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# Polymorphisms and distribution of South American manatees (*Trichechus* spp.)

**Cibele Rodrigues Bonvicino** [1] , **Maria Carolina Viana** [2] , **Edivaldo HC de Oliveira** [3] , **Renata Emin-Lima** [4] , **José de Sousa e Silva Júnior** [4] , **Maura Elisabeth M Sousa** [4] , **Salvatore Siciliano** Corresp. [5]

[1] Programa de Genética, Instituto Nacional de Câncer-INCA, Rio de Janeiro, RJ, Brasil
[2] Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, SP, Brasil
[3] Laboratório de Cultura de Tecidos e Citogenética-SAMAM, Instituto Evandro Chagas-IEC, Ananindeua, Pará, Brasil
[4] Coordenação de Zoologia, Setor de Mastozoologia, Museu Paraense Emílio Goeldi-MPEG, Belém, Pará, Brasil
[5] 6Laboratório de Enterobactérias, Instituto Oswaldo Cruz/Fiocruz, Rio de Janeiro, RJ, Brasil

Corresponding Author: Salvatore Siciliano
Email address: gemmlagos@gmail.com

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# Polymorphisms and distribution of South American manatees (*Trichechus* spp.)

**Cibele Rodrigues Bonvicino[1,2], Maria Carolina Viana[1,3], Edivaldo H. C. de Oliveira[4], Renata Emin-Lima[5], José de Sousa e Silva Júnior[5], Maura Elisabeth M. Sousa[5], Salvatore Siciliano[5,6,7]**

[1]Programa de Genética, Instituto Nacional de Câncer, Rua André Cavalcanti, 37, 4º. andar, Bairro de Fátima, Rio de Janeiro, RJ, 20231-050 Brazil

[2]Laboratório de Biologia e Parasitologia de Mamíferos Reservatórios Silvestres, Instituto Oswaldo Cruz/Fiocruz, Pavilhão Lauro Travassos, Av. Brasil, 4.365, Rio de Janeiro, RJ, 21940-900 Brazil

[3]Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas – UNICAMP, Campus Universitário Zeferino Vaz, R. Monteiro Lobato, 255, Campinas, SP 13083-862 Brazil

[4]Laboratório de Cultura de Tecidos e Citogenética-SAMAM – Instituto Evandro Chagas, Ananindeua, PA, Brazil and Faculdade de Ciências Naturais-ICEN, Universidade Federal do Pará, Belém, PA, Brazil

[5]Coordenação de Zoologia, Setor de Mastozoologia, Grupo de Estudos de Mamíferos Aquáticos da Amazônia (GEMAM), Museu Paraense Emílio Goeldi, and Programa de Capacitação Institucional. Av. Perimetral, 1.901, Terra Firme, 66077-830, Belém, PA, Brazil

[6]Laboratório de Enterobactérias, Instituto Oswaldo Cruz/Fiocruz, Pavilhão Rocha Lima, 3º. andar, Av. Brasil, 4.365, Manguinhos, Rio de Janeiro, RJ, 21940-900 Brazil

[7]Grupo de Estudos de Mamíferos Marinhos da Região dos Lagos (GEMAM-Lagos), Rua São José, 1.260, Praia Seca, Araruama, RJ, 28970-972, Brazil

Corresponding author: Salvatore Siciliano

Email address: gemmlagos@gmail.com

## Abstract

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Traditionally, the morphological attributes and the range of *Trichechus* species have been clearly established. However, we herein show that morphological traits, like belly and pectoral flipper coloration in South American manatees may be polymorphic. Karyotypic analysis of *T. manatus* allowed the precise identification of this species and confirmed the variability of the observed morphological findings. Molecular analysis based on cytochrome *b* DNA and the D-loop mitochondrial region showed shared haplotypes between *T. inunguis* and *T. manatus*, suggesting the presence of an ancestral polymorphism. These findings showed the need of improving the identification of these species before implementing conservation strategies. Finally, we present a complete report on the extant distribution of these species in South America.

Key words: aquatic mammals, Atlantic Ocean, Amazonas river basin, *Trichechus inunguis*, *Trichechus manatus*

**Introduction**

South America is unique in hosting two Trichechid species: The West Indian manatee, *Trichechus manatus*, and the Amazonian manatee, *T. inunguis* (**Husar, 1977**). These species have been considered to be easily identified by their belly coloration, the former being entirely gray and the latter with white patches. Moreover, *Trichechus manatus,* unlike *T. inunguis,* has nails on the flippers and a diploid number (2n) of 48 chromosomes (**Gray et al., 2002; Barros et al., 2016**) against the nailess *T. inunguis* with 2n = 56 (**Loughman et al., 1970; Assis et al., 1988**).

Interestingly, both species require abundant aquatic vegetation and freshwater. *Trichechus manatus* typically occupies grazing pastures in shallow coastal waters and adjacent freshwater ecosystems and is rare or absent from areas lacking these habitats (**Best, 1981**). Seasonal migrations have been documented in Florida (**Reid & O'Shea, 1989; Rathbun et al., 1990**), presumably in response to intra-annual weather change (**Raymond, 1981; Reid et al., 1991; Reid et al., 1995**).

Two subspecies have been proposed: the Florida manatee (*T. m. latirostris*), mostly restricted to the Florida peninsula (United States) and the Antillean manatee (*T. m. manatus*), distributed throughout the Caribbean, Central and South America (**Hatt, 1934; Domning & Hayek, 1986**). The Florida and the Antillean subspecies are very similar in external morphology but can be distinguished by morphometric analysis of cranial characters (**Domning & Hayek, 1986**). In recent decades, West Indian manatee populations have been affected by habitat degradation (**O'Shea & Ackerman, 1995**), exceptional cold weather (**O'Shea et al., 1985**), red tide outbreaks (**Buergelt et al., 1984; O'Shea et al., 1991**), hunting and incidental catch (**Thornback & Jenkins, 1982; Mignucci-Giannoni, 1989**) and collisions with boats (**O'Shea et al., 1985**). The manatee is a specialized feeder and reduction of seagrass beds due to coastal development poses a serious threat (**Reynolds et al., 1985**).

*Trichechus inunguis* is distributed throughout the major rivers of the Amazon basin, although it is rare in the Tocantins, Xingu and Tapajós rivers in Brazil (**Bertram & Bertram, 1973**), while information on its distribution in eastern Pará state, including the large Marajó Island, is deficient, imprecise, and limited to a few historical records (*e.g.* **Edwards, 1847; Ferreira, 1903; Domning, 1981**). Thousands of Amazonian manatees have been hunted for centuries in Brazil (**Domning, 1982**) and, in some regions, they continue to be hunted at a local scale. Due to their murky water habitat, accurate population estimates are difficult. The International Union for Conservation of Nature and Natural Resources (IUCN) presently recognizes *T. manatus* and *T. inunguis* as vulnerable taxa (**Thornback & Jenkins, 1982**). National management programs for the Amazonian manatee are presently going on in Colombia and Brazil. In Brazil, however, conservation actions are limited by the scarce knowledge of the potential occurrence of Amazonian manatees on the eastern Amazonia coast, a vast geographic region generally and frequently referred to as 'Marajó Island'. At present, key accounts of the distribution of the Amazonian manatee fail to provide a clear picture of the precise range of these elusive species (**Husar, 1977; Reeves et al., 1992; Jefferson et al., 1993; Jefferson et al., 2008; Emmons, 1997**).

In this paper we present (a) a review of former and current records of manatees (*Trichechus* spp.) in South America, mainly along Marajó Island, on the Eastern Amazon coast; (b) a gazetteer of published and unpublished records of *Trichechus* spp. in South America; (c) genetic data on karyotype, cytochrome *b* DNA and the D-loop mitochondrial region for elucidating the diversity of *Trichechus manatus* and *T. inunguis* along the Eastern Amazon coast; (d) a discussion on the conservation status of these species on the Eastern Amazon coast.

## Materials and Methods

### *Sample collection*

In order to investigate the presence of manatees in rivers in Eastern Amazonia and the Marajó Bay area, the Grupo de Estudos de Mamíferos Aquáticos da Amazônia (GEMAM) of the Museu Paraense Emílio Goeldi (MPEG) established a collaborative network of investigators and volunteers to search for stranded and live-rescued manatees in November 2005. A toll-free phone number and additional social media were available for reporting manatee strandings (facebook.com/bichodagua). These events were regularly monitored between 2005 and 2018. Every two weeks, a coastline stretch of approximately 300 km, including the Marajó Bay area and Eastern Pará State coast, were accessed by boat and a four-wheel vehicle or, alternatively, monitored on foot. At least three field assistants took part in surveys. Findings were validated with voucher samples, photographs, and other sources of original information. Marine mammal transportation, handling and necropsy licenses issued by ***Sistema de Autorização e Informação em Biodiversidade - SISBIO, Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio, Ministério do Meio Ambiente - MMA***, herein called SISBIO/ICMBio, to Grupo de Estudos de Mamíferos Aquáticos da Amazônia (GEMAM) of Museu Paraense Emílio Goeldi (MPEG) numbers 30327-1 (permit issued on 22/02/2013 expired 24/03/2014) and 54305-1 (permit issued 06/02/2019 expires 06/02/2020).

