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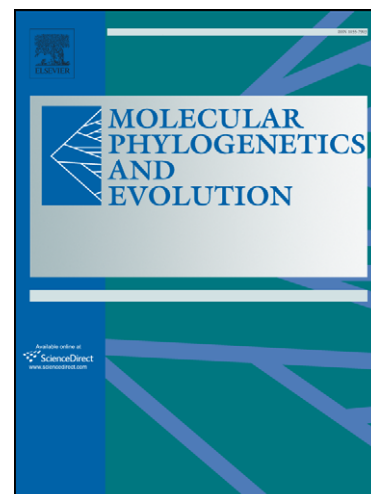
Evolutionary history and identification of conservation units in the giant otter,
Pteronura brasiliensis

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1 Evolutionary history and identification of conservation
2 units in the giant otter, *Pteronura brasiliensis*

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1

2 **Running title:** Phylogeography of *Pteronura*

ACCEPTED MANUSCRIPT

1 Abstract

2 The giant otter, *Pteronura brasiliensis*, occupies a range including the
3 major drainage basins of South America, yet the degree of structure that
4 exists within and among populations inhabiting these drainages is
5 unknown. We sequenced portions of the mitochondrial DNA (mtDNA)
6 cytochrome *b* (612 bp) and control region (383 bp) genes in order to
7 determine patterns of genetic variation within the species. We found high
8 levels of mtDNA haplotype diversity ($h = 0.93$ overall) and support for
9 subdivision into four distinct groups of populations, representing important
10 centers of genetic diversity and useful units for prioritizing conservation
11 within the giant otter. We tested these results against the predictions of
12 three hypotheses of Amazonian diversification (Pleistocene Refugia,
13 Paleogeography, and Hydrogeology). While the phylogeographic pattern
14 conformed to the predictions of the Refugia Hypothesis, molecular dating
15 using a relaxed clock revealed the phylogroups diverged from one
16 another between 1.69 and 0.84 Ma, ruling out the influence of Late
17 Pleistocene glacial refugia. However, the role of Plio-Pleistocene climate
18 change could not be rejected. While the molecular dating also makes
19 the influence of geological arches according to the Paleogeography
20 Hypothesis extremely unlikely, the recent Pliocene formation of the
21 Fitzcarrald Arch and its effect of subsequently altering drainage pattern
22 could not be rejected. The data presented here support the interactions

of both climatic and hydrological changes resulting from geological activity in the Plio-Pleistocene, in shaping the phylogeographic structure of the giant otter.

1. Introduction

The giant otter, *Pteronura brasiliensis*, is distributed throughout north and central South America, occurring in the drainage basins of the Amazon (including the Tocantins), Orinoco and Paraná–Paraguay as well as the Caribbean-draining Guianan river systems (Essequibo, Corentyne, Marowijne and Oyapock) and the eastern Brazilian São Francisco Basin (Fig. 1). The species is currently listed as Endangered in the IUCN Red List following population collapse as a result of over-hunting for its pelt in the last century (Duplaix et al. 2008). Although the threat of commercial hunting has declined, habitat degradation has increased. The environmental impact from mega-projects such as the canalization of the River Paraguay, the construction of hydroelectric dams throughout Amazonia and the transposition of the River São Francisco, combined with widespread gold mining and increasing river traffic, cast into doubt the recovery of the giant otter (Gottgens et al. 2001; Groenendijk et al. 2005; Duplaix et al. 2008). Due to these continuing pressures, there is an urgent need to determine the level of genetic diversity that exists within the

1 species and how this is distributed across the range. Understanding the
2 geographic distribution of genetic variation within a species enables the
3 pinpointing of regions of high conservation importance, such as
4 populations which display a history of reproductive isolation from one
5 another and within which there may exist local adaptations (Moritz 2002).
6 Such an understanding is fundamental for targeting conservation efforts
7 to successfully manage the recovery of endangered species such as the
8 giant otter (Awise 2000).

9

10 Previous taxonomic divisions have recognized two subspecies, *Pteronura*
11 *brasiliensis brasiliensis* occurring throughout Amazonia, the Orinoco and
12 Guianas, and *P. b. paranensis* restricted to the Pantanal and the rivers
13 Paraná and Paraguay of the southerly flowing Parana'-Paraguay
14 drainage basin (Rengger 1830). The Pantanal subspecies was considered
15 to have a broader skull and larger teeth (Harris 1968). However, the
16 validity of this subdivision has been questioned due to the abnormal
17 dentition of the type specimen (Carter and Rosas 1997). Garcia et al.
18 (2007) used partial mitochondrial control region, cytochrome *b* and
19 cytochrome *c* oxidase I sequence data to investigate genetic variation
20 among giant otter populations in the central Amazon, parts of the River
21 Negro, Tapajos and Pantanal. This study found some evidence of
22 phylogeographic structure between the Amazon and Parana-Paraguay

1 basins, but found an absence of structure within the central Amazon,
2 paving the way for the more extensive study we present here.

3

4 The giant otter is supported as being sister to the remaining Lutrinae, and
5 the two lineages are considered to have last shared a common ancestor
6 in the Miocene (Koepfli et al. 2008). Fossil evidence of *Satherium*
7 *piscinarium*, often considered a putative ancestor of the giant otter, has
8 been found in strata dated to the Pliocene (4.7 Ma) in North America
9 (Bjork 1973; Lindsay et al. 1984). However, resolving the exact relationship
10 of *Satherium* to *Pteronura* and the timing of the entry of the giant otter's
11 ancestor into South America and subsequent divergence of populations
12 has been confounded by a lack of fossil evidence. While Neotropical
13 otter (*Lontra longicaudis*) fossils dating to 0.98-1.8 Ma have been found in
14 Argentine deposits (Berman 1994 cited in Prevosti and Ferrero 2008), very
15 few giant otter fossils have been found in South America, the oldest of
16 which dates to the Pleistocene, 130,000-120,000 years before present (BP)
17 (Prevosti and Ferrero 2008). It is feasible that its ancestor may have crossed
18 to South America before the Great American Faunal Interchange (GAFI)
19 date of 3.5 Ma, as waif dispersal across the seaway had been occurring
20 for several million years beforehand (Flynn et al. 2005). Raccoons, for
21 example, are thought to have invaded mainland South America up to 7

1 Ma, before complete closure of the isthmus (Fulton and Strobeck 2007;
2 Koepfli et al. 2007; Coates et al. 2004).

3

4 South America, and Amazonia specifically, holds some of the greatest
5 species diversity on the planet (Chazdon and Whitmore 2002), and over
6 the last 40 years our understanding of the ecological changes occurring
7 within the continent and their impact on species diversification has greatly
8 improved. Nevertheless, there has been a lack of investigation into the
9 reason for phylogeographic patterns within species (Tchaika et al. 2007).

10 This study adds to our understanding of Amazonian processes and
11 important regions of genetic diversity by focusing on the semi-aquatic
12 giant otter, investigating the impact of three hypotheses as explanations
13 for shaping the phylogeography of the species. These relate to
14 geological, hydrological, and climatic change experienced by South
15 America over the course of the Pliocene and Pleistocene. While giant
16 otters are semi-aquatic and thus not confined to water bodies to the
17 same extent as fish, often travelling overland to reach isolated ponds,
18 they are among the most aquatically adapted of all lutrines and are not
19 thought to travel large distances overland. Therefore, we have tested only
20 those hypotheses that may be equally applicable to both aquatic and
21 terrestrial taxa.

