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Cranial and chromosomal geographic variation in manatees (Mammalia: Sirenia: Trichechidae) with the description of the Antillean manatee karyotype in Brazil

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Abstract

We employed landmark-based 3D geometric morphometrics to assess cranial size and shape diversification in *Trichechus manatus* and *T. inunguis* to compare it with patterns among all manatee taxa (*T. manatus latirostris*, *T. m. manatus*, *T. inunguis* and *T. senegalensis*), and to analyse geographic variation within American manatee populations, using a sample of 189 skulls. Chromosome G- and C-banding techniques were performed in *T. m. manatus* from Brazil. All taxa were statistically discriminated by skull shape. *Trichechus m. manatus* and *T. m. latirostris* have larger skulls than *T. inunguis*. A morphological discontinuity was noted within *T. m. manatus*, with the Brazilian population south of the Amazon discriminated from the *T. m. manatus* Caribbean and *T. m. latirostris* USA populations. Specimens from Suriname and Guyana had a skull shape more similar to the one from the Caribbean population. The Brazil Antillean manatee population exhibited morphological differences similar in magnitude to those found between the Amazonian and African species. Additionally, structural chromosome differences were detected between that population (chromosome pair 4 is metacentric and 10 is submetacentric) and manatees from Puerto Rico and Florida. Based on such morphological discontinuity and chromosomal divergence, we hypothesize that the Amazon River mouth may be acting as a reproductive barrier for the *T. m. manatus* population in Brazil, thus indicating that its taxonomic status and conservation strategies need an urgent reassessment.

Key words: Manatees – geographic variation – cytogenetics – geometric morphometrics – Sirenia – *Trichechus* – Trichechidae

Introduction

Currently, manatees (Trichechidae) include a single genus, *Trichechus* Linnaeus, 1758, with three species: *Trichechus manatus* Linnaeus, 1758, *Trichechus senegalensis* Link, 1795 and *Trichechus inunguis* (Natterer, 1883). The West Indian manatee (*T. manatus*) ranges from the Atlantic coastline and rivers in Florida (USA), Mexico, discontinuously along the Caribbean coast of Central and South America, also occurring in the Orinoco River Basin in Colombia and Venezuela, and its distribution reaches north-eastern Brazil, in Alagoas State (Lefebvre et al. 2001; Luna et al. 2008b; Castelblanco-Martínez et al. 2012; O'Shea 2014). The Amazonian manatee (*T. inunguis*) occurs in the freshwater systems in the Amazon Basin. Although *T. inunguis* is distributed throughout the Amazon basin, where there is little or no salinity even in the coastal regions, it has been suggested that this species may also sometimes occur in salt water, along the coast of Amapá State, Brazil (Domning 1981; Luna 2013; O'Shea 2014). The African manatee (*T. senegalensis*) is distributed along the rivers, estuaries and coastal regions of western Africa, from Senegal to Angola (Marsh et al. 2012; O'Shea 2014) (Fig. 1). In Brazil, *T. manatus* and *T. inunguis* appear to occur in parapatry near the Amazon River mouth (Domning 1981), where cases of hybridization between the two species have been reported (Vianna et al. 2006; Luna 2013).

The only species for which subspecies have been recognized is the West Indian manatee. Hatt (1934) proposed two subspecies of *Trichechus manatus* based on osteological characters, namely *T. m. manatus* Linnaeus, 1758 for Caribbean and South American populations, and *T. m. latirostris* (Harlan 1824) (Florida manatee) for populations in the United States and coasts of the Gulf of Mexico. Domning and Hayek (1986) confirmed two subspecies of *T. manatus* based mostly on the analysis of linear cranial measurements and suggested that the cool northern coast of the Gulf of Mexico and strong currents of the Straits of Florida have contributed to a restricted gene flow between the two taxa. Additionally, they adopted the name Antillean manatee for *T. m. manatus*, to clearly distinguish it from the Florida manatee. An additional fossil subspecies (*T. m. bakerorum*) is also known from the Late Pleistocene of North America, ranging from Florida to North Carolina (Domning 2005). Despite this taxonomic consensus, the diagnosis of species and living subspecies of *Trichechus* has been re-evaluated several times since the early diagnoses of Cuvier (Harlan 1824; Hartlaub 1886; Hatt 1934; Domning and Hayek 1986). Increased sample sizes and broader geographic sampling have significantly increased the magnitude of observed morphological variation. If the diagnosis of species of *Trichechus* is straightforward based on Domning and Hayek (1986), there are but few strict autapomorphies and many characters have overlapping ranges of variation or are polymorphic. The same is true for the subspecies of *Trichechus manatus*. Genetic data based on mitochondrial and nuclear markers show similar trends of high variability and geographic structuring of *T. manatus* and *T. senegalensis* populations (García-Rodríguez et al. 1998; Vianna et al. 2006; Hunter et al. 2012).

Molecular genetic studies of the West Indian manatee, however, are not concordant with the current subspecies designation

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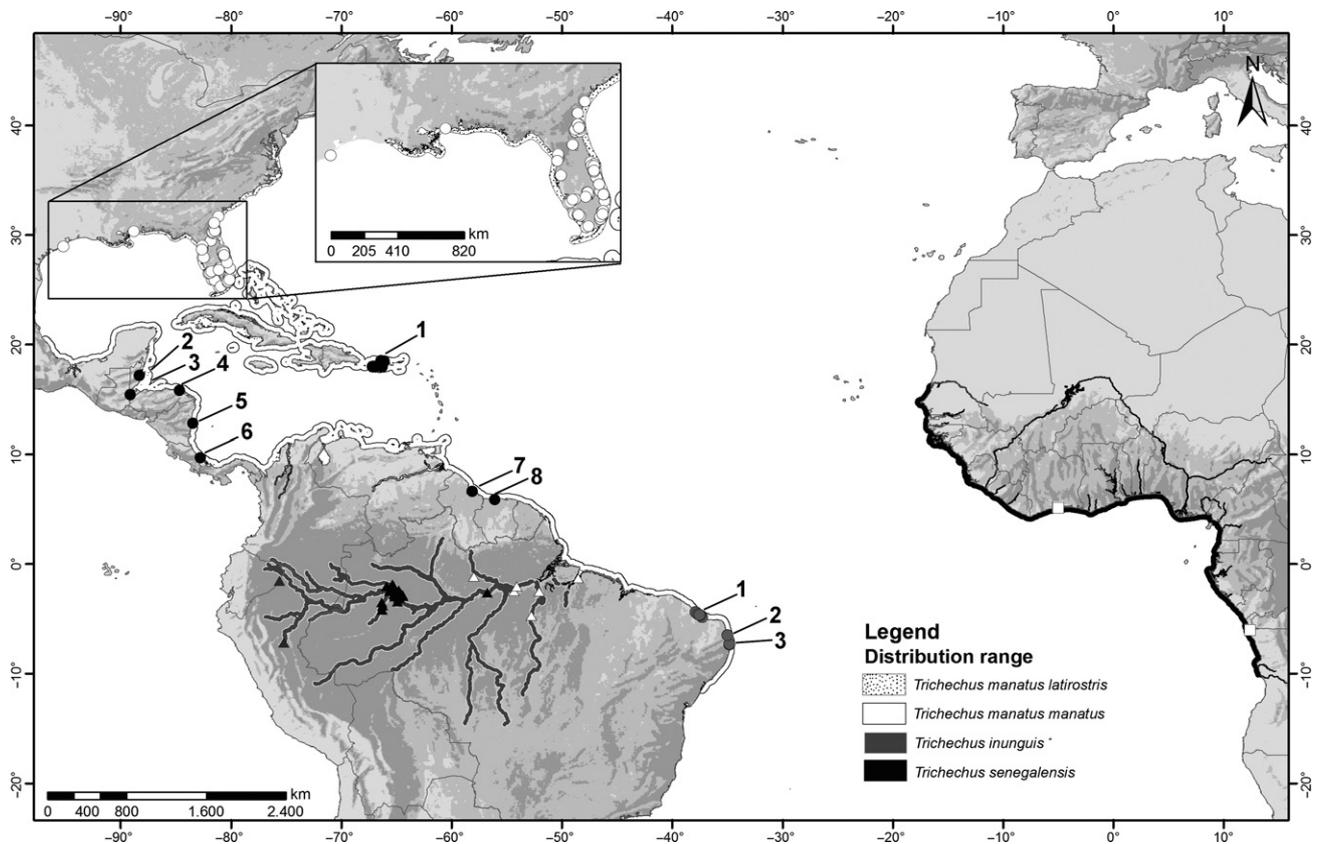


Fig. 1. Collection localities of the skulls and distribution range of the taxa included in the present study. Localities and geographic range are labelled according to current species and subspecies taxonomy, following Domning and Hayek (1986). White circles represent *Trichechus manatus latirostris* individuals from United States of America, black circles represent *Trichechus manatus manatus* Caribbean population (1: Puerto Rico, 2: Belize, 3: Guatemala, 4: Honduras, 5: Nicaragua, 6: Costa Rica, 7: Guyana and 8: Suriname), grey circles represent Brazilian *Trichechus manatus manatus* population (1: Ceará, 2: Paraíba and 3: Rio Grande do Norte), grey triangles represent *Trichechus inunguis* specimens from Peru, black triangles represent *Trichechus inunguis* specimens from Amazonas, white triangles represent *Trichechus inunguis* specimens from Pará, and white squares represent *Trichechus senegalensis* individuals.

based on morphological characters. Mitochondrial DNA (mtDNA) control region (D-loop) (García-Rodríguez et al. 1998; Vianna et al. 2006) revealed three, rather than two, distinctive lineages for this species that are geographically structured with a first cluster comprising samples from Florida, Dominican Republic, Puerto Rico, Mexico, Belize, Colombia and Venezuela; a second cluster with samples from Mexico, Belize, Colombia and Venezuela; and a third one from the Guianas and Brazil. The last lineage supports the Lesser Antilles barrier hypothesis during the Pleistocene, isolating those populations (Vianna et al. 2006). These authors also concluded that the Lesser Antilles divided *T. manatus* into two distinct evolutionarily significant units (ESUs), one including populations from the Guianas and Brazil and another including those from the rest of the range, both of which might deserve subspecific status.

