

Phylogeography and population genetics of the endangered Amazonian manatee, *Trichechus inunguis* Natterer, 1883 (Mammalia, Sirenia)

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Abstract

We used mitochondrial DNA control region sequences to examine phylogeography and population differentiation of the endangered Amazonian manatee *Trichechus inunguis*. We observe lack of molecular differentiation among localities and we find weak association between geographical and genetic distances. However, nested clade analysis supports restricted gene flow and/or dispersal with some long-distance dispersal. Although this species has a history of extensive hunting, genetic diversity and effective population sizes are relatively high when compared to the West Indian manatee *Trichechus manatus*. Patterns of mtDNA haplotype diversity in *T. inunguis* suggest a genetic disequilibrium most likely explained by demographic expansion resulting from secession of hunting and enforcement of conservation and protective measures. Phylogenetic analysis of *T. manatus* and *T. inunguis* haplotypes suggests that *T. inunguis* is nested within *T. manatus*, effectively making *T. manatus* a paraphyletic entity. Paraphyly of *T. manatus* and recent divergence times of *T. inunguis* and the three main *T. manatus* lineages suggest a possible need for a taxonomic re-evaluation of the western Atlantic *Trichechus*.

Keywords: Amazonian manatee, control region, mtDNA, phylogeography, population genetics, *Trichechus*

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Introduction

The order Sirenia is currently represented by two families with two living genera and five species. The family Dugongidae contains *Dugong dugon* and an extinct species *Hydrodamalis gigas*, and the family Trichechidae comprises *Trichechus senegalensis*, *Trichechus manatus*, and *Trichechus inunguis*. This order appeared approximately 45–50 million years ago (Ma) during the Middle Eocene, and had its maximum diversity with 12 genera during the Miocene (Domning 1981, 1982, 2001).

Domning (1982) speculated that the genus *Trichechus* descended from the now extinct genus *Ribodon*. Both genera

are characterized by supernumerary molars which continue to be replaced horizontally throughout life and thus are thought to constitute an adaptation to eating true grasses (Gramineae) which form the bulk of the diet of *T. inunguis* (Domning 1982, 2001). Domning (1982) hypothesized that *Trichechus* originated in brackish or freshwater lagoons and estuaries of South America, and then in the Late Pliocene to Early Pleistocene expanded their range throughout the Caribbean region followed by dispersal to West Africa. However, there are no morphological synapomorphies supporting the hypothesized sister taxon relationship of *T. manatus* and *T. senegalensis* (Domning 1982). Domning (1982) also hypothesized a Pliocene invasion of the western Amazon basin and morphological differentiation into modern *T. inunguis* because of temporary isolation of the progenitor of *T. inunguis* within the western Amazon basin by Andean

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orogenic events. This hypothesis is supported by two fragmentary Pleistocene fossil finds in the Brazilian state of Acre (western Amazon basin) of animals morphologically similar to *T. manatus*.

In Brazil, two species of manatees exist: the marine species *T. manatus* and *T. inunguis*, the Amazonian manatee, the smallest and the only exclusively freshwater sirenian, which is endemic to the Amazon River Basin. The mouth of the Amazon River is a zone of parapatric contact of these two species (Domning 1981, 1982). The Amazonian manatee occurs from the headwaters of rivers in Colombia, Peru and Ecuador that form the Amazon drainage system up to Marajó Island, Brazil (Best 1984). They move between lakes and rivers, returning to the várzea and igapó areas (flooded forests and meadows) during the high-water season, when food is more abundant (Best 1983). The seasonal cycle of floods in the Amazon drainage system has a strong influence on the production of aquatic macrophytes in the region, and consequently on the manatee's food. With increase of river water levels (December–June), there is an increase in the production of aquatic macrophytes in the várzea and igapó, and the manatees disperse into these feeding areas. During low-water season (July–November) they migrate to deep places, and remain there until the next high-water season when they once again move into the várzea and igapó areas (Best 1982, 1983, 1984).

Feeding seasonality apparently regulates reproduction. Copulation and birth happen when water starts rising at the end of December to June, with most births occurring between February and May. In this period, plants are abundant, thus allowing females to recover their physiological condition (Best 1982). Gestation lasts 12 months, suggesting synchronization between estrus of the female and food availability (Best 1982; Junk & da Silva 1997).

Historically, *T. inunguis* suffered extensive hunting which reduced its population dramatically in the Amazon. First records detailing the exploitation of Amazonian manatees are from 1542 (Best 1984), however, native human populations always utilized manatees as source of meat and hides. From 1935 to 1954, the species was hunted intensively for its hide when approximately 4000–7000 manatees were killed per year in Brazil alone. Hides were used for making machine belts, pulleys and hoses, and rest of the animal was rendered for oil and sold for meat (Best 1984). The species is listed in Appendix I-CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora), as 'Vulnerable' in the Official List of Species of Brazilian Fauna Threatened with Extinction (IBAMA 2001) and in the Red List of IUCN (International Union for Conservation of Nature and Natural Resources). As a result of the inclusion of the Amazonian manatee on the Endangered Species List, much effort has gone to understand their ecology in order to aid in their conservation (Best 1981; Rosas 1994; da Silva 1999; Sousa Lima & da Silva 2000).

While it is necessary to remove human aspects that increase manatee mortality, an elemental understanding of the manatee's population genetics would also greatly aid conservationist and managers in implementing effective management plans. With the advent and application of molecular techniques to conservation, we are now able to address questions which ecological or morphological approaches cannot answer. Genetic analysis of populations has therefore become very important in the development of management plans for threatened species (Frankham *et al.* 2002).