22

1 The Paleogeography Hypothesis considers that patterns of geological
2 uplift in the Andes foreland basin may have led to populations becoming
3 separated and diverging through vicariance (Haffer 1997). In particular,
4 the rising of geological arches in Western Amazonia is considered to have
5 altered flow regimes, splitting lineages on either side of the arches,
6 resulting in vicariant divergence (Da Silva and Patton 1998; Hubert and
7 Renno 2006). This hypothesis has been challenged by Rossetti et al. (2003)
8 and Wesselingh and Salo (2006). These authors suggest that these
9 structures are predominantly extremely ancient (Palaeozoic and
10 Mesozoic) and buried under subsequent sedimentary deposits to the
11 extent that they barely cause elevations and could not be responsible for
12 the phylogeographic pattern seen in the majority of taxa. However,
13 Roddaz et al. (2006), Espurt et al. (2007), Regard et al. (2009) and Roddaz
14 et al. (2010) have shown that in Western Amazonia the Fitzcarrald Arch is a
15 more recent geological feature, and that while the origins lie in the
16 Tertiary, the arch itself is no older than the Pliocene and had a
17 pronounced effect in shaping the flow of the rivers in western Amazonia
18 (Toivonen et al. 2007). The Paleogeography Hypothesis predicts that sister
19 lineages will be found on either side of a geological arch and that in most
20 cases divergence will date to Middle Miocene or earlier, whereas on
21 either side of the Fitzcarrald Arch, divergence will date to the Pliocene
22 (Table 1; important Amazonian geological arches shown in Fig. 1).

1

2 The Hydrogeology Hypothesis (Montoya-Burgos 2003) considers the role of
3 geological changes over the course of the Mio-Pliocene and Pleistocene
4 in the establishment of the modern drainage patterns and the vicariance
5 of populations of aquatic species (Lundberg et al. 1998). In this study the
6 definition of Hubert and Renno (2006) is used rather than the broader
7 definition of Montoya-Burgos (2003). Specifically, the hypothesis considers
8 a degree of isolation according to drainage basin, with watershed
9 breakdowns and headwater captures leading to a founding population
10 in the neighboring basin. Consequently, sister lineages will be found on
11 either side of watersheds in the headwaters of the catchments, and
12 lineages within one drainage basin will fall within a larger clade with
13 lineages from a neighboring basin. Evidence of populations being
14 structured by tributaries has been found in numerous species of
15 Neotropical fish (Montoya-Burgos 2003; Hubert and Renno 2006), frogs
16 (Garda and Cannatella 2007), and river turtles (Pearse et al. 2006).

17

18 The Refugia Hypothesis considers climatic change as the main driver of
19 diversification. The hypothesis states that episodic cooling events resulting
20 from Milankovitch cycles have caused rainfall over Amazonia to reduce,
21 leading to periodic reduction in river outflow and decline of rainforests.
22 Rainforest communities subsequently split into isolated refugia separated

by arid pampas (Haffer 1969; van der Hammen 1975). These refugia are considered to have led to divergence and areas of endemism from which new lineages arose. Phylogeographic patterns attributed to glacial refugia have been described in a variety of terrestrial taxa (Whitmore and Prance 1987), but also in Neotropical fish (Hubert et al. 2007). The hypothesis predicts that phylogeographic structure will agree with patterns of endemism identified by Whitmore and Prance (1987) and Hubert et al. (2007). While Aleixo (2004) considered predictions of the Refugia Hypothesis to include a demographic expansion which took place following the end of the Last Glacial Maximum (LGM) (20,000 years BP) along with low genetic variability and shallow phylogeographic structure, this only considers the influence of the LGM on shaping the pattern of phylogenies. By contrast, Haffer (1997) claimed that the Refugia Hypothesis is applicable to climatic change as far back as the Tertiary. Consequently rejection of Aleixo's predictions, and rejection of the role of the LGM, is not considered sufficient to reject the Refugia Hypothesis.

1.1 Specific aims

Research interest in Amazonian phylogeography has greatly increased in recent years (Antonelli et al. 2010). However, despite some thorough investigations conducted predominantly on the phylogeography of

1 ichthyological and avifaunal communities (Lundberg et al. 1998; Montoya-
2 Burgos 2003; Fjeldså 1994; Nores 1999; Aleixo 2004; Lovejoy and Aruajo
3 2000; Hubert and Renno 2006; Hubert et al. 2007), there remains a paucity
4 of studies which evaluate the geological, climatic and hydrological
5 processes which have shaped the present pattern of biodiversity. The
6 otter makes for an interesting study animal due to its semi-aquatic/semi-
7 terrestrial habits, which both restrict it to riparian and lacustrine
8 environments yet enable it to travel some distance overland. This study first
9 investigates the degree of mitochondrial genetic diversity within the
10 endangered giant otter and determines the degree to which populations
11 are structured. Second, it investigates the evolutionary history of the
12 species by testing for evidence of the three hypotheses of Amazonian
13 diversification outlined above.

16 2. Material and methods

18 2.1 Sample collection, storage and DNA extraction

19 Sequence data from a total of 70 genetic samples distributed among
20 eight countries and four drainage basins were used in this study (Fig. 1 and
21 Appendix A). The sequence data originate from 34 sites and comprise a
22 composite of fecal and museum samples collected for this study, and

sequences from the Brazilian Amazon and Pantanal reported by Garcia et al. (2007). The majority of samples collected for this study were fecal in origin, collected from the field in Bolivia, Peru and Guyana. These samples consisted of 3-5ml of fresh spraint taken from latrines shortly (<3hrs) after deposition and stored in 10ml of 90% ethanol. Museum samples consisted of dried skin clippings, adhered tissue to skulls, teeth and bones, and were collected and stored dry in sealed Eppendorf tubes or paper envelopes. Tissue or fecal samples from captive animals of known provenance were also collected and stored in 90% ethanol (the origin of each sample is given in Appendix A). Fecal samples were extracted using the QIAamp® DNA Stool Mini Kit (Qiagen) following the manufacturer's protocol with one modification; the incubation period with Buffer ASL was extended overnight with agitation at 37°C. Fresh tissue samples, museum skin clippings, and adhered skull tissue were extracted using the DNeasy® Tissue Extraction Kit (Qiagen). DNA was extracted from bone and teeth using the non-destructive guanidine method according to the protocol of Rohland and Hofreiter (2007). All extraction of DNA from museum samples was conducted in a dedicated room away from sources of contamination. Filter tips were used throughout the study and all equipment was regularly irradiated using UV light. Negative controls were used in each batch of DNA extractions and in each PCR and agarose gel run.

1

2 *2.2 DNA amplification and sequencing*

3 The entire giant otter cytochrome *b* (1140 bp) and control region (936 bp)
4 genes were amplified in six samples from disparate geographical sites
5 using the mustelid primers developed by Koepfli and Wayne (1998) and
6 primers developed in the Eurasian otter, *Lutra lutra*, by Mucci et al. (1999).
7 Variable regions were identified, resulting in 612 bp at the 3' end of
8 cytochrome *b* and 383 bp at the 5' end of control region being selected
9 for use in this study. Primers specific to the giant otter were then designed
10 to amplify this 995 bp sequence, amplifying six overlapping fragments of
11 ~300 bp each in order to obtain sequence from highly degraded fecal
12 and museum DNA (see Appendix B for primer sequences). Amplification
13 was carried out in 10 µl PCR reactions containing 3 µl template DNA, 3 µl
14 of 25 µM primer solution, 0.2 µg bovine serum albumen, autoclaved Milli-Q
15 water and 4 µl Qiagen® multiplex PCR kit (containing master mix,
16 HotStarTaq, MgCl₂, dNTPs and PCR buffer). PCR was performed using a 95
17 °C denaturing step for 12 min followed by 40 cycles of denaturing at 94 °C
18 for 30 s, annealing at 54 °C for 1 min 30 s, and extension at 72 °C for 1 min
19 30 s, with a 2 °C drop in annealing temperature every 10 cycles, and a
20 final extension period at 60 °C for 30 min. Following amplification, PCR
21 products were run on a 2% standard agarose gel stained with 0.02 µl of 10
22 mg/ml ethidium bromide and the bands excised before extraction of the

product using the QIAquick® Gel Extraction Kit (Qiagen). Sequencing reactions took place in a reaction volume of 15 µl with 5 µl PCR product, 1 µl BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), 5 µl Better Buffer (Web Scientific), 3 µl 0.8 µM individual primer solution and 1 µl ddH₂O. The sequencing reaction involved a 94 °C denaturing step for 3 min followed by 25 cycles of denaturing at 94 °C for 15 s, annealing at 50 °C for 10 s with extension at 60 °C for 4 min, and a final extension period at 60 °C for 5 min. Following sequencing, the sequenced product was cleaned using washes of 100% ethanol and 125 µM EDTA followed by 70% ethanol precipitation and centrifugation at 30000 x g for 30 min and 16,500 rpm for 15 min. Sequenced product (1.2 µl) was run on an ABI 3100 Sequencer (Applied Biosystems) with 9 µl formamide.