Contrasting with those phylogeographic studies, mtDNA control region and microsatellite data supported the manatees from Florida and from Puerto Rico as distinct populations, with no admixture or recent migrants between those localities (Hunter et al. 2012), supporting the traditionally recognized taxonomic classification at the subspecies level (Domning and Hayek 1986).

The comparison between genetically defined lineages and morphological groupings is not straightforward as sampling units overlap only partially. A critical comparison of the lineages defined by Vianna et al. (2006) with the most complete morphological analysis yet performed (Domning and Hayek 1986) is hampered by the small sample size of specimens from Brazil

($N = 3$, Daryl P. Domning [Howard University, Washington, DC], personal communication [October 2015]). Other data, such as cytogenetic markers, might also contribute to this comparison. If available, they could complement morphological, morphometric and molecular genetics data to provide an integrative assessment. However, until present, no detailed chromosome study had been performed in *T. m. manatus* from Brazil.

Here we compared the morphometric and karyological variation of *Trichechus* by including a larger sample size of the manatee population from Brazil. We employed three-dimensional geometric morphometric techniques to assess variation in size and shape of the skull in *Trichechus manatus* and *Trichechus inunguis* to compare it with patterns among all manatee taxa (*T. manatus latirostris*, *T. m. manatus*, *T. inunguis* and *T. senegalensis*). Also, we analysed the extent of geographic variation across the American manatee populations. Finally, we described the karyotype of the Antillean manatee from Brazil using GTG- and CBG-banding and AgNOR staining to investigate chromosomal differences between this taxon and other populations.

Materials and Methods

Morphological data

Samples

We analysed a total of 189 skulls from the four living taxa of manatees: *T. m. latirostris* ($N = 85$), *T. m. manatus* ($N = 26$), *T. inunguis* ($N = 73$)

and *T. senegalensis* ($N = 5$). Only adult individuals (as assessed by the fusion of the basioccipital–basisphenoid suture) were measured (Domning and Hayek 1986). These specimens were housed in the mammal collections of the following institutions: American Museum of Natural History (AMNH, New York, USA); Associação de Pesquisa e Preservação de Ecossistemas Aquáticos (AQUASIS, Caucaia, Brazil); Centro Nacional de Pesquisa e Conservação de Mamíferos Aquáticos, Instituto Chico Mendes de Conservação da Biodiversidade (CMA/ICMBio, Itamaracá, Brazil); Instituto de Desenvolvimento Sustentável Mamirauá (IDS, Tefé, Brazil); Florida Museum of Natural History, University of Florida (FLMNH, Gainesville, USA); Field Museum of Natural History (FMNH, Chicago, USA); Museu Paraense Emílio Goeldi (MPEG, Belém, Brazil); National Museum of Natural History, Smithsonian Institution (USNM, Washington, DC, USA); and US Geological Survey (USGS, Gainesville, USA).

The *Trichechus inunguis* sample included individuals from Peru (PER, $N = 2$), Amazonas, Brazil (AM, $N = 62$), and Pará, Brazil (PA, $N = 9$). The *Trichechus senegalensis* population was composed of individuals from Côte D'Ivoire ($N = 2$), Democratic Republic of the Congo ($N = 2$) and one specimen from Africa of unknown origin. The *Trichechus m. manatus* Caribbean population consisted of specimens from Puerto Rico ($N = 7$), Belize ($N = 1$), Guatemala ($N = 2$), Honduras ($N = 1$), Nicaragua ($N = 1$), Costa Rica ($N = 1$), Guyana ($N = 1$) and Suriname ($N = 1$). The Brazilian *T. m. manatus* population included individuals from Ceará ($N = 6$), Paraíba ($N = 2$) and Rio Grande do Norte ($N = 1$) and two specimens from Brazil of unknown locality. *Trichechus m. latirostris* individuals were from the United States of America (USA, $N = 85$) (Fig. 1). We pooled all specimens of *T. m. manatus* from Caribbean localities in one population and all those from Brazilian localities in

another due to the small sample sizes of each local population. This decision was based on the geographic region criteria (Caribbean population consisted of specimens from continental countries with Caribbean coastlines, including Suriname and Guyana, that border the Caribbean Sea), on the lack of geographic differentiation among the individuals in each region after performing preliminary morphometric analyses, and partially on geographic criteria based on population genetic studies of Vianna et al. (2006) and Hunter et al. (2012). This pooling might inflate differences between populations, although populations of *T. manatus* are patchy (Deutsch et al. 2008). A list of all examined specimens is presented in Appendix 1.

Data on sex were available for 82 specimens of *T. m. latirostris* (44 females and 38 males); 16 individuals of *T. m. manatus*, nine from Caribbean population (three females and six males) and seven from Brazil (four females and three males); 59 specimens of *T. inunguis* (25 females and 34 males); and only three individuals of *T. senegalensis*, all of them females. Therefore, sexual shape and size dimorphism analysis for this species could not be performed.

Landmarks, measurement error and sexual dimorphism

Geometric morphometrics were used to assess geographic variation in skulls of trichechid sirenians.

Three-dimensional coordinates were recorded twice for each skull, to assess measurement error, for 60 landmarks on both dorsal and ventral sides of the skulls (Fig. 2), using a MicroScribe 3DX digitizer. For 156 of 189 skulls, all landmarks were digitized. Dorsal and ventral views were combined into a single configuration of landmarks based on five common landmarks digitized in both views (1, 2, 3, 22 and 23) to fit

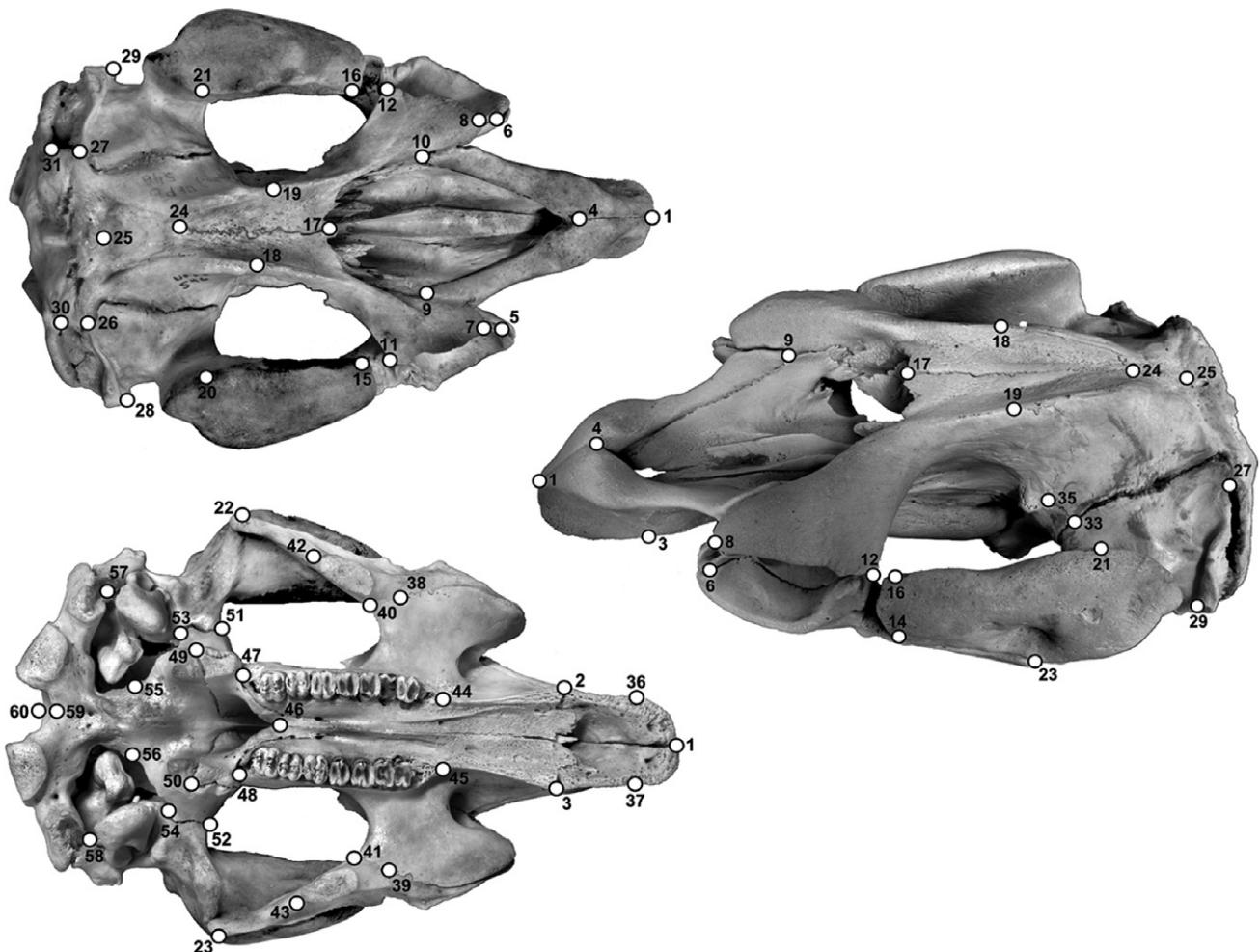


Fig. 2. *Trichechus manatus manatus* specimen from Brazil (UFPA 546), illustrating locations of landmarks and their respective numbers in dorsal, ventral and dorsolateral views of the skull. Landmark definitions are presented in Appendix 2.

them using FileConverter (available at <http://www.flywings.org.uk/>). Missing values were estimated for the 33 remaining specimens. For this, we used DVLR (Raaum 2006) to fit the two views because it allows missing landmarks to be entered. The same landmarks used in FileConverter to fit the dorsal and ventral views were used in this step. Then, a single file was imported to MorphoJ et al. (Slice 1999) where the final orientation was set to a Principal Component Analysis (PCA), and a Generalized Procrustes Analysis (GPA) was run to superimpose configurations through translation, scaling and rotation. After this, the scale was restored, so that landmarks of each specimen were aligned but size differences were preserved. Finally, missing landmarks were estimated by reflection of their homologs along the sagittal plane, which generally leads to negligible inaccuracies. A description of the landmarks is presented in Appendix 2, and a list of the steps to be followed for these procedures is available from the authors upon request.

