puerto Carreño and Puerto Inírida (Figure 1). Following the guidelines of Groenendijk *et al.* (2005), fieldwork was conducted during the low water season when latrines were accessible: in February and March 2010, and in April 2011. Giant otters use communal latrines and feces are mixed due to territorial marking. In order to minimize resampling scat material from the same individual, samples were collected in the periphery of latrines, and sampled stools were at least 1 m apart. Fifty-four fresh fecal samples were collected by random selection of stools in each of 22 latrines that were separated from each other by at least 5 km (see Supplementary Table 1 online). Samples were stored in 70% ethanol at 4 °C (Sambrook and Russell 2001). In addition to scat samples, we analyzed 3 dry skin samples obtained from indigenous hunters; 1 from Puerto Carreño, 1 from Puerto Inírida, and 1 from the Caucayá River in Puerto Leguizamo (Colombian Amazon) (Figure 1). Finally, tissue samples were taken from 2 giant otter pups in rehabilitation (Keiko and Rey). The pups had been confiscated by the local authorities around Puerto Carreño and were being rehabilitated by Fundación OMACHA in Bojonawi Private Nature Reserve, Vichada province, at the time of sample collection.

DNA Extraction, Amplification and Sequencing

Total DNA was extracted from feces and skin samples following a phenol-chloroform-isoamyl alcohol protocol after digestion with proteinase K (20 mg/mL) dissolved in Tris–HCl at 55 °C overnight (Sambrook and Russell 2001). A partial segment of approximately 250 bp of the mitochondrial *Control Region* was amplified by polymerase chain reaction (PCR), with the MTLPRO2 forward primer (5′-CACTATCAGCACCCAAAGCTG-3′) designed by Tchaicka *et al.* (2007), and the reverse primer LonCR-R1 (5′-ATGGTTTCTCGAGGCATGGT-3′) designed by Trinca *et al.* (2007). The amplification profile consisted of 2 min at 95 °C for predenaturation, followed by 20 cycles of 15 s at 95 °C for DNA denaturation, 45 s at 60 °C for primer annealing, and 1 min at 72 °C for elongation. This was followed by 35 cycles of 15 s at 95 °C for DNA denaturation, 45 s at 52 °C for primer annealing, and 1 min at 72 °C for elongation. The profile concluded with a 12-min final extension at 72 °C. The 3 tissue samples provided positive amplification controls due to high quality DNA. Purification was carried out using polyethyleneglycol and ethanol precipitation, and sequencing was conducted on an automated 3730XL DNA sequencer at Universidad de los Andes, where best results were obtained with a QV > 20 for all samples submitted.

Because we used mostly scat samples, a quality control was implemented to thoroughly check our results. Each sample was sequenced in both directions, finding an overlap of approximately 140 bp between forward and reverse strands, and all haplotypes were validated by at least 2 independent PCR and sequencing runs. No differences were detected in the runs of the same sample, which provided evidence of reliable sequencing. A Basic Local Alignment Search Tool for nucleotide (BLASTn) analysis was performed to confirm that, indeed, giant otter DNA was amplified and not neotropical river otter DNA.

