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VARIAÇÃO GEOGRÁFICA DE TRICHECHIDAE (MAMMALIA: SIRENIA): ANÁLISE MORFOMÉTRICA E CITOGENÉTICA

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VARIAÇÃO GEOGRÁFICA DE TRICHECHIDAE (MAMMALIA: SIRENIA): ANÁLISE MORFOMÉTRICA E CITOGENÉTICA

Tese apresentada ao Curso de Pós-Graduação em Biologia Animal da Universidade Federal de Pernambuco, como requisito parcial à obtenção do título de Doutor em Biologia Animal.

Orientador: Diego Astúa de Moraes

Co-orientadora: Neide Santos

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“It’s not the strongest, fittest, fastest or smartest that will outlast others and ultimately survive, but those who adapt to change”.

Charles Darwin

RESUMO

A família Trichechidae pertence à ordem Sirenia, mamíferos aquáticos preferencialmente herbívoros que habitam regiões costeiras. Atualmente, esta família inclui as três espécies viventes de peixes-bois: *Trichechus manatus*, encontrado na costa atlântica das Américas, ocorrendo descontinuamente desde a Flórida (Estados Unidos) até o Brasil (estado de Alagoas); *Trichechus inunguis*, endêmico da bacia amazônica; e *Trichechus senegalensis*, distribuído na costa atlântica da África, do Senegal a Angola. Adicionalmente, são descritas duas subespécies de *T. manatus*, *T. m. latirostris* (peixe-boi da Flórida), que ocorre na Flórida e Golfo do México, e *T. m. manatus* (peixe-boi das Antilhas), encontrado no México, Caribe e Américas Central e do Sul, até o nordeste do Brasil. Esta designação subespecífica difere quanto aos dados cranianos e moleculares. Neste estudo foram usadas abordagens morfométricas geométricas e citogenéticas para avaliar a existência de variação geográfica em *T. inunguis*, *T. m. manatus*, *T. m. latirostris* e *T. senegalensis*, com o propósito de analisar variações morfológicas do crânio intra e interespecíficas, e diferenças cariotípicas em *T. m. manatus* e *T. inunguis* do Brasil. Os resultados das análises de morfometria geométrica revelaram nenhum dimorfismo sexual de tamanho e forma do crânio em nenhuma das espécies e a separação da população de *T. inunguis* das populações de *T. senegalensis*, *T. m. manatus* e *T. m. latirostris* por mudanças de forma do crânio, havendo sobreposição entre alguns destes indivíduos. *T. m. manatus* e *T. m. latirostris* apresentaram maior tamanho de crânio que *T. inunguis* e presença de alometria (forma relacionada com tamanho) foi observada. As análises intraespecíficas de forma do crânio mostraram a separação da população brasileira de *T. m. manatus* das populações do Caribe de *T. m. manatus* e dos Estados Unidos de *T. m. latirostris*, as quais sobrepuseram-se parcialmente, enquanto que em *T. inunguis* não houve uma clara separação entre suas populações geográficas. Também não foi observada variação de tamanho do crânio entre as populações de nenhuma das espécies. O pequeno tamanho amostral não permitiu que uma análise intraespecífica fosse realizada em *T. senegalensis*. Considerando que somente indivíduos do nordeste do Brasil foram amostrados e que os espécimes do Suriname e Guiana apresentaram forma do crânio mais similar àquela da população do Caribe, é sugerido a hipótese de que a foz do rio Amazonas pode ter interrompido ou estar interrompendo o fluxo gênico nas populações de *T. m. manatus* do Brasil, condizente com modelos de especiação alopátrica. Além disso, a população do peixe-boi antilhano do Brasil mostrou-se tão diferenciada, em relação à forma do crânio, quanto são as duas espécies bem reconhecidas de peixes-bois (*T. inunguis* e *T. senegalensis*). Os resultados das análises cromossômicas também revelaram diferenças cariotípicas estruturais entre *T. m. manatus* (2n = 48) do Brasil e as populações do peixe-boi antilhano de Porto Rico e do peixe-boi da Flórida, reforçando as conclusões obtidas pela morfometria geométrica e sugerindo o isolamento reprodutivo da população do Brasil. A análise citogenética da população brasileira de *T. inunguis* (2n = 56) revelou a presença de um par cromossômico heteromórfico e polimórfico com relação à morfologia acrocêntrica e submetacêntrica, mostrando um padrão que pode ser indicativo de evolução cromossômica recente, devido à existência de variantes cariotípicas heterozigotas. Em adição, não foi verificada a presença de sítios teloméricos intersticiais nos cromossomos de *T. inunguis* e *T. m. manatus* pela técnica de hibridização *in situ* fluorescente. Os resultados obtidos neste estudo para a população brasileira de *T. m. manatus* suportam a evidência preliminar dos dados de DNA mitocondrial e estas conclusões combinadas não coincidem com a designação subespecífica de *T. manatus* atualmente aceita, baseada em medidas lineares do crânio.