Raw data (coordinates) were then imported into MorphoJ 1.05f (Klingenberg 2011) where most analyses were performed. Data were submitted to a Procrustes Fit, to remove the effects of isometric size and orientation. Centroid size (CS) was used to estimate skull size. It is defined as the square root of the sum of squared distances of each landmark from the centroid of the landmark configuration (Zelditch et al. 2004). As the main interest of the study is to quantify the variation between species and subspecies/populations, measurement error in size and shape was assessed with a Procrustes ANOVA (Klingenberg and McIntyre 1998) as the sum of means squares deviations between replicas. Sexual dimorphism in size and shape was tested for each taxon separately on the skulls that were sexed. Additionally, sexual shape dimorphism for each taxon was assessed using Hotelling's T^2 with permutation test, and sexual size dimorphism was assessed using a Student's t -test on CS for each taxon. Sex and taxon interaction for size and shape was tested with a Procrustes ANOVA.

Data analyses

We compared size among the taxa (species and populations) using one-way ANOVA, followed by a Tukey *post hoc* test. Skull size analysis among the populations of *T. m. manatus* compared populations from Brazil and the Caribbean and *T. m. latirostris* from USA. Also, skull size analysis among the populations of *T. inunguis* compared populations from Peru, and Amazonas and Pará states in Brazil (PER, AM and PA respectively). To assess the main shape variations, a PCA was first run with the complete data set, and afterwards within each species, to evaluate variation and possible intraspecific differences in shape. We used the symmetric component average (exported from MorphoJ) of the species and subspecies and populations of *T. manatus* to generate surface rendering of interlandmark polygons using the software Morphologika 2 v2.5 (O'Higgins and Jones 2006). Because of its small sample size, *T. senegalensis* was used only in analyses among the species and even in that context results must be taken with great caution. To test for shape differences between pairs of taxa we used permutation tests based on Procrustes and Mahalanobis distances. Canonical variates analysis (CVA) with leave-one-out cross-validation among all the species and populations of *T. m. manatus* (Brazil and Caribbean) and *T. m. latirostris* (USA) was performed on the shape variables to quantify shape differences between groupings previously suggested by the PCA, using all shape coordinates, and by population genetic data from independent studies. Mahalanobis and Procrustes distances among the groups from the CVA were used to measure the degree of skull shape differentiation

among the five taxa tested (*T. inunguis*, *T. senegalensis*, *T. m. manatus* Brazil population, *T. m. manatus* Caribbean population and *T. m. latirostris* USA population). A phenogram of the distance matrices between mean shapes for each taxon was generated by neighbour-joining (NJ), to depict graphically morphometric similarities and distances between them.

Karyological data

Blood samples were collected from captive Antillean manatee specimens (11 males and five females) from the Centro Nacional de Pesquisa e Conservação de Mamíferos Aquáticos (CMA/ICMBio), Itamaracá, Pernambuco State, Brazil. Samples were collected by the permanent veterinarian staff at the CMA, at the same time as the routine blood sampling performed to evaluate the health status of captive manatees. Handling of the specimens was performed following all internal procedures to minimize stress to the animals and all procedures follow the Guidelines of the American Society of Mammalogists for the Use of Wild Mammals in Research (Sikes et al. 2011). A list of the specimens sampled for blood is presented in Appendix 3.

Chromosome preparations were obtained by lymphocyte culture from peripheral blood samples collected using sodium heparin Vacutainer tubes and the cultures were incubated for 96 h at 37°C. GTG- and CBG-banding and silver nitrate staining (AgNOR) were performed following standard cytogenetic techniques, according to Seabright (1971), Sumner (1972) and Howell and Black (1980), respectively. Metaphases were examined with an Olympus optical microscope and the images were captured using a Leica IM50 imaging system.

Of the 16 individuals sampled, 11 produced good cultures for cytogenetic analyses. Three complete sets of solid stained chromosomes from these specimens were measured using ImageJ (Schneider et al. 2012), because solid staining maintains chromosomal integrity. Each arm was measured from the mid-point of the primary constriction to the end of the arm. Relative total length and arm ratios (q/p) were calculated based on the lengths of the short (p) and long (q) arms. Bi-armed chromosomes were classified as metacentric (m), submetacentric (sm) and subtelocentric (st), and one-armed chromosomes as acrocentric (a), following Levan et al. (1964). Chromosomes with no measurable short arm were classified as (a). Karyotypes were constructed according to the previously described Florida subspecies karyotype (Gray et al. 2002), and sex chromosomes were depicted separately.

Results

Morphological data

Measurement error effect (differences between replicates) in size and shape were non-significant and negligible as they accounted for $5 \times 10^{-4}\%$ and $2 \times 10^{-2}\%$ of the total variance and the same order of magnitude for interspecific or interpopulation variances. Individual values generated after Procrustes ANOVA were suitable for use in all subsequent analyses performed here.

Sexual dimorphism

No sexual dimorphism was detected in size and shape of the skull in any of the taxa (Table 1). The interaction term between

Table 1. Student's t -test and Hotelling's T^2 test results for sexual dimorphism in size and shape, respectively, of *Trichechus manatus manatus* (TMM), *Trichechus manatus latirostris* (TML) and *Trichechus inunguis* (TI) skulls. N: sample number; CS: centroid size (mm); SD: standard deviation; df: degrees of freedom; P: significance level for t -test and for Procrustes distance

Taxon	Student's t -test									Procrustes distance $\sigma_{\text{♀}}$ P
	N		Mean		SD			df	P	
	♂	♀	CS ♂	CS ♀	♂	♀	t-value			
TMM	9	7	809.66	854.90	69.87	83.30	-1.18	14	0.26	0.217
TML	38	44	824.72	842.81	87.37	82.58	-0.96	80	0.34	0.222
TI	34	25	732.41	748.29	82.12	51.85	0.85	57	0.40	0.570

each taxon and sex was not significant ($P = 1$, results not shown). Therefore, sexes were pooled in subsequent analyses, including those specimens with unknown sex.

Intertaxa shape and size analyses

The skull shape of *T. inunguis* is significantly different from the other species, and consequently, the populations of *T. inunguis* are clearly separated from the *T. senegalensis*, *T. m. manatus* and *T. m. latirostris* populations along the first two PCs from the PCA (Fig. 3). *Trichechus senegalensis* is separated from *T. m. latirostris* and there is some overlap between the *T. m. manatus* and *T. m. latirostris* populations along the axes (Fig. 3). *Trichechus inunguis* can be distinguished from the other

taxa by the presence of a more elongated and narrower rostrum, a less deflected snout, a more flattened supraoccipital region, and a narrower and a relatively smaller skull (orbits, basicranium and zygomatic arch regions) (Fig. 3).

Trichechus m. latirostris differs from *T. inunguis* by a relatively shorter and wider rostrum, a more deflected snout, a relative enlargement of the skull as a whole, with orbits, frontal, maxillary and jugal bones expanded laterally and more frontally oriented, expanded zygomatic arches, and a more rounded supraoccipital region. In addition, *T. senegalensis* presented some unique features, particularly a more slender rostrum, a broader zygomatic arch, a wider zygomatic process of the squamosal and a narrower superior region of the frontal (Fig. 3).

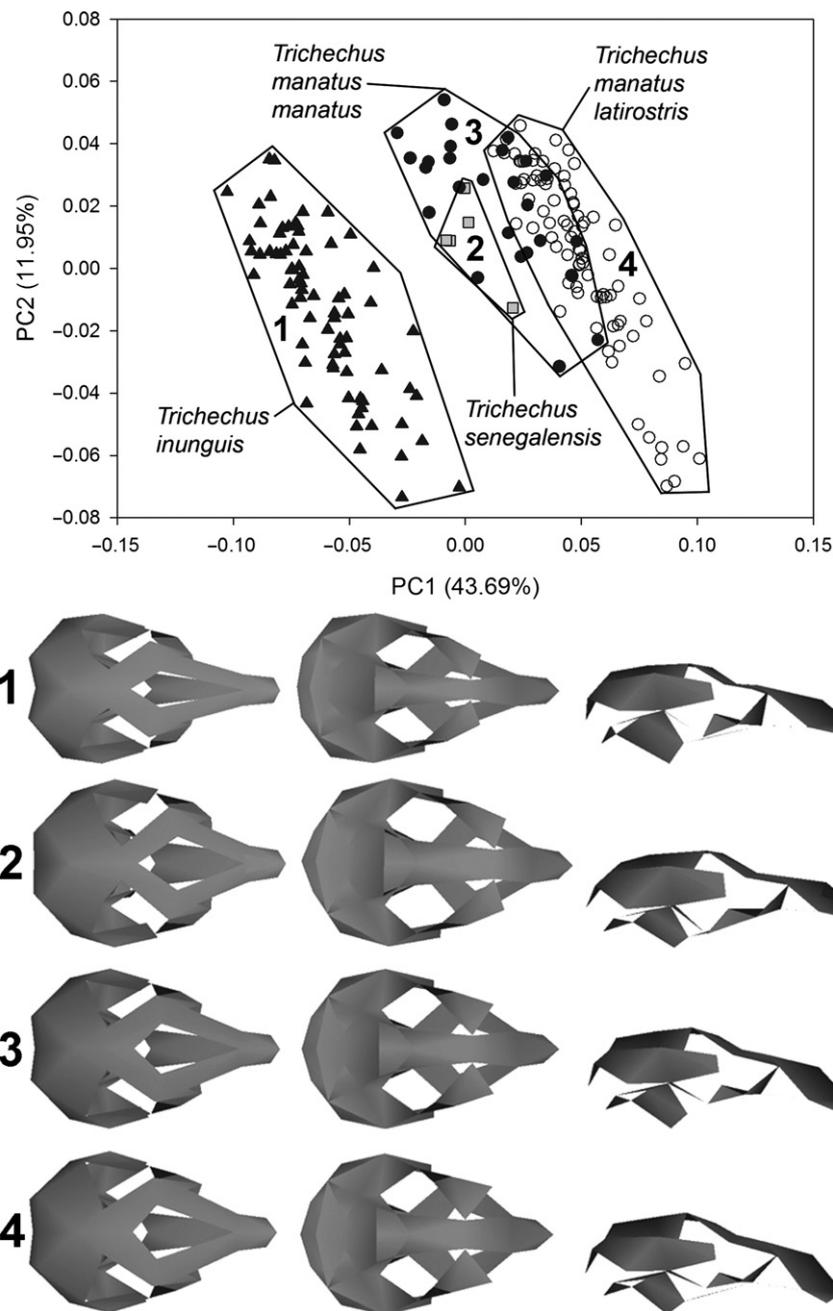


Fig. 3. Principal components (PC) analysis of Procrustes coordinates of the landmarks set on the cranium of specimens of all species studied, and surface rendering of interlandmark polygons showing shape changes, constructed from the average of symmetric components of each taxon. From left to right: dorsal, ventral and lateral views, respectively. Numbers in plot refer to cranial representations below