The analysis of mitochondrial DNA (mtDNA) of aquatic mammals, particularly of cetaceans, has greatly contributed to our knowledge of these enigmatic mammals (Baker *et al.* 1990; Baker *et al.* 1996; O'Corry-Crowe *et al.* 1997; Palsbøll *et al.* 1997; Secchi *et al.* 1998; Rosenbaum *et al.* 2000; Moller & Beheregaray 2001). Analyses of mtDNA have been applied to Amazon River dolphins as well (Banguera-Hinestroza *et al.* 2002). However, there are few genetic studies of sirenians, particularly of the Amazonian manatee. Genetic studies with *Trichechus manatus latirostris*, the Florida manatee, using protein electrophoresis (McClenaghan & O'Shea 1988) and mtDNA (Brandley *et al.* 1993; Garcia-Rodriguez *et al.* 1998) revealed low levels of genetic differentiation among samples collected around the peninsula of Florida. The authors suggested a recent population bottleneck or a recent colonization from the West Indies as the cause of low genetic diversity of the Florida manatees. This information formed the basis for implementation of management and conservation measures in the USA (Garcia-Rodriguez *et al.* 1998).

Considering the lack of genetic information on the Amazonian manatee, our goals are to estimate levels of genetic variability in different areas of the Amazon basin, to test association of mtDNA control region haplotypes with geography, and to investigate the phylogenetic status of the Amazonian manatee. The results of this study can be used to propose management and conservation policies for this species, as well as in efforts to re-introduce captive individuals to their natural habitat.

Materials and methods

Skin samples were obtained from 68 Amazonian manatees (*Trichechus inunguis*) from throughout the Amazon basin (Fig. 1). Samples were taken as a biopsy punch from the caudal flipper using a dermatome. The animals were mostly orphan calves donated by locals or seized by the Brazilian Agency of Conservation – IBAMA, and turned over to the Laboratory of Aquatic Mammals (INPA, Manaus, AM, Brazil), or to the Center of Conservation and Research of Aquatic Mammals (CPPMA, Eletronorte, Balbina, AM, Brazil). The samples were preserved in a solution of 20% DMSO saturated with NaCl and kept at room temperature (Amos & Hoelzel 1991).

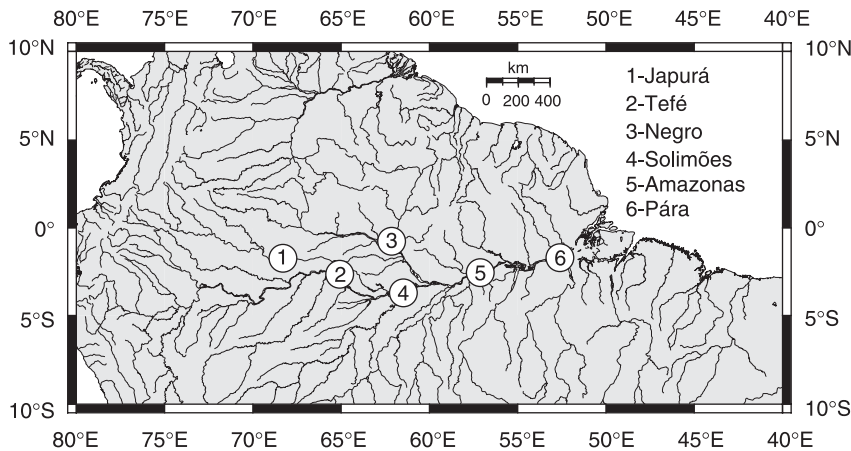


Fig. 1 Sample areas of *Trichechus inunguis* collected in the Amazon basin and used in the present study. The numbers correspond to: 1-Japurá ($n = 8$), 2-Tefé ($n = 16$), 3-Negro ($n = 10$), 4-Solimões ($n = 18$), 5-Amazonas ($n = 14$), and the samples from 6-Pará ($n = 9$).

Total genomic DNA was extracted using a phenol-chloroform-isoamyl alcohol protocol (Sambrook *et al.* 1989). A portion of the control region was amplified by polymerase chain reaction (PCR) with primers used by Garcia-Rodriguez *et al.* (1998) in the West Indian manatee (heavy strand primer: CR-5: 5'-TCACCATCAACACCCAAAGC-3'; light strand primer: CR-4: 5'-AGATGTCTTATTTAAGAGGAA-3'). This primer-pair amplifies the displacement loop of the mitochondrial control region. PCR was accomplished in 30 cycles: 5 cycles of 1 min at 94 °C for denaturation, 1 min at 58 °C for annealing, and 1.5 min at 72 °C for elongation. The remaining 25 cycles used the same temperature conditions of denaturation and elongation, but used an annealing temperature of 55 °C. PCR products were purified with Concert™ Rapid PCR Purification System kit (GibcoBRL), sequenced with amplification primers using manufacturer's recommended protocol for DYEnamic ET Terminator cycle sequencing kit from Amersham Bioscience, and resolved on a MegaBACE automatic DNA sequencer (Amersham Bioscience). Sequences were checked, edited and aligned in the program BIOEDIT (Hall 1999).

Phylogenetic analysis

All 31 unique haplotypes derived from the 68 individuals were used in phylogenetic analysis. We combined our haplotypes with additional two *T. inunguis* haplotypes found by Garcia-Rodriguez *et al.* (1998), and with *Trichechus manatus* haplotypes also from Garcia-Rodriguez *et al.* (1998). We removed the first 48 base pairs from haplotypes of Garcia-Rodriguez *et al.* (1998) because of ambiguities in their and our data sets. We used the Pacific sirenid *Dugong dugon* (GenBank accession no. AY075116) and the African elephant *Loxodonta africana* (GenBank accession no. AF106210) as outgroups; elephants are recognized as the closest sister taxon to the order Sirenia (Murphy *et al.* 2001). The program MODELTEST 3.06 (Posada & Crandall 1998) was used to determine the optimal model of nucleotide evolution for

the data set. A full heuristic maximum-likelihood analysis of the data was used to infer the most likely phylogeny. Robustness of the topology was tested with 200 maximum-likelihood bootstrap replicates. All phylogenetic analyses were performed in the program PAUP* 4.10b (Swofford 2001).