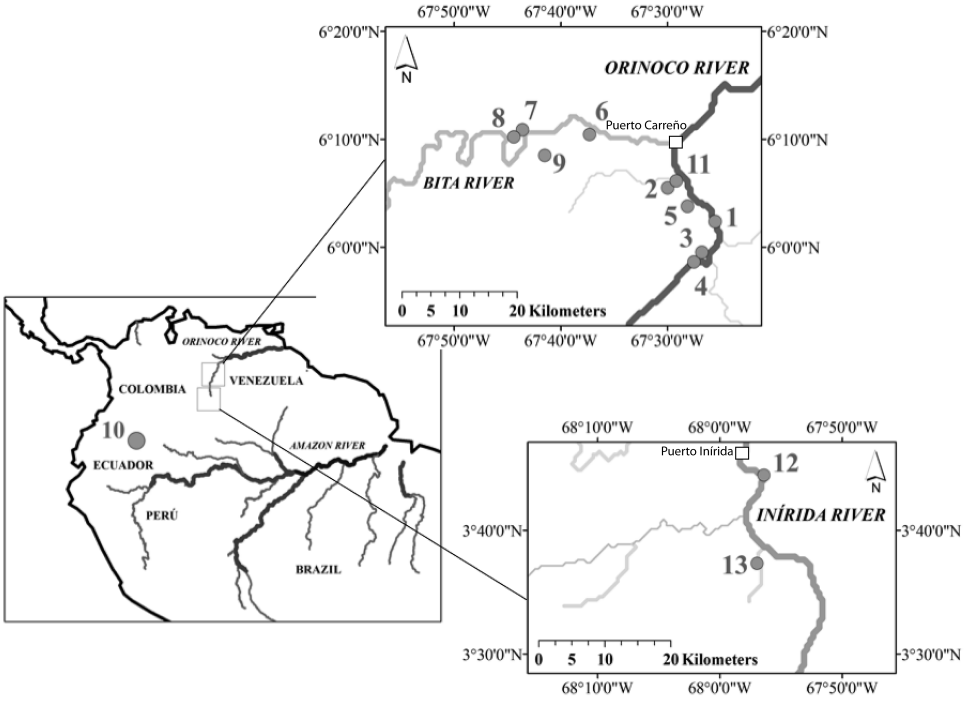

Figure 1. Sampling sites for giant otters in the Colombian Orinoco basin and the Colombian Amazon basin. Puerto Carreño and Puerto Inirida townships are shown as a white squares. Scat sampling sites (latrines) near Puerto Carreño township along the Orinoco River are labeled (1) San Borges, (2) Pañuelo lagoon, (3) La India lagoon, (4) Ángela lagoon, (5) San José. Along the Bita River: (6) Tres Bocas, (7) Paso de Ganado, (8) Tres Iglesias, (9) La Yuca. Scat sampling sites near Puerto Inirida township are labeled (11) Bojonawi Reserve (12) Caiman creek, Pajarito Creek and Clara Lagoon and (13) Vitina creek. Tissue samples were taken from 2 captive animals at (11) Bojonawi Reserve, from one skin from a hunted animal in the Colombian Amazon at (10) Caucayá River in Puerto Leguizamo, Putumayo province, and from one skin taken near Puerto Carreno and one near Puerto Inirida.

Estimates of Genetic Diversity, Phylogeography, and Population Structure

Sequences were checked by eye and base changes reviewed prior to alignment. We performed a multiple alignment using the MUSCLE algorithm (Edgar 2004) using the software Geneious 4.8.1 (Drummond *et al.* 2011). In order to obtain a wider phylogeographic comparison, quantify the levels of genetic diversity, and detect population structuring in the Colombian Orinoco, our sequences were compared with previously published sequences from 70 samples available on GenBank (http://www.ncbi.nlm.nih.gov/) for *P. brasiliensis*. These sequences originated in Pantanal, the Brazilian Amazon, the Black River, Guianas, and Iteñez (Brazil), Madre de Dios (Peru), and Madeira (Bolivia) [(accession numbers EF488532.1 to EF488561.1 (Garcia *et al.* 2007) and JN252256 to JN252295 (Pickles *et al.* 2011)]. These sequences had an approximate length between 812 and 948 bp of the genes cytochrome *b* and control region of mitochondrial DNA (mtDNA). We analyzed a total of 109 sequences and all sequences were cut to a consensus length of 258 bp of the control region of mtDNA amplified in this study to allow further comparisons. Haplotypes were identified using the software MacClade 4.0.8 (Maddison and Maddison 2000).