Table 2. Tukey test results (P -values) for skull size comparisons between *Trichechus manatus manatus* (TMM), *Trichechus manatus latirostris* (TML), *Trichechus senegalensis* (TS) and *Trichechus inunguis* (TI)

Taxon	TMM	TML	TS	TI
	Centroid Size Mean (mm)			
TMM	838.56	835.79	816.91	736.99
TML	–	0.99	0.94	<0.001
TS	0.99	–	0.95	<0.001
TI	0.94	0.952	–	0.11
	<0.001	<0.001	0.11	–

Table 3. Values of centroid size mean (mm), standard deviation and range (mm) for *Trichechus manatus manatus*, *Trichechus manatus latirostris*, *Trichechus senegalensis* and *Trichechus inunguis* skulls

Taxon	Centroid size mean	Standard deviation	Range
<i>Trichechus manatus manatus</i>	838.56	72.16	679.49–926.21
<i>Trichechus manatus latirostris</i>	835.79	83.99	599.24–1008.24
<i>Trichechus senegalensis</i>	816.91	70.18	696.99–882.52
<i>Trichechus inunguis</i>	736.99	70.79	564.34–874.03

Taxa of the genus *Trichechus* differ significantly in skull size (ANOVA, $F = 24.55$, degrees of freedom = 3, $P < 0.001$), and the a posteriori Tukey test revealed that *T. m. manatus* and *T. m. latirostris* are different from *T. inunguis* (Table 2). *Trichechus m. manatus* and *T. m. latirostris* are larger than *T. inunguis*, with *T. senegalensis* presenting an intermediate skull size (Table 3).

Intraspecific shape and size analyses: *Trichechus manatus manatus* populations and *Trichechus manatus latirostris*

The Brazilian *T. m. manatus* population is clearly separated from *T. m. manatus* Caribbean and *T. m. latirostris* USA populations, which in turn overlap partially along the first two PCs (Fig. 4). The particular cranial features of the Brazilian *T. m. manatus* population include the superior tip of premaxilla with a more posterior position, a more elongated and wider rostral region, the caudal end of the premaxilla in a more anterior position, a narrower superior region of the frontal and parietal, retracted orbits and zygomatic arch region, a wider zygomatic process of the squamosal, a less deflected snout, a wider median region of the rostrum on the ventral side, the superior point of foramen magnum (opisthion) in a more posterior position, and the inferior point of foramen magnum (basion) in a more anterior position (Fig. 4). The USA *T. m. latirostris* population presents the most extreme scores along the two axes, with morphological features such as the superior tip of the premaxilla with a more anterior position, a shorter and narrower rostral region, the caudal end of the premaxilla in a more posterior position, wider superior regions of the frontal and parietal, enlarged orbits and zygomatic region, with frontal, maxilla and jugal expanded laterally and more frontally oriented, a narrower zygomatic process of the squamosal, a more deflected snout, a narrower median region of the rostrum on the ventral side, the superior point of foramen magnum in a more anterior position, and the inferior point of foramen magnum in a more posterior position (Fig. 4). Although the Caribbean and USA populations are currently classified in separate subspecies, they overlapped partially along the first two PCs (Fig. 4).

The permutation tests from Procrustes and Mahalanobis distances between the two subspecies of *T. manatus* showed a significant discrimination ($P < 0.001$). Discriminant analysis yielded

92.9% and 84.6% of correct classification for *T. m. latirostris* and *T. m. manatus*, respectively, with cross-validation. When *T. m. manatus* population from the Caribbean and *T. m. latirostris* USA population were pooled and the test was run comparing them with the Brazilian *T. m. manatus* population, a significant difference from permutation tests from the Procrustes and Mahalanobis distances were also found ($P < 0.001$) with 100% correct classification of all the specimens in both groups from cross-validation. Mahalanobis and Procrustes distances were smaller between *T. m. latirostris* from USA and *T. m. manatus* from the Caribbean (16.49 and 0.04, respectively) than between those and *T. m. manatus* from Brazil (Mahalanobis: 29.99 and 28.26, respectively; Procrustes: 0.09 and 0.07, respectively). The shape changes that allowed discriminating among the populations are similar to those that separated the Brazilian *T. m. manatus* population from the others by the PCA.

The ANOVA showed no significant skull size variation among the populations of *T. m. manatus* from Brazil, Caribbean and *T. m. latirostris* ($F = 1.03$, degrees of freedom = 2, $P = 0.36$).

Intraspecific shape and size analyses: *Trichechus inunguis*

The distribution of PCs scores did not reveal any clear pattern for this species, with a wide overlap of the three populations established based on geographic origin. *Trichechus inunguis* populations (PER, AM and PA) differed significantly in shape (ANOVA, $F = 69.2$, degrees of freedom = 2), but they did not differ significantly in size (ANOVA, $F = 0.68$, degrees of freedom = 2, $P = 0.51$).

Disparity shape analyses between Trichechus species and populations of Trichechus manatus manatus and Trichechus manatus latirostris. – Permutation tests with the Mahalanobis and Procrustes distances were statistically significant among the five groups studied, *T. inunguis*, *T. senegalensis*, *T. manatus latirostris* population from USA, *T. manatus manatus* population from the Caribbean and *T. manatus manatus* population from Brazil (Table 4). The comparison of the degree of shape differentiation of *T. manatus manatus* from Brazil with other *T. manatus* populations is high when compared to the interspecific magnitudes of differences. Using Mahalanobis distances the intraspecific distances are smaller than interspecific distances but of similar magnitude. When using Procrustes distances some interspecific distances are equal to or inferior (*T. m. manatus* Caribbean/*T. senegalensis* = 0.08) to intraspecific distances within *T. manatus* (*T. m. manatus* Brazil/*T. manatus latirostris* USA = 0.09). The Caribbean *T. m. manatus* and *T. m. latirostris* USA populations are closer to one another than to the Brazilian *T. m. manatus* population (0.04) (Fig. 5). This distance pattern is very similar if *T. senegalensis* is excluded.

Karyological data

Chromosomes of the Antillean manatee were arranged into seven groups (A-G) according to the similarity of the G-banding pattern between *Trichechus manatus manatus* and *Trichechus manatus latirostris*. The diploid chromosome number was $2n = 48$, and the fundamental number (FN) was 90. This karyotype was composed of eight subtelocentric (pairs 1, 2, 3, 11, 12, 20, 21 and 22), nine metacentric (pairs 4, 7, 8, 9, 15, 16, 17, 18 and 19), five submetacentric (pairs 5, 6, 10, 13 and 14) and one acrocentric (pair 23) autosome pairs. The karyotype had 22 bi-armed pairs and one one-armed pair. The X chromosome was (sm) and Y was (a) (Fig. 6).

All homologs were identified using G-banding (Fig. 6). C-banding detected centromeric heterochromatin in all chromosomes (Fig. 7), and nucleolar organizer regions were observed on the short arms of chromosome pair 20st (Fig. 8).

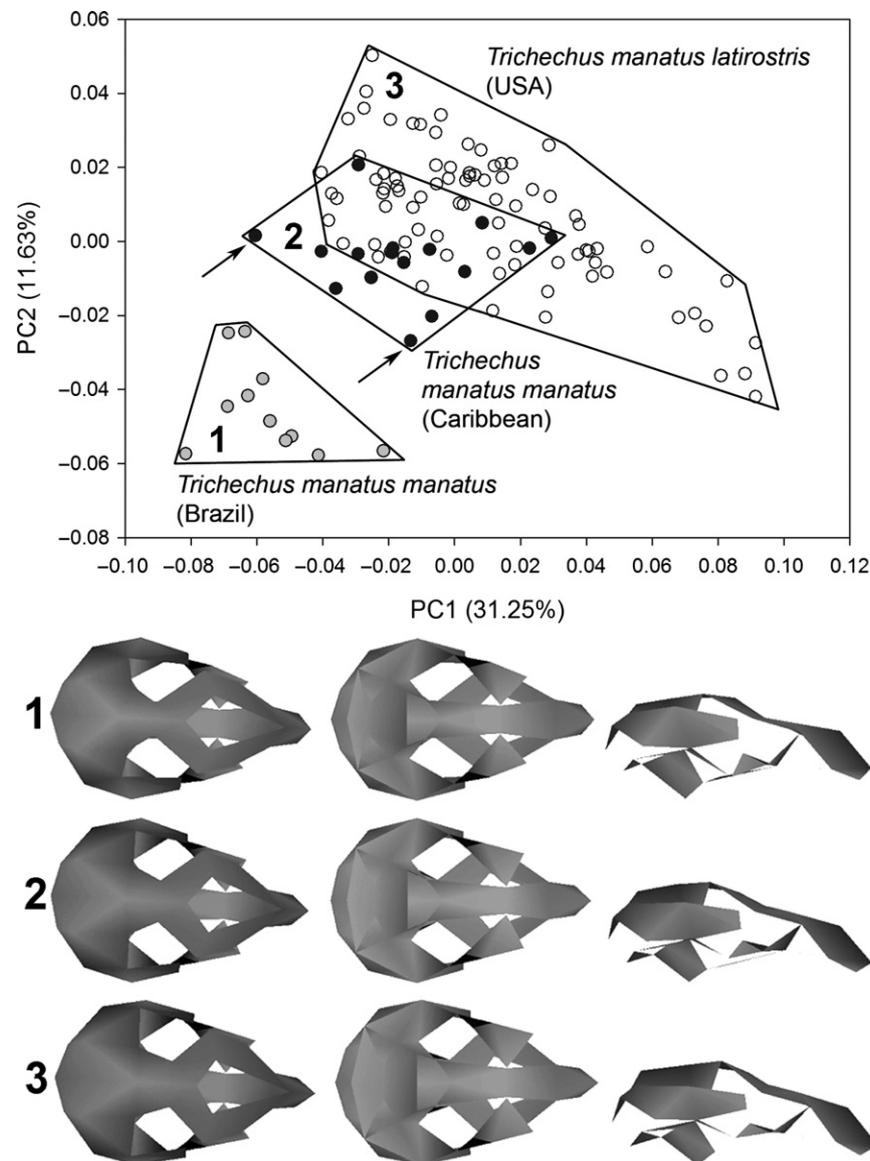


Fig. 4. Principal components (PC) analysis of Procrustes coordinates of the landmarks of the cranium of specimens from the Brazilian and Caribbean *Trichechus manatus manatus* populations and United States of America *Trichechus manatus latirostris* population (arrows represent individuals from Guyana – above; and Suriname – below), and surface rendering of interlandmark polygons showing shape changes, constructed from the average of symmetric components of each population. From left to right: dorsal, ventral and lateral views, respectively. Numbers in plot refer to cranial representations below.