2.3 Analysis of genetic variability and phylogeographic structure

The chromatograms of all sequences were checked by eye and edited using Sequencher 4.8 (Gene Codes Corporation). A conservative approach was taken in which a polymorphism had to be evident in both forward and reverse strands to be accepted. Sequences were checked against reference giant otter sequences using BLAST searches. The six fragments of control region and cytochrome *b* for each sample were concatenated and these sequences were aligned with the 30 sequences

1 from Garcia et al. (2007) in Sequencher. All new sequences have been
2 deposited in GenBank (accession numbers JN252256-JN252295).

3

4 Nucleotide and haplotypic diversity and F_{ST} values were calculated using
5 DNASP 5 (Librado and Rozas 2009). The program Modeltest 3.6 (Posada
6 and Crandall 1998) was used to select the model of nucleotide
7 substitution that best fit the data. Cytochrome *b* and control region
8 fragments were analyzed both separately and as concatenated
9 sequence. In each case the model of evolution selected by Akaike
10 Information Criterion was HKY+I+G with equal proportion of invariable sites
11 (*I*) and transition/transversion ratios (*ti/tv*) for both control region and
12 cytochrome *b* (*I* = 0.805 and 0.839, *ti/tv* = 7.684 and 7.353 respectively). To
13 establish that these genes did not have differing phylogenetic signals, ML
14 trees with 100 bootstrap replicates were generated separately for each
15 gene partition and the topology of the consensus trees compared. The
16 trees of both genes exhibited the same deep splits in topology, suggesting
17 that the two genes could be concatenated for phylogenetic and
18 network analysis.

19

20 Phylogenetic relationships among haplotypes were analyzed using
21 MrBayes 3.1 (Ronquist and Huelsenbeck 2003). Sequence data were run
22 under a variety of evolutionary models and the best-fitting model was

selected using Bayes factors (Kass and Raftery 1995). This selected the HKY model of evolution, corroborating the Modeltest result. Four simultaneous chains were run for 13 million generations, sampling every 100th generation and excluding the initial 13,000 runs as burn-in before plotting the consensus tree. The sea otter, *Enhydra lutris*, and the Eurasian otter, *Lutra lutra*, were used as outgroups to root the tree. The MrBayes analysis was run twice, yielding the same topology in each case. To test the accuracy with which phylogeny could be inferred using the sequence data, the Bayesian approach was compared with the ML method implemented using PhyML 3.0 (Guindon and Gascuel 2003) in which node support was verified by bootstrapping for 1000 replications. The relationships between populations were also resolved using the statistical parsimony approach implemented using the program TCS (Clement et al. 2000). Up to 947 bp of concatenated cytochrome *b*/control region was sequenced in each sample. However, the sequences from the study by Garcia et al. (2007) were 129 bp shorter than our own and one sample from the River Madeira was sequenced to 482 bp.

Phylogeographic structure was tested using SAMOVA 1.0 (Dupanloup et al. 2002). The program was run with the number of populations prior (*K*) of between 2 and 20, with 100 simulations per *K*. No populations were predetermined and each sampling site was considered alone as a

'population' for the sake of the test. The actual number of phylogeographic groups was identified using the difference between the measure of among-group variation for each K (ΔF_{CT}) (Dupanloup et al. 2002). To test for a pattern of isolation by distance, we first partitioned the samples into 11 sampling regions, determined by continuous aquatic provinces, such as the Essequibo basin, the River Negro and tributaries and restricted to 1000 km². The geographical coordinates were taken as the point equidistant from all sampling sites within the 'sampling region' and geographic distances among sampling regions calculated. A Mantel test was performed in Arlequin 3.0 (Excoffier et al. 2005) using geographic distance and $F_{ST}/(1-F_{ST})$.

2.4 Molecular clock calibration and estimation of phylogroup divergence times

Evidence of fixed rate evolution among lineages was also tested for using the likelihood ratio test. This was conducted in PAUP*4.0 (Swofford 2002) using the model of evolution determined by Modeltest to compare the likelihood scores of strict clock and non-clock models. The significance of the resulting test statistic was determined using a Chi-squared test, which produced a highly significant result ($P < 0.001$), rejecting the strict clock model.

1

2 Timing of phylogroup divergence was resolved using Bayesian coalescent
3 analysis implemented with the software BEAST 1.5.3 (Drummond and
4 Rambaut 2007). The protocol of Lebarbenchon et al. (2010) was followed
5 in which the program was run using three different molecular clock
6 models (strict clock, relaxed uncorrelated exponential, and relaxed
7 uncorrelated lognormal) and three different coalescent priors (constant
8 size, exponential growth, and expansion growth). Results were compared
9 against the null model (strict clock, constant size) using Bayes factor in
10 Tracer 1.4 (Drummond and Rambaut 2007), in which a log₁₀ Bayes factor
11 of greater than 1 was considered evidence in favor of the alternative
12 model (Kass and Raftery 1995).

13

14 Three outgroups were used to root the phylogenetic tree. These
15 comprised two lutrines (the sea otter and Eurasian otter) and one
16 musteline (the stoat, *Mustela erminea*). Taxonomic groupings were
17 specified using the phylogenetic relationships of the Mustelidae reported
18 by Koepfli et al. (2008). In setting nodal calibration points, the lognormal
19 distribution date range was used as recommended by Ho (2007). In
20 setting constraints using the lognormal distribution, the fossil date was
21 considered an absolute lower bound, with the 95% highest posterior
22 density (HPD) lying between the fossil date and a soft upper bound two

million years earlier. The entire tree was constrained by a lognormal distribution with an absolute lower bound of 5.3 Ma, considered the earliest fossil record of the genus *Mustela* (The Paleobiology database <http://paleodb.org>). The earliest appearance of the genus *Lutra*, dating to 3.6 Ma, was used as a minimum lower bound to date the split between the sea otter and the Eurasian otter (Willemsen 1992). The split between the giant otter clade and the sea otter and Eurasian otter clade is considered to have occurred early in the evolutionary history of the lutrines (Koepfli and Wayne 1998; Koepfli et al. 2008). However, the earliest fossil record of the giant otter's putative ancestor, *Satherium piscinarium*, dates to no earlier than 4.7 Ma in North America (Bjork 1973; Lindsay et al. 1984). Despite morphometric analysis supporting a close relationship between *Satherium* and *Pteronura* (Prevosti and Ferrero 2008), the ancestry of *Satherium* to *Pteronura* remains controversial. Consequently, fossil records of *Satherium* were not used to constrain the tree. BEAST was run for 10 million generations, sampling every 1000th iteration with a burn-in of 10%. Two chains were run and convergence checked using Tracer. Runs were performed for each clock model and coalescent priors and Bayes factor between the likelihoods determined using a bootstrap of 1000.

Evidence of recent population expansion, as predicted by the Pleistocene Refugia hypothesis, was investigated by testing Fu's F_s (Fu 1997) in the control region sequences of each of the phylogroups resolved using SAMOVA. A significantly negative F_s is considered to be evidence against population stasis. The nature of the demographic change was then characterized by comparing mismatch distributions of sequence differences (Rogers 1995). Both Fu's F_s and mismatch distributions were calculated using Arlequin. The dates of population expansions were estimated using the method of Rogers and Harpending (1992). The demographic expansion parameter tau (τ), was estimated in Arlequin and used to calculate time of expansion (t) using the formula $\tau = 2ut$, in which $u = \mu k$ where μ is the mutation rate per site per year and k is the length of the sequence. We used the 1.92% per million years sequence divergence rate between the weasel (*Mustela nivalis*) and ermine (*Mustela erminea*) calculated by Marmi et al. (2004), but due to evidence that mutation rates are higher in tropical species (Weir and Schluter 2007), we also used the faster 10% per million years divergence rate calculated by Vila et al. (1999) for the split between grey wolves (*Canis lupus*) and coyotes (*Canis latrans*).