Population analyses

In all population analyses we included 68 individual represented by 32 haplotypes. One individual from Mojú River in Pará was excluded since its haplotype H27 was most closely related to *T. manatus* haplotype cluster 3. We also included additional 16 individuals from the Tefé region analysed by Garcia-Rodriguez *et al.* (1998). Three of these individuals had two new haplotypes, while the remaining 13 individuals shared haplotypes with our samples. Individuals were grouped in to six populations defined according to macro-geographical areas from which the animals came: Japurá ($n = 8$), Tefé ($n = 16$), Solimões ($n = 18$), Negro ($n = 10$), Amazonas ($n = 14$), and individuals from the state of Pará ($n = 8$ of 9); geographical origin of nine additional individuals was uncertain (Fig. 1).

Nested clade analysis

A haplotype network was constructed based on the statistical parsimony method of Templeton *et al.* (1992). This method gives an estimate of the maximum number of differences among the haplotypes as a result of single substitutions with the statistical confidence of 95% (Posada & Crandall 2001). This analysis was performed with the program rcs using predictions from the coalescent theory to calculate root probabilities and relative haplotype ages (Clement *et al.* 2000). Ambiguous connections resulting from homoplastic mutations were resolved using information from the maximum-likelihood topology.

A priori analysis to infer whether or not a significant association of geography and nested haplotype is a result

of some level of restricted gene flow was implemented using nested clade analysis (NCA) developed by Templeton *et al.* (1995). The NCA takes into account gene genealogies, haplotype frequencies and geographical data to inferentially discriminate between historical events (such as fragmentation or range expansion events) and ongoing processes (such as gene flow). To implement the NCA, the haplotype network was manually nested into increasingly inclusive clades following the rules described by Templeton and collaborators (Templeton *et al.* 1987; Templeton & Sing 1993). The NCA estimates the clade distance, which measures the geographical spread of a clade, and the nested clade distance, which measures how a clade is geographically distributed relative to other clades in the same higher-level nesting category. Both distances were calculated and tested using the method of random permutations (Templeton & Sing 1993) implemented in GEODIS 2.0 (Posada *et al.* 2000). The interpretation of the results is following the Inference Key for the Nested Haplotype Tree Analysis of Geographical Distance [http://www.cals.ncsu.edu/plantpath/Faculty/carbone/NCA-key(24Oct01).htm].

Population diversity analysis

Genetic diversity within localities was measured as the number of DNA mitochondrial haplotypes, haplotype diversity (\hat{H}), nucleotide diversity (π) calculated by Nei's method (Nei 1987) and number of polymorphic sites.

We also performed Tajima's D test (Tajima 1989) and the more sensitive Fu's F_s test (Fu 1997) to examine whether samples from different localities are at equilibrium with respect to mtDNA, considering that a significant deviation from mtDNA genetic equilibrium is presumably a result of recent population expansion or bottleneck in situations where no selective advantage among haplotypes exists (Rand 1996). Tajima's test compares the number of segregating sites with nucleotide diversity (defined as the average number of nucleotide differences per site between any two sequences), while Fu's F_s test takes into account the polarity of mutations and it estimates θ based on the number of derived unique mutations (singletons). Additional information regarding past and present population sizes was derived from estimators of historical θ_0 and current θ parameters (Rogers & Harpending 1992) as implemented in the program ARLEQUIN (Schneider *et al.* 2000).

Population subdivision and structure was estimated using an analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992), and pairwise population F_{ST} significance test (Cockerham & Weir 1993) as implemented in the program ARLEQUIN (Schneider *et al.* 2000). For AMOVA analysis of the haplotypic data, one group of populations' model was used. The Mantel test (Mantel 1967) as implemented in ARLEQUIN (Schneider *et al.* 2000) was used to assess the sig-

nificance of association between genetic and geographical distances. Statistical significance was tested using 10 000 random permutations.

Estimates of diversification

To estimate persistence of the investigated populations and lineages, we calculated the time to coalescence T , utilizing Templeton's (1993) formulation of Tajima's (1983) formula

$$T = 1(1 + k) / [2\mu(1 + L\theta)]$$

where k represents the maximum pairwise divergence between haplotypes expressed in number of nucleotide differences in a sample of sequences, L represents the length of sequence in base pairs (i.e. number of nucleotides sampled), μ is the mutation rate in substitutions/base pair/year assumed to be equivalent to 2×10^{-8} substitutions/site/year which is an estimated mutation rate for the sirenid *D. dugon* (Garcia-Rodriguez *et al.* 1998) and also observed in cetaceans (Rooney *et al.* 2001), and Watterson's θ is an estimator of genetic variation (Watterson 1975). Tajima (1983) showed that even if one knows all parameters without error, and the molecular clock works perfectly, the time to coalescent T is a random variable with a variance of

$$\sigma^2 = \theta^2(1 + k) / [4\mu^2(1 + L\theta)^2]$$

In addition to Tajima's (1983) method of coalescence time estimation, we also used divergence estimates based on a linearized – two-cluster test – neighbour-joining topology (Takezaki *et al.* 1995) under the Kimura 2-parameter model of molecular evolution (Kimura 1980) as implemented in the program MEGA2 (Kumar *et al.* 2001). The linearized topology inferred with the Kimura 2-parameter model was chosen since Garcia-Rodriguez *et al.* (1998) used this method in their study. The parameters θ and μ were also used to infer the female variance effective population size N_{ef} (Wright 1951).