Discussion

The understanding of the genetic structure of *Trichechus manatus* has significantly improved by the proposal of a new hypothesis of three distinctive and geographically structured mtDNA lineages (Garcia-Rodriguez et al. 1998) and two ESUs, composed of individuals from (1) Florida, Dominican Republic, Puerto Rico, Mexico, Belize, Colombia and Venezuela; (2) Mexico, Belize, Colombia and Venezuela; and (3) the Guianas and Brazil (Vianna et al. 2006) based on mtDNA control region. Detailed population genetic analyses seem to support further structuring between Puerto Rico and Florida (Hunter et al. 2012); Belize and Florida, and Belize City Cayes and the Southern Lagoon system in Belize (Hunter et al. 2010); the Gulf of Mexico and Chetumal Bay, on the Caribbean coast (Nourisson et al. 2011); and among Colombian rivers, revealing five different populations (Satizábal et al. 2012), based on mtDNA, microsatellite DNA or

both. These cases of population structuring show the need of conservation and management actions for the Antillean manatee populations; thus, the use of proper systematics would allow a correct and unambiguous designation of metapopulations. The correspondence of available taxon names (Harlan 1824; Hatt 1934) with new proposed lineages was hampered by the lack of a comprehensive morphological analysis encompassing population representatives for these lineages. Our results partially support the conclusions from molecular studies (Garcia-Rodriguez et al. 1998; Vianna et al. 2006), which showed a differentiation of the Brazilian *T. m. manatus* population together with the population from Guyana. In addition, the Antillean manatee from Brazil had not been properly analysed previously, and cytogenetic differences confirm the distinctiveness of this population. Furthermore, the inclusion of the other species of the genus *Trichechus* allowed us to evaluate the degree of morphological

Table 4. Mahalanobis distances and *P*-values (above diagonal) and Procrustes distances and *P*-values (below diagonal) from the CVA among the five groups studied, *Trichechus inunguis* (TI), *Trichechus manatus latirostris* USA population (TML USA), *Trichechus manatus manatus* Brazilian population (TMM BRA), *Trichechus manatus manatus* Caribbean population (TMM CA) and *Trichechus senegalensis* (TS)

Groups	TI	TML USA	TMM BRA	TMM CA	TS
TI	0	21.99	21.57	20.90	24.66
		<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001
TML USA	0.11	0	18.17	7.76	19.66
	<i>P</i> < 0.001		<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001
TMM BRA	0.09	0.09	0	16.84	21.53
	<i>P</i> < 0.001	<i>P</i> < 0.001		<i>P</i> < 0.001	<i>P</i> = 0.001
TMM CA	0.10	0.04	0.07	0	17.98
	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001		<i>P</i> < 0.001
TS	0.10	0.10	0.09	0.08	0
	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001	

differentiation among populations, subspecies and species. These results are discussed in the light of the systematics of the group and of hypotheses about the adaptive significance of shape changes inferred from geometric morphometrics.

Sexual dimorphism

Our geometric morphometrics data indicated that *Trichechus* species are not sexually dimorphic in cranial size or shape, as concluded in previous traditional morphometric analyses on skulls of *T. manatus* and *T. inunguis* (Domning and Hayek 1986). This can be related to the manatee mating system, which involves male and female polygamy, with several males mating with individual females during a single oestrous period, forming mating herds (Marsh et al. 2012). Sperm competition apparently exists (Marsh et al. 2012) and physical confrontations during mating are rare (Reynolds and Powell 2002), with no defence of harems or territories nor any kind of resource, thus supporting similarities in cranial robustness across sexes.

Interspecific differences

Separation of *T. inunguis* from *T. senegalensis* and the two *T. manatus* subspecies is supported by shape differences of the skull (Fig. 3). Results involving *T. senegalensis*, however, have to be considered as preliminary, because of the small sample size we examined. They support previous findings by Domning and Hayek (1986), who, based on a suite of characters that included cranial data, showed that *T. inunguis* was the most divergent species, compared to the other manatees, with several characters they considered apomorphic, such as smaller and more complex molars, lack of nails on the flippers, reduced number of dorsal vertebrae, thickened supraoccipital bone and higher diploid chromosome number (although adequate studies on sirenian chromosomal evolution are still lacking). The probable origin of *T. inunguis* is through allopatric speciation due to isolation in the Amazon Basin during the Pliocene, following the Andean uplift but prior to the establishment of the modern Amazon drainage into the Atlantic. It has a more ancient origin than the other manatees (Domning and Hayek 1986; Domning 1994; Vianna et al. 2006), but it also might have diverged more rapidly than coastal manatees in response to the new freshwater environment of the Amazon and new food resources (Domning 1982; Vianna et al. 2006).

Some of the cranial features observed in this study were also reported by Domning and Hayek (1986), who considered some

of them likely autapomorphies, including a long and narrow rostrum, and supraoccipital more flattened and swollen, allowing the skull almost always to stand upright on its posterior surface (for *T. inunguis*); broader zygomatic arches (for *T. senegalensis*); and more deflected snout (for *T. m. manatus* and *T. m. latirostris*, particularly manatees from Florida).

Manatees in general seem to prefer feeding on submerged vegetation (Domning 1980). The little deflected snouts of the Amazonian and African manatees are possibly an adaptation for feeding instead on emergent and floating vegetation, often available in their freshwater habitats (Domning 1982; Marsh et al. 2012). On the other hand, with wider feeding niches (freshwater, estuarine and marine environments), Antillean and Florida manatees, especially the latter, have more deflected snouts. This is probably related to their bottom feeding habit, required because the vegetation actually available to them in many areas (especially Florida) consists mainly of bottom-growing marine seagrasses (Domning 1982; Domning and Hayek 1986; Marsh et al. 2012).

There are significant differences in skull size between *T. inunguis* and *T. m. manatus* and *T. m. latirostris*. *T. m. manatus* and *T. m. latirostris* have significantly larger skulls than *T. inunguis*. *Trichechus senegalensis* has a size intermediate between the other two species, and as such, no significant size difference was found among *T. senegalensis*, *T. m. manatus* and *T. m. latirostris*, nor between *T. senegalensis* and *T. inunguis*. The lack of differences could however be due to low power due to the small sample size of *T. senegalensis*. Based on body length and weight, the Amazonian manatee is the smallest and lightest of manatees, measuring up to 3.0 m and weighing up to 450 kg. Antillean and Florida manatees are larger and heavier, reaching lengths of up to 3.5 m, and weights of up to 1620 kg (Marsh et al. 2012).

Intraspecific shape variation

Trichechus manatus

The shape of the skull in the Brazilian *T. m. manatus* population south of the Amazon is statistically distinct from populations from the USA and the Caribbean. Although Suriname and Guyana are geographically closer to Brazil, individuals from those localities presented skull shapes more similar to those of specimens from the Caribbean population (Fig. 4).

The Amazon continental shelf is highly energetic and dynamic, reflecting a combination of different influences, including tides and tidal currents, waves, the North Brazil current, a large amount of freshwater (causing low salinity) and sediment from the Amazon River discharge, and wind stress from the trade winds (Geyer et al. 1996). These peculiarities distinguish this region from other environments, and the relationship of these factors controls the hydrodynamic mechanisms, processes of sedimentation, sediment supply and reworking, physico-chemistry of waters and biota organization (Lima et al. 2001). As a result, the Amazon River outflow has been suggested to act as a major barrier between the Brazilian and Caribbean biogeographic provinces, mainly separating the shallow water faunas (Floeter and Gasparini 2000; McCartney et al. 2000; Lessios et al. 2003; Rocha 2003; Robertson et al. 2006; Floeter et al. 2008; Rocha et al. 2008; Nunes et al. 2011). The Amazon River is considered an important barrier to dispersal for passerine birds, because of its width at the mouth (ca. 300 km) (Hayes and Sewlal 2004). Those factors may also be somehow adverse for the Antillean manatee, hindering its occurrence at the Amazon River mouth, which offers more favourable environmental conditions for the occurrence of the Amazonian manatee (Luna et al. 2008a).