3. Results

1

2 *3.1 Genetic variation and phylogeographic structure*

3 Fifty-nine polymorphic sites were identified in the mtDNA sequence
4 spanning up to 946 bp of cytochrome *b* and the control region. Overall
5 haplotype diversity was high at 0.93 with 41 haplotypes resolved, and
6 nucleotide diversity was also high ($\pi = 0.015$). Genetic diversity was high in
7 all of the phylogroups (described below) except for the Pantanal ($h =$
8 0.44), where a single haplotype dominated 75% of all sequences (A table
9 of mutational differences among haplotypes is given in Appendix C)

10

11 Four phylogroups were resolved in SAMOVA using the ΔF_{CT} method,
12 comprising the Iténez and River Uruá (1); the Madre de Dios and Madeira
13 (2); the Pantanal (3); and the Amazon, the Orinoco and the Guianas (4).
14 This grouping explained 65% of the variation, with 18% explained by
15 among populations within-group variation, and 17% due to within-
16 population differences. The phylogeographic clustering resolved with
17 SAMOVA was supported by the statistical parsimony network (Fig. 2),
18 which revealed three differentiated clusters in the south-west of the giant
19 otter's range. Genetic distances among phylogroups were high, with F_{ST}
20 values ranging from 0.64 to 0.74, implying a strong degree of structuring
21 (Table 2).

22

1 Within the Amazon/Orinoco/Guianas phylogroup, three weakly-supported
2 sub-groups were identified with haplotype network analysis, namely the
3 Central Amazon and Guianas; the Orinoco; and the Upper Amazon (Fig.
4 2). However, Bayesian phylogenetic reconstruction only differentiated two
5 subclades: the Guianas and Central Amazon, and the Orinoco and Upper
6 Amazon. Maximum likelihood analysis failed to resolve subdivisions within
7 the phylogroup (Fig. 3).

8
9 The relationship of the Madre de Dios and Madeira populations within
10 their phylogroup was unresolved. They cluster as a single phylogeographic
11 group in the network analysis. However, there are a number of mutational
12 differences between the Madre de Dios and Madeira sequences (Fig. 2,
13 Appendix C). When K in SAMOVA is increased to five, the Madre de Dios
14 and Madeira sequence sets are split into two distinct phylogeographic
15 groupings. The relationship is also not well resolved either with Bayesian or
16 maximum likelihood phylogenetic reconstructions (Fig. 3), nor is the
17 relationship of the phylogroup to the Iténez, becoming paraphyletic in ML
18 and BEAST phylogenetic reconstructions (Fig. 3, Fig. 4).

19
20 Repeated analysis using different mustelid outgroups (*Eira barbara*,
21 *Mustela erminea*) produced the same topology, as did performing

1 unrooted phylogenetic analyses, implying that the resulting topology is
2 unlikely to be due to long branch attraction to the outgroup sequences.

3

4 *3.2 Phylogroup divergence times*

5 The null clock model: strict clock (likelihood -2995.11), was rejected by
6 both the relaxed exponential (-2984.36) and the relaxed lognormal (-
7 2993.25) clocks with Bayes factors >20. By contrast, the comparison of
8 coalescence priors did not lead to the rejection of the null model:
9 constant growth (-2995.11) by either expansion growth (-2994.67) or
10 exponential growth (-2994.92) (Bayes factors <2). Consequently, the most
11 appropriate model used to estimate timings of phylogroup divergence
12 was the relaxed exponential clock with constant growth.

13

14 Two stages of divergence can be observed from the molecular dating.
15 Firstly, the Iténez and Madre de Dios/Madeira phylogroups diverged from
16 the rest of the giant otter populations during the Early Pleistocene 1.24-
17 1.69 Ma (0.72-3.1 Ma 95% HPD). Subsequently, the Pantanal and
18 Amazon/Orinoco/Guianas phylogroups diverged from one another 0.88
19 Ma (0.2-1.6 Ma 95% HPD) during the Middle Pleistocene (Fig. 4, Appendix
20 D).

21

22 *3.3 Demographic expansion*

Analysis of the demographic history of the phylogroups produced ambiguous results. The neutrality test suggested expansions had occurred in all four phylogroups ($F_s = -3.58$ to -26.66 , $P = 0.01$). Characterizing the expansion further with mismatch analysis, a smooth wave with an expected distribution close to the observed was recorded for the Amazon/Orinoco/Guianas and Pantanal phylogroups, indicative of a population which had recently expanded (Fig. 5). The rough, multimodal distribution of the Madre de Dios/Madeira and Iténez phylogroups, by contrast suggests a lack of recent population growth. However, low sample sizes in three of the phylogroups may have reduced the power of the test to discern a demographic expansion. The phylogroups were calculated to have expanded between 672,000 and 407,000 years BP during the Middle Pleistocene using the mustelid divergence rate of Marmi et al. (2004). The faster canid divergence rate of Vila et al (1999) yielded a lower bound of between 130,000 and 66,000 years BP.

3.4 Isolation by distance

A weak relationship between the geographic and genetic distances among the 11 sampling regions ($R^2 = 0.11$) was not found to be significant ($P = 0.06$), indicating that isolation by distance is unlikely to have generated the observed phylogeographic structure.

1

2 4. Discussion

3

4 4.1 Genetic variation and phylogeographic structure in the
5 giant otter

6 This study reveals a high degree of genetic diversity in the giant otter
7 compared with other lutrines. Ferrando et al. (2004) found levels of
8 haplotype diversity of 0.16 (number of samples [n] = 73) with a single
9 haplotype dominating throughout Europe and Russia in the Eurasian otter,
10 *Lutra lutra*. Similarly, the heavily persecuted sea otter, *Enhydra lutris*, shows
11 a haplotypic diversity of only 0.18-0.4 (n = 24-36) (Larson et al. 2002). This
12 disparity may also reflect the faster rate of molecular evolution and lower
13 extinction rate of lineages recorded in many tropical species, including
14 mammals (Weir and Schluter 2007; Gillman et al. 2009). However,
15 haplotypic diversity in the giant otter is also higher than that so far
16 recorded in the Neotropical otter, *Lontra longicaudis*, h = 0.82 n = 20
17 (Trinca et al. 2007) and southern river otter, *Lontra provocax*, h = 0.71, n =
18 25 (Centron et al. 2008). Our results also contrast with the lower haplotype
19 and nucleotide diversities previously found in the giant otter by Garcia et
20 al (2007), (h = 0.87, π = 0.006), which was limited geographically to the
21 central Amazon and one site in the Pantanal and restricted in terms of

sample size ($n = 30$). The level of diversity found in our study was also high throughout three of the phylogeographic groups of populations (Table 3) and this remains the case when museum samples dating to the middle of the last century are removed. The high levels of mitochondrial genetic diversity we observed in the Amazon/Orinoco/Guianas, Iténez, and Madre de Dios/Madeira phylogroups are somewhat surprising given the extent of the demographic decline known to have occurred, and may be partly attributed to the giant otter's longevity (up to 13 years recorded in the wild) and long generation time (reported as seven years in Groenendijk et al. 2004) buffering attrition of genetic diversity (Hailer et al. 2006). However, the lower haplotype and nucleotide diversity seen in the Pantanal phylogroup, with a single dominant haplotype found in nine of the twelve individuals sequenced, may be due to a more sustained level of persecution. Between 1960 and 1969 12,390 skins were exported from the Brazilian Pantanal alone, constituting 24% of the total Brazilian export during this time (Harris et al. 2005).

The genetic diversity is not evenly geographically distributed and despite the smaller total area, lower sample numbers, and number of sampling sites, the south-western fringe of the giant otter's range comprises three of the four phylogroups and contains a similar level of mean haplotypic diversity ($h = 0.87$, $\pi = 0.015$) to the entirety of northern Amazonia ($h = 0.88$,

$\pi = 0.0067$). No strong support is given to the previous subspecies division between the giant otter populations of the Parana'-Paraguay drainage, *P. b. paranensis*, and the Amazon basin, *P. b. brasiliensis*, agreeing with Garcia et al. (2007). In contrast, the phylogroups of the Iténez and Madre de Dios/Madeira within the Amazon basin itself appear distinct from other Amazonian populations. Sample 2.11 (origin: River Negro, Garcia et al. 2007) is unusual in that it exhibits Haplotype 1, the most common haplotype in the Pantanal. The most parsimonious explanation is likely to be that of convergent evolution rather than representing evidence of migration between the Pantanal and River Negro.