In view of lack of accurate data on manatee locations in previous published records, we produced a gazetteer of *Trichechus* spp. in South America. We used all available peer-reviewed data, theses and newspaper files with photos for generating a map of manatee distribution in South America. The Gazetteer included new records of *Trichechus* spp. of osteologic material and/or tissue samples deposited in the mammal collection of the Museu Paraense Emílio Goeldi (MPEG) in Belém.

*Morphology and karyotype*

We examined external morphology, including coloration, of *Trichechus* specimens of all available photographs and *in loco* observations during stranding events along Eastern Amazon coast. Taxonomic identification was carried out based on external morphological characters as described in **Domning & Hayek (1986)**. Stranded specimens were checked for (a) presence of nails, (b) body coloration (black or grey), (c) skin texture, and (d) presence of white (or pinkish) patch(es) on breast, belly, fluke and/or flippers. We examined all available specimens of *Trichechus* spp. deposited in MPEG (Table 1).

Karyotyping was carried out with whole-blood cultures according to **Moorhead et al. (1960)**. Briefly, 10 drops of blood collected in heparinized tubes were added to 5ml of RPMI 1640 medium supplemented with fetal bovine serum (Gibco) and phytohemagglutinin (Sigma), incubated at 37°C for 72 hours. Two drops of colchicine were added to each tube 90 min before harvesting. Cell suspensions were centrifuged, the supernatant discarded, and 10 ml of KCl hypotonic solution were added and subsequently incubated at 37°C for 20 min. Cells were thrice fixed with cold fixative (3:1 methanol:acetic acid). Cell suspensions were dropped onto slides and conventionally stained with a 5% Giemsa's solution for 5 min. Ten good quality metaphases were captured using a CCD video-camera connected to a Leica DM1000 microscope equipped with a 100x immersion lens. Chromosomes were analyzed with GenASIs software (Applied Spectral Imaging).

*Molecular studies: DNA Isolation, Amplification and Sequencing*

Tissue samples preserved in 100% ethanol (Table 2) were used for molecular analysis. DNA was isolated with phenol-chloroform (**Sambrook et al., 1989**) and its quality assessed by electrophoresis in 0.8% agarose gels and quantification in a NanoDrop® 1.000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). PCR amplifications of the, mitochondrial cytochrome *b* (MT-CYB) gene following HGNC rules (**Eyre et al., 2006; HGNC, 2009**) and the control and non-coding (D-loop) region were carried out. MT-CYB was amplified with MVZ03, MVZ04 primers (**Smith et al., 1992**), L14724 (**Irwin et al., 1991**) and citb-REV (**Casado et al., 2010**), and D-loop with H16498 (**Meyer et al., 1990**) and L15926 (**Kocher et al., 1989**). Amplifications were carried out, for both MT-CYB and D-loop, with a pre-denaturation step at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 42°C for 45 sec, extension at 72°C for 1 min, and a final extension of 72°C for 10 min. PCR products were purified with GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Brazil). Sequencing reactions were performed with PCR primers and Cytb1F, Cytb1R, Cytb2R (**Vianna et al., 2006**), CB-in1 and CB-in2 (**Cassens et al., 2000**), labeled with XL and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and loaded to an ABI Prism™ 3130 platform.

*Phylogenetic reconstructions and analyses*

Sequences were edited and assembled with Chromas (**McCarthy, 1988**) and subjected to Basic Local Alignment Search Tool (BLAST-NCBI) to confirm molecular identification. Assembled MT-CYB sequences were aligned with MEGA v.6 (**Tamura et al., 2013**) and D-loop data converted to FASTA format, aligned with MUSCLE (**Edgar, 2004**) and curated considering Gblocks (**Castresana, 2000**) using PHYLOGENY.FR, following a step-by-step mode (**Dereeper et al., 2008**).

We included MT-CYB data available in GenBank from *T. inunguis* (AY965887, AY965888, AY965889, AY965890), *T. manatus* (AM904728, AY965883-86, D83050, JF489120), *T. senegalensis* (AY965880–82), with Dugong dugon (U07564) as outgroup. Only sequences with at least 615 bp were included. For D-loop analysis, we included GenBank data from *T. inunguis*, *T. manatus, T. senegalensis*, and *Hydrodamalis gigas* as outgroup (Tables 3 and 4).

Genetic p-distances were estimated with complete deletion using Kimura's two parameters with MEGA v.6. The best fit model of nucleotide evolution was chosen with MODELGENERATOR v.0.85 (**Keane et al., 2006**). The HKY modl with gamma distributed Rate according to AIC and BIC criteria was used for MT-CYB analysis.

Phylogenetic reconstructions based on maximum-likelihood (ML) were performed with MEGA v.6 with bootstrap estimates based on 1,000 replicates. Bayesian analysis (BA) was carried out with MrBayes 3.2.1

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(**Ronquist et al., 2003**), and posterior probabilities estimated by sampling every 100th generation over a total of 1,000,000 with a burn-in of 25% generations (2,500 trees). Topologies were generated and edited with FigTree v1.4.0 (**Rambaud, 2002**).

Intraspecific analyses were carried out with DNAsp 5 for estimating haplotype and nucleotide diversity (**Librado & Rozas, 2009**). Median-joining (MJ) and network (**Bandelt et al., 1999**) analyses were carried out with NETWORK (version 4.6.1.1; available at http://www.fluxusengineering.com) for evaluating population structure and geographic distribution patterns using variable sites only.

## Results

### *Geographic distribution*

The most complete map of the geographic distribution of South American manatees (*Trichechus* spp.) is herein presented, including Brazil, French Guiana, Suriname, Guyana, Venezuela, Colombia, Peru and Ecuador (Figure 1). Additional file 1 shows localities in detail. In this study, *T. inunguis* has been recorded along the Eastern Amazon coast, in the Marajó Bay area, nearby inlets and channels in sympatry with *T. manatus* in several localities (Table 1). Amazonian manatees have also been reported along the eastern coast of Marajó Island (Table 1, Records #21, #22, #24, #32, #39, #40, #41, #42) and adjacent eastern coastline (Table 1, Records #26, #35). Presently, 33 specimens of *T. inunguis*, and nine *T. m. manatus* have been deposited in the Museu Paraense Emílio Goeldi (MPEG) (Table 1).

### *Morphology and genetics*

A total of 15 specimens, stranded, live-captured or photographed, were examined. Twelve specimens presented typical *T. inunguis* or *T. manatus* morphological attributes (Table 1, Fig. 2), including two live specimens with a characteristic *T. inunguis* phenotype (Fig. 2A1-A3), with whitish/pinkish patches on chest and belly, black body coloration, and nailless flippers. This was the case of MPEG42157 recovered in Colares, Marajó Bay, on 31 July 2012, with a large white patch on the chest and belly and rounded paddle, and Tito (GEMAM748) – a rescued and released orphan, from Furo das Marinhas, Santa Bárbara do Pará, eastern Marajó Bay. Another specimen showed the typical *T. manatus* phenotype, like Udi (MPEG44496) (Fig. 2B1-B3). However, three other specimens with nails (characteristic of *T. manatus*) showed white belly patches (characteristic of *T. inunguis*). This was the case of one male manatee (Omar, MPEG42229, Fig. 2C) with the characteristic *T. manatus* 2n = 48 karyotype (Figure 3), but with atypical coloration. This male manatee calf is held in captivity in Salvaterra, Marajó Island since 20 July 2013, presenting a characteristic Amazonian phenotype, including a white narrow belly patch and another small one in the caudal fluke. Another specimen, Leleco (MPEG44491, Figure 2D1-D2), a calf rescued December 13, 2014, in Vila do Pesqueiro, Soure, Marajó Island, also showed an Amazonian phenotype, including a round paddle, large pinkish patch on chest and belly, and pinkish patches on the flippers. In addition, a third specimen with nails and an Amazonian phenotype, was stranded alive on Soure, Marajó Island.

In *T. inunguis,* analysis of cytochrome *b* DNA showed four haplotypes (Table 2), three ones (HC2, HC3, HC4) restrict to this species and another (HC1) shared by six *T. inunguis* and three *T. manatus* (one in GenBank AM904728 and two herein reported: Omar (MPEG42229), Leleco (MPEG44491). In *T. manatus,* cytochrome *b* DNA comprised six haplotypes (HC5, HC6, HC7, HC8, HC9, HC10) restrict to this species, and one (HC1) shared with *T. inunguis. T. senegalensis* comprised three haplotypes (HC11, HC12, HC13; Table 2). Phylogenetic analyses were coincident in showing the monophyly of *Trichechus* spp. and *T. senegalensis* (Figs. 4, 5, supplementary material) but *T. inunguis* was paraphyletic, with three independent lineages: a basal offshoot accounting for the shared haplotype with *T. manatus* (HC1), a second leading to a single haplotype (HC2) and a third leading to HC3 and HC4. Furthermore, *T. manatus* and *T. senegalensis* grouped in a trichotomy, one branch leading to *T. manatus* HC9 and HC10, a second to *T. manatus* HC5, HC6, HC7 HC8, and a third one to *T. senegalensis* HC11, HC12, HC13.