Results

Phylogenetics

A total of 361 base pairs of the mtDNA control region were aligned in 68 samples of *Trichechus inunguis* with 14 haplotypes of *Trichechus manatus* (obtained from Garcia-Rodriguez *et al.* 1998), and one sequence each of *Dugong dugon* and *Loxodonta africana*, used as outgroups. The complete alignment contained a total of 154 variable positions with 64 parsimony informative sites. Within the family Trichechidae, a total of 63 variable positions were observed of which 49 sites were parsimony informative (Table 1). In

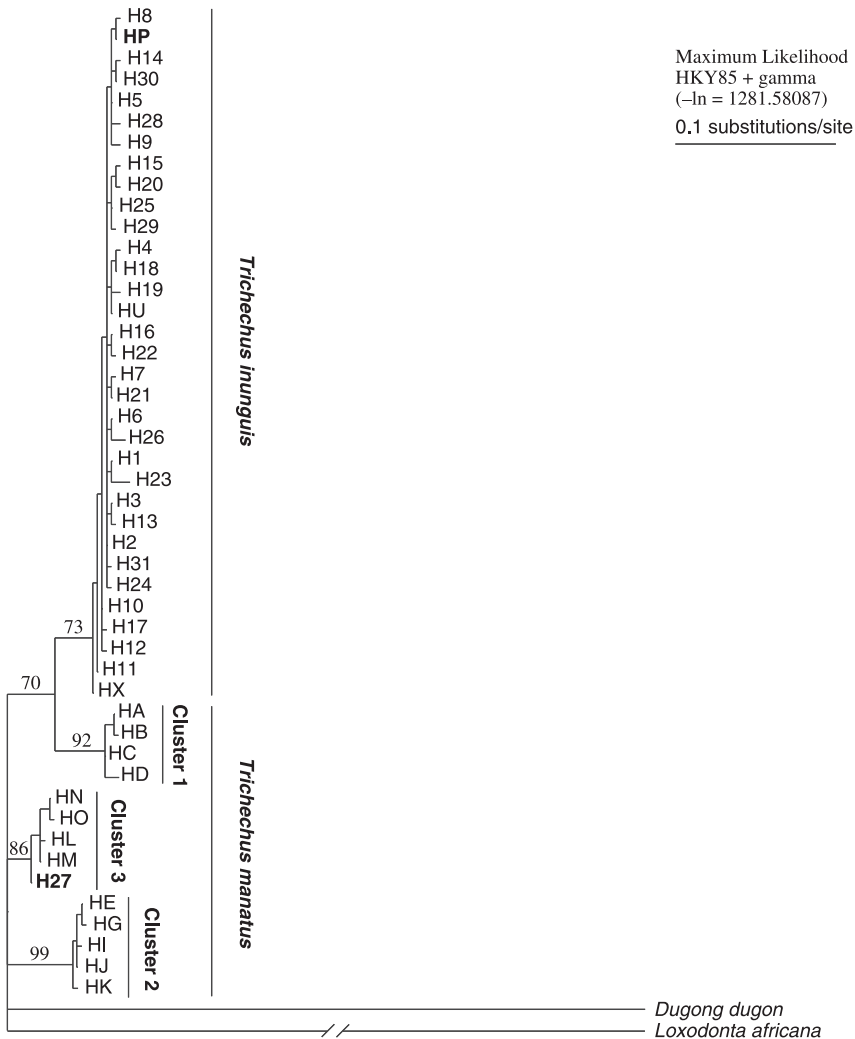


Fig. 2 Maximum-likelihood estimate of phylogenetic relationships 33 haplotypes of *Trichechus inunguis*, and 14 haplotypes of *Trichechus manatus*. Maximum-likelihood topology (-ln = 1281.58087) was estimated under the HKY85 + Gamma model of molecular evolution. Bootstrap values for *T. inunguis* and the three clades of *T. manatus* are indicated above nodes. Haplotypes HP and H27 are shared between species.

T. inunguis, 31 haplotypes were observed and are available in GenBank (accession nos AY738549–AY738579).

Fifty-six evolution models were tested using the program MODELTEST 3.06 (Posada & Crandall 1998). The model selected as the best fit among the sequences was the HKY 85 model (Hasegawa *et al.* 1985), with a gamma-distributed mutation probabilities. The maximum-likelihood topology (-ln = 1281.58087) showed no clustering of haplotypes that are also geographically close to each other (Fig. 2), and the topology clearly indicates that *T. manatus* is paraphyletic with respect to *T. inunguis*. Cluster 1 of *T. manatus* from Florida, the Greater Antilles and Colombia is more closely related to *T. inunguis* from the Amazon basin than it is to either of the other two *T. manatus* clades (Fig. 2). This topology is weakly supported by bootstrap values, but is not significantly more likely than an alternate topology with *T. manatus* constrained in to monophyly (S-H-test, diff -ln = 1.44175, $P = 0.56$). Similar to the findings of Garcia-Rodriguez *et al.* (1998), we also find interspecific haplotype

sharing. In our study, haplotype H27 from the Mojú River close to the estuary of Amazon River is a member of *T. manatus* haplotype cluster 3 from Brazil and the Guyana coast (Garcia-Rodriguez *et al.* 1998).

