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Phylogeography, phylogeny and hybridization in trichechid sirenians: implications for manatee conservation

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Abstract

The three living species of manatees, West Indian (*Trichechus manatus*), Amazonian (*Trichechus inunguis*) and West African (*Trichechus senegalensis*), are distributed across the shallow tropical and subtropical waters of America and the western coast of Africa. We have sequenced the mitochondrial DNA control region in 330 *Trichechus* to compare their phylogeographic patterns. In *T. manatus* we observed a marked population structure with the identification of three haplotype clusters showing a distinct spatial distribution. A geographic barrier represented by the continuity of the Lesser Antilles to Trinidad Island, near the mouth of the Orinoco River in Venezuela, appears to have restricted the gene flow historically in *T. manatus*. However, for *T. inunguis* we observed a single expanding population cluster, with a high diversity of very closely related haplotypes. A marked geographic population structure is likely present in *T. senegalensis* with at least two distinct clusters. Phylogenetic analyses with the mtDNA cytochrome *b* gene suggest a clade of the marine *Trichechus* species, with *T. inunguis* as the most basal trichechid. This is in agreement with previous morphological analyses. Mitochondrial DNA, autosomal microsatellites and cytogenetic analyses revealed the presence of hybrids between the *T. manatus* and *T. inunguis* species at the mouth of the Amazon River in Brazil, extending to the Guyanas and probably as far as the mouth of the Orinoco River. Future conservation strategies should consider the distinct population structure of manatee species, as well as the historical barriers to gene flow and the likely occurrence of interspecific hybridization.

Keywords: conservation, hybridization, manatees, phylogeography, Sirenia, *Trichechus*

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Introduction

Comparative studies of phylogeographic patterns among closely related species can reveal hidden aspects of their evolutionary history. We have applied this comparative approach in the spatial analysis of genetic diversity of extant manatee species. Manatees belong to the order Sirenia, which is composed of the only herbivorous, strictly aquatic mammals found in shallow tropical and subtropical waters. Sirenians are divided into two families. In the family Dugongidae the single living member is the dugong

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(*Dugong dugon*), which inhabits tropical and subtropical latitudes of the Indian and Pacific Oceans. The Steller sea cow (*Hydrodamalis gigas*) was another Dugongidae that became extinct in the 18th century due to predatory hunting by humans. The family Trichechidae comprises three living species of manatees. The West Indian manatee (*Trichechus manatus*) is distributed from Florida (USA) to the northeast coast of Brazil. The Amazonian manatee (*Trichechus inunguis*) is the only trichechid restricted to the freshwater environment in the Amazon Basin. The West African manatee (*Trichechus senegalensis*) is distributed along the rivers, estuaries, and coastal regions of western Africa from Senegal to Angola. Morphologically, all manatees are similar. However, *T. inunguis* is smaller in size, does not have nails on its flippers and consistently displays a white pigmented patch on the chest or abdomen.

Based on quantitative cranial characters, the West Indian manatee has been tentatively divided into two subspecies: the Florida manatee (*T. manatus latirostris*) and the Antillean manatee (*T. manatus manatus*) (Domning & Hayek 1986). This species subdivision has been questioned by the first phylogeographic study of *T. manatus* (Garcia-Rodriguez *et al*. 1998), which presented evidence of sharing of mitochondrial DNA (mtDNA) haplotypes between the Florida manatee population and the regional populations occurring in the Greater Antilles, such as Puerto Rico and the Dominican Republic.

All three living manatee species are considered vulnerable by the International Union for the Conservation of Nature and Natural Resources (IUCN) and are listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), with the exception of *T. senegalensis* which is included in Appendix II. However, manatees are still being hunted in many countries. In Brazil, *T. manatus* is considered critically endangered by the Brazilian Action Plan for Aquatic Mammals (IBAMA 2001) where their numbers have been reduced due to historic intensive hunting (Lima 1997). Today, current population estimates of around 500 individuals are projected (Lima 1997; Luna 2001) and could explain a serious reduction in the historical distribution of this species along the Brazilian coast. Documents from 1935 to 1954 indicate that approximately 200 000 Amazonian and West Indian manatee deaths attributed to harvesting were reported in Brazil (Best 1982). In addition to illegal hunting throughout their distribution, threats to manatee species include exposure to red tide outbreaks (Bossart *et al*. 1998), hypothermia due to cold stress (Bossart *et al*. 2003), collisions with water vessels (Marmontel *et al*. 1997), habitat destruction and alteration (de Thoisy *et al*. 2003) and incidental ingestion and entanglement in fishing gear and other debris (Beck & Barros 1991; Marmontel *et al*. 1997; Mignucci-Giannoni *et al*. 2000; de Thoisy *et al*. 2003). In West Africa, manatees are killed as agricultural pests and they frequently die in gill nets and other types of fishing gear (J. Powell, unpublished).

The most-studied sirenian species is *T. manatus*, especially the population inhabiting the USA. The first genetic study using alloenzymes suggested the genetic homogeneity of all manatees in Florida (McClenaghan & O'Shea 1988). That finding was also supported by the first intraspecific phylogeographic study using the mtDNA D-loop (Garcia-Rodriguez *et al*. 1998). The only previous phylogenetic study involving all five sirenian species was based on morphology and palaeontology (Domning 1994), and suggested that the West African manatee and the West Indian manatee share a more recent common ancestor than either does with the Amazonian manatee. Recently, Cantanhede *et al*. (2005) studied 361 bp of mtDNA D-loop from 68 Amazonian manatees and suggested a recent demographic expansion in this species resulting from cessation of hunting and enforcement of conservation measures. The present evolutionary genetics study is the first to include all manatee species. Here we conducted a comparative analysis using phylogeographic and phylogenetic methods with mtDNA sequence data from populations of the three manatee species. We have also employed two autosomal microsatellite loci and karyotype analysis to investigate likely cases of hybridization observed between *T. manatus* and *T. inunguis*.

To devise adequate conservation and management strategies for the species of concern, it is important to incorporate a reliable understanding of their population structure and history, investigate the likely existence of demographic partitions throughout their geographic range (Moritz 1995), and to characterize the hierarchical distribution of genetic diversity (Excoffier *et al*. 1992). Assessing biodiversity within and among regions, and focusing on within-country populations that are generally tailored to meet management unit needs, makes it possible to identify and prioritize populations for monitoring, management and future protection measures (Moritz 1995).

Materials and methods

Sample collection and DNA extraction

Muscle, skin, blood or bones were obtained from 291 individuals representing all four species of sirenians: 189 from the West Indian manatee, 93 from the Amazonian manatee, 6 from the West African manatee and 3 samples from the dugong; this latter species was used as an outgroup for comparisons with Trichechidae. We have also included previously reported sequence data from 42 *Trichechus manatus* individuals (Garcia-Rodriguez *et al*. 1998). During a review of the original sequence data, inconsistencies were found between the published sequences and those in GenBank. The corrections resulted

in a change of some haplotype assignments. Our analyses included *T. manatus* individuals sampled from 10 locations: Florida, USA ($n = 28$), Puerto Rico ($n = 62$), Dominican Republic ($n = 6$), Mexico ($n = 14$), Belize ($n = 43$), Colombia ($n = 33$), Venezuela ($n = 4$), Guyana ($n = 7$), French Guiana ($n = 3$) and Brazil ($n = 31$). *Trichechus inunguis* individuals were sampled from the Brazilian ($n = 89$), Colombian ($n = 3$) and Peruvian ($n = 1$) Amazon. *Trichechus senegalensis* samples were collected from four locations including Niger ($n = 1$), Guinea-Bissau ($n = 2$), Ghana ($n = 1$) and Chad ($n = 2$). Dugong samples came from Australia. We used country populations because they generally represent effective management units, but other geographically based divisions were also tested. We also pooled samples of each species together in order to analyse logical population grouping patterns.