Cytochrome *b* and D-Loop networks were similar in showing three haplogroups, one with *T. senegalensis,* a second grouping the majority of *T. manatus*, and a third one with six *T. inunguis* sharing haplotype HC1 with three *T. manatus* (Figs. 4, 5). These haplogroups were separated by at least one median vector and three nucleotide substitutions. All three haplogroups presented at least one internal median vector, with *T. manatus* and *T. senegalensis* with more than one nucleotide substitution between some haplotypes. D-loop median joining also showed *T. inunguis* with more than one nucleotide substitution between some haplotypes (Fig. 5). The two median joining networks differed in the position of haplogroups; D-loop placed the *T. inunguis* haplogroup in a central position and connected with the two other haplogroups, while cytochrome *b* placed *T. manatus* in a central position (Fig. 4, 5).

## Discussion

### Geographic distribution

The map and gazetteer provide insights on the distribution of manatees (*Trichechus* spp.) in South America, poiting to Brazil and Colombia as the only two countries with both *T. inunguis* and *T. m. manatus.* The occurrence of two trichechid species within the boundaries of these countries represents a unique situation worldwide and deserves special attention for conservation strategies. According to two reviews (**Reeves et al., 1992; Jefferson et al., 1993**), Antillean manatees in mainland South America are presently distributed from Alagoas (10˚S), in the Northeast of Brazil, to the Colombian coastline and major inland rivers. Their former southern range in South America having extended further south to the mouth of Rio Doce (19°37'S), Espírito Santo State, in the Southeast of Brazil. More recently, a manatee rib found amid a pile of weathered bones of humpback whales in Barra de Caravelas (17°43'S), Bahia State, in September 1990, posed the intriguing possibility that manatees have been recently present at this latitude. The Caravelas record has been included in our map and gazetteer, accounting for the likely southern limit of *T. manatus* in South America. These records are further discussed by **Siciliano & Barbosa-Filho (2016)**. Detailed examination of the map indicates two gaps in the distribution of *T. m. manatus* in South America, one along the Venezuelan coastline, in accordance with **Lefebvre et al. (1989)** who proposed that this major discontinuity was likely due to unsuitable habitats. Another gap, herein reported, was found along a vast stretch of the northern coast of Brazil, known as Lençóis Maranhenses and Pequenos Lençóis, a continuum of sand dunes along the seashore. Our findings also provided compelling evidence of recent recolonization around Marajó Island, in agreement with a previous report on the mouths of the Amazon and the vicinity of Belém where *T. inunguis* and *T. manatus* had apparently been heavily hunted to the verge of extintion (**Domning, 1981**).

Our review indicated that *Trichechus inunguis* is widely distributed in the Amazon basin, following the main course of the Amazon River and its major tributaries to Colombia, Peru and Ecuador. Records of *T. inunguis* in French Guiana, Suriname, Guyana and Venezuela are presently unavailable, although Amazonian manatees have been found to occasionally penetrate southern Guyana close to the boundary with Brazil (**Bertram & Bertram, 1973**). This finding has been confirmed by a recent manatee rescue in the Rio Takutu (=Tacutu), on the border of Brazil and Guyana (**Marmontel et al., 2016**).

As above stated, previous reports on the potential distribution of Amazonian manatees in eastern Pará have been incomplete because several authors restricted their potential occurrence in eastern Amazonia to a vast geographic area frequently referred as 'Marajó Island' (see Table 1), failing to define the actual range of these elusive species. Along the Eastern Amazon coast, *T. inunguis* has been herein confirmed in the Marajó Bay area and nearby inlets and channels. The influx of abundant freshwater during the rainy season (December-June) may favor the spreading of Amazonian manatees througout this vast territory, when the mixed waters in Marajó Bay undergo reduced salinity as attested by recent regular records in Furo das Marinhas (1°21'4"S and 47°34'20"W, Mosqueiro (1°9'49S and 48°28'15W), and Ourém (1°33'0S and 47°6'0W) localities. These findings are in accordance with previous work that reported the presence of *T. inunguis* in the coast of French Guiana (Vilaça et al. 2019), with strong influence of fresh water, and then with low salinity. The records herein presented revise the distribution map of Amazonian manatees in Brazil (Fig. 1).

We found *Trichechus inunguis* throughout the lower Amazon and its tributaries, including Marajó Island and its Atlantic coast and coastal islands, and from the northern (Amapá) to the southern shore of the Amazon estuaries (Pará). These findings contradicted **Domning's (1981)** assertion that *Trichechus inunguis* would not inhabit regions with a high inflow of salt water (Table 1, Fig. 1). **Domning (1981)** stated *Trichechus manatus* as apparently "exterminated from the Atlantic coast of Pará", assuming that this species, and not *T. inunguis*, formerly occurred in this region. This author concluded that any zone of sympatry between *T. manatus* and *T. inunguis* was highly restricted, if ever present, and that these species had mutually exclusive (*i.e.*, parapratic) distributions. However, both manatee species are presently common and have been recorded by us in a wide area of sympatry on the eastern cost of Marajó Island, providing the first evidence that *T. inunguis* and *T. manatus* were syntopic. Although some populations have been reduced with respect to historical levels (**Lefebvre et al., 1989**), these findings suggest that manatee populations are recovering, as was the case of other marine species like the humpback whale and the northern elephant seal (**Thomas et al., 2016; Stewart et al., 1994**). Similar findings were also reported for *T. inunguis* (**Cantanhede et al., 2005**) in disagreement with a previous postulation that a low reproductive rate imposed a limit to the ability of manatees to recover from population reduction (**Thornback & Jenkins, 1982; Marmontel, 1995**).

***Morphology and Karyotype***

In this study, external morphology and coloration in live *Trichechus* specimens examined in the field and the Salvaterra rehabilitation center revealed that these traits were variable. **Domning & Hayek (1986)** stated that nails represented primitive mammalian traits, and that their loss in *T. inunguis* was a derived trait, in accordance with findings of vestigial nails in *T. inunguis* newborns which were subsequently absent in adults (**Luna, 2013**). Noteworthily, **Domning & Hayek (1986)** described an atypical ventral coloration in a female manatee from Rio Arari, Marajó Island, together with a detailed and comparative description of *Trichechus* spp coloration. In that report, "*T. senegalensis* and *T. manatus* normally are uniformly dark in color without distinct markings … *T. inunguis*, in contrast to the other species, is said to be constantly characterized by … a white breast patch" (**Domning & Hayek, 1986, p. 91**). This description, however, was only valid for the majority of individuals rather than for all; in fact, unmarked specimens had been reported by **Stannius (1845, p. 2)**, **Edwards (1847, p. 149)** and **Pereira (1944, p. 39)**, as well as two of the three specimens in Natterer's original type-series (**v. Pelzeln 1883, pp. 91-92**). **Domning & Hayek (1986)** further stated that "Of 23 individuals examined by one of us (DPD), three were completely unmarked, three had only a tiny and inconspicuous belly patch, ten had a single, "normal" patch or a pair of patches confined to the chest and/or abdomen, and seven had multiple ventral patches extending onto the throat and/or tail. In one of the later, the patches consisted of white mottling on broad areas of the belly and tail, and in one of the second category there was also some gray speckling on the belly and indistinct pinkish areas on the throat and chest (Fig. 1 in **Domning & Hayek, 1986**). The belly patches may be either white or bright pink, possibly depending on the visibility of vascularized layers through the skin". When referring to nails, these authors mentioned: "Small nails on the lateral surfaces of the tips of one or more digits normally characterize *T. manatus* and *T. senegalensis*; their absence gave *T. inunguis* its name. However, **Stannius (1845, p. 2)** found a single nail on each flipper in a young Amazonian manatee from "Para [*sic* Pará]". Generally, conventional descriptions of *Trichechus* spp. coloration are vague, stating that Amazonian manatees were grey to black, while most of them showed white or pink belly and chest patches (**Jefferson et al., 1993; Jefferson et al., 2008**). In West Indian manatees "the colour of the skin is generally grey to brown, often with a green tinge caused by algal growth. The short hairs are colourless. Calves appear to be a darker shade of grey, almost black" (**Jefferson et al., 1993; Jefferson et al., 2008**). According to **Emmons (1997)**, *T. manatus* was "similar to Amazonian manatee except larger; flippers with large, flat nails on their tips; underparts gray or with pink blotches, without white patches". However, previous work reported one karyotyped specimen of *T. inunguis* (Vitória) with nails on one flipper (**Luna, 2013**).

Our data on the coloration and external morphology of recovered *T. manatus* along the Eastern coast of Pará contradicted these previous reports and provided strong evidence of color polymorphism. We noted that some *T. manatus* specimens had nails but also whitish or pinkish patches on the chest and/or belly, including flippers, with typical coloration of *T. inunguis*. Two of these *T. manatus*, named Omar (MPEG42229) and Leleco (MPEG44491), shared the same cytochrome *b* haplotype with some *T. inunguis.* It has been postulated that colored body patches in mammals might be useful for intraspecific signaling (**Caro, 2005**) in mate selection, which can lead to reproductive isolation, even in sympatry (**White, 2016**). It is notable that *T. inunguis* also showed an apparent polymorphism with respect to nails, which were detected in at least two specimens (**Luna, 2013; Domning & Hayek, 1986**).