4.2 Support for the hypotheses of divergence

4.2.1 Paleogeography Hypothesis

The impact of the formation of geological arches in driving vicariance in South American species has been largely unacknowledged until recently (Räsänen et al. 1990) and remains controversial (Rossetti et al. 2005; Wesselingh and Salo 2006). Da Silva and Patton (1998) provide evidence of phylogeographic pattern being determined by tectonic forces in terrestrial mammals on either side of the Iquitos Arch. The uplift of the Vaupes Arch in separating the Amazon and Orinoco drainages has also been considered a driver of the divergence of piranhas in the genus *Prochilodus* by Sivasundar et al. (2001). Within the giant otter, the strong

pattern of divergence seen between the phylogroups of the Upper Madeira and the Amazon/Orinoco/Guianas might be considered evidence of a vicariance event in which any freshwater contact between the Amazon tributary, the River Ucayali, and the Upper Madeira tributary, the River Madre de Dios, would have been broken by the formation of the Fitzcarrald Arch. This region has remained geologically active into the Late Quaternary (Latrubesse and Rancy 2000), with uplift of the Fitzcarrald Arch continuing into the Pliocene (Roddaz et al 2010). Da Silva and Patton (1998) resolved divergence times between lineages of between 1-3 million years, which they considered to have been driven by the impact of Andes foreland dynamics on the landscape of western Amazonia rather than by climatic change. The rivers of the region are particularly dynamic (Toivonen et al. 2007), with striking shifts in flow recorded over very recent geological time. The River Beni for instance, one of the principal tributaries of the River Madeira, has migrated anticlockwise over the course of the Holocene leading to a change in flow orientation of 45 degrees over no more than 10,000 years in response to the uplifting Andes piedmont (Dumont 1996). Wilkinson et al. (2007) have pointed out that such dynamic behavior by river courses and periodic river-switching events may lead to fragmentation and a degree of isolation of riparian populations.

1 Molecular dating of the divergence time between giant otter lineages in
2 the Upper Amazon and Upper Madeira cannot rule out the role of the
3 Paleogeography Hypothesis, with complete divergence of the
4 phylogroups occurring around 1.24 Ma. However, the likelihood of the
5 Vaupes Arch influencing the divergence of Amazon and Orinoco
6 subclades or the Michicola Arch influencing the Pantanal/Iténez split is
7 extremely low due to the very old (Mesozoic) age of these structures
8 (Limarino and Spalleti 2006; Rossetti et al. 2005).

9

10 4.2.2 Hydrogeology Hypothesis

11 The Hydrogeology Hypothesis, according to the definition of Hubert and
12 Renno (2006), states that a headwater capture event or watershed
13 breakdown will have led to the colonization of a neighboring drainage
14 basin and subsequent retraction of the headwaters will lead to
15 vicariance. It predicts that lineages within one drainage basin will fall
16 within a larger clade with lineages from a neighboring basin. The
17 relationships among the subclades of the Amazon/Orinoco/Guianas
18 phylogroup suggest that the giant otter populations of the Orinoco,
19 Guianas, and Upper Amazon were founded from a Central Amazon
20 population and subsequently diverged from one another through partial
21 isolation. While there are freshwater connections among all three basins,
22 climatic change may have both facilitated movement among the basins

during warmer, wetter periods and reduced inter-basin dispersal during colder, more arid periods. However, this hypothesis is insufficient to explain the divergence of the Madre de Dios/Madeira, the Amazon/Orinoco/Guianas, and the Iténez phylogroups from one another. While the Amazon/Paraná–Paraguay watershed is considered to have formed in the Late Miocene (Lundberg et al. 1998; Montoya-Burgos 2003), much younger ages of divergence between fish populations in Amazon and Paraná–Paraguay basins of between 0.8 and 2 Ma have been found in both the catfish *Pseudoplatysoma* (Torrico et al. 2009) and black-bellied piranha *Serrasalmus* (Hubert et al. 2007), which are considered to have been the result of headwater capture. These dates are broadly consistent with those derived from the divergence of the giant otter Pantanal phylogroup from the Amazon/Orinoco/Guianas within the giant otter (0.88 Ma, 1.60–0.38 Ma 95% HPD) and could constitute evidence for the Hydrogeology Hypothesis.

4.2.3 Refugia Hypothesis

The phylogeographic pattern identified in the giant otter does partially correlate with a pattern of Pleistocene refugia identified in terrestrial fauna and flora by Whitmore and Prance (1987) (Fig. 6). The Iténez phylogroup overlaps the Aripuanã Refugium on the Brazilian Shield, and the Madre de Dios sub-group falls within the Inambari Refugium in the

1 immediate Andes forebasin. The Orinoco and Guianas sub-groups may
2 also overlap with refugia. The Pantanal phylogroup does not correspond
3 with any refugium. Hubert et al. (2007) and Renno et al. (2006) considered
4 the sub-basins of the Iténez and Madre de Dios to have been aquatic
5 refugia during the Pleistocene, leading to divergence in piranha
6 (*Serrasalmus*), and bass, (*Cichla*).

7
8 The divergence dates inferred among the giant otter phylogroups or sub-
9 groups do not correlate with the last glaciation, the Würm glaciation
10 (10,000-80,000 years BP), and evidence from the mismatch analysis in the
11 giant otter phylogroups does not suggest a recent expansion following the
12 LGM, despite accounting for a possible rapid rate of evolution using the
13 faster canid rate. Instead, the expansions are likely to have occurred
14 during the Middle Pleistocene. Other studies of mammals in Amazonia
15 have similarly failed to find evidence of divergence of phylogroups during
16 the last glaciation and range expansion succeeding it (Gonzalez et al.
17 1998; Culver et al. 2000; Lessa et al. 2003). While discounting the role of the
18 LGM, the dates of phylogroup divergence do not rule out the possibility of
19 Plio-Pleistocene climate change driving the observed pattern. While the
20 climate of South America and the corresponding vegetation structure
21 over the last five million years are still debated, evidence from a variety of
22 sources seems to suggest that whereas the Early-Middle Pliocene was

wetter and warmer by as much as 3 °C, exhibiting constant El Niño-type conditions with sea level up to 35 m above present levels, global cooling began in the Late Pliocene as early as 3 Ma (Sloan et al 1996; Molnar and Cane 2007). In contrast to evidence initially put forward by Zarate and Fazenda (1989) of a warm, wet Plio-Pleistocene, Ortiz Jaureguizar et al. (1995) in Vizcaino et al (2004) cite a reduction in the number of fossil rodent burrows in the Argentine strata around 2.4 Ma concordant with the onset of cooler glacial conditions. Leroy and Dupont (1994) provide evidence of aridity from North Africa during the Late Pliocene (3.7-1.7 Ma) in the pollen record, suggesting a reduction in forest type assemblages. This is backed up by evidence of a significant cooling of global ocean surfaces around 2.1 Ma from planktonic discoaster fossils (Chapman and Chepstow-Lusty 1997). Several studies of antarctic sediments suggest the onset of cyclical glaciation began during the Late Pliocene 2.2-2.9 Ma (Whitehead and McMinn 2002; Rebesco and Camerlenghi 2008), while deep sea sediment cores provide evidence of a marked decline in sea surface temperature from 2.1-1.9 Ma (Marlow et al. 2000).

Comparative phylogeographic studies in other large mammalian taxa are lacking. Nevertheless there are several studies which reveal similar divergence times to the giant otter among deep lineage splits. Divergence of major lineages within the crab-eating fox (*Cerdocyon*

1 *thous*) (Tchaitcka et al. 2007), several small South American cat species
2 (Johnson et al. 1999), and brocket deer (genus *Mazama*) (Barbante
3 Duarte et al. 2008) occurred during the Plio-Pleistocene between 3.7 and
4 0.4 Ma, again suggested in response to climate shifts.