Blood and skin biopsies were collected from live individuals, whereas muscles and bones were obtained from carcasses. The blood samples were preserved in EDTA or lysis solution [10 mM NaCl, 100 mM EDTA, 100 mM Tris (pH 8), and 1% (w/v) SDS]. Muscle and skin tissues were preserved in 70% ethanol or in a saturated salt SED [saturated NaCl, 250 mM EDTA (pH 7.5) and 20% v/v DMSO] buffer. Extraction of DNA from tissue samples was performed using the DNeasy Tissue Kit (QIAGEN) following the manufacturer's instructions or by standard phenol–chloroform protocol (Sambrook *et al*. 2001). DNA quality was visualized by electrophoresis in 0.8% agarose gels. DNA extractions from bones were performed according to Tuross (1994) and Holland *et al*. (2003).

PCR analysis and sequencing

A ~550-bp fragment of the mtDNA control region (D-loop) was amplified by the polymerase chain reaction (PCR) using the primers L15926 and H16498 (Kocher *et al*. 1989), of which 410 bp were used for the analyses. Each PCR mix contained 20 ng of genomic DNA, 1 × *Taq* reaction buffer 1B [Phoneutria® — 1.5 mM de $MgCl_2$, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.1% Triton X-100], 200 μM dNTPs, 0.5 μM of each primer, and 1 U of *Taq* polymerase (Phoneutria). The cycling reaction for *T. manatus* was performed as follows: 94 °C for 5 min, followed by 35 cycles of 42 °C for 45 s, 72 °C for 1 min and 94 °C for 30 s, and a final extension period of 72 °C for 10 min. The annealing temperature used for the *T. inunguis*, *T. senegalensis* and *Dugong dugon* was 47 °C. Some samples were amplified and sequenced with the protocol described by Garcia-Rodriguez *et al*. (1998), but was modified by adjusting the PCR annealing temperature to 50 °C. We have also amplified and sequenced 615 bp of the cytochrome *b* mtDNA gene using the same conditions as for the control region with universal primers MVZ3 and MVZ4 (based on Kocher *et al*. 1989) and new primers designed according to the dugong GenBank sequence (NC_003314): Cytb1F 5′-ATTCTCACAGGATTATTCCT-3′; Cytb1R 5′-AATAGGCCTAGGAGGTCTTTGA-3′ and Cytb2R 5′-GGTGTGTAGTTGTCTGGGTCTCC-3′. Amplified products were cleaned with ExoI/SAP (Amersham-Biosciences) at 37 °C for 45 min followed by 80 °C for 15 min. Both strands were sequenced, usually from two distinct PCR amplifications per individual to assure high quality consensus sequences. These were sequenced in a MegaBACE® 1000 (Amersham-Biosciences) using the DYEnamic ET® Dye Terminator Kit (Amersham-Biosciences). All sequence data have been deposited in GenBank (AY963840–AY963899 and AY965880–AY965890).

Seven microsatellite loci (TmaH11, A09, A02, E02, E11, M79, A03) identified by Garcia-Rodriguez *et al*. (2000) were analysed according to the authors' protocol. Two of these, TmaH11 and TmaA09, were selected because they showed informative alleles and larger heterozygosity (*h*). PCR annealing temperatures were adjusted to 58 °C and 61 °C, respectively. The PCR products were run on native 6% polyacrylamide gels in 1 × TBE buffer at 100 V for 3 h, and then visualized by silver staining (Santos *et al*. 1996). They were genotyped on an ALF sequencer (Amersham-Biosciences).

Cytogenetic analysis

Three West Indian manatee blood samples were collected from captive individuals in Brazil, including a likely hybrid that exhibited a mixture of morphological traits from both *T. manatus* and *T. inunguis* species. Vacutainers containing sodium heparin were used to draw blood. Samples were transported as soon as possible to the laboratory. The cytogenetic analysis followed the protocol described by Assis *et al*. (1988) developed to study *T. inunguis* karyotypes.

Data analyses

Sequence chromatograms were base called with PHRED version 0.20425 (Ewing & Green 1998). The mtDNA sequences were aligned and edited to produce a high-quality consensus sequence for each individual using PHRAP version 0.990319 (www.genome.washington.edu/UWGC/analysistools/phrap.htm) and CONSED version 12.0 (Gordon *et al*. 1998). Consensus sequences from all individuals were aligned in MEGA version 3.0 (Kumar *et al*. 2004) to identify polymorphic nucleotide sites and to assign haplotypes.

We estimated substitution models for each set of sequences with MODELTEST version 3.06 (Posada & Crandall 1998) to be used in further analyses. We calculated several standard and molecular diversity indexes with the DNASP version 4.0 (Rozas *et al*. 2003) and ARLEQUIN version 2.0 (Schneider *et al*. 2000). Effective population sizes (N_e) were

calculated using the indexes *NS*, *h* and π according to the Wright–Fisher model. We have also used ARLEQUIN to calculate pairwise Φ_{ST} and to perform a Mantel test with 10 000 permutations to assess the correlation significance using geographic distances calculated in two different ways: (i) the direct linear distance between populations; and (ii) considering that the animals migrate exclusively along the continental coast. The latter measure was obtained through the sum of linear distances between the closest pairs of studied populations along the coastline, with Puerto Rico at one extreme and Brazil at the other. A specific test was devised to suggest historical barriers to gene flow between populations using the program BARRIER version 2.2 (Manni *et al*. 2004), which uses the geographic coordinates of each population and the Φ_{ST} genetic distances calculated in ARLEQUIN as input. Gene flow between populations was estimated as the number of migrant females (N_{mf}) per generation (Slatkin 1995). ARLEQUIN was also used to perform the exact test of population differentiation and the analysis of molecular variance, AMOVA (Excoffier *et al*. 1992), to calculate the distribution of genetic variation at different hierarchical levels of a defined population structure. For *T. manatus*, all 10 sampling localities (countries) were tested. According to geographic coordinates of individual samples, we investigated other population divisions in tests using ARLEQUIN AND SAMOVA (Dupanloup *et al*. 2002), an approach to define groups of populations that are geographically homogeneous and maximally differentiated from each other. For *T. inunguis*, all of the sample localities were determined by GPS. After plotting on a satellite image map (data not shown) several groupings of populations were tested considering the potential for connectivity by migratory use of rivers, lakes and channels. Mismatch distributions were calculated with ARLEQUIN to estimate the demographic expansion parameter τ, which was used to date the onset of demographic expansion (Rogers & Harpending 1992). Through τ the time of the recent bottleneck (*t*) was calculated ($\tau = 2ut$) using the $u = \mu k$, where μ is the mutation rate per site per year and *k* is the sequence length. We have used a mutation rate of 2% per million years (Myr), which was previously calculated for the dugong mtDNA control region based on the fossil record (Tikel 1997). We have also used the 'expansion coefficient', the ratio S/d, to assess historical and recent population sizes, where *S* is the number of polymorphic sites and *d* is the mean number of pairwise nucleotide differences between haplotypes within a taxon (von Haeseler *et al*. 1996). Relationships among control region haplotypes were inferred by a median-joining network (MJN) analysis using the program NETWORK version 4 (Bandelt *et al*. 1999). Phylogenetic analyses were performed with MEGA version 3 (Kumar *et al*. 2004) to generate trees by different methods with 1000 bootstrap replicates.