Karyotyping of the *T. manatus* with atypical skin coloration showed 2n = 48, with the same chromosome morphology attributed to *T. m. latirostris* from Florida (**Gray et al., 2002**) and Puerto Rico (**Hunter et al., 2012**), but different from the *T. m. manatus* karyotype from the Brazilian Northeast (**Barros**

**et al., 2016**). Chromosome pairs 4 and 10 were metacentric and submetacentric in the Northeastern Brazilian manatee, while they were submetacentric and subtelocentric in Puerto Rico Antillean and Florida manatees (**Gray et al., 2002; Hunter et al., 2012; Barros et al., 2016**). These differences occurred between individuals from distant geographic regions, Pará/Maranhão and Northeast Brasil (Piauí to the east), separated by a gap of *ca.* 500 km of coastal dunes, known as Lençóis Maranhenses and Pequenos Lençós. That coastline can be considered an unsuitable habitat for manatees.

Interestingly, a 2n = 50 karyotype has been previously alleged for a hybrid specimen (Poque) found on the Northern Brazilian coast (**Luna, 2013**). However, a close analysis of this report indicated that this was actually a variant of the standard *T. manatus* karyotype, differing in fundamental number by an apparent fission event of a biarmed chromosome pair (n.9). The fact that this specimen showed a typical *T. inunguis* cytochrome *b* haplotype lead to the postulation that it was an F2 hybrid (**Luna, 2013**). Indeed, the haplotype sharing herein demonstrated between *T. manatus* and *T. inunguis* clearly indicated that cytochrome *b* haplotyping cannot be considered a reliable trait for species identification. The karyotype appear to be the single trait that unquestionably confirm manatee species identification.

***Genetic analysis***

*Trichechus* species distributed in the western Atlantic comprise several lineages inhabiting saltwater and freshwater habitats. In South America, *T. inunguis* is distinguished from *T. manatus* by morphology, although these two species were not reciprocally monophyletic (Fig. 4, additional file 2), because the markers used in phylogenetic reconstructions showed ancestral polymorphisms. In fact, the presence of shared mitochondrial D-loop haplotypes between *T. manatus* and *T. inunguis* has been reported in a previous publication (**Cantanhede et al., 2005**), further indicating the existence of an ancestral polymorphism, commonly found in vertebrates (**Wu, 1991**). Other studies using cytochrome b as marker also identified haplotypes shared by *T. inunguis* and *T. manatus* (**Santos et al., 2016**), however these authors did not use other marker to confirm species identification, whereas herein we confirm the identification of *T. manatus* that share haplotype with *T. inunguis* by karyotype. Another study, based on ddRAD SNP data and sequences of nuclear and mtDNA, found mtDNA haplotypes shared by *T. inunguis* and *T. manatus* and suggested that this is result from introgression or later generation interspecific hybrids (**Vilaça et al., 2019),** discarting the possibility of ancestral polymorphism. However, **Vilaça et al.** (**2019**) study is based on few samples, they used only three *T. inunguis* and two *T. manatus* for comparison, the genetics of other specimens are considered result from introgression or hybrids. Furthermore, the locality of *T. inunguis* used as control is farm from the locality of the other *T. inunguis* localities used in their analysis and considered hybrids or introgressed, and the same occur in relation to *T. manatus*. It was previously found that the manatee skull shape of specimens from Suriname and Guyana are more similar to the one from the Caribbean population than to the population south of Rio Amazonas (CE and PI), showing a difference between these populations (**Barros et al., 2017**), and suggesting that the comparison of *T. manatus* from Guiana with those of Ceará and Rio Grande do Norte used by **Vilaça et al**. (**2019**) is not adequated. Furthermore, these authors comment that "hybridization may be a local phenomenon" of the Brazilian Amapá coast and French Guiana coast.

Two of three individuals with an unusual morphology herein studied, with nails and belly and flipper patches, showed a *T. manatus* karyotype. Similar specimens with these morphological attributes have been postulated to be hybrids (**Luna, 2013**), while a young *T. inunguis* with 2n = 56 from eastern Amazonia was found to present a small nail subsequently lost in adult life (**Luna, 2013**). These data showed that external morphological traits may not be distinctive enough for the identification of *Trichechus* species. We disagree with the postulation that a specimen with 2n = 50 called Poque, captured in Oiapoque, Brazilian Amapá state, with nails and a small white patch, might have been a *T. manatus* - *T. inunguis* hybrid (**Luna, 2013**) because an interspecific hybrid should be expected to show a diploid chromosome number of 52. Another study also reported hybrids in the mouth of the Amazon in Brazil, extending to the Guyanas and probably as far as the mouth of the Orinoco River (**Vianna et al., 2006**). We do not refute the possibility of hybrids, however we consided that morphology and mitochondrial DNA markers were not enough to confir the presence of hybrids as showed by our specimens with cytochrome *b* of *T. inunguis*, morphological characters shared by *T. inunguis* and *T. manatus*, but karyotype of *T. manatus*.

**Threats to manatees on Eastern Amazon coast**

We identified several threats to manatees during field work along the Eastern Amazon coast since 2005 and we rescued 35 stranded manatees between November 2005 and January 2018 along Eastern Pará. Threats resulted from incidental catches in gillnets and fixed trap nets as well as illegal hunting, mainly of calves, and water contamination. The use of fishing corrals built in submerged aquatic vegetation beds in Pará accounted for serious threats to manatees. Very recently, in the spring of 2018, an intentional killing of a stranded manatee was reported in Joanes, Marajó Island. Necropsy and histopathologic findings in a newborn *T. inunguis* from Goianésia do Pará confirmed chronic systemic bacterial infection caused by *Salmonella enterica* subsp. *enterica* (**Cardoso et al., 2017**).

Moreover, one large Amazonian manatee, incidentally caught in gillnets in Rio Arari, Cachoeira do Arari, Marajó Island on 16 July 2007 was killed for food consumption and later recovered for genetic analysis (MPEG44485, Table 1). A young male *T. inunguis* named Tito (GEMAM 748) was caught by a small gillnet set off Furo das Marinhas, in Santa Bárbara do Pará, on 23 June 2017. It was disentangled alive and rescued by our team and forwarded to the MPEG rehabilitation center in Belém. Being in good health, it was released at the same spot a week later.

**Implications for conservation**

The haplotypic diversity of Brazilian *T. manatus* and *T. inunguis* is low with respect to the other populations, as previously reported (**Garcia-Rodriguez, 1988; Vianna et al., 2006**). These authors, together with **Domning (1981)** and **Lefebvre et al. (1989)**, pointed to the relatively small size of the Brazilian population at the limit of their range. This scenario, together with our findings, show the need of precise species identification by karyotyping and distinctive molecular markers before implemrnting conservation strategies.

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**Abbreviations**

°C = degrees Celsius

2n= diploid number

AMNH= American Museum of Natural History,

Aquasis= Associação de Pesquisa e Preservação de Ecossistemas Aquáticos

ca.= *circa*

CMA= Centro Mamíferos Aquáticos.

D-Loop= derived from displacement loop, is a noncoding gene region in mitochondrial DNA

DNA= deoxyribonucleic acid

e.g. = *exempli gratia*

et al. = *et alii*

F = female

GB= GenBank accession number

GEMAM = Grupo de Estudos de Mamíferos Marinhos da Amazônia

H= haplotype

I = indeterminate

IBAMA = Instituto Brasileiro do Meio Ambiente

IDSM= Instituto de Desenvolvimento Sustentável Mamirauá

KCl= potassium chloride

km= kilometers

M = male

MPEG = Museu Paraense Emílio Goeldi

*MT-CYB* = mitochondrial gene cytochrome b

No. = number

PCR = polymerase chain reaction

PNUMA = Programa das Nações Unidas para o Meio Ambiente

s = sex

S= South

Sec= seconds

spp. = species

T. = *Trichechus*

TS= this study

UFPB= Universidade Federal da Paraíba

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**Figure legends**

**Figure 1.** Map showing all available records of manatees in South America. Black circles indicate *Trichechus inunguis* localities, gray circles indicate *T. manatus* localities, and white circles indicate *Trichechus* sp. localities. Numbers refer to names of localities listed in additional file 1.

**Figure 2.** Variation in coloration of *Trichechus* spp. along the eastern coast of Marajó: A1, A2 and A3 represent typical *T. inunguis* specimens live rescued in Marajó Is., Pará, Brazil; Fig. B1, B2 and B3 represent 'Udi', a typical *T. manatus* rescued in Marajó Is., note the greyish coloration and the presence of nails; Fig. 2C reprent 'Omar' (MPEG42229), a live rescued *T. manatus* in rehabilitation in Salvaterra, Marajó Is., note the black coloration, presence of white spots in the belly and tail, round paddle tail, and the

presence of nails; and Fid. 2D1-D2 represent 'Leleco' a rescued *T. manatus* in Marajó Is.: note the blackish coloration, the presence of white coloration in the belly and flippers and also presence of nails.

**Figure 3.** Conventional Gimsa stained karyotype of *T. manatus* male (Omar, MPEG42229)).

**Figure 4.** Maximum Likelihood and network analyses of *Trichechus* based on *MT-CYB* data. Circles represent haplotypes. Haplotype size is related to number of shared sequences. Black circles represent median vectors. Haplotypes are listed in Table 2.