5

6 4.3 Conclusion

7 Following testing of three hypotheses of Amazonian diversification, it
8 appears that the phylogeographic pattern observed in the giant otter
9 might be the result of multiple drivers. The molecular dating suggests that
10 due to the long time span over which the four phylogroups diverged from
11 one another (1.69-0.61 Ma, 3.1-0.25 95% HPD), no single paleoclimatic or
12 paleoenvironmental event was likely to be responsible for the pattern. The
13 continued uplift of the Fitzcarrald Arch during the Plio-Pleistocene and the
14 ensuing reordering of fluvial systems driven by continuing geological
15 activity may have been a factor in driving the deepest phylogenetic split
16 in the giant otter. But these alterations in drainage pattern would also
17 have occurred during a time in which the climate of South America
18 became periodically cooler and more arid (Ortiz Jaureguizar et al. 1995;
19 Leroy and Dupont 1994; Chapman and Chepstow-Lusty 1997). Reduced
20 rainfall would have led to rivers changing from predominantly
21 depositional to high energy erosional. The preference of the giant otter for
22 slow moving creeks and ponds over fast-flowing rivers (Duplaix 1980;

1 Zambrana Rojas 2004) suggests that a shift to the high energy rivers would
2 have reduced optimal habitat for the species. Furthermore, the number of
3 inter-drainage connection routes would have shrunk, leading to partial
4 isolation of some populations. The phylogeographic pattern is then likely
5 to have been preserved through isolation by distance and a male
6 exogamous dispersal system (Groenendijk and Hajek 2006).

7

8 *4.4 Conservation*

9 In terms of prioritizing conservation effort within the species, this study has
10 identified four distinct lineages within the giant otter which broadly
11 correspond to four geographic regions. Preservation of genetic diversity
12 and evolutionary potential within the species will rely on ensuring
13 populations within each of these four regions are targeted for
14 conservation.

15

16 Further analysis using nuclear markers will be required to fully resolve
17 whether the four lineages constitute evolutionary significant units (Moritz
18 2002). However, focusing attention on the southern fringe of the range,
19 where a disproportionate amount of the species' total mitochondrial DNA
20 diversity is found, may preserve the greatest amount of diversity in the
21 smallest geographical area. Some recent experimental evidence supports
22 the view that conservation planning targeted to preserve the deepest

1 branched lineages within a species is an effective way of ensuring the
2 survival of that species (Rauch and Bar Yann 2004). This study has
3 determined that the giant otter exhibits high total mitochondrial
4 haplotype diversity and that possibly owing to a long generation time and
5 a number of small, geographically disparate, surviving populations, it has
6 not been left genetically depauperate in three of the four phylogroups
7 despite the population crash of the last century. However, despite the
8 fact that the giant otter population appears to be recovering in the
9 Pantanal (Tomas et al. 2000), the low haplotypic diversity seen there is of
10 concern and may suggest that this phylogroup is the most fragile of them
11 all. Our study lends support to previous research showing evidence of
12 endemism in fish (Hrbek et al. 2005; Renno et al. 2006) and river dolphins
13 (Banguera-Hinestroza et al. 2002) in the tributaries of the Upper Madeira
14 and south-western Amazonia, highlighting the biogeographical
15 importance of this biome for aquatic species across a broad range of
16 taxa.

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5

6

7 Figure Legends.

8

9 Fig. 1 The drainage system of South America and the geographic origin of
10 samples used in this study. Watersheds are outlined by dashed lines and
11 the positions of important geological arches are indicated. Black circles
12 refer to sequences from this study, grey circles to sequences from Garcia
13 et al. (2007).

14

15 Fig. 2 Statistical parsimony haplotype network based on concatenated
16 cytochrome *b* and control region sequence data. Nodes are colored to
17 reflect clustered phylogroups, with sub-clusters within each phylogroup
18 identified. Node size corresponds to haplotype frequency. Grey nodes
19 represent inferred mutational steps between haplotypes.

20

21 Fig. 3 Phylogenetic relationships reconstructed using Bayesian and
22 maximum likelihood methods of concatenated cytochrome *b* and

1 control region dataset. Bootstrap support values greater than 40 and
2 Bayesian posterior probability values greater than 0.50 are shown for
3 phylogroup-level splits. The geographic origin for each of the taxa (coded
4 1.01-8.08) is given in Appendix A.

5

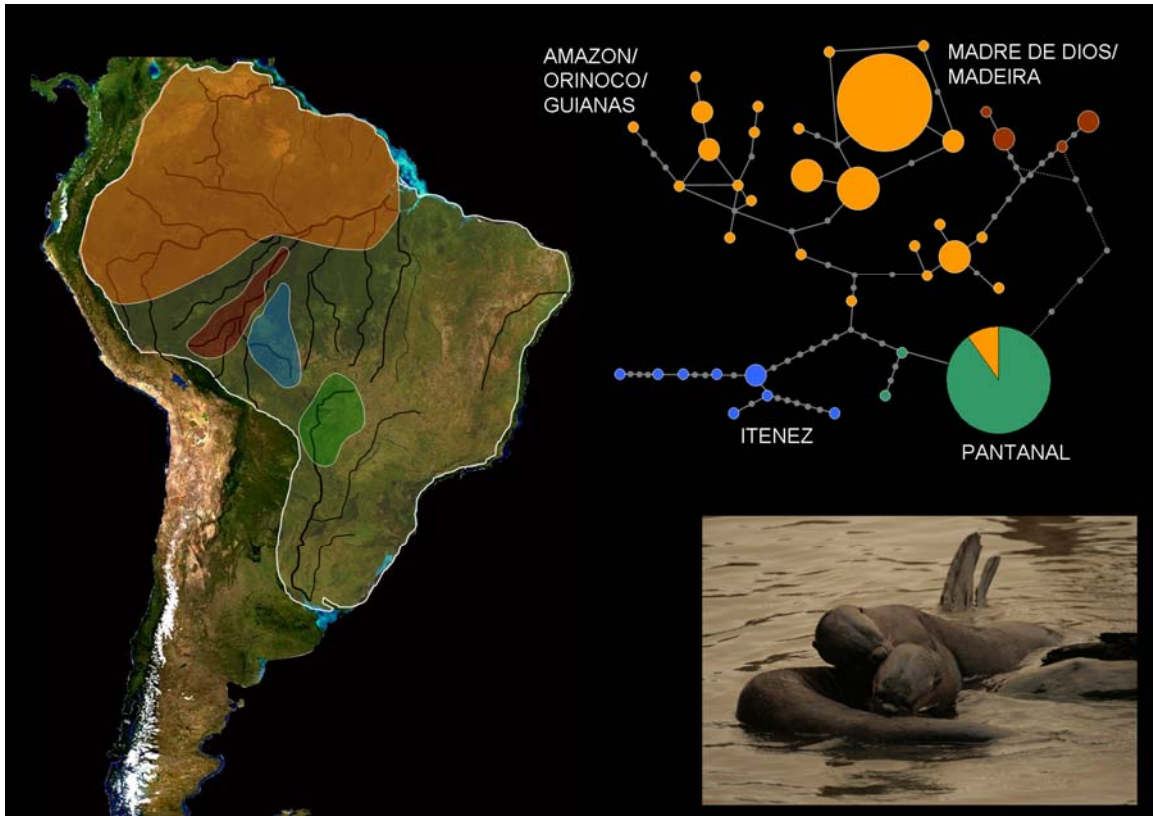
6 Fig. 4 Divergence times of phylogroups as resolved in BEAST. Node bars
7 display the 95% HPD. Time in the axis is given in millions of years before
8 present. Phylogroups resolved by SAMOVA are highlighted.

9

10 Fig. 5 Mismatch distribution of pairwise nucleotide differences among
11 individuals within the four phylogroups. Histograms represent the observed
12 differences, while lines represent the expected distribution.

13

14 Fig. 6 Important regions of genetic diversity within the giant otter
15 correlated with Pleistocene rainforest refugia in grey identified by
16 Whitmore and Prance (1987) based on data gathered from birds,
17 butterflies, and plants and accounting for soil and precipitation. The
18 geographic extent of the four phylogroups are highlighted and the
19 historical extent of the species' range is also shown.



1 Highlights of MPE-10-312R1

2 Evolutionary history and identification of Conservation Units in the giant
3 otter, *Pteronura brasiliensis*

4
5 >In this paper we investigate the phylogeography of the endangered
6 giant otter.

7 >We sequence mitochondrial control region and cytochrome b genes.

8 >We resolve four distinct phylogroups, constituting conservation units.