Results

Patterns of intraspecific variability

West Indian manatee. Analysis of the 410-bp mtDNA control region sequences from 10 countries revealed 20 typical haplotypes for the West Indian manatee (Fig. 1), as well as four additional haplotypes related to the Amazonian manatee haplotypes ($n = 7$). Among the typical West Indian haplotypes, there were 45 polymorphic sites consisting of 42 transitions and five transversions; the positions 328 and 332 contained both a transition and a transversion. The highest haplotype diversity was recorded from Colombia, followed by Mexico; higher diversities were seen in Guyana and Venezuela, but sample sizes were very small (Table 1). Only one haplotype (A) was identified in Florida, USA. Brazil also showed low haplotype and nucleotide diversity, displaying only three haplotypes, with one haplotype appearing only once (M3) and another (T) related to the *Trichechus inunguis* haplotypes. Samples collected from French Guiana and classified morphologically as *Trichechus manatus* produced sequences that are all related to *T. inunguis* haplotypes. The observation of haplotypes identical or closely related to the other species' mtDNA sequences has been considered the primary evidence of likely interspecific hybridization. In this study, we observed four instances (three from French Guiana and one from Brazil) of *T. manatus* bearing *T. inunguis* mtDNA haplotypes, and one instance (from Brazil) of *T. inunguis* with *T. manatus* mtDNA. We have also included in the analyses, three individuals with haplotype P, related to *T. inunguis*, observed in the population of Guyana by Garcia-Rodriguez *et al*. (1998).

The MODELTEST program indicated the HKY85 model (AIC based) was ideal for the control region, but we used

Table 1 Population parameters for all *Trichechus manatus* populations: (*n*) sample size, HT (number of haplotypes), *S* (polymorphic sites), *NS* (number of nucleotide substitutions), *h* (haplotype diversity) and π (nucleotide diversity)

Population	(*n*)	HT	*S*	*NS*	*h*	π
USA	28	1	0	0	0.0000	0.000000
Puerto Rico	62	3	2	2	0.5431	0.001473
Dominican Republic	6	2	1	1	0.5333	0.001314
Mexico	14	3	27	27	0.6154	0.040541
Belize	43	3	28	28	0.5581	0.036740
Colombia	33	8	31	32	0.7803	0.031248
Venezuela	4	3	3	3	0.8333	0.003708
Guyana	4	4	4	4	1.0000	0.005769
Guyana*	7	5	30	31	0.8571	0.048923
French Guiana*	3	2	3	3	0.6667	0.004969
Brazil	30	2	1	1	0.0667	0.000164
Brazil*	31	3	28	28	0.1269	0.005201

*Populations including the likely hybrids.

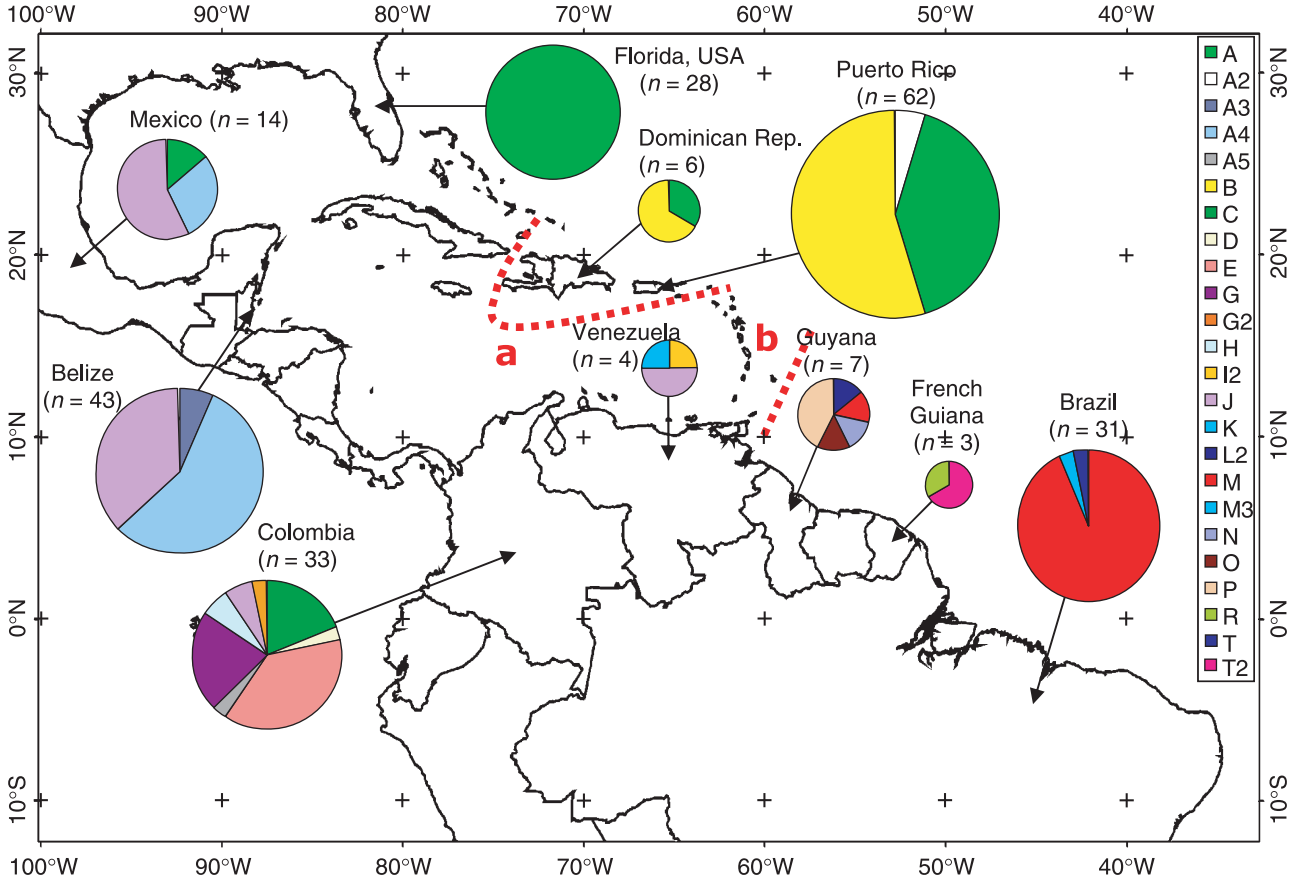

Fig. 1 Control region mtDNA haplotypes found in *Trichechus manatus* populations. The circle area reflects the country sample size and disk sectors are proportional to haplotype frequencies depicted as distinct colours. The discontinuous lines are the two likely genetic barriers (a and b) suggested by the algorithm Barrier (Manni *et al*. 2004).

the similar Tamura–Nei model in ARLEQUIN and MEGA due to software limitations. Negligible differences were observed in the results when using different models (data not shown). High population differentiation was found for nine populations (Φ_{ST} = 0.658) as well as for 10 populations (including French Guiana) with the addition of the likely hybrids (Φ_{ST} = 0.634). No differentiation was found between the populations from Puerto Rico and the Dominican Republic, but a high differentiation was found among Florida, Puerto Rico and the Dominican Republic when compared to the populations from Brazil, Guyana and Venezuela in South America (Table 2). The BARRIER program was used to assign likely barriers to gene flow. One barrier was assigned isolating the two Greater Antilles islands (Puerto Rico and the Dominican Republic) and the next barrier separated Guyana and Brazil, suggesting their relative isolation from all other tested populations (Fig. 1). The A haplotype, found in all Florida samples, has also been observed in Mexico, and in Puerto Rico and the Dominican Republic despite the potential geographic barrier to gene flow around the Antilles.

The association test between geographic and genetic distances using direct linear distances presented no significant correlation (Mantel value: Z = 52.65, r = 0.273, P = 0.2734). We have also tested the geographic distances along the coastline because manatees live and usually disperse along shallow waters, avoiding the open sea and potential wave action. The correlation between genetic and coastline geographic distances was positive and significant (Mantel value: Z = 109.74, r = 0.641, P = 0.0002) supporting the idea that manatees migrate along the coast.

Our analysis detected a significant difference between populations from Guyana and Brazil as compared to populations from Colombia and Venezuela (Table 2). We suggest that the continuity of the Caribbean islands, from Trinidad Island to north of the Lesser Antilles, could have operated as a land barrier to gene flow between those populations at least during times when ocean levels were lower during the Pleistocene. Removing Brazil and Guyana from the Mantel test, and considering the distance along the coast, resulted in a regression line that is steeper than the previous analysis indicated (Mantel value: Z = 43.33, r = 0.687, P = 0.005).