**Figure 5**. Network analysis of *Trichechus* based on *D-loop* data. Circles represent haplotypes. Haplotype size is related to number of shared sequences Black circles represent median vectors. Haplotypes are listed in Table 3.

Tables legends

**Table 1.** Specimens of *Trichechus* deposited in the collection of Museu Paraense Emílio Goeldi (MPEG). Record number (Rec), sex (S), female (F), male (M), indeterminate (I), Pará state (PA).

**Table 2.** Manatee samples used in molecular analysis based on *MT-CYB* data. Hhaplotype (H), field (museum) or GenBank accession number (ID/GB). Numbers in brackets following locality names refer to Figure 1 and gazetteer.

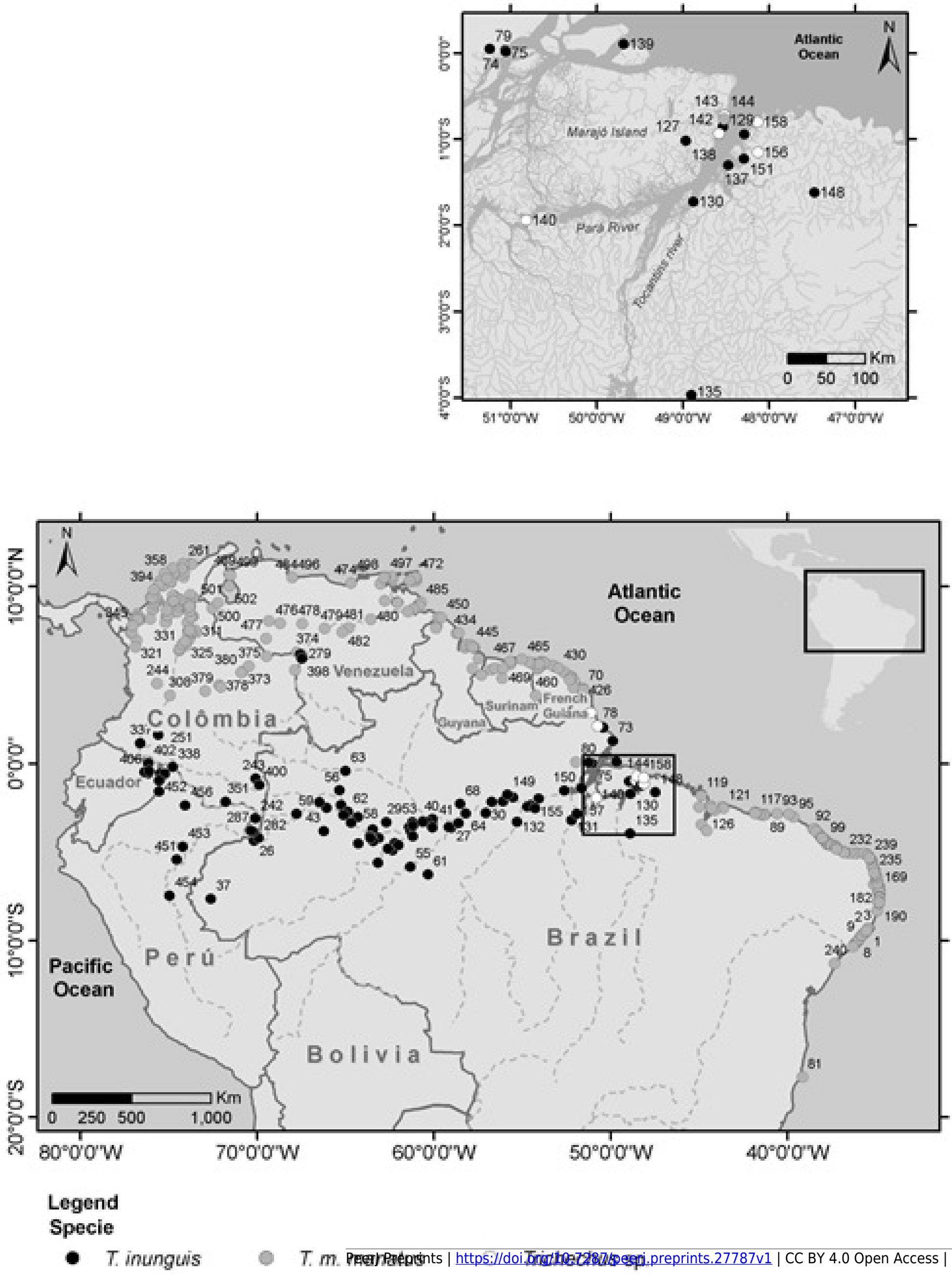
**Table 3.** Genetic distance estimates (%) between *MT-CYB* haplotypes (H) of *Trichechus*ID = museum or field number, GB = GenBank number. Localities are listed in Table 2.

**Table 4.** Manatee samples used in molecular analysis based on in D-Loop data. Field and GenBank accession numbers (ID). Localities are shown in Tables 1 and 2

## Figure 1(on next page)

Map showing all available records of manatees in South America.

Black circles indicate *Trichechus inunguis* localities, gray circles indicate *T. manatus* localities, and white circles indicate *Trichechus* sp. localities. Numbers refer to names of localities listed in additional file 1.


Atlantic Ocean
Marajó Island
Pará River
Tocantins river
Km
0 50 100
51°0'0"W 50°0'0"W 49°0'0"W 48°0'0"W 47°0'0"W
Atlantic Ocean
Pacific Ocean
Colômbia
Venezuela
Guyana
Surinam
French Guiana
Ecuador
Perú
Brazil
Bolivia
Km
0 250 500 1,000
80°0'0"W 70°0'0"W 60°0'0"W 50°0'0"W 40°0'0"W
10°0'0"N 0°0'0" 10°0'0"S 20°0'0"S
Legend
Specie
T. inunguis
T. m. manatus
Trichechus sp.

## **Figure 2**(on next page)

Variation in coloration of *Trichechus* spp. along the eastern coast of Marajó

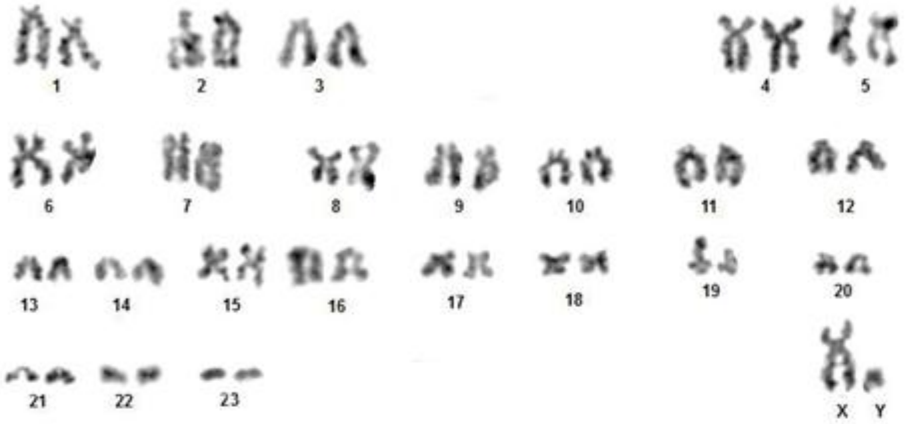
A1, A2 and A3 represent typical *T. inunguis* specimens live rescued in Marajó Is., Pará, Brazil; Fig. B1, B2 and B3 represent 'Udi', a typical *T. manatus* rescued in Marajó Is., note the greyish coloration and the presence of nails; Fig. 2C reprent 'Omar' (MPEG42229), a live rescued *T. manatus* in rehabilitation in Salvaterra, Marajó Is., note the black coloration, presence of white spots in the belly and tail, round paddle tail, and the presence of nails; and Fid. 2D1-D2 represent 'Leleco' a rescued *T. manatus* in Marajó Is.: note the blackish coloration, the presence of white coloration in the belly and flippers and also presence of nails.


A
B
C
D

## Figure 3(on next page)

Conventional Gimsa stained karyotype of *T. manatus*

Conventional Gimsa stained karyotype of *T. manatus* male (Omar, MPEG42229)).


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
X
Y

## Figure 4 (on next page)

Maximum Likelihood and network analyses of *Trichechus* based on *MT-CYB* data.

Circles represent haplotypes. Haplotype size is related to number of shared sequences. Black circles represent median vectors. Haplotypes are listed in Table 2.