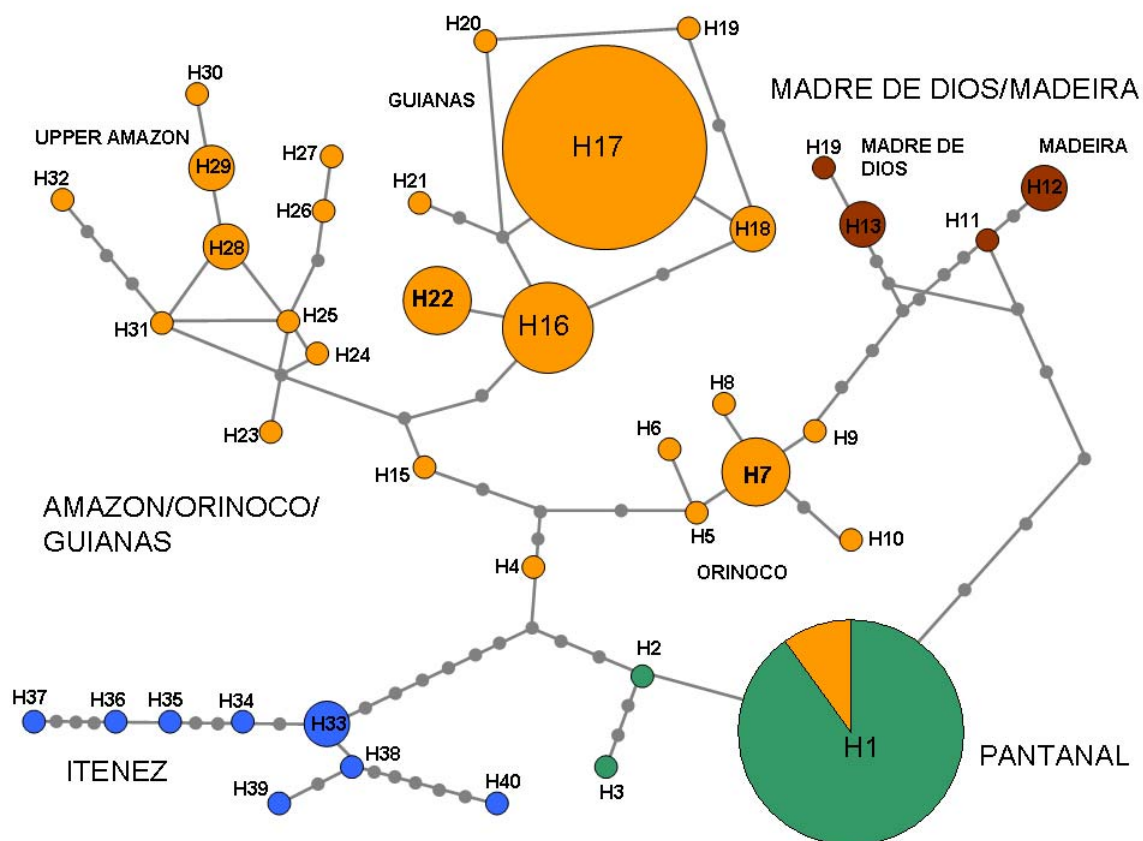
9 >We tested three hypotheses of Amazonian diversification.

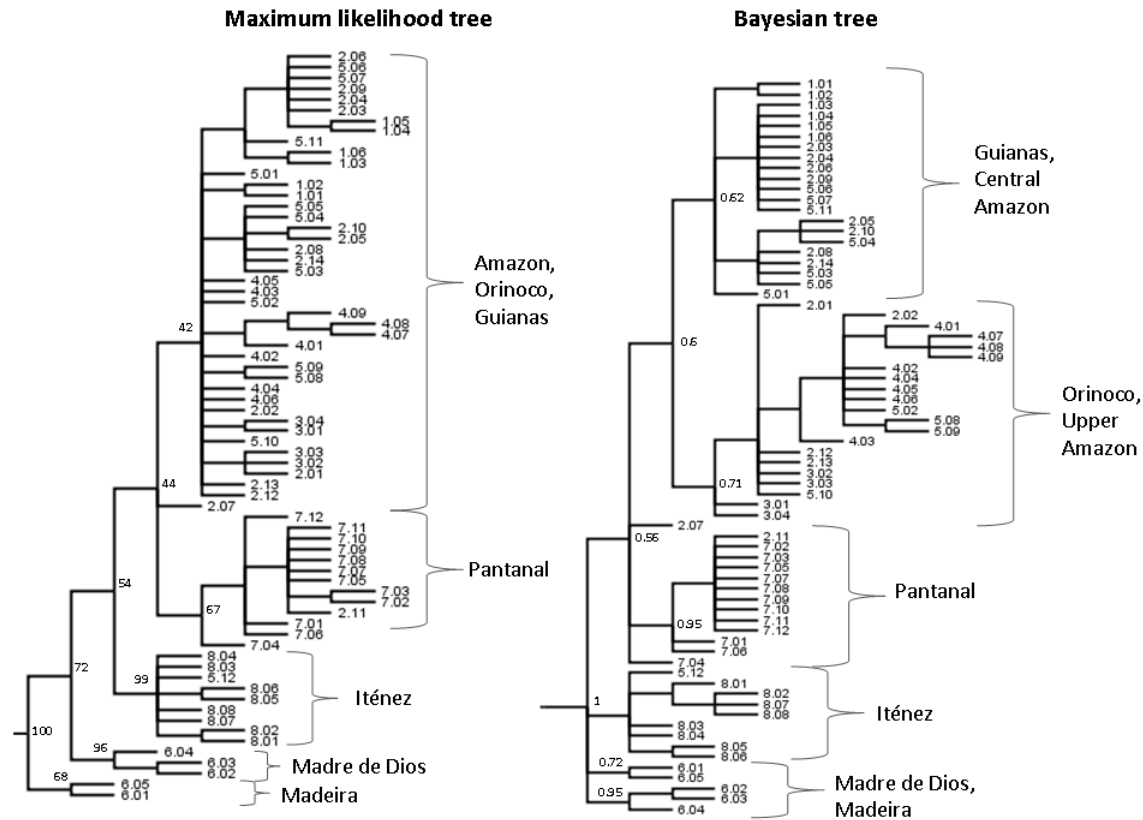
10 >The Plio-Pleistocene origin of the phylogroups appears driven by
11 changes in climate and drainage.