Because Garcia-Rodriguez *et al.* (1998) used the Kimura 2-parameter algorithm (Kimura 1980) to calculate sequence divergence, we applied the same algorithm for comparison (see Table 2). Within the ingroup, the average genetic distance calculated for *T. inunguis* was $1.0 \pm 0.2\%$ excluding the haplotype H27. H27, which corresponds to a *T. inunguis* morphotype, was excluded since it shows $6.9 \pm 1.4\%$ divergence with respect to other *T. inunguis* haplotypes, but only $1.0 \pm 0.4\%$ divergence with *T. manatus* cluster 3 (Garcia-Rodriguez *et al.* 1998) which includes haplotypes from Guyana and the coast of Brazil. The genetic divergence between *T. inunguis* and *T. manatus* individuals varied from $6.2 \pm 1.3\%$ to $8.3 \pm 1.5\%$ (Table 2), and correspond to 3.1 ± 0.65 – 4.0 ± 0.65 million-year divergence based on a 2% substitutions/site/year rate (Table 3). The *T. manatus*

Table 2 Average pairwise divergence and standard errors among groups and within groups based on the Kimura 2-parameter algorithm (Kimura 1980). Following the nomination of Garcia-Rodriguez *et al.* (1998) *Trichechus manatus* used in present study is represented by cluster 1 haplotypes from Florida, the Caribbean and Colombia; cluster 2 haplotypes from Mexico, Colombia and Venezuela; and cluster 3 haplotypes from Guyana (except HP haplotype). Haplotype H27 was found in a morphological *Trichechus inunguis* but is a member of haplotype cluster 1 of *T. manatus*, and haplotype HP (Garcia-Rodriguez *et al.* 1998) was found in morphological *T. manatus*, but it clusters with *T. inunguis* haplotypes

	Among-group % sequence divergence						<i>Dugong dugon</i>	Within-group % sequence divergence
	Amazon	H27	Cluster 1	Cluster 2	Cluster 3	HP		
Amazon	—							1.0% ± 0.2
H27	6.9 ± 1.4	—						—
Cluster 1	6.2 ± 1.3	6.6 ± 1.4	—					0.9% ± 0.4
Cluster 2	8.3 ± 1.5	5.4 ± 1.3	7.9 ± 1.6	—				0.6% ± 0.3
Cluster 3	7.8 ± 1.5	1.0 ± 0.4	6.3 ± 1.3	6.4 ± 1.4	—			0.6% ± 0.3
HP	0.9 ± 0.3	7.4 ± 1.5	6.5 ± 1.4	8.3 ± 1.6	8.1 ± 1.6	—		—
<i>D. dugon</i>	21.4 ± 2.6	19.8 ± 2.6	23.0 ± 2.8	22.1 ± 2.9	21.0 ± 2.7	21.7 ± 2.7	—	—
<i>Loxodonta africana</i>	36.5 ± 4.0	35.7 ± 3.9	37.7 ± 4.2	37.8 ± 4.2	36.8 ± 4.0	36.8 ± 4.1	40.9 ± 4.4	—

Table 3 Estimates and standard deviations of Watterson's θ (1975), linearized divergence time (Takezaki *et al.* 1995), coalescent time (Tajima 1983; Templeton 1993) and female variance effective population size (Wright 1951) of *Trichechus* lineages. All estimates are based on $\mu = 2 \times 10^{-8}$ substitutions/site/year

Lineages	Theta θ (S)	Linearized divergence time	Coalescent time	Females effective population size
<i>T. inunguis</i>	6.564 ± 2.007	~500 000 ± 100 000	~692 200 ± 85 440	~454 600
<i>T. manatus</i> Cluster 1	1.365 ± 0.657	~450 000 ± 200 000	~483 800 ± 156 500	~94 540
<i>T. manatus</i> Cluster 2	1.324 ± 0.702	~300 000 ± 150 000	~345 500 ± 134 300	~91 700
<i>T. manatus</i> Cluster 3	1.414 ± 0.861	~300 000 ± 150 000	~345 600 ± 130 000	~97 920
<i>T. manatus</i> Cluster 3 + H27	2.049 ± 1.111	~350 000 ± 150 000	~415 000 ± 118 400	~141 900
<i>T. manatus</i> Clusters	8.257 ± 0.656	—	~1 938 000 ± 127 500	—
<i>T. manatus</i> Cluster 1 + <i>T. inunguis</i>	9.701 ± 2.604	~3100 000 ± 650 000	~1 869 000 ± 115 500	—
<i>T. manatus</i> Cluster 2 + 3	6.313 ± 2.206	~3200 000 ± 700 000	~1 731 000 ± 137 700	—
<i>T. inunguis</i> + <i>T. manatus</i>	11.393 ± 2.887	~4000 000 ± 650 000	~2 146 000 ± 114 200	—

haplotype HP was not included in our estimates of genetic divergence between *T. inunguis* and *T. manatus* because it clusters closely with *T. inunguis*. Individuals bearing this haplotype were morphologically identified as *T. manatus* and therefore were considered by Garcia-Rodriguez *et al.* (1998) to be of hybrid origin. The genetic divergence between the family Dugongidae and family Trichechidae was $21.5 \pm 2.6\%$.

Population analysis

A total of 361 base pairs of the mtDNA control region were aligned in 74 samples of *T. inunguis* containing a total of 42 variable positions with 18 parsimony informative sites. In the sample of 74 individuals, a total of 33 haplotypes were found; frequency of each haplotype is shown in Table 4. Since haplotype H27 clusters with *T. manatus*, we excluded this sample from population analyses. Nine *T. inunguis*

samples from unknown localities were also excluded from NCA, AMOVA and Mantel test analyses.

The parsimony network of *T. inunguis* haplotypes (Fig. 3) revealed that the most likely ancestral haplotype is haplotype H2. The two most frequent haplotypes are H2 and H5. Ambiguities in the parsimony network were resolved by maximum likelihood. Parsimony network and nesting design are shown in Fig. 3. Nested contingency analysis revealed no significant association between genetic variability and geographical distribution at all clade levels, with the exception of the final nested clade (Permutational $X^2 = 49.3899$, $P = 0.0011$). In this level, the inferences made from Templeton's key suggest restricted gene flow and/or dispersal with some long-distance dispersal as the main force underlying distribution of mtDNA haplotypes. Because this clade represents the nested-most clade, the interpretation applies to all haplotypes and Amazonia as a whole.