Palavras-chave: Peixes-bois. Morfometria geométrica. Morfologia craniana. Cromossomo. Diferenciação populacional. Brasil.

ABSTRACT

The family Trichechidae belongs to the mammalian order Sirenia, which includes the only herbivorous aquatic mammals, inhabiting coastal regions. Currently, this family comprises three living manatee species: *Trichechus manatus*, found on the Atlantic coast of the Americas, occurring discontinuously from Florida (United States) to Brazil (Alagoas state); *Trichechus inunguis*, endemic to the Amazon River Basin; and *Trichechus senegalensis*, occurring on the Atlantic coast of the West Africa, from Senegal to Angola. In addition, *T. manatus* is divided into two subspecies, *T. m. latirostris* (Florida manatee), which occupies waters of Florida and Gulf of Mexico, and *T. m. manatus* (Antillean manatee), which is found in Mexico, the Caribbean and Central and South America to northeastern Brazil. This subspecies designation differs when are used cranial characters and molecular data. Here, both geometric morphometric and cytogenetic approaches were used to assess the existence of geographical variation in *T. inunguis, T. m. manatus, T. m. latirostris* and *T. senegalensis*, in order to analyze cranial intra- and interspecific morphological variations, and karyotype differences in *T. m. manatus* and *T. inunguis* from Brazil. Geometric morphometric analyses showed no sexual dimorphism in skull size and shape in any of the species and a separation of *T. inunguis* population from the *T. senegalensis*, *T. m. manatus* and *T. m. latirostris* populations based on skull shape changes, with some overlap between these individuals. *T. m. manatus* and *T. m. latirostris* had larger skulls than *T. inunguis*, and presence of allometry (shape associated with size) was observed. Intraspecific analyses of skull shape showed a clear separation of the Brazilian *T. m. manatus* population from the *T. m. manatus* Caribbean and *T. m. latirostris* United States populations, which partially overlapped, whereas there was no clear separation among *T. inunguis* populations. Skull size variation was not observed among the populations in any of the species. Because of its small sample size, intraspecific analyses in *T. senegalensis* could not be performed. Considering that only individuals of northeastern Brazil were sampled and specimens from Suriname and Guyana had skull shape more similar to that one from the Caribbean population, it is suggested the hypothesis that the Amazon River mouth may have interrupted or it may be interrupting gene flow in *T. manatus* populations from Brazil, consistent with models of allopatric speciation. Moreover, Brazil Antillean manatee population was as differentiated as are the two well-recognized species of manatees (*T. inunguis* and *T. senegalensis*) based on skull shape. Results from karyotype analyses also revealed structural chromosome differences between *T. m. manatus* (2n = 48) from Brazil and Puerto Rico Antillean manatee and Florida manatee populations, reinforcing the conclusions from the geometric morphometric technique and suggesting reproductive isolation of the Brazilian population. Cytogenetic analyses of Brazil *T. inunguis* (2n = 56) detected the presence of one heteromorphic and polymorphic chromosome pair regarding to morphological variations (acro- and submetacentric), showing a pattern that may be indicating recent chromosomal evolution due to the existence of heterozygous karyotypic variants. In addition, presence of interstitial telomeric sequences in *T. inunguis* and *T. m. manatus* chromosomes was not revealed by fluorescence *in situ* hybridization. The results obtained in the present study for the Brazilian *T. m. manatus* population support preliminary evidence from mitochondrial DNA data and these combined conclusions do not coincide with the currently accepted subspecies designation for *T. manatus* based on skull linear measurements.