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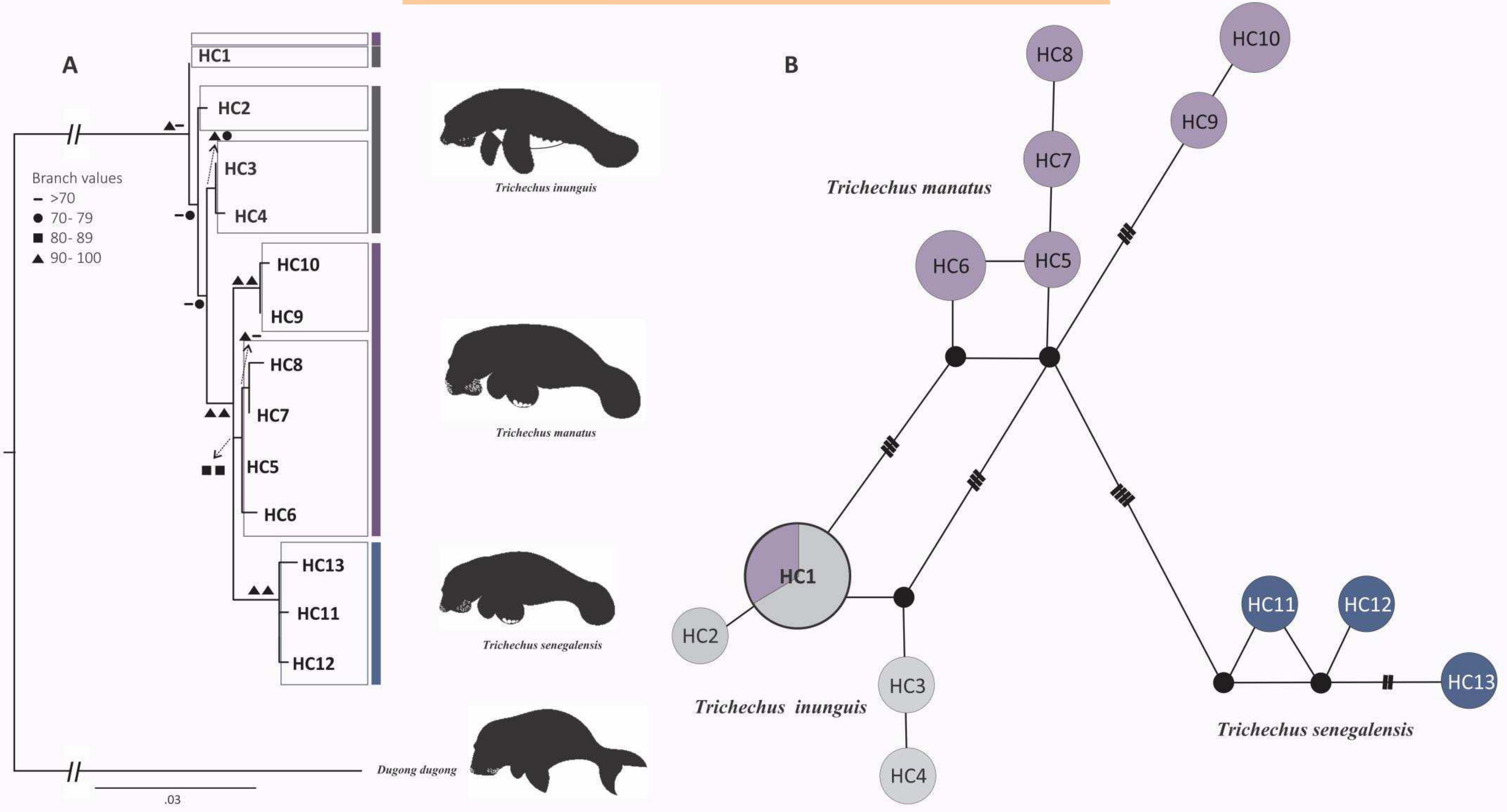


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# Table 1(on next page)

Specimens of *Trichechus* deposited in the collection of Museu Paraense Emílio Goeldi (MPEG)

Table 1. Specimens of *Trichechus* deposited in the collection of Museu Paraense Emílio Goeldi (MPEG)

| Rec | Species | Museum no. | Localities | Name | s | Material | Remarks |
|---|---|---|---|---|---|---|---|
| #01 | *T. inunguis* | MPEG1518 | Brazil, PA, Belém, Icoaraci | - | M | Skeleton | - |
| #02 | *T. inunguis* | MPEG4636 | Brazil, PA, Santarém, Taperinha, rio Ayaya | - | I | Skull, mandible | - |
| #03 | *T. inunguis* | MPEG4637 | Brazil, PA, Santarém, Taperinha | - | F | Skull, mandible | - |
| #04 | *T. inunguis* | MPEG4638 | Brazil, PA, Santarém, Taperinha, rio Ayaya | - | F | Skull, mandible | - |
| #05 | *T. inunguis* | MPEG4639 | Brazil, PA, Santarém, Taperinha, rio Ayaya | - | F | Skull, mandible | - |
| #06 | *T. inunguis* | MPEG4640 | PA, rio Curuá | - | F | Skull, mandible | - |
| #07 | *T. inunguis* | MPEG4641 | Brazil, PA, Monte Alegre, Lago Grande de Maycurú | - | I | Skull, mandible | - |
| #08 | *T. inunguis* | MPEG6491 | Brazil, PA, Jardim Zoológico de Belém | - | F | Skull, mandible | - |
| #09 | *T. manatus* | MPEG6492 | Brazil, PA, Belém, Jardim Zoológico do MPEG | - | F | Skull, mandible | - |
| #10 | *T. inunguis* | MPEG6493 | Brazil | - | M | Skull, skeleton | - |
| #11 | *T. inunguis* | MPEG6494 | Brazil, PA, Belém, Jardim Zoológico do MPEG | - | M | Skull, skeleton | - |
| #12 | *T. inunguis* | MPEG6495 | Brazil | - | F | Skull | - |
| #13 | *T. inunguis* | MPEG07959 | Brazil, PA, Oriximiná, rio Trombetas | - | F | Skull | - |
| #14 | *T. inunguis* | MPEG8845 | Brazil, PA, Jardim Zoológico de Belém | - | F | Skull | - |
| #15 | *T. inunguis* | MPEG09138 | Brazil, PA, Belém, Jardim Zoológico do MPEG | - | I | Skeleton | - |
| #16 | *T. inunguis* | MPEG11298 | Brazil, PA, Rio Guamá, mouth of igarapé Tucunduba | - | F | Skeleton | |
| #17 | *T. inunguis* | MPEG11838 | Brazil, PA, Cametá, rio Tocantins | - | I | Skin | |
| #18 | *T. inunguis* | MPEG12755 | Brazil, PA, Belém, Parque Zoobotânico do MPEG | - | M | Skeleton | - |
| #19 | *T. inunguis* | MPEG22428 | Brazil, unknown locality | - | M | Skull, mandible | - |
| #20 | *T. manatus* | MPEG37815 | Brazil, PA, Marajó island, Salvaterra, Porto dos | - | I | Skull | - |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | Pescadores | | | | |
| #21 | *T. inunguis* | MPEG44485 | Brazil, PA, Marajó island, Cachoeira do Arari (GEMAM216) | - | F | Pectoral | Female with fetus |
| #22 | *T. inunguis* | MPEG44486 | Brazil, PA, Marajó island, Salvaterra, Ponta do Pilão | Vitória | F | tissue | rehabilitation |
| #23 | *T. manatus* | MPEG42043 | Brazil, PA, Marajó island, Soure, vila Caju-una | - | F | Skeleton | stranded dead |
| #24 | *T. inunguis* | MPEG42148 | Brazil, PA, Abaetetuba (GEMAM419) | - | I | Skeleton | hunted and confiscated |
| #25 | *T. inunguis* | MPEG42156 | Brazil, PA, Jardim Zoológico de Belém | - | F | Skeleton | - |
| #26 | *T. inunguis* | MPEG42157 | Brazil, PA, Colares, igarapé do Cedro (GEMAM380) | - | F | tissue | stranded |
| #27 | *T. manatus* | MPEG42229 | Brazil, PA, Salvaterra, praia do Salazar (GEMAM585) | Omar | M | tissue | Alive, in rehabilitation |
| #28 | *T. manatus* | MPEG44487 | Brazil, PA, Santo Antônio do Tauá, vila Jutaí (GEMAM581) | Jutaí | M | tissue | Died in rehabilitation |
| #29 | *T. manatus* | MPEG44488 | Brazil, PA, Salvaterra, praia do Salazar | | | tissue | |
| #30 | *T. inunguis* | MPEG44489 | Brazil, PA, Gurupá (GEMAM586) | Gurupá | M | tissue | Died in rehabilitation |
| #31 | *T. manatus* | MPEG44478 | Brazil, PA, Salvaterra, praia de Joanes | | | tissue | carcass |
| #32 | *T. inunguis* | MPEG44490 | Brazil, PA, Salvaterra, praia de Joanes (GEMAM617) | | | tissue | carcass |
| #33 | *T. manatus* | MPEG44491 | Brazil, PA, Soure, vila do Pesqueiro (GEMAM645) | Leleco | M | Skeleton | Died in rehabilitation |
| #34 | *T. inunguis* | MPEG44492 | Brazil, PA, Melgaço, FLONA Caxiuanã | | | tissue | |
| #35 | *T. inunguis* | MPEG44493 | Brazil, PA, Vigia (GEMAM671) | | | tissue | stranded dead |
| #36 | *T. inunguis* | MPEG44494 | Brazil, PA, Goianésia do Pará | Vitória | F | Skeleton | |
| #37 | *T. inunguis* | MPEG44495 | Brazil, PA, Melgaço, FLONA Caxiuanã (GEMAM649) | Kaluan ã | F | tissue | Alive, in rehabilitation |
| #38 | *T. manatus* | MPEG44496 | Brazil, PA, Salvaterra, vila Água Boa (GEMAM741) | Udi | F | Skeleton | Hydrocephalus |
| #39 | *T. inunguis* | MPEG44497 | Brazil, PA, Salvaterra, praia de Joanes | Joeny | F | tissue | Alive, in rehabilitation |
| #40 | *T. inunguis* | MPEG44498 | Brazil, PA, Salvaterra, Ponta do Pilão | - | | Skeleton | - |
| #41 | *T. inunguis* | MPEG44499 | Brazil, PA, Salvaterra, praia de Joanes (GEMAM746) | - | M | Skeleton | - |
| #42 | *T. inunguis* | GEMAM748 | Brazil, PA, Santa Bárbara do Pará, Furo das Marinhas | Tito | M | tissue | Rescued and released |

Legend: record number (Rec), sex (s), female (F), male (M), indeterminate (I), Pará state (PA).