Table 4 Frequency of the 33 haplotypes of *Trichechus inunguis* in the six areas of the Amazon basin sampled which include samples from the Tefé region (Garcia-Rodriguez *et al.* 1998)

Haplotype	Amazonas	Negro	Tefé	Japurá	Solimões	Pará	Unknown Origin	Total
1	1							1
2	5		2	1	7	1	3	19
3	2							2
4	1							1
5	2	4	8	1	2	2	4	23
6	1							1
7	1							1
8				1				1
9				1				1
10			1	2			1	4
11			2	1				3
12				1				1
13	1							1
14		1						1
15		1						1
16		1						1
17		1						1
18		1			1			2
19		1			1			2
20					1			1
21					2	1		3
22					1			1
23					1			1
24					1			1
25					1			1
26						1		1
27						1		1
28						1		1
29						1		1
30						1		1
31							1	1
Hu			1					1
Hx			2					2
Total	14	10	16	8	18	9	9	84

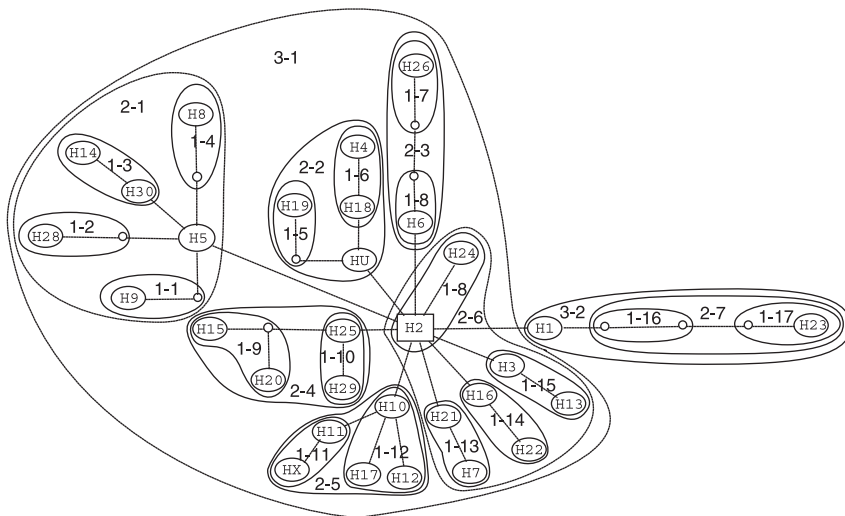


Fig. 3 Maximum parsimony network and corresponding nested design used in nested clade analysis. Sizes of the circles are not proportional to the haplotype frequency. Each line indicates one mutation between the haplotypes and the small empty circles represent hypothetical haplotypes that were not sampled. Haplotype H2 is the most likely ancestral haplotype.

Table 5 Measures of mitochondrial DNA diversity observed control region of *Trichechus inunguis* within five areas sampled in the Amazon basin. Standard deviation is given for haplotype (gene) diversity (\hat{H}) and percent nucleotide diversity per site (π); S represents number of segregating sites. For comparison the same calculations were done for *Trichechus manatus* (data from Garcia-Rodriguez *et al.* 1998)

Species	Sampled areas	Sample size	N° of haplotypes	(\hat{H})	(π)	S	Tajima's <i>D</i> -test	Fu's <i>F_s</i> test
<i>T. inunguis</i>	Japurá	8	7	0.964 ± 0.077	0.663 ± 0.140	7	-0.5409	-3.9414*
	Tefé	16	6	0.929 ± 0.084	0.485 ± 0.330	5	0.5318	-1.0517
	Solimões	18	10	0.850 ± 0.077	0.592 ± 0.147	13	-1.6207*	-4.7613*
	Negro	10	7	0.867 ± 0.107	0.726 ± 0.167	14	-1.4581	-2.4348*
	Amazonas	14	9	0.868 ± 0.076	0.478 ± 0.104	10	-1.5804*	-5.1586*
	Pará	8	7	0.964 ± 0.077	0.752 ± 0.163	9	-1.0681	-3.5473*
	All	74	32	0.887 ± 0.026	0.624 ± 0.384	31	-2.0852*	-27.0345*
<i>T. manatus</i>	Cluster 1	46	4	0.521 ± 0.068	0.248 ± 0.190	6	-0.8853	0.5195
	Cluster 2	25	5	0.727 ± 0.047	0.521 ± 0.340	5	1.2169	0.8712
	Cluster 3	10	3	0.533 ± 0.180	0.313 ± 0.250	3	-1.0204	-0.9618
	Cluster 3 + H27	11	4	1.000 ± 0.126	0.611 ± 0.143	5	-0.5619	-2.8620
	All	81	13	0.815 ± 0.031	0.409 ± 0.205	40	2.3304*	2.0448*

*Significant at the 0.05 level.

Table 6 Estimates of pairwise F_{ST} and their uncorrected *P*-values (below diagonal), and Raymond and Rousset (1995) exact population differentiation probabilities (below diagonal) estimated for populations of *Trichechus inunguis*. Significance level after Bonferroni correction $P = 0.003$

Areas	Japurá	Tefé	Solimões	Negro	Amazonas	Pará
Japurá	—	$P = 0.113 \pm 0.026$	$P = 1.000 \pm 0.000$	$P = 0.314 \pm 0.022$	$P = 1.000 \pm 0.000$	$P = 1.000 \pm 0.000$
Tefé	$F_{ST} = -0.026$ $P = 0.546$	—	$P = 0.081 \pm 0.016$	$P = 0.435 \pm 0.031$	$P = 0.054 \pm 0.011$	$P = 0.255 \pm 0.035$
Solimões	$F_{ST} = 0.099$ $P = 0.015$	$F_{ST} = 0.072$ $P = 0.032$	—	$P = 0.707 \pm 0.029$	$P = 1.000 \pm 0.000$	$P = 1.000 \pm 0.000$
Negro	$F_{ST} = 0.043$ $P = 0.148$	$F_{ST} = 0.043$ $P = 0.147$	$F_{ST} = 0.036$ $P = 0.125$	—	$P = 0.651 \pm 0.018$	$P = 0.647 \pm 0.025$
Amazonas	$F_{ST} = 0.126$ $P = 0.007$	$F_{ST} = 0.108$ $P = 0.016$	$F_{ST} = -0.002$ $P = 0.466$	$F_{ST} = 0.093$ $P = 0.015$	—	$P = 1.000 \pm 0.000$
Pará	$F_{ST} = 0.071$ $P = 0.094$	$F_{ST} = 0.078$ $P = 0.090$	$F_{ST} = 0.016$ $P = 0.273$	$F_{ST} = -0.036$ $P = 0.840$	$F_{ST} = 0.069$ $P = 0.051$	—