Key-words: Manatees. Geometric morphometrics. Skull morphology. Chromosome. Population differentiation. Brazil.

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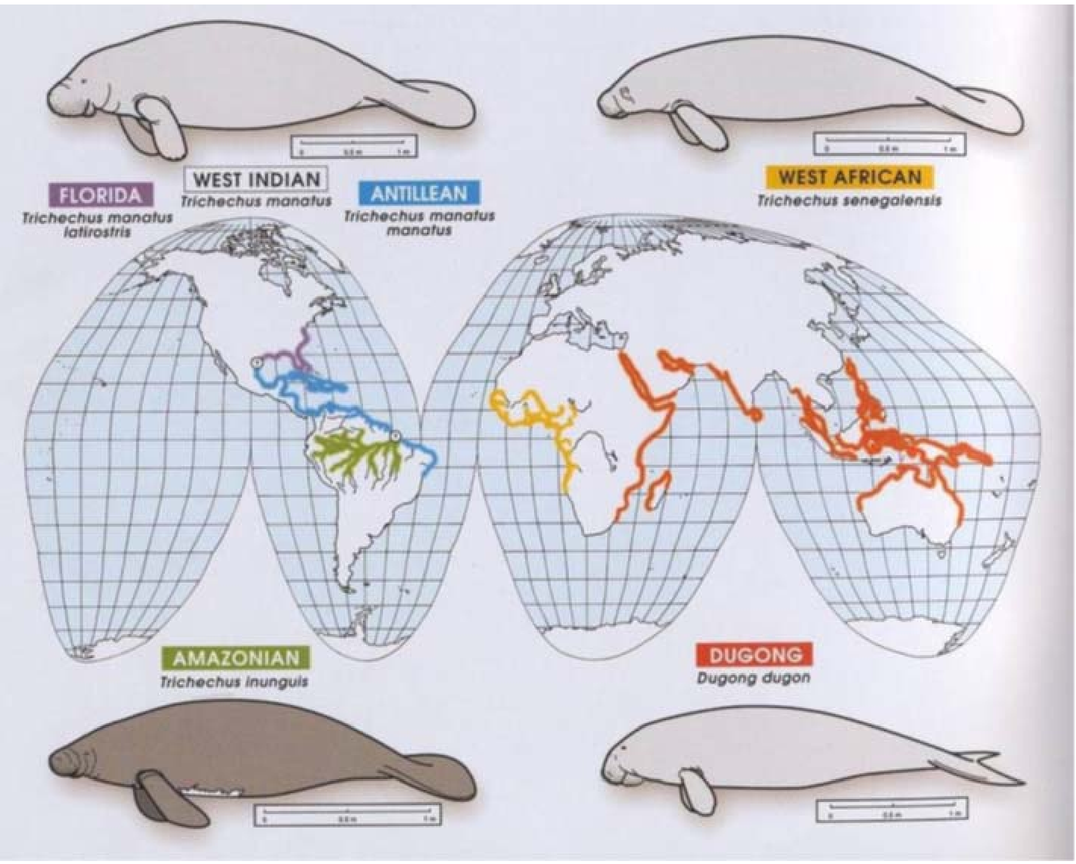
CAPÍTULO 1. INTRODUÇÃO GERAL

A ordem Sirenia (peixes-bois e dugongos) constitui a única linhagem de mamíferos aquáticos totalmente herbívoros. Atualmente, esta é composta de duas famílias: Trichechidae, que inclui as espécies de peixe-boi, *Trichechus manatus* Linnaeus, 1758, *Trichechus senegalensis* Link, 1795 e *Trichechus inunguis* (Natterer, 1883), e Dugongidae, com uma espécie vivente (*Dugong dugon* Müller, 1776) e outra recentemente extinta no século XVIII (*Hydrodamalis gigas* Zimmermann, 1780). As espécies viventes de sirênios habitam rios, estuários e águas oceânicas costeiras rasas nas regiões tropical e subtropical (Reynolds III e Odell, 1991) (Figura 1).