Table 2 Pairwise Φ_{ST} estimates for nine *Trichechus manatus* populations (excluding likely hybrids) and *P* values for the exact test of population differentiation (below diagonal) calculated with ARLEQUIN

Population	USA	Puerto Rico	Dominican Republic*	Mexico	Colombia	Venezuela*	Guyana*	Belize	Brazil
USA	0.0000	0.7078	0.0956	0.2529	0.1839	0.0028	0.0055	0.9115	0.0008
Puerto Rico	0.4131	0.0000	*inf*	0.1680	0.1325	0.0124	0.0168	0.6501	0.0105
	$P = 0.0000$								
Dominican Republic*	0.8395	−0.0680	0.0000	0.6662	0.3217	0.0175	0.0302	1.7107	0.0034
	$P = 0.0006$	$P = 1.0000$							
Mexico	0.6657	0.7533	0.4282	0.0000	5.9587	2.0075	0.4847	15.2071	0.1637
	$P = 0.0000$	$P = 0.0000$	$P = 0.0006$						
Colombia	0.7317	0.7926	0.6077	0.0696	0.0000	5.3752	0.3481	1.401	0.1884
	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$					
Venezuela*	0.9953	0.9795	0.9713	0.2032	0.0824	0.0000	0.0483	0.7033	0.0051
	$P = 0.0000$	$P = 0.0000$	$P = 0.0179$	$P = 0.0979$	$P = 0.0042$				
Guyana*	0.9904	0.9714	0.9495	0.4995	0.5838	0.9235	0.0000	0.4765	0.2380
	$P = 0.0000$	$P = 0.0000$	$P = 0.0389$	$P = 0.0027$	$P = 0.0007$	$P = 0.4261$			
Belize	0.3545	0.4343	0.2221	0.0315	0.2573	0.4187	0.5020	0.0000	0.2762
	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$	$P = 0.0427$	$P = 0.0000$	$P = 0.0032$	$P = 0.0000$		
Brazil	0.9985	0.9816	0.9941	0.7446	0.7189	0.9912	0.6772	0.6329	0.0000
	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$	$P = 0.0039$	$P = 0.0000$	

The number of female migrants (N_{mf}) per generation between pairwise populations are depicted in the above diagonal matrix; *inf*, infinite.
*Comparisons with these populations result sometimes in insignificant values likely due to their small sample sizes.

Table 3 Parameters for the three manatee species: sample size (*n*), number of haplotypes (HT), haplotype (*h*) and nucleotide diversity (π), polymorphic sites (*S*), mean number of pairwise nucleotide differences between haplotypes (*d*), the ratio *S*/*d* and the Tajima test values of neutrality (*D*)

Species/cluster	(*n*)	HT	*h*	π	S	*d*	*S*/*d*	*D*
T. senegalensis	6	5	0.9333	0.019581	15	7.533	1.991	0.905
T. inunguis	92	31	0.8772	0.005353	34	2.127	15.982	−2.119*
T. manatus	224	20	0.8554	0.038648	45	13.580	3.314	2.354
T. m. cluster *I*	137	8	0.7104	0.002655	10	1.007	9.934	−1.007
T. m. cluster *II*	53	7	0.6531	0.002566	7	1.038	6.745	−0.856
T. m. cluster *III*	35	6	0.2689	0.001112	6	0.450	13.321	−1.899*

*Significant Tajima *D* values at $P < 0.01$.

Phylogenetic reconstruction using nucleotides to draw MJN (Fig. 2) and NJ tree (Fig. 3), among the control region haplotypes of *T. manatus*, showed three very distinct lineage clusters: (*I*) A, A2, A3, A4, A5, B, C, D; (*II*) E, G, G2, H, I, J, K; and (*III*) M, M2, M3, N, O, L. These clusters display a heterogeneous geographic distribution (Fig. 1): (*I*) Florida, Mexico, Greater Antilles, Central America and Caribbean coast of South America; (*II*) Mexico, Central America and Caribbean coast of South America; and (*III*) the northeastern coast of South America (Brazil and the Guyanas).

Haplotypes from cluster *III* were identified only in Guyana and Brazil, which also supports the Lesser Antilles barrier hypothesis. Haplotypes from Florida, Puerto Rico and the Dominican Republic revealed only cluster *I* haplotypes. Individuals sampled from Mexico, Belize, Colombia and Venezuela presented haplotypes belonging to clusters *I* and *II*.

The mismatch distribution analysis for *T. manatus* (data not shown) presented a multimodal pattern that confirms the observed population structure. Population analysis of samples from Colombia, Mexico and Belize demonstrated bimodal distribution due to the presence of two control region mtDNA clusters. The remaining populations presented unimodal distribution with a single cluster containing few divergent haplotypes. While the shape of mismatch analysis indicates recent expansion, the Tajima test presented no significant results for any of the populations included in this study. However, when we analysed the three control region clusters observed in *T. manatus* separately, we obtained a negative and significant *D* only for cluster *III* (Table 3). This result indicates a bottleneck followed by population expansion in cluster *III* that occurred exclusively east of the Lesser Antilles. Using the average of

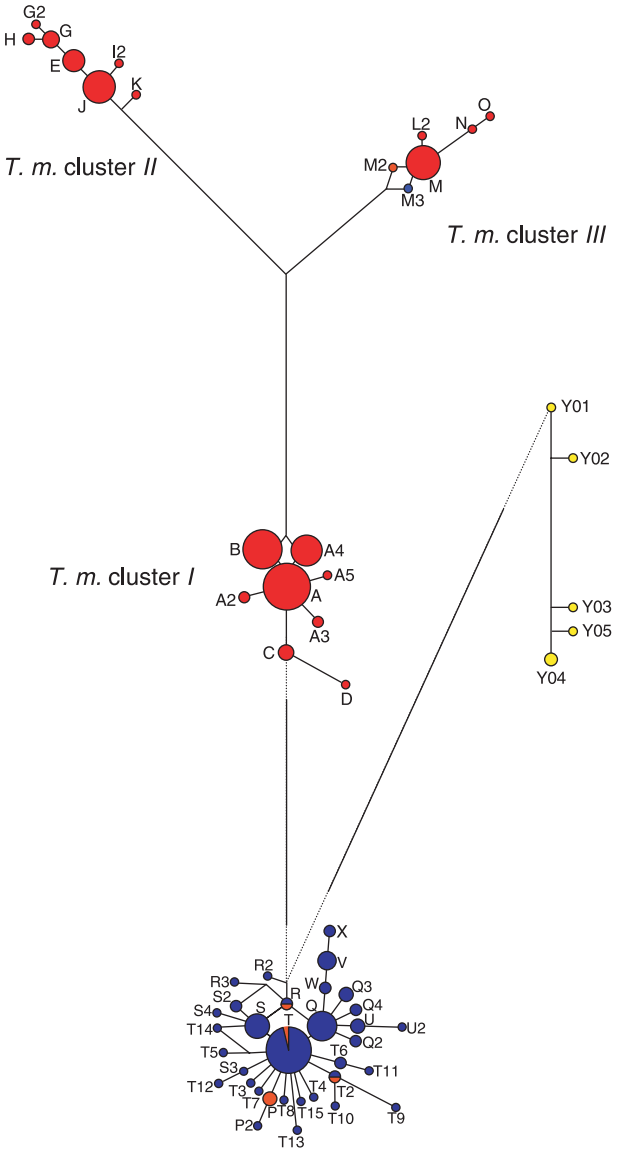

Fig. 2 Median-joining network (MJN) of all control region haplotypes for the three manatee species. In red colour, *Trichechus manatus* haplotypes and their three clusters (*I*, *II* and *III*); in blue, the *Trichechus inunguis* haplotypes and in yellow the *Trichechus senegalensis* haplotypes. Circle area is proportional to the number of individuals with each haplotype. Dashed lines indicate that interspecific connections should be considered with caution due to long branches (Bandelt *et al*. 1999).

pairwise differences ($\tau = 3.0$), we calculated the time of the beginning of the expansion to be about 182 926 years before present (BP). Cluster *III* also presents the highest *S*/*d* values when compared to clusters *I* and *II*, which is indicative of recent expansion (Table 3). This also suggests the existence of two evolutionary significant units (ESUs), east and west of the Lesser Antilles barrier, with the eastern ESU (Guyana and Brazil) resulting from an expansion following a bottleneck in the late Pleistocene.