Genetic analysis revealed that populations were characterized by few common haplotypes and by a great number of rare haplotypes (Table 4). Among the 33 observed haplotypes, only eight were found in more than one individual; the remaining 25 haplotypes occurred just once. Haplotype 27 was found in only one individual, and in the nine individuals without known origin, only H31 represents a new haplotype. Tajima's *D*-statistic and Fu's *F_s* were significantly negative in most instances (Table 5), i.e. both tests show a significant excess of the number of segregating sites and singletons compared to the average pairwise sequence divergence. These tests show that the species is at a mutation–migration–drift genetic disequilibrium with respect to mtDNA alleles. The data also suggest that *T. inunguis* increased from 2.23×10^4 ($\theta_0 = 0.337$) to 4.55×10^5 ($\theta = 6.565$) female effective individuals. The female effective population size of *T. inunguis* is much larger than

any of the three *T. manatus* clusters, and so are corresponding coalescent times (Table 3).

AMOVA demonstrated that 94.6% of genetic variability is attributable to the variance within populations, and only 5.4% is accounted for by differences among populations; but the overall inferred difference among populations is significant ($F_{ST} = 0.054$, $P = 0.007$). After sequential Bonferroni correction for multiple comparisons (Rice 1989) no significant differences were found between localities as measured by F_{ST} (Table 6). Raymond and Rousset's (Raymond & Rousset 1995) test of exact population differentiation also revealed no significant pairwise locality differences (Table 6), and global test of differentiation among samples was nonsignificant ($P = 0.433 \pm 0.127$). Mantel test indicates no significant correlation between genetic and geographical distance exists ($r = -0.085$, $P = 0.591$).

Discussion

Population genetics of Trichechus inunguis

Despite the unrestrained hunting of the Amazon manatee for centuries, the species appears to have maintained a relatively high level of genetic diversity when compared to the Caribbean manatee. The total nucleotide diversity is $\pi = 0.75\%$ and total haplotype $\hat{H} = 0.909$ (Table 5). Similar values were also found in 16 samples and eight haplotypes of *Trichechus inunguis* from central Amazon (Garcia-Rodriguez *et al.* 1998) where $\pi = 0.50\%$ and $\hat{H} = 0.875$.

Inference based on the only significant result of the NCA (Templeton *et al.* 1995) supports the hypothesis of restricted gene flow and/or dispersal with some long distance dispersal. This inference pertains to the highest nesting level, and applies to Amazonia as a whole. As discussed by Masta *et al.* (2003), a false positive signal of long-distance dispersal may be inferred in situations where local extinction occurred. This is certainly possible given history of over-exploitation of the Amazonian manatee, although manatees have also been observed to move over wide areas (AMC and VMFS pers. obs.). Thus multiple factors may result in the NCA signal of long-distance dispersal. The NCA inference is supported by an overall significant differentiation among localities based on AMOVA ($F_{ST} = 0.054$, $P = 0.007$), but is not supported by the global test of exact population differentiation ($P = 0.433 \pm 0.127$).

Results of AMOVA also indicate that 94.6% of observed genetic variability is found within localities, and only 5.4% of genetic variability is found among localities. Thus among-locality genetic differentiation is very small. Pairwise F_{ST} analysis, as well as Raymond & Rousset's (1995) exact test of differentiation show no pair of populations to be significantly differentiated (Table 6). Mantel test analysis of correlation of genetic and geographical distances does not support the hypothesis of a linear pattern of differentiation ($r = -0.085$, $P = 0.591$).

These results would seem to imply that while a pattern of restricted gene flow and/or dispersal throughout the whole of the Amazon basin appear to exist – although this pattern may be confounded by haplotype extinctions – this pattern is unlikely to be a very strong population-structuring factor. *T. inunguis* most likely behaves as a panmictic population, but because of the vastness of its geographical range, some restricted gene flow and/or dispersal within the Amazon basin probably exists.

This inference is consistent with observations that *T. inunguis* has seasonal movements synchronized with the flood regime of the Amazon River basin. During the flood season when food is abundant, manatees disperse in to várzea and igapó areas. During the low-water season, they seek protection in deep lakes and channels (Best 1981; Junk & da Silva 1997) with little evidence of phylopatry

(VMFS pers. obs.). Várzea and igapó areas form highly interconnected aquatic ecosystems, thus manatees have many opportunities for extensive movement and migration, thereby increasing the possibility of gene flow, and consequently of phenotypic and genetic homogenization.

Relatively high genetic diversity and possible recovery of T. inunguis

Tajima's and Fu's tests have been formally designed to test for genetic disequilibrium resulting from selection. However, a significant deviation from genetic equilibrium in mtDNA alleles may result from demographic events, such as population expansions or bottlenecks in situations where no selective advantage among haplotypes exists (Rand 1996). Since selective advantage among haplotypes is normally not observed for mtDNA control region (Avice 2004), alternate hypotheses for the genetic disequilibrium are a recent genetic bottleneck, or a recent population expansion at a broad geographical scale (Rand 1996). Significant deviation will also be observed in situations when deeply divergent lineages are assumed to be part of an interbreeding entity, but in reality are not.