Das três espécies de triquequídeos existentes, duas são encontradas no Brasil: o peixe-boi amazônico (*Trichechus inunguis*), que habita ambiente de água doce na bacia Amazônica (Domning, 1981), e o peixe-boi marinho (*Trichechus manatus*) que está amplamente distribuído, ocorrendo desde a costa atlântica e muitos rios da Flórida (EUA), México, Antilhas, América Central até norte e nordeste da América do Sul (Lefebvre *et al*., 1989). O peixe-boi africano (*Trichechus senegalensis*) é encontrado em rios, estuários e regiões costeiras da África Ocidental, entre o Senegal e Angola (Perrine e Ripple, 2002).

A única espécie para a qual subespécies têm sido descritas é *T. manatus*. O estudo de Hatt (1934) foi o primeiro a reconhecer o peixe-boi da Flórida como uma subespécie de *T. manatus*, *Trichechus manatus latirostris* (Harlan, 1824). O termo específico *latirostris* pertencia anteriormente ao gênero *Manatus* (*Manatus latirostris* Harlan, 1824), tendo como localidade tipo a costa leste da Flórida. A outra subespécie é *Trichechus manatus manatus* Linnaeus, 1758, cuja localidade tipo são as Índias Ocidentais. Hatt (1934) propôs uma distribuição para *T. m. manatus* ao longo do mar do Caribe até a costa e rios do nordeste da América do Sul, e da costa e rios de Beaufort (Carolina do Norte) para as Florida Keys, Estados Unidos, e regiões costeiras do Golfo do México, para *T. m. latirostris*. Essa divisão subespecífica foi baseada em caracteres osteológicos. Domning e Hayek (1986) confirmaram a existência das duas subespécies de *T. manatus*, com base principalmente na análise de medidas cranianas lineares. Estes autores sugerem que as águas frias da costa norte do Golfo do México e as fortes correntes do Estreito da Flórida estariam atuando como barreiras, isolando reprodutivamente os peixes-bois da Flórida da outra subespécie, contribuindo para um fluxo gênico restrito entre os dois táxons. Adicionalmente, tem sido proposto o nome de peixe-boi das Antilhas para *T. m. manatus* (Domning e Hayek, 1986).

Figura 1. Distribuição mundial dos representantes viventes da ordem Sirenia.



Fonte: Medina, 2008.

A separação subespecífica de *T. manatus* tem sido questionada por estudos moleculares utilizando sequências da região controle do DNA mitocondrial (DNAmt), os quais revelaram para a espécie três linhagens distintas (Garcia-Rodriguez *et al*., 1998; Vianna *et al*., 2006) e duas unidades evolutivamente significativas (ESUs) através da barreira das Antilhas Menores (Vianna *et al*., 2006). A categoria taxonômica de subespécie para o peixe-boi da Flórida tem sido contestada devido o seu compartilhamento de haplótipos com outras populações (República Dominicana, Porto Rico e México), e o peixe-boi Antilhano das Guianas e Brasil apresentou haplótipos identificados somente nestas localidades (Garcia-Rodriguez *et al*., 1998; Vianna *et al*., 2006).

O peixe-boi africano também mostrou uma marcante estruturação populacional geográfica, com pelo menos dois grupos distintos de haplótipos (Vianna *et al*., 2006). No entanto para o peixe-boi amazônico foi observado um único grupo que apresentou expansão populacional (Cantanhede *et al*., 2005; Vianna *et al*., 2006).