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## Figure 5 (on next page)

Network analysis of *Trichechus* based on *D-loop* data.

Circles represent haplotypes. Haplotype size is related to number of shared sequences Black circles represent median vectors. Haplotypes are listed in Table 3.

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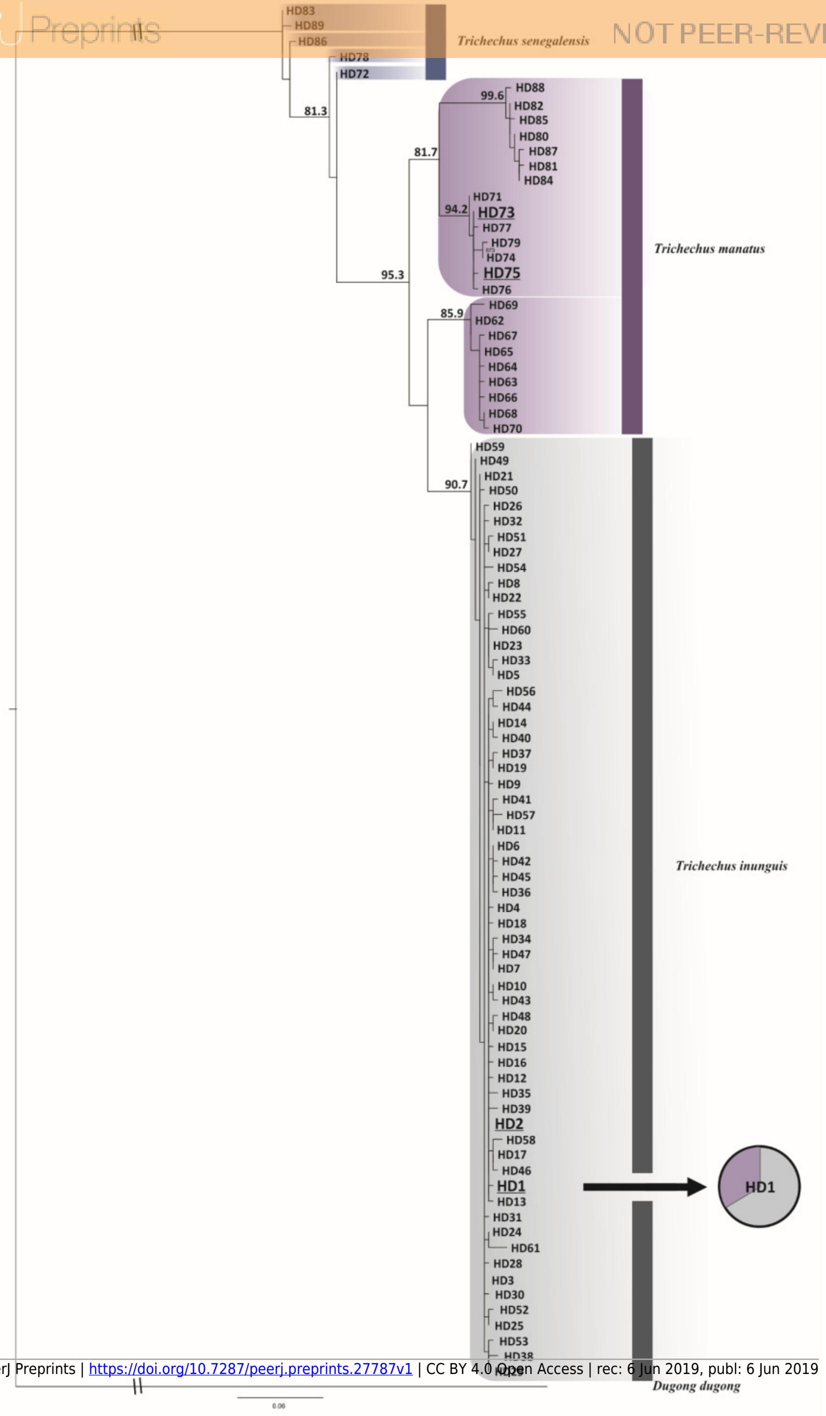
HD83
HD89
HD86
Trichechus senegalensis
HD78
HD72
81.3
99.6
HD88
HD82
HD85
HD80
HD87
HD81
HD84
81.7
HD71
94.2
HD73
HD77
HD79
HD74
HD75
HD76
Trichechus manatus
95.3
85.9
HD69
HD62
HD67
HD65
HD64
HD63
HD66
HD68
HD70
HD59
HD49
90.7
HD21
HD50
HD26
HD32
HD51
HD27
HD54
HD8
HD22
HD55
HD60
HD23
HD33
HD5
HD56
HD44
HD14
HD40
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HD9
HD41
HD57
HD11
HD6
HD42
HD45
HD36
Trichechus inunguis
HD4
HD18
HD34
HD47
HD7
HD10
HD43
HD48
HD20
HD15
HD16
HD12
HD35
HD39
HD2
HD58
HD17
HD46
HD1
HD1
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HD24
HD61
HD28
HD3
HD30
HD52
HD25
HD53
HD38
Dugong dugong
0.06

## Table 2 (on next page)

List of manatee samples used in molecular analysis with Cytochrome b gene.

List of manatee samples used in molecular analysis with Cytochrome b gene.

1 Table 2. List of manatee samples used in molecular analysis with Cytochrome b gene.

2

| H | field (museum) or GenBank accession number | Species | Locality | F |
|---|---|---|---|---|
| 01 | GEMAM585 (Omar, MPEG42229) | *T. manatus* | Pará state, Salvaterra, Passagem Grande (129) | TS |
| 01 | GEMAM645 (Leleco, MPEG44491) | *T. manatus* | Pará, Soure, praia do Pesqueiro | TS |
| 01 | GEMAM216 (MPEG44485) | *T. inunguis* | Pará, Cachoeira do Arari | TS |
| 01 | GEMAM419 (MPEG42148) | *T. inunguis* | Pará, Abaetetuba | TS |
| 01 | GEMAM586 (MPEG44489) | *T. inunguis* | Pará, Gurupá | TS |
| 01 | GEMAM617 (MPEG44490) | *T. inunguis* | Pará, Salvaterra, praia de Joanes | TS |
| 01 | GEMAM604 (MPEG44478) | *T. inunguis* | Pará, Salvaterra, praia de Joanes | TS |
| 01 | Ti_T_S_S2_STi04/ AY965890 | *T. inunguis* | Unknown | 1 |
| 01 | AM904728 | *T. manatus* | Unknown | 2 |
| 02 | Ti_T_STi03/ AY965889 | *T. inunguis* | Unknown | 1 |
| 03 | Ti_R_R2_U_Q_STi02/ AY965888 | *T. inunguis* | Unknown | 1 |
| 04 | Ti_V_X_STi01/ AY965887 | *T. inunguis* | Unknown | 1 |
| 06 | GEMAM298 (MPEG42043) | *T. manatus* | Pará, Soure, vila Caju-una | TS |
| 06 | GEMAM581(MPEG44487) | *T. manatus* | Pará, Santo Antônio do Tauá | TS |
| 05 | Tm_M_M2_M3_STm03/ AY965885 | *T. manatus* | Maranhão state | 1 |
| 07 | Tm_J_STm04/ AY965884 | *T. manatus* | Maranhão to Piauí states | 1 |
| 08 | D83050 | *T. manatus* | Unknown | 3 |
| 09 | Tm_A3_A4_STm02/ AY965883 | *T. manatus* | Unknown | 1 |
| 10 | Tm_A_STm01/ AY965886 | *T. manatus* | Piauí to Alagoas states | 1 |
| 10 | AM-3/ JF489120 | *T. manatus* | Unknown | 4 |
| 11 | AY965880 | *T. senegalensis* | Unknown | 1 |
| 12 | AY965882 | *T. senegalensis* | Unknown | 1 |

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| | | | | |
|---|---|---|---|---|
| 13 | AY965881 | *T. senegalensis* | Unknown | 1 |

Legend: haplotype (H), font (F), TS=this study, 1= (Vianna et al., 2006), 2= (Arnason et al., 2008), 3= (Ozawa et al., 1997), 4= (Naidu et al., 2012). Number between brackets after locality name refers to figure 1 and gazetteer.

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## Table 3(on next page)

Genetic distance estimates (%) among haplotypes (H) of *Trichechus* with Cytochrome b sequences.