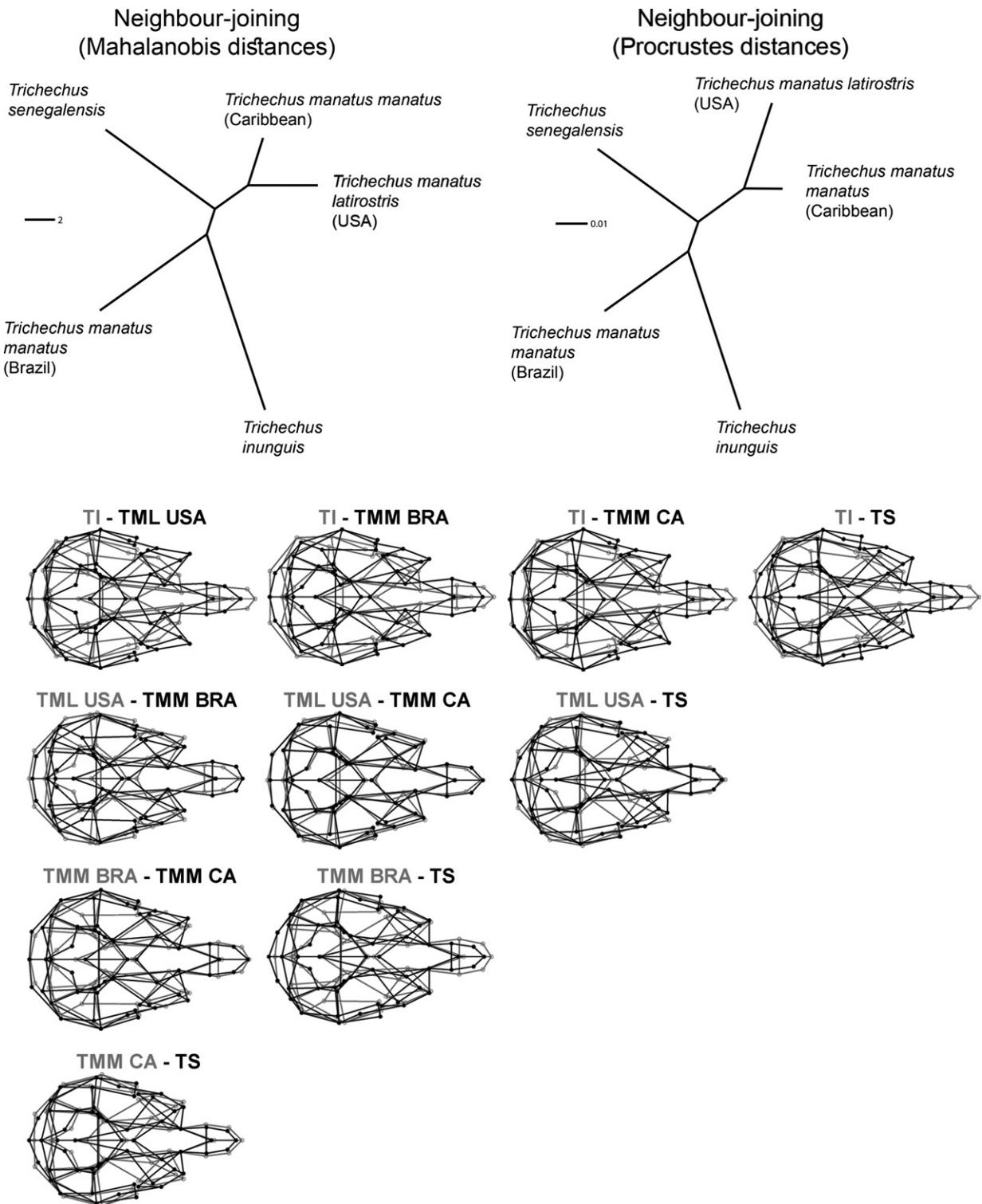


Fig. 5. Neighbour-joining trees based on the Mahalanobis and Procrustes distances matrices from the CVA, expressing the degree of morphological similarity among the five groups studied, *Trichechus inunguis* (TI), *Trichechus manatus latirostris* USA population (TML USA), *Trichechus manatus manatus* Brazil population (TMM BRA), *Trichechus manatus manatus* Caribbean population (TMM CA) and *Trichechus senegalensis* (TS) (above), and superimposition of skull shape between each pair of groups (wireframe in grey represents starting shape and wireframe in black represents target shape) (below)

During the Late Pliocene–Early Pleistocene, the drainage barrier between the inland Amazon waters and the Atlantic Ocean was breached, establishing a connection between these two regions (Domning 1982; Marsh et al. 2012). This period coincides with the dispersal of coastal manatees from South America

to the Caribbean and North America. Thus, it is possible that some manatee populations were isolated in Brazil by the Amazon barrier, suggesting a vicariant event at the Amazon River mouth, which may have led to skull shape differences observed in the Brazilian population.

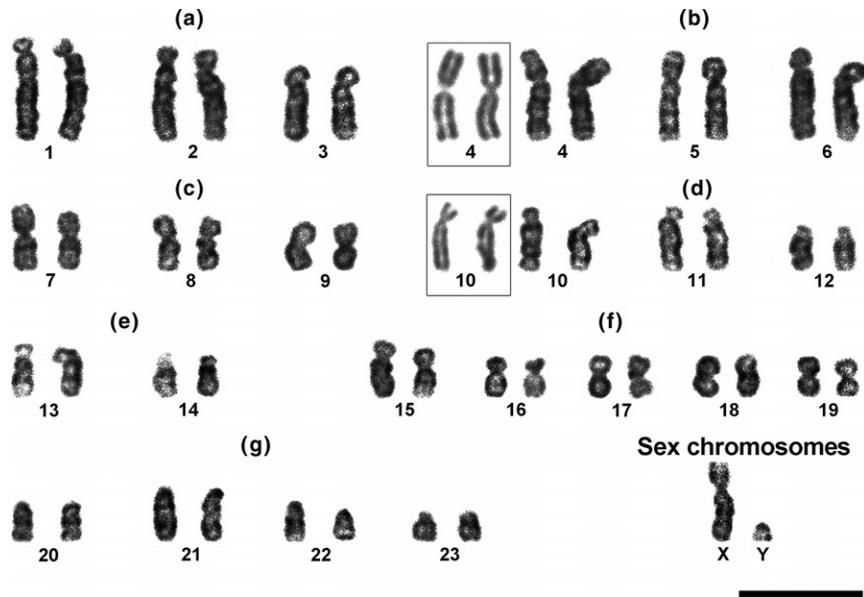


Fig. 6. GTG-banded karyotype of *Trichechus manatus manatus* from Brazil ($2n=48$, XY) (inserts represent solid stained chromosome pairs 4m and 10sm allowing better visualization of chromosome morphology). Letters from a to g indicate chromosome groups as defined Gray et al. (2002). Bar = 10 μ m

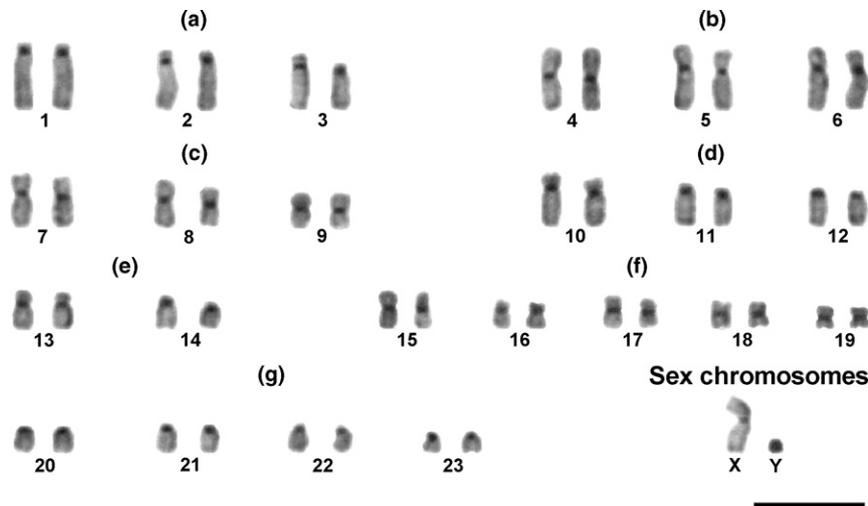


Fig. 7. CBG-banded karyotype of Brazilian Antillean manatee; bar = 10 μ m

The previous morphological study using qualitative and quantitative cranial characters (Domning and Hayek 1986) confirmed the existence of two subspecies of *T. manatus* (*T. m. latirostris* and *T. m. manatus*), initially proposed by Hatt (1934), suggesting that the cool northern coast of the Gulf of Mexico and strong currents of the Straits of Florida were effective barriers to gene flow between Florida and Antillean manatees. However, phylogeographic analyses of *T. manatus* based on mtDNA control region sequences revealed three lineages (Garcia-Rodriguez et al. 1998; Vianna et al. 2006) and two ESUs (Vianna et al. 2006) for this species. As there is some overlap between clusters 1 and 2 (individuals from Florida, Mexico, Central and South America), and cluster 3 was found only in the Guianas and Brazil, Vianna et al. (2006) proposed that a geographic barrier represented by the continuity of the Lesser Antilles with Trinidad would have restricted gene flow historically in *T. manatus*. As a consequence populations from the Guianas and Brazil seem to have been separated by this barrier, with haplotypes found in these localities only. These authors also suggested that further

analysis could indicate a subspecies status for both ESUs. Further structuring might occur south of the Amazon, as Luna et al. (2012) found three haplotypes in the northernmost states of north-eastern Brazil (Maranhão and Piauí), and only a single haplotype in its southern portion (Ceará, Rio Grande do Norte, Paraíba, Pernambuco and Alagoas states), suggesting the presence of two genetic groups with a central mixing zone in Piauí State.

The skull shape differences distinguishing the Brazilian *T. m. manatus* population from *T. m. manatus* Caribbean and *T. m. latirostris* USA populations partially support conclusions from molecular studies. Probably both barriers, the Lesser Antilles and the Amazon River mouth, may be involved in the process of phenotypic and genetic differentiation of the South American Antillean manatee populations.

The phenogram including two species of manatees (*T. inunguis* and *T. senegalensis*) and three populations of *T. m. manatus* and *T. m. latirostris* (Brazil, Caribbean and USA) showed somewhat unexpected results. Differences in skull shape of the

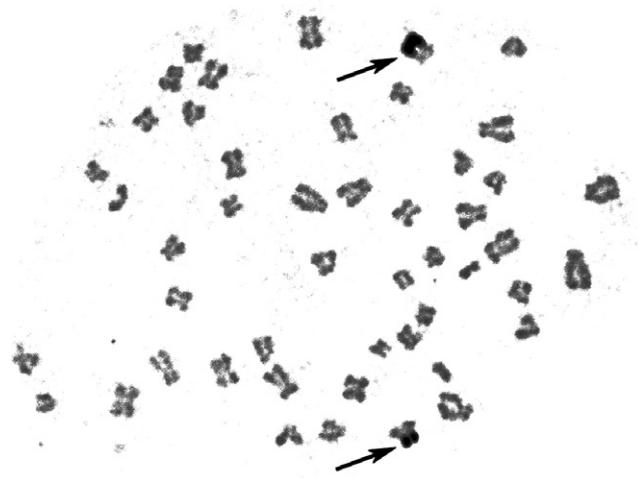


Fig. 8. AgNOR staining of Brazilian *Trichechus manatus manatus* (arrows indicate positive NOR regions)

Brazilian *T. m. manatus* population are considerable, and they are in magnitude as large as are those found between *T. inunguis* and *T. senegalensis*, although these differences might in part be due to the small sample size of the latter. On the other hand, the Caribbean *T. m. manatus* and *T. m. latirostris* USA populations are closer to each other, reflecting the overlapping observed in the PCA. Therefore, this scenario supports conclusions from molecular studies that suggest the existence of three distinct lineages and two ESUs within *T. manatus*, besides the differentiation of the population from Brazil (Garcia-Rodriguez et al. 1998; Vianna et al. 2006).