Atualmente a técnica de morfometria geométrica tem sido utilizada com sucesso para descrever variações morfológicas intra e interespecíficas, mostrando-se, em muitos casos, uma ferramenta mais robusta para este fim do que abordagens morfométricas baseadas em medidas lineares (Marcus *et al*., 2000; Figueirido *et al*., 2012). Os métodos de morfometria geométrica permitem avaliar a variação de tamanho e forma separadamente, representar graficamente as mudanças de forma em estruturas biológicas, além de indicar com maior precisão em quais regiões concentra-se esta variação (Monteiro e Reis, 1999; Moraes, 2003). Esta metodologia tem contribuído para o entendimento de aspectos evolutivos, ecológicos, filogenéticos e taxonômicos em vários grupos de organismos (Marroig, 2007; Bornholdt *et al*., 2008; Camargo *et al*., 2008; Nunes *et al*., 2008; Vieira *et al*., 2008; Amaral *et al*., 2009), inclusive detectando descontinuidades morfológicas que revelaram-se posteriormente relacionadas aos eventos de especiação (Monteiro-Filho *et al*., 2002; Gündüz *et al*., 2007; Oliveira *et al*., 2008).

Dados obtidos em estudos cariotípicos podem ser utilizados para auxiliar no esclarecimento de problemas sistemáticos e no entendimento de determinados processos evolutivos. Alterações nos cromossomos podem ajudar na identificação de espécies, principalmente, quando estas são morfologicamente similares, mas apresentam divergências cariotípicas (Seebeck e Johnson, 1980; Ao *et al*., 2007; Romanenko *et al*., 2012), auxiliando na resolução de incertezas taxonômicas (Sharman *et al*., 1989). Diferenças cromossômicas fixadas normalmente proporcionam uma evidência definitiva para o *status* de espécie distinta (Lee *et al*., 1993), uma vez que muitas diferenças cromossômicas resultam em certa esterilidade nos indivíduos heterozigotos, indicando isolamento reprodutivo dos táxons (Frankham *et al*., 2008).

O presente estudo teve como objetivo avaliar a existência de variação geográfica em *Trichechus inunguis*, *Trichechus manatus manatus*, *Trichechus manatus latirostris* e *Trichechus senegalensis*, através das técnicas de morfometria geométrica e citogenética, com o propósito de analisar variações morfológicas do crânio intra e interespecíficas, e diferenças cariotípicas em *T. m. manatus* e *T. inunguis* do Brasil.

CAPÍTULO 2. REVISÃO BIBLIOGRÁFICA

2.1. EVOLUÇÃO DE TRICHECHIDAE: ÊNFASE PARA SISTEMÁTICA E TAXONOMIA DO GÊNERO *Trichechus* LINNAEUS, 1758

Esta família é composta de duas subfamílias: a extinta Miosireninae e Trichechinae, que inclui as espécies de peixes-bois atuais (*Trichechus manatus*, *T. inunguis* e *T. senegalensis*). O primeiro triquequíneo conhecido, *Potamosiren magdalenensis*, apareceu no meio do Mioceno na Colômbia (Domning, 1982) e possivelmente ocupava ambientes de água doce e estuarinos (Domning, 2001). Muito da história dos triquequíneos foi provavelmente passada na América do Sul, de onde espalharam-se para a América do Norte e África somente no Plioceno ou Pleistoceno. Durante o final do Mioceno e início do Plioceno, o soerguimento dos Andes criou uma erosão e nutrientes dissolvidos escoaram para dentro dos sistemas dos rios, o que aumentou consideravelmente a abundância de gramíneas abrasivas. Os peixes-bois adaptaram-se a esse novo recurso alimentar por meio de uma inovação ainda usada por seus descendentes modernos: dentes extras são adicionados à série molar, ao longo da vida, e como os dentes desgastados caem na frente, toda a fileira de dentes lentamente desloca-se para frente, dando espaço para a erupção de novos dentes na parte de trás (Domning, 2002).