Amazonian manatee. We have identified 31 typical haplotypes in *T. inunguis*. In addition, we identified one haplotype (M2) that is more closely associated with *T. manatus* haplotypes. Our analysis revealed 34 substitutions consisting of 32 transitions and 2 transversions. The *T. inunguis* haplotypes are closely related (Figs 2 and 3), despite the fact that the individuals analysed originated from different countries and regions in Brazil.

The location of Brazilian samples were referenced by GPS and plotted on a satellite image map (data not shown). Using this plot map and considering the connectivity of the rivers, lakes and channels, we defined 18 subpopulations of *T. inunguis*. Other alternative groupings were defined for the first 18 subpopulations, and we observed a moderate interpopulational differentiation ($\Phi_{ST} = 0.10$ to 0.22). Adding 11 individuals without GPS data from Colombia, Peru and Brazil, we observed a $\Phi_{ST} = 0.18$. We have also observed some unequal distribution of haplotypes. The basal haplotypes R, R2 and R3 (Figs 2 and 3) were reported only in the more eastern localities, closer to the mouth of the Amazon. Despite the lower differentiation (compared to *T. manatus*) among *T. inunguis* populations, it is enough to be further considered for species management, especially in cases where animal translocation between distant areas is being suggested.

The presence of a single compact cluster (Figs 2 and 3) in the Amazonian manatee allowed us to estimate more precisely the effective female population size (N_{ef}) for the species. The N_{ef} was estimated as 133 200 females [estimated from $\theta(\pi)$], 479 588 females (estimated from h), and 510 823 females [estimated from $\theta(S)$], all of which are likely overestimated (see Discussion). The mismatch distribution pattern of *T. inunguis* was approximately unimodal and wave-like (data not shown), which is compatible with a population bottleneck followed by expansion in the recent past (Rogers & Harpending 1992). The mismatch observed fits perfectly on the expected Wright–Fisher model. Using the average of pairwise differences ($\tau = 2.12$), we calculated the time of the recent bottleneck at 129 216 BP. The Tajima's test of neutrality was significant (Table 3), showing an excess of rare haplotypes and a strong indication of past population expansion ($D = -2.09$), excluding the possibility of purifying selection in the control region. The *D* values for *T. manatus* populations and *Trichechus senegalensis* were not significant.

West African manatee. Five haplotypes from four different locations were identified in our sample of *T. senegalensis* ($n = 6$). These haplotypes were characterized by 15 polymorphic sites consisting of 14 transitions and 1 transversion. No haplotype was shared between locations. Two clusters were found for the African manatee (Figs 2 and 3): (*I*) Y1 and Y2; and (*II*) Y3, Y4 and Y5. The first cluster was composed of Guinea-Bissau haplotypes and the second

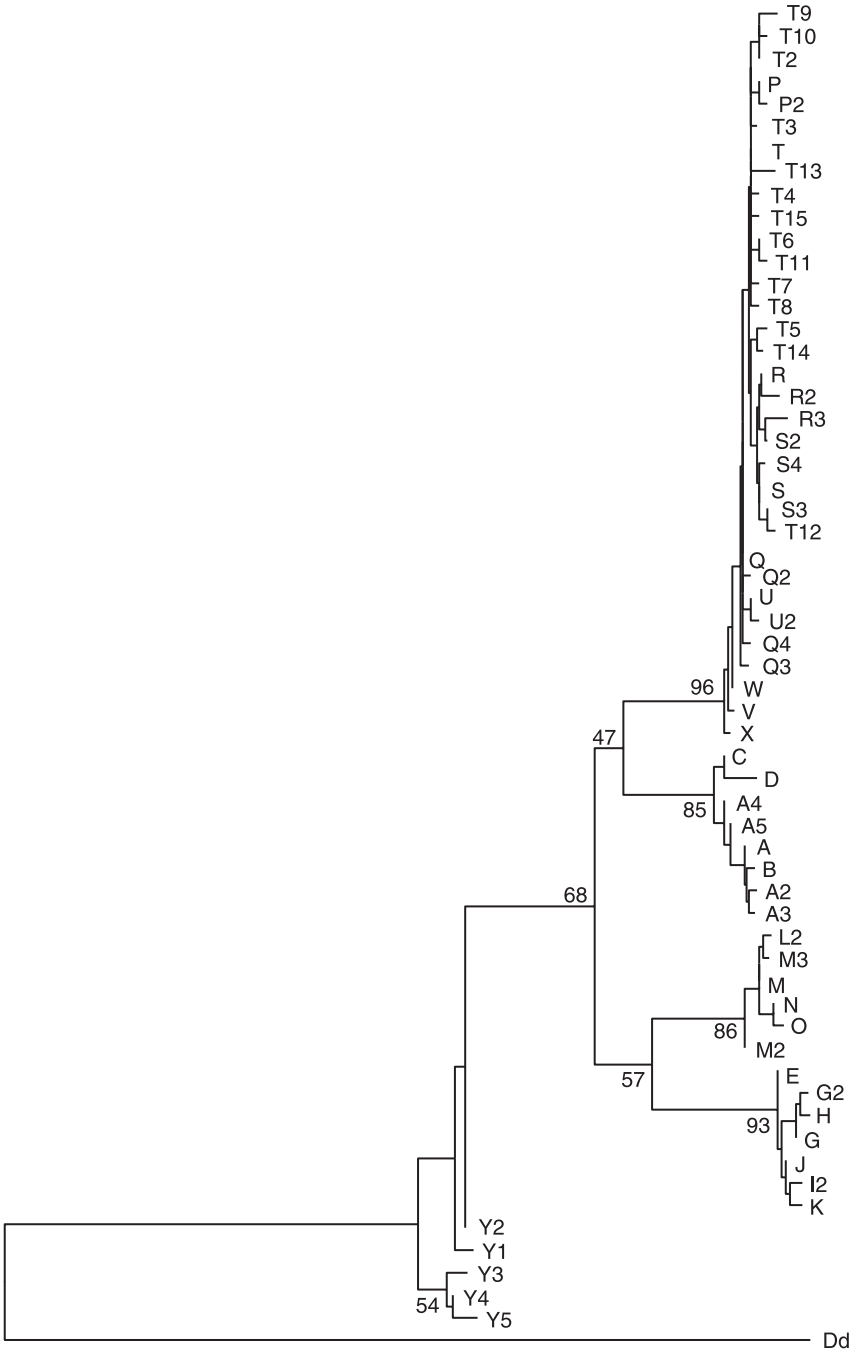

Fig. 3 Neighbour-joining (NJ) tree of control region sequences from all three manatee species. Haplotypes A to O, *Trichechus manatus*, P to X, *Trichechus inunguis*, Y1 to Y5, *Trichechus senegalensis*. *Dugong dugon* (Dd) was used as outgroup. Bootstrap supporting values are shown on the main branches only. It was used Tamura–Nei model, alpha = 0.19114 available in MEGA. Distinct models or alpha parameters have not changed the topology.

of Ghana (haplotype Y3), Chad (haplotype Y4), and Niger (haplotype Y5) haplotypes. The two samples from Chad (both haplotype Y4) were collected from Lake Léré on the southwest border of the country, over 1000 miles (1600 km) from the ocean. Lake Léré is part of the Niger River drainage basin, communicating with the Benué River. The Niger sample was collected from the Niger River area. Low differentiation was observed between haplotypes from the interior rivers and lakes of Africa and the Ghana coast.