A haplotype network was constructed using the statistical parsimony methodology as implemented in the software TCS vers. 1.21 (Clement *et al.* 2000). This method estimates an unrooted tree, providing a 95% plausible set for all sequence type linkages within the tree and considering gaps as a fifth character state. An analysis of population structure with estimates of the correlation of variance was calculated using the program SAMOVA 1.2 (Dupanloup *et al.* 2002) with the number of population prior (K) between 2 and 25 from 47 geographic coordinates from all sampling sites [(including those from Pickles *et al.* (2011)], with 100 simulations per K. The haplotype frequencies and the number of partitions selected among phylogeographic groups were tested with the optimal Φ_{CT} (F_{ST}). A pair-wise comparison between the phylogroups obtained in the SAMOVA analysis was conducted using an AMOVA of F_{ST} and Φ_{ST} (Excoffier *et al.* 2006). Nucleotide (π) and haplotype diversity (h) indexes were calculated with Arlequin 3.5.1.2 (Excoffier *et al.* 2006).

Data Archiving

In fulfillment of data archiving guidelines (Baker 2013), haplotypes identified in this study were submitted to GenBank as accession numbers KF322040 to KF322054.

Results

Phylogeography and Population Structure

Thirty-nine sequences were obtained out of 59 stored samples (54 scat samples and 5 tissue samples). We were unsuccessful in amplifying 20 scat samples, possibly due to DNA being highly degraded. When combined with previously published data of Garcia et al. (2007) and Pickles *et al.* (2011), the total dataset included 109 samples representing 41 control region haplotypes (Table 1). Haplotypes defined in previous studies by substitutions in the cytochrome *b* region were not included in this analysis (H2, H6, H18, H19, H20, H24, H27, H28, H29, H31, H35, H36, H41). The large number of fixed differences and indels showed that there is a marked differentiation among maternal lineages of giant otters in the Amazon and Orinoco basins. Within the 41 haplotypes we detected 55 variable sites, including 40 transitions, 11 transversions, and 10 indels or insertions. The 39 new sequences were assigned to 15 haplotypes, including 2 previously reported haplotypes (7 and 10) and 13 new haplotypes (42–54). Haplotype 7 was identified in 1 of the 2 animals being rehabilitated, and haplotype 45 in the other (Table 1 and see Supplementary Table 2 online).

The haplotype network (Figure 2) retained previously described phylogeographic relationships among giant otter haplotypes in other South American regions. The majority of the Colombian Orinoco samples, particularly those collected around Puerto Carreño, have a set of substitutions in the control region, including an 8 bp insertion. Interestingly, among samples collected around Puerto Carreño, those collected in the latrine of San José showed high haplotype heterogeneity: 5 haplotypes were identified for 5 samples. Some samples collected around Puerto Carreño and Puerto Inírida and the 1 Caucayá (Amazon) sample had haplotypes (H7, H10, H42, H43, H44) more similar to those previously described for Guianas, the Amazon, and the Black River (Figure 2) (Pickles *et al.* 2011).

In the SAMOVA analysis, we obtained a value of Φ_{ST} (F_{IT}) = 0.843 (P = 0.012), indicating a high correlation of haplotypes into subpopulations relative to random pairs of total populations. Also Φ_{SC} (F_{IS}) = 0.050 (P = 0.023) showed an average correlation of haplotypes within the demos for the subpopulation or phylogroup. The Φ_{CT} (F_{CT}) = 0.835 (P = 0.003) provided evidence of a high correlation of haplotypes within a subpopulation or phylogroup compared with the total population and therefore highly significant differences between subpopulations (F_{ST} > 0.25).

On the basis of the SAMOVA results, 5 phylogroups were defined: 1) Puerto Carreño (grouping the majority of samples collected at latrines around Puerto Carreño except those from latrine San José, Figure 1), 2) Amazon, Orinoco, and Guianas (including the sample from the Caucayá River, Colombian Amazon, and a small number of samples collected around latrine San José, Colombian Orinoco), 3) Pantanal, 4) Iténez, and 5) Madre de Dios and Madeira (Figure 2). Significant population differentiation was found among all phylogroups at the F_{ST} and Φ_{ST} levels except between the Itenéz and Madre de Dios phylogroups at the F_{ST} levels (Table 2).