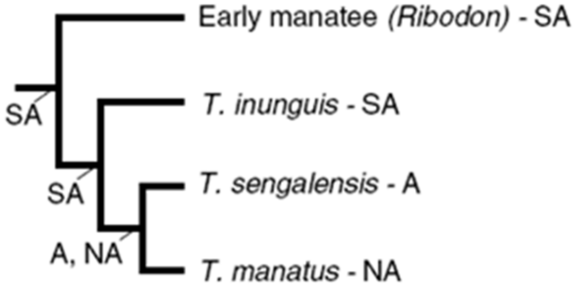
Este novo padrão de substituição dos dentes para a dieta de gramíneas de água doce é encontrado no gênero *Ribodon* da Argentina (Mioceno) e Carolina do Norte (Plioceno). Por conta disto, *Ribodon* e *Trichechus* são considerados grupos-irmãos, sendo o período Plioceno-Pleistoceno, a possível época de surgimento dos primeiros representantes do último gênero, que expandiram-se e alcançaram o ambiente marinho (Figura 2) (Domning, 1982, 2002; Berta *et al*., 2006).

Domning (1982) sugeriu que provavelmente os peixes-bois entraram na bacia amazônica através de sua saída para o oceano Pacífico ou por meio de conexões intermitentes entre a bacia e drenagens do Atlântico ou Caribe, que existiram no Mioceno médio. Com o soerguimento dos Andes, a saída para o Pacífico foi fechada durante o início do Plioceno, isolando temporariamente os peixes-bois na bacia amazônica das populações costeiras. O acesso restrito às águas marinhas proporcionou o surgimento da espécie amazônica (*T. inunguis*), com o isolamento terminando no final do Plioceno-início do Pleistoceno, através do estabelecimento da conexão com o oceano Atlântico (Marsh *et al*., 2012). Os peixes-bois costeiros durante o Plioceno-Pleistoceno expandiram sua distribuição através do Caribe e depois para a América do Norte, dando origem a *T. manatus* e esta espécie é conhecida a partir do Pleistoceno no sudeste dos Estados Unidos (Domning, 1982). Não há registro fóssil que mostre a dispersão de *Trichechus* a leste para a África Ocidental (*T. senegalensis*) (Berta *et al*., 2006), porém, a dispersão pelo Atlântico da América do Sul para a África no final do Plioceno ou início do Pleistoceno é a hipótese mais aceita para o surgimento desta espécie, sustentada no fato de que *T. manatus* e *T. senegalensis*, que são morfologicamente mais similares e são unidos por sinapomorfias que são ausentes na espécie amazônica, compartilham um ancestral comum mais recente entre si do que com *T. inunguis* (Figura 2). Entretanto, as três espécies provavelmente separaram-se em um curto intervalo de tempo (Domning, 1982; Domning e Hayek, 1986). Similarmente a estes achados baseados em dados morfológicos, estudos moleculares com dados imunológicos mostraram uma semelhança muito próxima entre as três espécies de peixes-bois e sugeriram que o peixe-boi africano derivou através de uma migração trans-Atlântica do Novo Mundo (Rainey *et al*., 1984).

Em adaptação ao novo ambiente, a bacia Amazônica, e às novas fontes de alimento de água doce, *T. inunguis* evoluiu mais rapidamente do que as populações remanescentes nas regiões costeiras, consequentemente exibe caracteres apomórficos em relação a *T. manatus* e

T. senegalensis, como: molares menores e mais complexos, ausência de unhas nas nadadeiras, número reduzido de vértebras dorsais, osso supraoccipital mais grosso e um maior número diploide (2n=56) (Domning, 1982; Domning e Hayek, 1986; Assis *et al*., 1988). *T. manatus* possui 2n=48 (Gray *et al*., 2002) e o número diploide de *T. senegalensis* ainda não foi descrito. Apesar de *T. manatus* e *T. senegalensis* serem unidos por certos caracteres derivados, estas espécies apresentam poucas mudanças dos seus representantes ancestrais até o presente, sendo consideradas mais conservadas que *T. inunguis* (Domning e Hayek, 1986). Levando-se em consideração que *D. dugon* apresenta 2n = 50 (Short, 1984) e que até o momento não estão disponíveis na literatura trabalhos sobre evolução cromossômica dos sirênios, a apomorfia de um maior número diploide deve ter sido baseada no fato de que *T. inunguis* apresenta caracteres derivados (Domning e Hayek, 1986). Entretanto, esta espécie apresentou-se basal em relação a *T. senegalensis* e *T. manatus* em ambas as filogenias, baseadas em caracteres morfológicos (Domning, 1994) e moleculares (Vianna *et al*., 2006), ocasionando uma certa contradição no que diz respeito a *T. inunguis* possuir caracteres derivados e uma posição evolutiva basal, e *T. manatus* e *T. senegalensis* apresentarem caracteres conservados e estarem em um clado mais apical. Claramente, a sistemática de Trichechidae é um tópico que requer estudos adicionais.