Patterns of interspecific variability

Eighty polymorphic sites for the mtDNA control region, including 74 transitions and 11 transversions were detected among *T. manatus*, *T. inunguis* and *T. senegalensis*. Both transitions and transversions were detected at positions 192, 267, 328, 331, and 332. The West African species displayed the highest haplotype diversity ($h = 0.9333$, $n = 6$) followed by *T. inunguis* ($h = 0.8772$, $n = 92$) and then

T. manatus ($h = 0.8554$, $n = 224$), excluding the likely hybrids. However, the Amazonian manatee presented the lowest nucleotide diversity ($\pi = 0.005353$) as demonstrated by the compact haplotype cluster found for this species. The West Indian manatee displayed the highest nucleotide diversity ($\pi = 0.038648$) reflecting the three clusters found, followed by the West African manatee with two clusters ($\pi = 0.019581$). The values of S/d ratio (Table 3) were much higher for *T. inunguis* (15.98) when compared to *T. manatus* (3.31) and *T. senegalensis* (1.99).

The phylogenetic relationship among the three species based on the NJ tree of the control region mtDNA sequences (Fig. 3) suggests that *T. inunguis* and *T. manatus* form a monophyletic clade, with *T. inunguis* more related to the cluster *I* of *T. manatus*. This implies that *T. manatus* is a paraphyletic species, a conclusion that has also been recently proposed by Cantanhede *et al*. (2005) using shorter control region sequences. However we have also analysed 615 bp of the cytochrome *b* (cyt *b*) gene in all sirenian species (including several individuals with distinct control region haplotypes). The NJ and maximum-parsimony (MP) trees of the corresponding cyt *b* amino acids (Fig. 4) also suggest a monophyly of the trichechid species, but show *T. inunguis* as the basal species in this clade with even higher supporting values than the control region trees indicate. The same topology is also observed in the cyt *b* trees using nucleotides with different methods and evolutionary models (trees not shown), as well as using the PAUP program (Swofford 1998). When a linearized approach implemented in MEGA (Kumar *et al*. 2004) was utilized, we were able to date the ancestral nodes for each species using the nucleotide divergence. We used an evolutionary rate of 0.0066/site/Myr for the cyt *b* calculated from the average divergence between *Dugomg dugon* and *Trichechus* spp., and assuming a common ancestry between those lineages from at least 20 million years ago (Ma) (Domning 1994). We obtained dates of 621 000, 371 000 and 308 900 BP for *T. manatus*, *T. inunguis* and *T. senegalensis*, respectively.

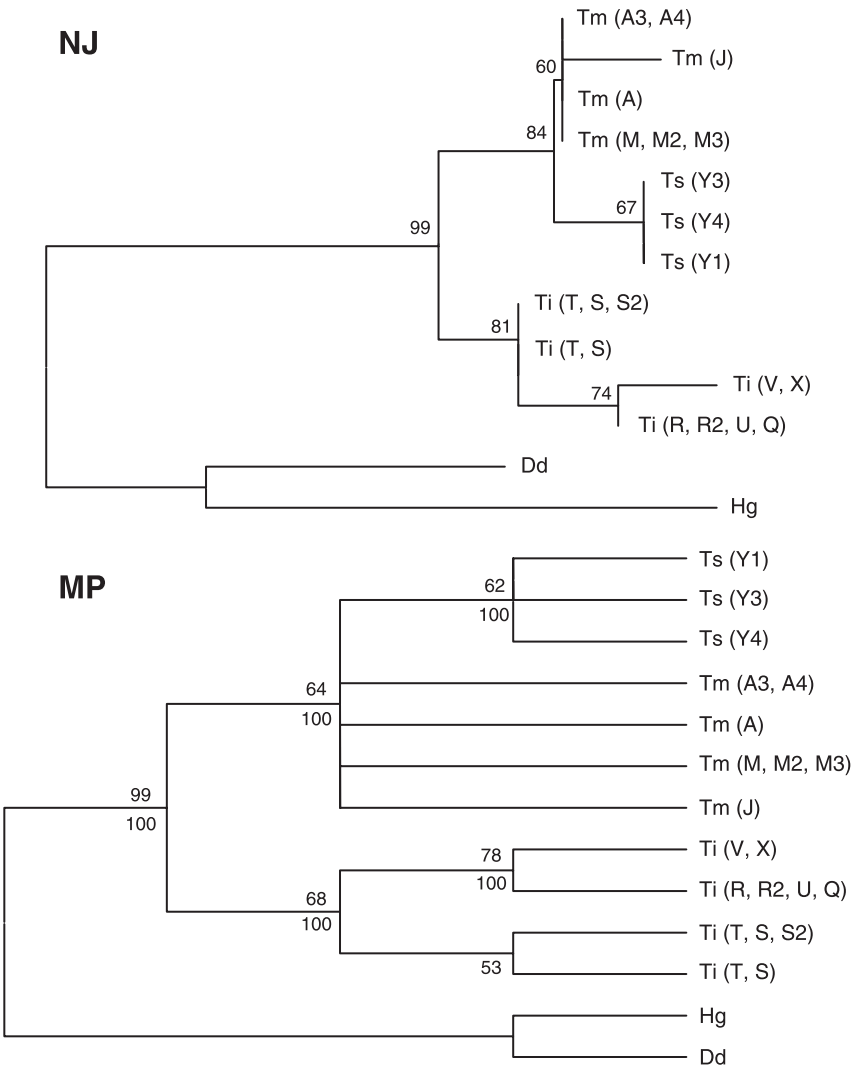

Fig. 4 Neighbour-joining (NJ) and maximum parsimony consensus (MP) trees of the cyt *b* gene for the five sirenian species using amino acids generated by the MEGA software. The trichechid control region haplotypes related to each cyt *b* sequence is depicted together the species name (Tm, *Trichechus manatus*; Ti, *Trichechus Inunguis*; and Ts, *Trichechus senegalensis*). As outgroups we have used sequences of *Dugong* (Dd) and *H. gigas* (Hg), the latter was obtained from GenBank (D83049). Bootstrap supporting values are shown above the main branches and in the MP tree are also shown the frequencies of branches (below) appearing in individual trees (43) to build the consensus.

However these dates should be taken with caution, as we have not included cyt *b* sequences from all individuals for all species, only those included in Fig. 4.

Hybridization between American manatee species

A sample identified as *T. inunguis* found near the mouth of the Amazon, presented a *T. manatus*-related mtDNA haplotype (M2). Three individuals from French Guiana, and one from northern Brazil, identified as *T. manatus*, presented haplotypes that are affiliated with those of the Amazonian manatee (R, T, T2). Garcia-Rodriguez *et al.* (1998) also found three specimens from Guyana (haplotype P) with the same characteristics. Karyotype analyses in *T. manatus* and *T. inunguis* demonstrate a large difference in chromosome numbers, 48 chromosomes for *T. manatus* from Florida (Gray *et al*. 2002) and 56 for *T. inunguis* (Assis *et al*. 1988). From our analysis, a karyotype of a Brazilian *T. manatus* with a typical control region haplotype (M) displays $2n = 48$. However, an individual from northern Brazil, previously classified as *T. manatus* but with an Amazonian manatee mtDNA (T), showed a diploid number of $2n = 50$ (data not shown), intermediate between *T. manatus* and *T. inunguis*. This chromosome number can be the result of an F_2 backcross, possibly due to breeding between an F_1 hybrid female with a male *T. manatus*.