Estimates of Genetic Diversity

Overall haplotype diversity ranged from 0.16 to 0.90 (Table 3). The highest value was found for the Madre de Dios phylogroup, although sample size was small. Haplotype diversity for the Puerto Carreño phylogroup was relatively high, considering also the large number of samples analyzed. Nucleotide diversity ranged from 0.07% (Pantanal) to 2.91% (Puerto Carreño) (Table 3).

Discussion

The aim of this study was to investigate the genetic diversity and population structure of giant otters in the Colombian Orinoco, in order to provide baseline information for management, reintroduction, and captive breeding programs in the country.

Genetic Diversity

This study revealed high genetic diversity and population structure among giant otters from this region. The haplotype diversity was relatively high in most phylogroups, particularly in the Puerto Carreño and Orinoco-Amazon-Guianas phylogroups, and was consistent with the values reported by Pickles *et al.* (2011) for giant otters. However, because the short sequences we analyzed may have led to

Table 1. Variable sites over 258 bp of *Control region* gene of mitochondrial DNA. Dashes (-) represent an indel, dots (•) represent a match to haplotype 1. Haplotypes from 42 to 54 correspond to new assignments of this study

Control region (258pb)

```
                  1111111111111111111111111111111122222222222
      24555666888900011111112222222222333455889999000011111123
      30528346256156824567890123456789017359893679267801349 67
H1    CTTTTTTTTTGCTAGTCTACG-CGCA--------TTACCTAGGCAACGGCCCCAC
H3    ..........A..........-....--------.....................
H4    .....................-....--------..G.T...........T....
H5    .....................-....--------..............TTT...
H7    ..................C...-....--------..............TTT...
H8    ..................C...-.A..--------..............TTT...
H9    ..................C...-....--------..............TTTT..
H10   ...C.............C...-....--------..............TT...
H11   .....................-....--------........AT.......TTGT
H12   .....................-....--------........AT.......TTG.
H13   T....................-....--------.........T.......TT..
H14   .....................-....--------.........T.......TT..
H15   .....................-....--------....T.........A.TTT...
H16   .....................-....--------......G..T...A.TTT...
H17   .....................-....--------.........T...A.TTT...
H21   .....................-....--------........AT...A.TTT...
H22   .....................-....--------......G..T.G.A.TTT...
H23   ..................C...-....--------.............A.TTT...
H25   ..................C...-T...--------.............A.TTT...
H26   ..................C...-....--------............TA.TTT...
H30   .....................-....--------.............A.TTT...
H32   ..................C...-....--------........A.G..A.TTT...
H33   .....................-...--------CC...T...AT...........
H34   .....................-...GC------CC...TC..AT...........
H37   .....................-...GC------C....TC..AT...........
H38   .....................-.C.--------CC...T...AT...........
H39   ......C..............-.C.--------CC...TC..AT...........
H40   ....................G...C-----CCCC...T...AT...........
H42   .C..............C...-....--------..............TTT...
H43   ...C.............C...-....--------..............TTT...
H44   ..................C...-....--------.............T.TTT...
H45   ..C.CA.CCCAA...GT.TTC-TTTTTTCTTCCT.C.TT..AAT.GTAC.TT...
H46   ........CCAA...GT.TTC-TTTTTTCTTCCT.C.TT..AAT.GTAC.TT...