Table 3. Genetic distance estimates (%) among haplotypes (H) of *Trichechus* with Cytochrome b sequences.

| H | Field no. / GB | TAXA | 01 | 02 | 03 | 04 | 05 | 06 | 07 | 08 | 09 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H01 | GEMAM 216, 419, 586, 604, AY965890 | *T. inunguis* | | | | | | | | | | | | |
| H01 | GEMAM 585, 645, AM904728 | *T. manatus* | | | | | | | | | | | | |
| H02 | AY965889 | *T. inunguis* | 0.2 | | | | | | | | | | | |
| H03 | AY965888 | *T. inunguis* | 0.3 | 0.5 | | | | | | | | | | |
| H04 | AY965887 | *T. inunguis* | 0.5 | 0.7 | 0.2 | | | | | | | | | |
| H05 | AY 965885 | *T. manatus* | 0.8 | 1. | 0.8 | 1. | | | | | | | | |
| H06 | GEMAM 298, 581 | *T. manatus* | 0.8 | 1. | 1.2 | 1.3 | 0.3 | | | | | | | |
| H07 | AY965884 | *T. manatus* | 1. | 1.2 | 1. | 1.2 | 0.2 | 0.5 | | | | | | |
| H08 | D83050 | *T. manatus* | 1.2 | 1.3 | 1.2 | 1.3 | 0.3 | 0.7 | 0.2 | | | | | |
| H09 | AY965883 | *T. manatus* | 1.2 | 1.3 | 1.2 | 1.3 | 0.7 | 1. | 0.8 | 1. | | | | |
| H10 | AY965886, JF489120 | *T. manatus* | 1.3 | 1.5 | 1.3 | 1.5 | 0.8 | 1.2 | 1. | 1.2 | 0.2 | | | |
| H11 | AY965880 | *T.senegalensis* | 1.5 | 1.7 | 1.5 | 1.7 | 1. | 1.3 | 1.2 | 1.3 | 1.3 | 1.5 | | |
| H12 | AY965882 | *T.senegalensis* | 1.7 | 1.8 | 1.7 | 1.8 | 1.2 | 1.5 | 1.3 | 1.5 | 1.5 | 1.7 | 0.3 | |
| H13 | AY965881 | *T.senegalensis* | 1.8 | 2.0 | 1.8 | 2.0 | 1.3 | 1.7 | 1.5 | 1.7 | 1.7 | 1.8 | 0.5 | 0.5 |

Legend: analysis carried out with complete deletion using Kimura's two parameters. GB= GenBank number. For sample localities see table 2.

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## Table 4 (on next page)

List of manatee sample used in *D-Loop* data analysis specifying the haplotype (H).

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1 Table 4. List of manatee sample used in *D-Loop* data analysis specifying the haplotype (H).

| H | **Field** or GenBank accession number | TAXON | F |
|---|---|---|---|
| HD01 | GEMAM216, GEMAM429, GEMAM585, GEMAM586, GEMAM604, GEMAM617, GEMAM645 | *T. inunguis* | TS |
| HD02 | GEMAM419/ AY963876, AY963870, AY738553, AY963882/ JX982641 | *T. inunguis* | TS/2 /3 |
| HD03 | AY738550/ AY963863, AY963867 | *T. inunguis* | 1/2 |
| HD04 | AY738560/ AY963873 | *T. inunguis* | 1/2 |
| HD05 | AY738566/ JX982643 | *T. inunguis* | 1/3 |
| HD06 | AY738578/ AY963881 | *T. inunguis* | 1/2 |
| HD07 | AY96386 | *T. inunguis* | 2 |
| HD08 | AY738570 | *T. inunguis* | 1 |
| HD09 | AY963871 | *T. inunguis* | 2 |
| HD10 | AY963872 | *T. inunguis* | 2 |
| HD11 | AY963877/ AM904728 | *T. inunguis* | 2/8 |
| HD12 | AY963878 | *T. inunguis* | 2 |
| HD13 | AY963879 | *T. inunguis* | 2 |
| HD14 | AY963889 | *T. inunguis* | 2 |
| HD15 | AY963883 | *T. inunguis* | 2 |
| HD16 | AY963890 | *T. inunguis* | 2 |
| HD17 | JX982642 | *T. inunguis* | 3 |
| HD18 | JX982646 | *T. inunguis* | 3 |
| HD19 | JX982647, JX982648 | *T. inunguis* | 3 |
| HD20 | JX982650 | *T. inunguis* | 3 |
| HD21 | AY738558/ AY963892 | *T. inunguis* | 1/2 |
| HD22 | AY738564 | *T. inunguis* | 1 |
| HD23 | AF046160/ AY963874 | *T. inunguis* | 4/2 |
| HD24 | AY738549/ AY963866 | *T. inunguis* | 1/2 |
| HD25 | AY738551 | *T. inunguis* | 1 |
| HD26 | AY738554 | *T. inunguis* | 1 |
| HD27 | AY738569 | *T. inunguis* | 1 |
| HD28 | AY738572 | *T. inunguis* | 1 |
| HD29 | AY738573 | *T. inunguis* | 1 |
| HD30 | AY963864 | *T. inunguis* | *2* |
| HD31 | AY738579 | *T. inunguis* | *1* |
| HD32 | AY963865 | *T. inunguis* | *2* |
| HD33 | AY738552 | *T. inunguis* | 1 |
| HD34 | AY738556 | *T. inunguis* | 1 |
| HD35 | AY738557 | *T. inunguis* | 1 |
| HD36 | AY738562 | *T. inunguis* | 1 |
| HD37 | AY738568 | *T. inunguis* | 1 |
| HD38 | AY738563 | *T. inunguis* | 1 |
| HD39 | AY738576 | *T. inunguis* | 1 |
| HD40 | AY963880 | *T. inunguis* | 2 |
| HD41 | AY963885 | *T. inunguis* | 2 |
| HD42 | AY963886 | *T. inunguis* | 2 |
| HD43 | AY963887 | *T. inunguis* | *2* |
| HD44 | JX982639 | *T. inunguis* | 3 |

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|---|---|---|---|
| HD45 | JX982640 | *T. inunguis* | 3 |
| HD46 | JX982644 | *T. inunguis* | 3 |
| HD47 | JX982645 | *T. inunguis* | 3 |
| HD48 | JX982649 | *T. inunguis* | 3 |
| HD49 | AY738559/ AY963891 | *T. inunguis* | 1/2 |
| HD50 | AY738565 | *T. inunguis* | 1 |
| HD51 | AY738555 | *T. inunguis* | 1 |
| HD52 | AY963868/ AY738561 | *T. inunguis* | 2/1 |
| HD53 | AY738577 | *T. inunguis* | 1 |
| HD54 | AY963869 | *T. inunguis* | 2 |
| HD55 | AY963875 | *T. inunguis* | 2 |
| HD56 | AY738574 | *T. inunguis* | 1 |
| HD57 | AY963884 | *T. inunguis* | 2 |
| HD58 | AY963888 | *T. inunguis* | 2 |
| HD59 | AY963893 | *T. inunguis* | 2 |
| HD60 | AY738567 | *T. inunguis* | 1 |
| HD61 | AY738571 | *T. inunguis* | 1 |
| HD62 | AY963846 | *T. manatus* | 2 |
| HD63 | AY963843 | *T. manatus* | 2 |
| HD64 | AY963844 | *T. manatus* | 2 |
| HD65 | AY963840/ AF046157/ KJ022760 | *T. manatus* | 2/4/ 5 |
| HD66 | AY963841 | *T. manatus* | 2 |
| HD67 | AY963842 | *T. manatus* | 2 |
| HD68 | AY963845 | *T. manatus* | 2 |
| HD69 | AY963847 | *T. manatus* | 2 |
| HD70 | JX564997 | *T. manatus* | 2 |
| HD71 | AY963857 | *T. manatus* | 6 |
| HD72 | AY963895 | *T.senegalensis* | 2 |
| HD73 | AY963856/ GEMAM581 | *T. manatus* | 2/TS |
| HD74 | AF046159 /AY963859 | *T. manatus* | 4/ |
| HD75 | GEMAM298/ AY963855 | *T. manatus* | TS/2 |
| HD76 | AY963858 | *T. manatus* | 2 |
| HD77 | JX171295 | *T. manatus* | 7 |
| HD78 | AY963894 | *T.senegalensis* | 2 |
| HD79 | AY963860 | *T. manatus* | 2 |
| HD80 | AY963848 | *T. manatus* | 2 |
| HD81 | JX982651 | *T. manatus* | 3 |
| HD82 | AY963853 | *T. manatus* | 2 |
| HD83 | AY963897 | *T.senegalensis* | 2 |
| HD84 | AY963849, AY963851 | *T. manatus* | 2 |
| HD85 | AY963852 | *T. manatus* | 2 |
| HD86 | AY963896 | *T.senegalensis* | 2 |
| HD88 | AF046158 / AY963854 | *T. manatus* | 4/2 |
| HD87 | AY963850 | *T. manatus* | 2 |
| HD89 | AY963898 | *T.senegalensis* | 2 |

Legend: font (F), TS= this study, 1= [56], 2= [39], 3= [72], 4= [68], 5= [73], 6= [65], 7= [74], 8= [69].

For localities of specimens see tables 1 and 2.