The most likely explanation for the significant excess of recent mutations in the mtDNA of *T. inunguis* is an overall population expansion of this species. *T. inunguis* was historically exploited for local as well as the export market from 1780s to 1954 (Domning 1982). In 1967 this species became formally protected by Brazilian legislature, and since 1973 it has been protected under the IUCN agreement. Although some illegal and traditional hunting continue, this species has been relatively free of exploitation for the last 30–40 years.

T. inunguis females give first birth between 5 and 7 years of age, and have a 2 to 3 years interval between births; they live to approximately 40 years in captivity (AMC and VMFS pers. obs.). Conservatively one can estimate a generation time of 10 years with lifetime reproductive output of three female and three male offspring. With no predation or other forms of premature death, after 40 years of protection one female would leave behind 27 female offspring. This would represent a 95% increase in population size. Estimates based on historical and current values of θ suggest that present-day population of 455 000 female effective individuals increased from an initial population of 22 300 female effective individuals, which represents a 95% increase in population size. Therefore it is quite likely that the observed disequilibrium in mtDNA of *T. inunguis* is a genetic signature of a population undergoing a demographic expansion as would be expected in a species released from hunting pressure.

The genetic diversity in *T. inunguis* is relatively high and higher than in any one of the three major clusters of *T. manatus* (Garcia-Rodriguez *et al.* 1998) (Table 5), as is the

female variance effective population size of *T. inunguis* compared to clusters of *T. manatus* (Table 3). These results are not contrary to evidence of population reduction resulting from over-exploitation. Rather they support the hypothesis that either the historical reduction in population size has not resulted in a severe genetic bottleneck, or the effects of any genetic bottleneck that *T. inunguis* might have experienced have been mitigated by a subsequent demographic expansion. Thus *T. inunguis* appears to be well on a road to genetic and demographic recovery, although this conclusion must be viewed with caution and verified by other studies.

Population history of *T. inunguis* and the status of *T. manatus*

Coalescent estimate of diversification of the New World *Trichechus* is estimated at 2.15 ± 0.11 Ma. Because no correction for back mutations was applied to this estimate, it represents the lower minimum estimate of divergence time. Using a linearized phylogenetic estimate of $8.0 \pm 1.3\%$ obtained from the program MEGA2 (Kumar *et al.* 2001), and the same 2% per site/per million year mtDNA d-loop substitution rate, we arrive at an estimate of 4.00 ± 0.65 Ma divergence. These results are in accord with the hypothesis of a Plio-Pleistocene origin and diversification of *Trichechus* (Domning 1982).

As noted by Garcia-Rodriguez *et al.* (1998), *T. manatus* is composed of three deeply divergent lineages. Maximum-likelihood phylogeny indicates that cluster 1 of *T. manatus* is sister to *T. inunguis*, effectively making *T. manatus* a paraphyletic assemblage (Fig. 2). However, this topology is not significantly more likely than the best topology where *T. manatus* is monophyletic (S-H-test, diff $-\ln = 1.44175$, $P = 0.56$) or where relationships between *T. inunguis* and the three clades of *T. manatus* are represented as a polytomy (S-H-test, diff $-\ln = 9.16346$, $P = 0.07$).

The genus *Trichechus* of the western Atlantic may therefore best be viewed as comprising four lineages; three of these lineages inhabit saltwater while one lineage inhabits freshwater. The lineages diverged within a relatively short period during the Plio-Pleistocene (Table 3). *T. inunguis* is easily differentiated morphologically from *T. manatus*, although genetically it is no more different from the three clades of *T. manatus* than the three *T. manatus* lineages are from each other. Whether or not the three marine lineages of *T. manatus* represent three different species or whether the western Atlantic *Trichechus* is only one species with four distinct lineages is outside the scope of this study and must be tested by other criteria. However, it is worth noting that the three marine lineages show near perfect allopatry (Garcia-Rodriguez *et al.* 1998), and they occupy separate marine biogeographical provinces (Briggs 1974), a situation one would not expect to observe in cases of

incomplete lineage sorting of ancestral polymorphism. Additionally, genetic equilibrium exists within each separate lineage, but not when all three are considered to form a single species *T. manatus* (Table 5) suggesting lack of genetic exchange among these mitochondrial lineages. However, similar to our study, Garcia-Rodriguez *et al.* (1998) observed haplotype sharing between cluster 3 of *T. manatus* and *T. inunguis*, and suggested that haplotype sharing might be a result hybridization. This hypothesis and species status of *Trichechus* lineages need further testing.

Implications for conservation of *T. inunguis*

The results of phylogeographical and population analyses demonstrate near absence of geographical structuring in *T. inunguis*, with 94.6% of all genetic variation being shared between analysed areas. In other words, the Amazonian manatee seems to effectively form one large nearly panmictic population and an individual's origin should not limit future programs of re-introduction of captive manatees. Although classified on the Endangered Species List, the Amazonian manatee has maintained relatively high genetic variability, and the genetic signature of an expanding population might be an indication of recovery in the last 30–40 years after a 200-year period of heavy exploitation.

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- Research of Andréa Cantanhede focuses on conservation of aquatic mammals and particularly on manatees; this paper is a result of her Masters Thesis project. Vera da Silva studies ecology and ethology of aquatic mammals, and was Andréa's Master's Thesis advisor. Stella Lazzarini works in the manatee rescue centre in Balbina. Izeni Farias, José Alves-Gomes and Tomas Hrbek study phylogenies, phylogeography, population and conservation genetics of Amazonian aquatic vertebrates (fishes, reptiles and mammals) particularly those living in the flooded forest ecosystem; Izeni is also Andrea's Ph.D. advisor. Andrea is currently undertaking microsatellite studies of paternity and fine-scale population genetic structure of manatees.
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