Trichechus inunguis

The pattern observed for the Amazonian manatee, which lacks geographic differentiation in skull shape among the populations studied, agrees with the results obtained by mtDNA control region sequences, which reported no geographic structure of this species in Brazilian rivers of the Amazon Basin, with this species most likely behaving as a panmictic population, constituting a single expanding population cluster (Cantanhede et al. 2005; Vianna et al. 2006). However, differences among *T. inunguis* populations were supported by analysis using D-loop sequences and microsatellite data, which demonstrated structure between the Peruvian and Colombian Amazon populations (Satzíbal et al. 2012). Unfortunately, more precise information on specimens' collection localities was unavailable for testing additional hypotheses, such as differences between populations from different rivers.

Karyological data

Although the diploid chromosome number was the same ($2n = 48$) as previously established (Vianna et al. 2006), the Brazil Antillean manatee karyotype presented divergences from Puerto Rico Antillean and Florida manatees. Chromosome pairs 4 and 10 were (m) and (sm), respectively (Fig. 6) in the Brazil Antillean manatee, while they were (sm) and (st), respectively, in the other two groups (Gray et al. 2002; Hunter et al. 2012). Unfortunately, G-banding alone was insufficient to ascertain the rearrangement types (probably balanced rearrangements) that led to the observed structural chromosome differentiation between these three populations.

Gray et al. (2002) suggested that the evolution of the morphological karyotypic differences between *T. m. latirostris* and *T.*

inunguis might have occurred by a variety of both interchromosomal and intrachromosomal rearrangement mechanisms. Therefore, the chromosomal rearrangements that have occurred among trichechid sirenians seem to be subtle, and they will probably be better revealed using comparative chromosome painting, hopefully permitting the proposition of a scenario of Trichechidae chromosome evolution.

The chromosomal differentiation of *T. m. manatus* from Brazil supports our geometric morphometrics results for this population, as well as the conclusions from analyses of mtDNA (Garcia-Rodriguez et al. 1998; Vianna et al. 2006). Although traditionally classified in the Antillean subspecies, the manatees occurring in Puerto Rico showed a karyotype more similar to the Florida manatee karyotype (Hunter et al. 2012) than to the karyotype found in the same subspecies from Brazil.

Taxonomic and conservation implications

By exploring levels of morphometric differentiation between species and populations within Trichechidae, we were able to show clear differences in skull shape among the groups studied, while a detailed chromosome study for *T. m. manatus* from Brazil also revealed divergences in karyotype between this population and the others. Within Trichechidae, *Trichechus inunguis* is clearly distinct in skull shape from the remaining species, while *T. senegalensis*, *T. m. manatus* and *T. m. latirostris* skulls are more similar to each other in shape. *Trichechus m. manatus* and *T. m. latirostris* have larger skulls than *T. inunguis*, and there is no sexual dimorphism in skull size and shape in any of the species (although our sample for *T. senegalensis* was too restricted for any adequate evaluation).

The most interesting results, however, were obtained for *Trichechus manatus*: the skull shape of the Brazilian *T. m. manatus* population is clearly different from that of *T. m. manatus* Caribbean and *T. m. latirostris* USA populations (which overlap partially, in spite of the fact that these latter two are traditionally considered different subspecies). In addition, the few individuals available to us from Suriname and Guyana are more similar to Caribbean individuals, although these countries are geographically closer to Brazil. Our results suggest that the Amazon barrier may have interrupted, or may be interrupting, gene flow in *T. m. manatus* populations between the Caribbean and Brazil. It is also noteworthy that the degree of morphological differentiation observed in the Brazil Antillean manatee population might be similar to that found between the Amazonian and African species (Fig. 5).

Additionally, the karyological differences detected between *T. m. manatus* from Brazil and Puerto Rico and Florida manatees reinforce the conclusions from the geometric morphometrics analyses. All these results strongly indicate that the current taxonomy of *T. manatus* needs to be revised, as our results support preliminary evidence from molecular data and contrast with the currently accepted subspecies designation. All these results also show that Brazilian manatees need to have their current management and conservation strategies reviewed. In contrast, there is no geographic differentiation in skull shape among *T. inunguis* populations, while a greater number of *T. senegalensis* specimens are still needed to properly assess skull size and shape variation throughout its distribution.

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Appendix 1. Specimens analysed in this study

Trichechus inunguis

AMNH: 73594, Peru, Loreto, Maynas, Curaray River mouth; 76813, Brazil, Amazonas, Tefé, Amazon River, mouth of Tefe Lake; 93124, Brazil, Amazonas, Parintins, Villa Bella Imperatriz, Santa Clara, Amazon River, South bank; 94167, Brazil, Pará, Faro, Amazon River, North bank, Nhamundá River; 98691, Peru, Loreto, Ucayali, Tapiche River. CMA: 01S0121/293, Brazil, Pará. IDSM: 00/01, Brazil, Amazonas, Lago Aranucú; 00/04, Brazil, Amazonas, Ilha do Machado; 00/06, Brazil, Amazonas, Uarini, São Raimundo do Jarauá, comunidade Jarauá; 00/07, Brazil, Amazonas, Uarini, Lago Mamirauá; 00/09, Brazil, Amazonas, Maraã, São Francisco do Boia, Rio Aranapú, boca do Lago Gigante; 01/01_01/46, Brazil, Amazonas, Maraã, Panauá, acima da boca do Lago Guedes; 01/13, Brazil, Amazonas, Uarini, enseada do Rio, setor Horizonte; 01/14, Brazil, Amazonas, Ilha da Zizi, setor Aiucá; 01/34, Brazil, Amazonas, Uarini, Lago do Cipó; 01/39, Brazil, Amazonas, Maraã, comunidade do Curupira, próximo Porto Alegre; 01/43, Brazil, Amazonas, Maraã, Paranã do Castanho; 01/44, Brazil, Amazonas, Maraã,

Trichechus manatus latirostris

AMNH: 100152, USA, Florida; 100323, USA, Florida, Miami-Dade County, Miami; 24295, USA, Florida, Collier County, Marco; 70364, USA, Florida, Miami-Dade County, Miami, Biscayne Bay; 90178, USA, Florida, Palm Beach County, Lake Worth; 91096, USA, Florida, Palm Beach County, Lake Worth. FLMNH: 13877, USA, Florida, Duval County, Jacksonville, power plant, West bank; 15111, USA, Florida, Putnam County, in boat canal, just S of Rodman dam, approximately 100 yards from lock; 15112, USA, Florida, Martin County, Stuart, St. Lucie canal at Buoy 50, near St. Lucie lock (downstream); 15114, USA, Florida, Brevard County, Cocoa Beach, E shore

Banana River at Highway 520 and 390, Coca Beach Boulevard; 15115, USA, Florida, Duval County, Jacksonville, E shore St. Johns River, at University Park; 15120, USA, Florida, Duval County, Jacksonville, N shore of St. Johns River, near Broward point; 15121, USA, Florida, Duval County, Jacksonville, Clapboard Creek; 15159, USA, Florida, Glades County, Indian Prairie canal, 0.5 miles upstream of Highway 78 Bridge; 15162, USA, Florida, Martin County, Jupiter Island, Intracoastal Waterway, Hobe Sound; 15169, USA, Florida, Collier County, Remuda Ranch canal; 15174, USA, Florida, Broward County, Fort Lauderdale, 2 miles W of I-95 at SR-84; 15177, USA, Florida, Glades County, Lake Okeechobee, 1 mile SW Indian Prairie canal; 15186, USA, Florida, Monroe County, Everglades National Park, Joe River; 15187, USA, Florida, Martin County, St. Lucie Waterway at Phipps Park; 15190, USA, Florida, Lee County, Fort Myers, Punta Rassa, Caloosahatchee River, Big Shell Island, W side of ICW; 15191, USA, Florida, Lee County, Caloosahatchee River, E side of entrance of Deep Side Lagoon Marine; 15193, USA, Florida, Broward County, Dania, Dania cut off canal at Florida power and light plant; 15195, USA, Florida, Broward County, Fort Lauderdale, Lauderdale Yacht Basin on New River canal, Stranahan River; 15200, USA, Florida, Collier County, Everglades City at Chokoloskee Bay & Halfway Creek on bank; 15202, USA, Florida, Lee County, Fort Myers at end of Coon Road 100 ft out into Caloosahatchee River; 15203, USA, Florida, Broward County, Port Everglades, berth 8A in Turning Basin; 15206, USA, Florida, Collier County, Remuda Ranch (Port of the Islands) in Finger Impoundments, SE of Marina and S of M81-2; 15207, USA, Florida, Broward County, Port Everglades, berth 24; 19134, USA, Florida, Collier County, Port of the Islands, in canal, 0.25 mile downstream from the Finger canals; 19135, USA, Florida, Lee County, Fort Myers, Caloosahatchee River, near Deep Lagoon Marina; 20594, USA, Florida, Citrus County, Homosassa River, just downstream from Homosassa Springs Attraction; 20595, USA, Florida, Duval County, Jacksonville, E shore St. Johns River, at 13280 Mandarin Road; 20598, USA, Florida, Brevard County, W shore Indian River, just S of Highway 405 Causeway; 20600, USA, Florida, Brevard County, Indian River, N of Sebastian on Spoil Island just offshore of Miner's Marina; 20601, USA, Florida, Nassau County, Amelia Island, American Beach, 1 mile S American Avenue in surf; 20602, USA, Florida, Brevard County, East shore Indian River (Mullet Creek), Spoonbill canal near Honest John's Fish Camp; 20608, USA, Florida, Citrus County, Crystal River; 20609, USA, Florida, Palm Beach County, Jupiter Inlet; 24954, USA, Florida, St. Johns County, Intracoastal Waterway, 0.8 mile N of Palm Valley landing; 24955, USA, Georgia, Chatham County, Ossabaw Island, 1 kilometre NE of Southernmost tip of Island, just offshore; 24958, USA, Florida, Hillsborough County, Tampa, W shore of Hillsborough Bay at Ballast point; 24959, USA, Florida, Duval County, Neptune Beach, Pablo Creek, Intracoastal Waterway, just S of marker 22; 24960, USA, Florida, Brevard County, Port Canaveral, NW shore of W Turning Basin; 24961, USA, Florida, Duval County, E shore of St. Johns River, near City Marina; 24965, USA, Florida, Duval County, Little Talbot Island State Park, ocean side, 1 kilometre N of picnic area; 24966, USA, Florida, Nassau County, Nassauville, N shore Nassau River, 1 kilometre W of Seymour point at Thomas Fish Camp; 24967, USA, Florida, Indian River County, Indian River, Sebastian, Indian River near ICW marker 69; 24969, USA, Florida, Duval County, W bank of Fanning Island near buoy 24, St. Johns River; 24971, USA, Florida, Levy County, Inglis, Cross Florida Barge Canal, Inglis lock, outside of W gate; 24972, USA, Florida, Indian River County, Roseland, Sebastian River, W of Railroad Bridge; 24980, USA, Florida, Brevard