We also analysed autosomal microsatellites to test the hybridization hypothesis. We genotyped TmaH11 alleles in 26 *T. manatus* individuals from Brazil (including 1 likely hybrid), 2 from Florida, 3 from Belize and 2 likely hybrids from French Guiana, and 84 *T. inunguis* (including 1 possible hybrid from Brazil). We also used the TmaA09 microsatellite to analyse the likely hybrids as before and additional 25 *T. manatus* from Brazil, 2 from Florida and 1 from Belize, and 57 *T. inunguis* individuals.

The TmaH11 microsatellite was shown to have species-specific alleles, so was a good marker to identify the alleles derived from the different American species. Seven alleles were found for the Amazonian manatee ($h = 0.5576$), with the 22 repeats allele occurring most frequently. Only one allele was found for the West Indian manatees ($h = 0$) from Brazil (25 repeats). Three of the likely hybrids had one allele characteristic of the Amazonian manatee (22 repeats) and one from the West Indian manatee (25 repeats), supporting the hybridization hypothesis (Table 4). The manatee from the river mouth presented both of the Amazonian manatee alleles (22 and 24 repeats). However, it could possibly be an F_2 backcross or a later generation hybrid. The TmaA09 microsatellite presented several alleles and could not be used to discriminate between West Indian and Amazonian manatee origin. Ten alleles were found in the Amazonian manatees ($h = 0.8245$) and two alleles were found in the West Indian manatees from Brazil ($h = 0.2155$). Both autosomal microsatellites display much higher diversities (h) in *T. inunguis* than in *T. manatus* populations from Brazil.

Discussion

Population structure, present species distribution and Palaeoecological correlation

Phylogeographic analysis showed a marked population structure for *Trichechus manatus*, with limited distribution in the Northern and Southern hemispheres around the 24 °C mean annual isotherm (Whitehead 1977). The inter-population differentiation and within-population genetic diversity present today seems to be the result of a combination of the linear stepping-stone model of dispersal along the shallow coastal waters and the influence of the species'

Table 4 Morphological identification, country of origin, mtDNA control region haplotype, microsatellite alleles and karyotype for the suspect hybrids ($n = 8$). The TmaH11 microsatellite alleles 22 and 24 repeats are typical from *Trichechus inunguis* and the 25-repeat allele is typical from *Trichechus manatus*. The mtDNA control region haplotype (P) from three suspected hybrids from Guyana were identified by Garcia-Rodriguez *et al.* (1998)

			Microsatellite alleles (number of repeats)				
Morphological identification	Country	mtDNA haplotype	TmaH11		TmaA09		Karyotype
T. inunguis	Brazil	M3	22	24	18	18	—
T. manatus	Brazil	T	22	25	14	16	2n = 50
T. manatus	French Guiana	T2	22	25	16	16	—
T. manatus	French Guiana	R	22	25	13	16	—
T. manatus	French Guiana	T2	—	—	—	—	—
T. manatus	Guyana	P	—	—	—	—	—
T. manatus	Guyana	P	—	—	—	—	—
T. manatus	Guyana	P	—	—	—	—	—

Data not available are marked with a hyphen.

latitudinal distribution. According to the linear stepping-stone or isolation-by-distance models, the most extreme populations would be the most genetically distinct in this case, the population from Puerto Rico when compared to Brazil. We have also observed that genetic diversity is decreased in the extremes of their latitudinal distribution, with Florida and Brazil presenting the less diverse populations. This is likely the result of a founder effect, which may be due to a process of recolonization by few individuals from lower latitudes to the more northern and southern regions following glaciation, assuming that during the most recent glacial period (about 30 000–12 000 BP) the tropical Atlantic Ocean was 5 °C cooler than it is today (Guilderson *et al*. 1994). During the same period, the ocean level was also approximately 100 m lower (Colman & Mixon 1988). Therefore, some areas that are currently submersed might have served as natural barriers for manatee distribution during the last glaciation. Mantel tests have supported the idea that West Indian manatees migrate along the coast and tend to avoid the open sea, as they depend on the availability of fresh water and vegetation as afforded by local habitats (Lefebvre *et al*. 2001). Furthermore, it also suggests that manatees may not migrate through the shallow water corridor of the Lesser Antilles between North and South America. Indeed, manatees have not been documented to occur in significant numbers in the Lesser Antilles since the 18th century (Ray 1960). Thus, it seems the Lesser Antilles may not be working as stepping stones for the occasional establishment of manatees crossing the open waters between South and North America, which was also likely true during the Pleistocene. This conclusion is reinforced by our findings of the large genetic differentiation between populations (Table 2) from South America (Venezuela, Brazil and Guyana) and the ones from Florida and Greater Antilles (Puerto Rico and Dominican Republic). In this study we suggest that the continuity of the Lesser Antilles from Trinidad towards the north, was and may still be a likely geographic barrier to West Indian manatees and possibly also to the hybrids. Although this barrier would have been much more prominent during the last glaciation when sea levels were lower. The existence of a gene flow barrier was supported by compelling evidence from distinct analyses including the measure of population differentiation, the Mantel tests, the phylogeographic network analysis and the test to detect historical barriers to gene flow.

We have analysed *T. manatus* diversity according to the country affinities because this reflects effective management units for conservation as previously discussed (Garcia-Rodriguez *et al*. 1998). To evaluate whether we have a bias due to these country designations, we used other population divisions according to the geographic positions of each individual sample, particularly for populations of countries with extensive coastlines. Using GPS localities (data not shown) we have further divided populations from Brazil, Mexico and Colombia in two localities each. Using SAMOVA (Dupanloup *et al*. 2002), the grouping of geographically redefined populations with GPS data is very congruent with the grouping using our country populations. Indeed, the two populations of Brazil and the one from Guyana cluster together in all major groupings (3, 4 and 5 groups) suggested by SAMOVA. We have only observed some heterogeneity in the samples from Mexico (data not shown), where the population found in lakes in the Mexico Gulf is homogeneous, and the one from the Caribbean coast is more similar to the ones from Belize and Greater Antilles. However, the heterogeneity in Mexico is also observed using the phylogenetic reconstruction (see Results). Furthermore, Mantel tests using the *T. manatus* GPS localities resulted in the same conclusion of a larger significant correlation using coastline than direct line geographic distances. This means that our population designation by country seems to represent natural divisions well. This is likely due to the nature of this species that displays a very restricted migration along coastlines.

The West African manatee is restricted to rivers, lakes and the coast of tropical and subtropical waters. However, many manatees travel over 1000 miles (1600 km) from the ocean up the Niger River to reside in Lake Léré, which has in the past connected with the Benué River. Traditional allegations of morphological differences between inland and coastal manatees in Africa persist (Kleinschmidt 1982). Although we analysed only six samples, we found a phylogeographic structure with two divergent clusters that do not agree with the simple separation of riverine and coastal populations as suggested. Domning & Hayek (1986) used morphological analysis on three specimens from the mouth of Niger River and two from northern Nigeria and also were not able to discern any significant differences between coastal and inland specimens. The inland samples from Chad and Niger form a monophyletic group with the coastal sample from Ghana, while the samples from Guinea-Bissau formed another cluster. The manatees inhabiting the inland Niger basin are isolated from the coast due to waterfalls, rapids and very recently by dams on the Niger River. Additionally, there may be another separation between populations from the north (Guinea-Bissau) and south that includes the specimens from the Niger River basin (Ghana, Chad and Niger). These separations may occur between hydrographic basins and their coastal proximity from the river mouth. Indeed, Parr (2000) reported previously the existence of three clusters (Guinea Bissau, Chad, and Cameroon–Gabon–Ghana) in her mtDNA analysis of 17 *Trichechus senegalensis*.

The effective numbers of females (N_{ef}) that we calculated for *Trichechus inunguis* using the mtDNA control region are probably overestimated, although the present Amazonian manatee population size has not been directly estimated.