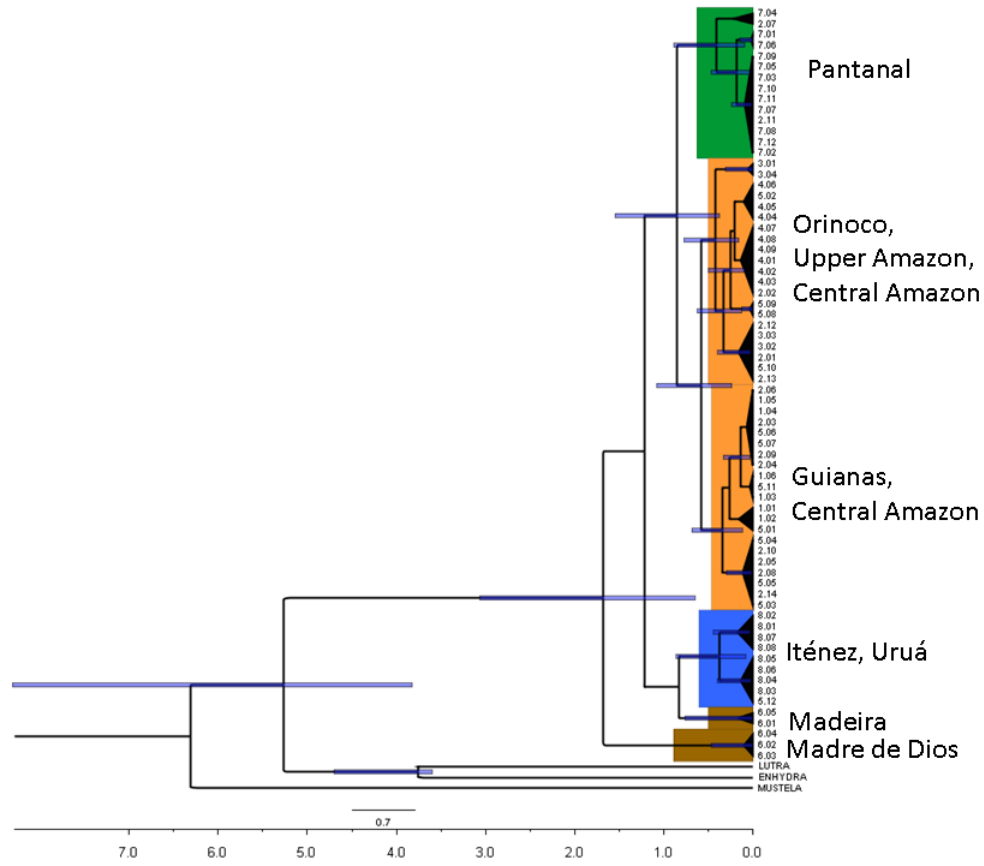
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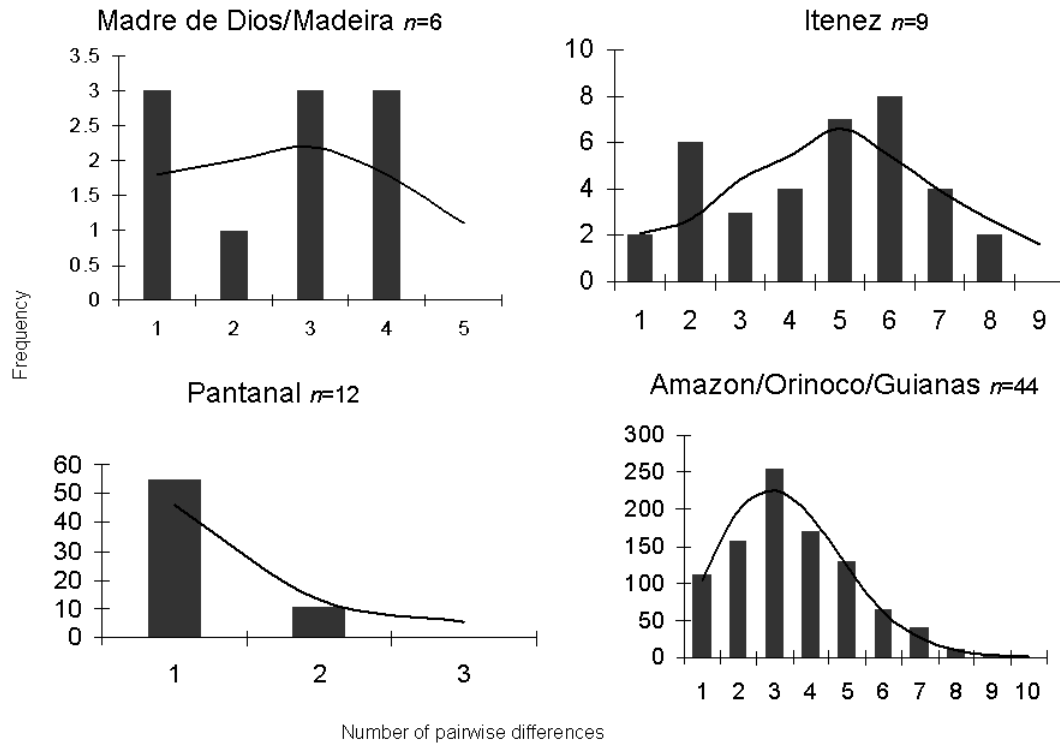
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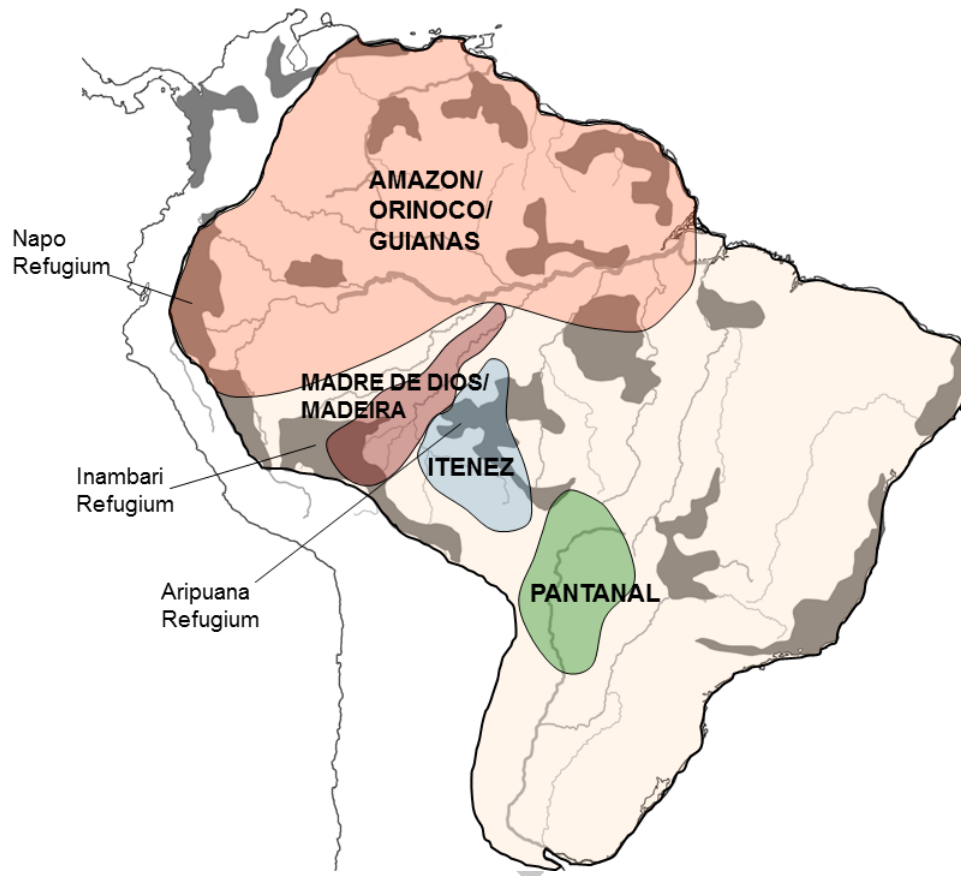












Tables

Table 1 Three hypothesized drivers of diversification in Amazonia and the predictions they make regarding the phylogeographical structure of the giant otter.

Palaeoecological drivers of divergence	References	Prediction
Palaeogeography	Da Silva and Patton 1998; Hubert et al. 2007	Sister lineages will be found either side of a geological arch. Divergence dates will be pre-Pliocene except in the case of the Fitzcarrald Arch, a more recent formation.
Hydrogeology	Lundberg et al. 1998; Montoya-Burgos 2003; Hubert and Renno 2006	Populations in adjacent drainage basins will exhibit a pattern of ancestry directly across the watersheds, with samples from one drainage clustering within the larger clade of its neighbor.
Pleistocene glacial refugia	Haffer 1969; Whitmore and Prance 1987; Marquez et al. 2006; Conn and Mirabello 2007; Mucci et al. 2010	Discrete regions of genetic diversity will be seen, not necessarily correlated with geological features or altitude. The phylogeographical pattern may agree with that of rainforest refugia identified by Whitmore and Prance (1987). For the last glaciation to have been a driver, divergence times between phylogroups will be <80,000 BP

Table 2 Genetic distance among phylogroups (F_{ST})

	Iténez	Madre de Dios/Madeira	Pantanal	Amazon/Orinoco/Guianas
Iténez				
Madre de Dios/Madeira	0.64			
Pantanal	0.70	0.74		
Amazon/Orinoco/Guianas	0.69	0.63	0.73	

Table 3 Genetic diversity of each phylogroup identified by SAMOVA

Phylogroup	Number of samples	Number of Sampling sites	Number of haplotypes	Haplotype diversity (h)	Number of polymorphic sites	Nucleotide diversity per site (π)
Iténez	9	4	8	0.97	12	0.0073
Madre de Dios/Madeira	6	3	6	0.93	7	0.0076
Pantanal	12	4	3	0.44	2	0.0015
Amazon/Orinoco/Guianas	44	23	17	0.88	18	0.0067