County, Titusville, Indian River, S shore of SR 402 Causeway; 24993, USA, Florida, Brevard County, Banana River, Merritt Island, just NW of Nassau Causeway; 24996, USA, Florida, Hernando County, mouth of Chassahowitzka River, on Double Key Rock, 0.5 mile SW of John's Island; 25001, USA, Florida, Brevard County, W shore Banana River, Merritt Island, behind residence 1642, S Banana River Boulevard; 25003, USA, Florida, Brevard County, 100 yards E of Canaveral lock, in Basin; 25005, USA, Florida, Brevard County, Melbourne, Eau Gallie River at 1317 Richmond Drive; 25008, USA, Florida, Broward County; 25009, USA, Florida, Dade County; 25010, USA, Florida, Brevard County, Cocoa, Indian River, W side of Bennett Causeway; 25014, USA, Florida, Dade County, Miami, NE 100th Street at canal, in N Miami; 25017, USA, Florida, Brevard County, Port Canaveral locks; 25018, USA, Florida, Dade County, Miami, Maule Lake, NE 163rd Street, N Miami; 25019, USA, Florida, Dade County, Miami River at flood control dam near Legeuine Road, at airport; 25021, USA, Florida, Dade County, Snapper Creek flood control dam; 25025, USA, Florida, Palm Beach County, Loxahatchee River, Palm Point; 25026, USA, Florida, Palm Beach County, Loxahatchee River, Palm Point; 25028, USA, Florida, Dade County, Miami, storm sewer at Biscayne Boulevard & NE 87th Street; 25030, USA, Florida, St. Lucie County, Port St. Lucie, 615 Whitmore Drive; 25031, USA, Florida, Lee County; 25032, USA, Florida, Palm Beach County, Jupiter, Frederick Small Road Bridge in S Jupiter; 25033, USA, Florida, Monroe County, Everglades National Park, Whitewater Bay, Joe River; 25035, USA, Florida, Dade County, Snapper Creek automatic flood control dam; 25036, USA, Florida, Dade County, Miami River, flood control dam (S-258), downstream; 25037, USA, Florida, Dade County, Miami, Miami International Airport, canal behind airline hanger; 25038, USA, Florida, Dade County, Miami, Greynolds Park flood control dam; 25040, USA, Florida, Charlotte County, Punta Gorda, Myrtle Creek, 1-1.5 mile upstream from junction of Shell Creek & RT 17. USNM: 530313, USA, Florida, Glades County, Moorehaven; 551656, USA, Florida, Brevard County, Frontenac, Florida power and light, Cape Canaveral plant, 200 yards N; 551665, USA, Florida, Brevard County, Merritt Island, W shore Banana River, 3 miles N Pineda Causeway; 551671, USA, Florida, Brevard County, Banana River, Cape Canaveral, in Turn Basin N of Highway 528; 551672, USA, Florida, Brevard County, Lake Shepard, Satellite Beach; 551681, USA, Georgia, Glynn County, Brunswick, Terry Creek at Hercules Inc.; 554180, USA, Mississippi, Harrison County, Mississippi Sound, 0.25 mile E of tip of Ship Island, on N side; A 1375, USA, Texas, Brazos.

Trichechus manatus manatus

AMNH: 35566, Puerto Rico. AQUASIS: 2, Brazil, Ceará, Beberibe, Praia de Parajuru; 10, Brazil, Ceará, Aracati, Praia de Quixaba; 24, Brazil, Ceará, Icapuí, Praia do Ceará; 33, Brazil, Ceará, Aracati, Praia de Majorlândia; 36, Brazil, Ceará, Icapuí, entre as Praias de Redonda e Peroba; 38, Brazil, Ceará, Icapuí, Praia de Retiro Grande. CMA: 01S0111/13, Brazil, Paraíba, Conde, Praia de Coqueirinho; 01S0111/14, Brazil, Rio Grande do Norte, Baía Formosa, Praia do Sagi; 01S0110/57, Brazil; 01S0112/135, Brazil, Paraíba, Conde, Praia de Jacumã; 01S0111/152, Brazil. FMNH: 13888, Guatemala, Izabal; 13890, Guatemala, Izabal. USNM: 257674, Costa Rica, Colorado Bay; 257675, Nicaragua; 258298, Honduras, Gracias a Dios, Cannon Island (Isla Canones), Breivers Lagoon; 550416, Puerto Rico, Vega Baja, N shore, between Punta Puerto Nuevo and Isleta D. Garzas; 550417, Puerto Rico, Cabo Rojo, La Parguera, S coast; 550418,

Puerto Rico, Guayanilla, Playa Puntilla, at Union Carbide plant; 550419, Puerto Rico, Old San Juan, La Perla, near The Old Cemetery; 550420, Puerto Rico, Salinas, Playa Salinas, Cayo Mata; 554181, Puerto Rico, Peñuelas, El Boquete, E of Bahía de Tallaboa and Euayanilla chemical plant, E of Río Tallaboa; A 13009, Suriname, Surinam; A 20948, Guyana, Demerara. USGS: SB-Belize I, Belize.

Trichechus senegalensis

AMNH: 53939, Democratic Republic of the Congo, Banana. FMNH: 81513, Democratic Republic of the Congo; 81514, Africa. USNM: 571420, Côte D'Ivoire, Lagunes, Grand Lahou, Lagoon Tagba; 571421, Côte D'Ivoire, Lagunes, Grand Lahou, Bandama River.

Appendix 2. Landmark definitions

1. Anteriormost tip of premaxilla, midline; 2–3. most ventral maxilla–premaxilla suture, both sides; 4. superior tip of premaxilla as viewed dorsally, midline; 5–6. anteriormost point of the zygo-maxillary superior suture (between jugal and maxillary), both sides; 7–8. anteriormost tip of the supraorbital process of frontal, both sides; 9–10. posteriormost point of premaxilla as viewed dorsally, both sides; 11–12. posteriormost tip of the postorbital process of frontal, both sides; 13–14. inferiormost tip of the zygomatic process of squamosal, anterior position, both sides; 15–16. superiormost tip of the zygomatic process of squamosal, anterior position, both sides; 17. anteriormost point of suture between frontals, midline; 18–19. anteriormost point of suture between frontal–parietal, both sides; 20–21. Most dorsal point of the zygomatic process of squamosal, posterior position, both sides; 22–23. Most ventral zygo-squamosal suture (inferior suture between jugal and squamosal), both sides; 24. bregma,

frontal–parietal suture, midline; 25. most dorsal superior point of parietal, midline; 26–27. Occipital–parietal–squamosal junction, both sides; 28–29. posteriormost tip of squamosal as viewed laterally, both sides; 30–31. Posteriormost point of suture between supraoccipital–exoccipital, both sides; 32–33. Squamosal–alisphe-noid–parietal junction, both sides; 34–35. frontal–parietal–alisphe-noid junction, both sides; 36–37. Anteriormost point of premaxilla as viewed ventrally, both sides; 38–39. Maxilla–jugal suture, most-anterior position, both sides; 40–41. Zygo-maxillary inferior suture (between jugal and maxillary), both sides; 42–43. Posteriormost point of jugal, both sides; 44–45. Anterior-most point of tooth row at the bone, both sides; 46. palatine limit with the mesopterygoid fossa, midline; 47–48. Posterior-most point of tooth row, both sides; 49–50. posteriormost sphenoid–palatine suture on the pterygoid process, both sides; 51–52. Squamosal–sphenoid junction, anteriormost position, both sides; 53–54. Squamosal–sphenoid junction, posteriormost position, both sides; 55–56. Basisphenoid–basioccipital suture at the limit with middle lacerate foramen, both sides; 57–58. Jugular process of occipital bone, right after the jugular (posterior lacerate) foramen, both sides; 59. Basion, inferior point of foramen magnum, midline; 60. Opisthion, superior point of foramen magnum, midline.

Appendix 3. Specimens sampled for blood for karyological data, with respective sex

Specimens indicated with * yielded adequate cell cultures for cytogenetic analyses. Names as used at the Centro Nacional de Pesquisa e Conservação de Mamíferos Aquáticos (CMA/ICMBio), Itamaracá, Pernambuco State, Brazil, to designate individuals

Pintado ♂*; Telinha ♀*; Sereno ♂*; Ariel ♀*; Filho de Carla ♂*; Canoa ♀; B6 ♂; Clara ♀*; Artur ♂; Joana ♀*; Tupã ♂; Zoé ♂; Quitéria ♀*; Miriri ♂*; Tião ♂*; Arati ♂*.