H47   ........CCAA...GT.TTC-TTTTTTCTTCCT.C.TT...AT.GTAC.TT...
H48   ..............GT.TTC-TTTTTTCTTCCT.C............CTTT...
H49   ............CTTGT.TTC-TTTTTTCTTCCT.C.TT..AAT.GTAC.TT...
H50   ........C......GT.TTC-TTTTTTCTTCCT.C.TT...AT.GTAC.TT...
H51   ........C...CTTGT.TTC-TTTTTTCTTCCT.C.TT..AAT.GTAC.TT...
H52   ........C......GT.TTC-TTTTTTCTTCCT.C.TT..AAT.GTAC.TT...
H53   ........C..A...GT.TTC-TTTTTTCTTCCT.C.TT..AAT.GTAC.TT...
H54   ........CCAA...GT.TTC-TTTTTTCTTCCT.C.TT..AAT...AC.TT...
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Previously published haplotypes H2, H6, H18, H19, H20, H24, H27, H28, H29, H31, H35, H36, and H41 are defined by cytochrome *b* and are not included here.

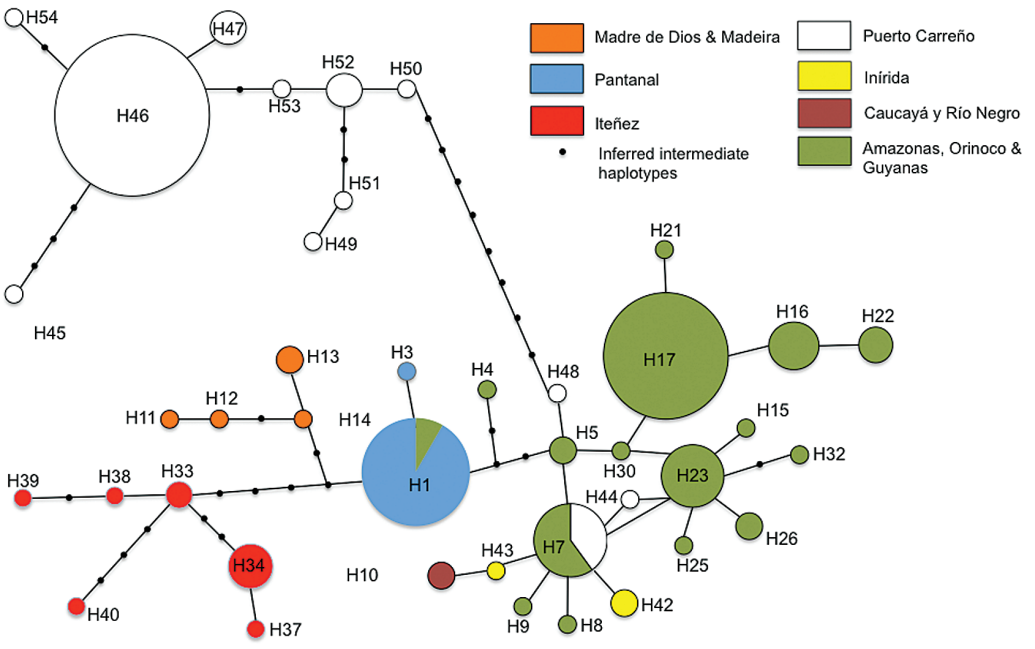

Figure 2. Haplotype network based on mtDNA *Control Region* sequence data of giant otter constructed using the statistical parsimony methodology as implemented in the software TCS Vs. 1.21 (Clement *et al.* 2000). We include haplotypes defined in samples from the Colombian Orinoco (around Puerto Carreño and Puerto Inírida) and the Colombian Amazon (Caucayá River), as well as haplotypes identified in phylogroups previously defined by Pickles *et al.* (2011). New haplotypes reported in this study correspond to numbers 42–54.

Table 2. Conventional F_{STs} (above diagonal) and pairwise Φ_{STs} (below diagonal) for mtDNA control region among phylogroups of giant otters

Φ_{ST} \ F_{ST}	Puerto Carreño	Amazon, Orinoco, and Guianas + Inírida + Caucayá	Pantanal	Iténez	Madre de Dios and Madeira
Puerto Carreño, *n* = 35	—	**0.197**	**0.492**	**0.224**	**0.231**
Orinoco, Amazon and Guianas + Inírida + Caucayá, *n* = 48	**0.830**	—	**0.370**	**0.106**	**0.101**
Pantanal, *n* = 12	**0.890**	**0.611**	—	**0.506**	**0.586**
Iténez, *n* = 9	**0.780**	**0.751**	**0.801**	—	0.106
Madre de Dios and Madeira, *n* = 5	**0.779**	**0.500**	**0.830**	**0.685**	—

Significant differentiation values among phylogroups shown in bold ($P < 0.05$).

Table 3. Sample sizes (*n*), number of haplotypes and CR mtDNA diversity (haplotype (*h*) and nucleotide (π) diversity ± standard deviation) for giant otters from the Puerto Carreño phylogroup and 4 other phylogroups presented in this study

Phylogroup	Sample size (*n*)	Number of haplotypes	Haplotype diversity (*h*)	% Nucleotide diversity (π)
Puerto Carreño	35	12	0.7008 ± 0.0836	2.9068 ± 1.5466
Orinoco, Amazon and Guianas + Inírida + Caucayá	48	17[a]	0.8980 ± 0.0300	1.098 ± 0.6581
Pantanal	12	2	0.1667 ± 0.1343	0.0669 ± 0.1086
Iteñez	9	6	0.8889 ± 0.0910	1.2905 ± 0.8305
Madre de Dios and Madeira	5	4	0.9000 ± 0.1610	0.8835 ± 0.6809
Total	109	41	0.7108 ± 0.0818	1.2500 ± 0.6090

[a]This study + Pickles *et al.* (2011).

under-detection of haplotypes, the values that we report in this study may be low estimates of diversity. The nucleotide diversity for all phylogroups was lower than or equal to values reported for other mustelids listed as Endangered (*EN*) by the IUCN (Davison *et al.* 2001; Michaux *et al.* 2005; Valqui *et al.* 2010). Analogous to haplotype diversity, nucleotide diversity is also dependent on the length of the sequences. We used a 258 bp hyper variable region of the mtDNA control region, resulting in a higher estimate of nucleotide diversity than the values obtained for a longer sequence by Pickles *et al.* (2011), which ranged from 0.015% to 0.76%. Relatively high haplotype diversity was found in the Puerto Carreño phylogroup; this may be due to the high heterogeneity of the haplotypes reported