The estimated N_{ef} obtained using mtDNA data could be a portrait of the past effective female size of the species without accounting for the recent population reduction in many regions of the Amazon. Thus, our main conclusion is that the remaining genetic diversity is still large, likely equivalent to a time before the last century of hunting in the Amazon Basin. For *T. inunguis*, the MJN starlike pattern with a single cluster, the negative Tajima *D*-test value, the mismatch distribution analysis, and the high S/d value suggest a recent expansion after a bottleneck. Considering that the most basal Amazonian manatee haplotypes (R, S) were usually found in the eastern localities studied, we suggest the species expanded from east to west, i.e. from the Amazon mouth to the inner Amazon Basin. If we consider the coalescence time of present-day mtDNA lineages as a rough approximation of the speciation event, these data support that the most recent taxon is the Amazonian species, followed by the West Indian and the West African manatees.

The mtDNA control region phylogeny (Fig. 3) suggests *T. inunguis* is the sister group to the *T. manatus* cluster *I*, indicating that *T. manatus* would be a paraphyletic species, with some haplotypes (cluster *I*) more related to *T. inunguis*, as recently suggested by Cantanhede *et al.* (2005). However, our analysis of the cyt *b* gene (Fig. 4), a region less prone to homoplasy than the control region, suggests instead that *T. inunguis* is basal related to *T. manatus* and *T. senegalensis*, and the two later species are likely derived from the same marine ancestor. Our interpretation, which relies on the genetic analyses of all three extant species, agrees with the conclusions of Domning (1994) who proposed the monophyly of the marine species, implying a long time of separation for *T. inunguis*. Considering that *T. inunguis* is a more recent taxon compared to the other marine trichechids (see Discussion above), it may be the only surviving species of an ancient lineage adapted to fresh water. Intraspecific analyses in Colombian *T. manatus* resulted in two haplotype clusters and also showed the highest diversity. Thus, we suggest that Colombia may be most likely the place of origin for *T. manatus*. However, the haplotype distribution (Figs 1 and 2) indicates that this species likely dispersed from at least two distinct regions located, respectively, east and west of the Lesser Antilles. Those regions could be two Pleistocenic refuges from where *T. manatus* populations recovered during the Holocene leading to the present-day distribution, which indicates at least two distinct ESUs.

Management, conservation and interspecific hybrids

The marked genetic structure and geographic subdivision of *T. manatus* should be considered in its management and conservation. We suggest avoiding translocation and captive breeding between individuals from distant regions. Management strategies should consider particularly the distinctiveness of manatees separated by the Lesser Antilles which isolate the populations in the Guyanas–Brazil from other locations. Indeed, we conclude that the Lesser Antilles divide *T. manatus* into two distinct ESUs, and perhaps, further analysis will indicate a subspecies status for both units. Also, independent evidence (mtDNA and autosomal microsatellites) indicates a much lower diversity in the Brazilian population of *T. manatus* as compared to *T. inunguis*. This emphasizes the need for careful management of *T. manatus* in Brazil, where the population is under especially serious threat due to its small population size, low occurrence throughout a large area, low genetic diversity and interspecific hybridization (see below).

Although the *T. inunguis* did not show the remarkable spatial population structure evident in *T. manatus*, we recommend a similarly careful analysis of any animal being considered for translocation. We also encourage the implementation of a larger population study that would include other Amazonian regions to elucidate the population structure of this species at the macro-regional level. Because of a significant population divergence (Φ_{ST} ~0.20), the mating of captive individuals from different Amazonian regions at the rehabilitation centres should also be monitored with caution. The high genetic diversity observed in *T. inunguis* was also reported in the Cantanhede *et al.* (2005) study. The authors suggested that a recent demographic expansion, perhaps during the last 30–40 years of protection in the Amazon, could have mitigated the effect of hunting in the last century. As they discuss, this conclusion must be viewed with caution. In our opinion, the most likely explanation is that the population reduction by hunting has not erased the genetic signal of the expansion beginning in the Pleistocene.

The observed diversity in our few samples of *T. senegalensis* indicates a stronger population structure than what exists in *T. inunguis*. A larger population study in Africa, including samples from northern rivers such as the Senegal, and from the southern limit such as the Cuanza River, would be very informative. However, in the last 40 years, several dams on the inland waterways of Chad have created landlocked, isolated populations of manatees (J. Powell, unpublished). These dams serve as gene-flow barriers that may lead to significant differences in *T. senegalensis* diversity distribution in the future. Thus, landlocked populations will be worth monitoring as a comparison to the other natural populations of this species.

Hybridization can be a very important conservation problem (Allendorf *et al.* 2001), particularly when it involves populations of few individuals like the *T. manatus* from the Brazilian coast. Other analyses reinforced the hypothesis of hybridization between the American species, indicated initially by the mtDNA results. Our study revealed that hybridization might occur with a relatively

high frequency, as suggested by the identification of eight individuals that are likely hybrids from our sample set. We also found evidence that interspecific hybrids result from at least two generation (F_2) backcrosses between *T. manatus* and *T. inunguis*, whose F_1 hybrids mated with one of the parental species. In the microsatellite analysis, particularly using the TmaH11 locus, we would expect 50% occurrence of both parental alleles at backcross F_2 hybrids. This probability would be decreased by half with each subsequent generation. Three of four analysed hybrids presented both parental TmaH11 alleles, which indicate they are the results of recent hybridization. The hybridization process likely follows Haldane's law, which states that only hybrid males are infertile in mammals. However, no study could be done to confirm this possibility.

Anatomically, the likely hybrid from the Brazilian coast is very similar to a West Indian manatee, but it also displays some characteristics of an Amazonian manatee, such as lacking some nails on both pectoral flippers, its caudal fin is completely rounded, and there are two light spots on its abdomen. The individual is also shorter for its age than a typical *T. manatus* with the size being more consistent with *T. inunguis* (F. P. Colares, personal communication). Domning & Hayek (1986) reported finding specimens with similar morphological characteristics near the mouth of the Amazon River, including an Amazonian manatee from the Pará state of Brazil with a single nail on each flipper and two apparently normal *T. manatus* from Suriname lacking nails entirely.

All of these hybrids were found around an area of sympatry located at the mouth of the Amazon River. The hybridization area likely spans from the mouth of the Amazon River west towards Guyana. In this case, the area would be influenced by the ocean currents pushing great volumes of Amazon water from the river mouth towards the west. The annual average of 175000 m^3/s of freshwater discharge into the ocean (Tundisi *et al.* 1999) would also create a good environment for these hybrids, as they are likely to not be so well adapted to salt water. High salinity water is a limiting factor for *T. inunguis*, whereas fresh water generally is not a limiting factor to *T. manatus*, which is frequently found in the mouths and interiors of rivers (Lefebvre *et al.* 2001). Hybrids may also occur on the extreme eastern coast of Venezuela and east of Trinidad Island near the mouth of the Orinoco River with the Lesser Antilles serving as a barrier to hybrids. All four samples from Venezuela display typical *T. manatus* control region sequences. According to Domning & Hayek (1986) only *T. manatus* is known to occur in the Orinoco basin, but a specimen apparently without nails was initially identified as *T. inunguis* (von Humboldt 1838). This early account could be the result of past instances of hybridization.

The region of sympatry and hybridization deserves special management attention. We also advise against the translocation of animals from these areas to areas where the hybridization was not genetically detected, such as the northeastern part of the Brazilian coast or the interior of the Amazon Basin. Allowing or introducing new regions of possible hybridization would be very costly for conservation efforts, especially for *T. manatus* populations in Brazil where the population size is estimated at about 500 individuals (Lima 1997; Luna 2001) and could be further reduced due to hybridization with the Amazonian manatee. Finally, we suggest that more attention should be given to populations of manatees in Guyana where 43% of the individuals analysed were suspected hybrids, and especially in French Guiana, where all three manatees studied appear to be products of hybridization. The possibility of a long-term hybridization history between both species cannot be discarded, especially in the Guyanas region, where introgression through recurrent backcrosses with one of parental species seems also a likely scenario, carrying genes from one species to another.

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