for the San José latrine (5 haplotypes found for 5 samples analyzed). Genetic diversity for the Pantanal phylogroup was underestimated because due to the short sequences analyzed, haplotypes 2 and 41 were not found in this study. Additionally, we did not detect haplotypes 6, 18, 19, 20, 24, 27, 28, 29, 31, 35, and 36, but the relationships among phylogroups were maintained.

Phylogeographic Patterns of Colombian Giant Otters

The haplotype network generated clear phylogeographic relationships among the 39 sequences. Many of the haplotypes found around Puerto Carreño and Puerto Inírida (44–54) had not been reported previously in South America. One previously described haplotype (7), found in the Manaués River (Amazonas State, Brazil), the Takutu River (Roraima State, Brazil), and the Venezuelan Orinoco (Garcia *et al.* 2007; Pickles *et al.* 2011) was also found in the San José latrine (Puerto Carreño) and in one pup (Keiko) that was being rehabilitated at Bojonawi Reserve. Taking into consideration other information available regarding Keiko´s origin, it was suggested that the phylogeographic origin of Keiko was around Puerto Carreño. The pup Rey was reported as having a new and unique haplotype (45). This haplotype was closely related to haplotype 46, the most common haplotype found in the Orinoco basin around Puerto Carreño, and suggests that Puerto Carreño was also Rey's potential phylogeographic origin. A new haplotype (44) reported from a latrine in San José was related to haplotypes from the Orinoco-Amazon and Guianas, showing that it is phylogenetically differentiated from other haplotypes found in the Colombian Orinoco. Three samples from Puerto Inírida had new and unique haplotypes reported as 42 and 43, which seem to be more closely related to the Orinoco-Amazon and Guianas phylogroup proposed by Pickles *et al.* (2011). There is evidence of haplotype heterogeneity in the latrine of San José, where haplotype 48 connected to our proposed phylogroup with the Orinoco-Amazon and Guianas phylogroup proposed by Pickles *et al.* (2011). Furthermore, haplotype 10 [called haplotype 2 in Garcia et al. (2007)] and reported previously in the Black River of Brazil, was found in the sample from the Caucayá River, Colombian Amazon (Figure 2).

The finding of Colombian haplotypes in the Orinoco-Amazon and Guianas phylogroup allows us to suggest 3 hypotheses regarding gene flow and connectivity among giant otter populations. The first hypothesis suggests migration events without being able to discriminate if these occurred in the past or are currently occurring. The second hypothesis suggests past fragmentation that resulted in isolation of maternal lineages that have been maintained over time (e.g., haplotypes 7 and 10). A third hypothesis to explain the absence of some haplotypes (haplotypes 42, 43, and 44) could be the past occurrence of fragmentation events that resulted in these haplotypes becoming extinct. Alternatively, insufficient sampling throughout the range might have caused a similar result.

Pickles *et al.* (2011) reported a sub-phylogroup, weakly supported in their haplotype network that included samples from the Upper Amazon, Orinoco, Central Amazon, and Guianas. We now present evidence of shared haplotypes between the Upper Amazon and Orinoco including haplotype 7, and haplotypes closely related among them and found in the Orinoco (haplotypes 5, 8, 42, 43, and 44) and Amazon (haplotypes 9 and 10 from the Black River). This could be considered initial evidence of gene flow and possible migration events of giant otters between these basins in the past or the present. The northwestern region of South America has a complex paleogeographic history that has had an influence in the diversification of tropical species (Lovejoy and De Araújo 2000; Hoorn *et al.* 2010). Also, giant otters can be found in sediment-rich (white) waters, clear waters, and humic-rich (black) waters (Carter and Rosas 1997), suggesting a propensity for broad dispersal. However, under stressful conditions, such as the intensive hunting period they were subjected to between 1940 and 1970, these populations may take refuge in certain water bodies and adapt to restrictive localities as has been found for other mammals affected by habitat fragmentation (Coster and Kovach 2012).

The Black River, the Amazon's largest tributary, characterized by black waters, and the Orinoco River, which has white water, are connected by the Casiquiare River, which acts as a corridor for many fish as well as dolphin species (Banguera-Hinestroza *et al.* 2002; Willis *et al.* 2010; Winemiller and Willis 2011). Our results suggest genetic connectivity of giant otter populations between the Orinoco and Amazon basins through the Casiquiare River. In fact, there are reports of giant otters in the Casiquiare River (Mondolfi and Trebbau 1978).

Haplotype 46 was present in almost all sampling points in this study and was the most common haplotype found in the Colombian Orinoco region (Figure 2). This provides evidence of connectivity among giant otter populations around Puerto Carreño, probably facilitated by their social behavior and also hydroclimatic cycles (Schenck and Staib 1998; Gómez 1999; Velasco 2004), as during the high water season the populations are connected. We cannot entirely rule out confounding by pseudoreplicates of haplotype 46 in our study because the distance among some sampled latrines in El Pañuelo Lagoon, Puerto Carreño, was less than 5 km (Valqui *et al.* 2010). Thus, the detection of haplotype 46 in most locations might be partially explained by a wide-roaming individual.

The San José latrine showed high genetic differentiation and haplotype heterogeneity compared with other latrines. However, it is close to Puerto Carreño and there are no obvious ecological barriers between these locations. We hypothesize that the San José latrine results may be due to the sampled latrine being used by unrelated solitary males, which sometimes form groups. An exogamous dispersal system, with males leaving their natal groups to look for mates, has been suggested for giant otters and our finding seems to support such an idea (Duplaix 1980; Groenendijk 1998; Groenendijk *et al.* 2005).

The 15 haplotypes defined for the Colombian Orinoco correspond to a large sample of wild free-ranging giant otters. These divergent haplotypes suggest that in this region there is a confluence of several local maternal lineages presumably isolated by anthropogenic barriers but punctuated by occasional mixing due to specific gregarious behavior as suggested by previous studies (Carter and Rosas 1997). Because of the high diversity in the Colombian Orinoco and the need for greater certainty about this species' phylogeography, giant otter phylogroup partitioning in South America should be reevaluated with complementary studies of the various factors involved. For example, in our study we did not find any shared haplotype between the Orinoco and Guianas, but some of our network clusters could be artifacts of the lack of sampling in intermediate geographic regions rather than the result of isolation by distance. Even if this is the case, isolation by distance may also be occurring due to ecological, social, paleoclimatic, paleogeological, or other fragmentation processes, including creation of anthropogenic barriers (Trujillo-González *et al.* 2006; Zambrana Rojas 2007; Ottaviani *et al.* 2009; Clément and Thoisy 2010; Bezerra *et al.* 2011). Additionally, in certain populations interdrainage migratory routes produced during seasonal hydroclimatic cycles may maintain

intermittent connectivity between animals with haplotypes from different phylogroups.

Conservation and Management Implications of this Study

Directed efforts are underway in Colombia and other countries in the Amazon and Orinoco regions to protect and restore giant otter populations in the wild and ex-situ, via captive breeding programs. Maintenance of the long-term genetic integrity and viability of giant otter populations requires consideration of their genetic makeup (e.g., their phylogroup assignation), especially if animals are to be reintroduced to their natural habitat. Our genetic results are a first step in the National Action Plan for the conservation of this semi-aquatic mammal in Colombia (Trujillo *et al.* 2013). Our study shows relatively high genetic diversity in the giant otter populations around Puerto Carreño, Puerto Inírida and Puerto Leguízamo. We propose that the new phylogroup described in this study, as well as the previously described Amazon, Orinoco and Guianas phylogroup, to which many of this study's samples belong, should be considered as separate management units for giant otters, as their genetic component is sufficiently distinct to require separate genetic management (Frankham *et al.* 2010). Two management units should be considered in Colombia: one around Puerto Carreño and one including Puerto Inirida and the Amazon. Our findings can help streamline conservation efforts for this species. First, the most likely maternal genetic origin of animals in rehabilitation can be determined by sequencing a short fragment of the control region sequence, making it possible to define the optimal release location. Second, to promote maintenance of these maternal lineages, we recommend working with the environmental authorities to design protected areas encompassing the sampling areas included in this study. It is also necessary to collect samples south of Puerto Carreño to resolve questions concerning possible isolation by distance, population structuring, and connectivity among other locations in the Colombian Amazon and Orinoco.

Our results show that noninvasive sampling of this species is feasible and should continue and be improved. Ideally, samples need to be collected fresh and processed in the shortest possible time, to prevent further DNA degradation. Other sampling strategies also need to be implemented to obtain fresh tissue samples; for example, remote biopsy sampling has been tried for some populations in Brazil (Ribas 2012). Tissue sampling would allow more detailed genetic analyses (i.e., nuclear markers, genotyping), enabling study of sex-biased dispersal, the genetic composition of latrines, and relatedness among individuals in particular groups, as well as allowing for comparisons between genetics and behavioral data. Such studies would provide valuable information on the social structure, social interaction, and mating system of giant otters. This knowledge would help improve husbandry conditions in captive breeding programs, such as the one led by Zoológico de Cali in Cali, Colombia (Corredor 2013).

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/

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