Figura 2. História biogeográfica dos peixes-bois. A = África; NA = América do Norte; SA = América do Sul (baseada em Domning, 1982). Calendário dos eventos: SA, 5 Ma – peixes-bois habitavam rios e estuários da América do Sul. Soerguimento dos Andes, aumento de sedimentos e gramíneas, correspondendo às mudanças na dentição: substituição dos dentes, aumento no número de dentes, diminuição no tamanho dos dentes, aumento na complexidade do esmalte dentário. SA, 3 Ma – peixes-bois obtiveram acesso à bacia Amazônica, especiação localizada de *T. inunguis*. A, NA, 1-2 Ma – dispersão dos peixes-bois: A (*T. senegalensis*) e NA (*T. manatus*).

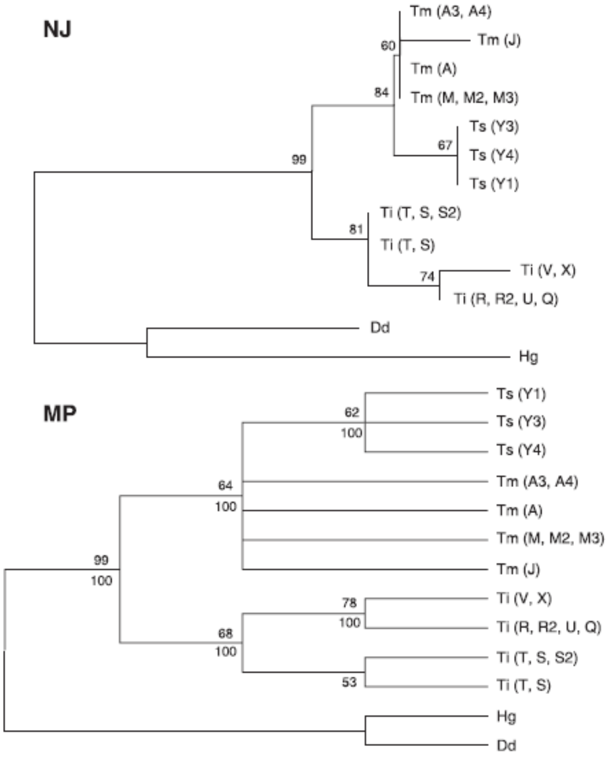


Fonte: Berta *et al*., 2006.

Análises filogenéticas para investigar as relações evolutivas entre *T. manatus, T. inunguis* e *T. senegalensis,* baseadas em dados moleculares, foram investigadas por Vianna *et al*. (2006). Através da árvore usando o método de *neighbour-joining* da região controle do DNA mitocondrial (DNAmt), apesar deste método expressar similaridade podendo não refletir exatamente ancestralidade comum, foi revelado que *T. inunguis* é grupo irmão do cluster I de *T. manatus* (espécie parafilética), como tem sido sugerido por Cantanhede *et al*. (2005), e *T. senegalensis* apresentou-se basal. As árvores de *neighbour-joining* e de consenso de máxima parcimônia utilizando aminoácidos correspondentes ao gene *citocromo b* (Figura 3), mostraram que a linhagem de *T. inunguis* separou-se há mais tempo da linhagem comum que depois deu origem a *T. manatus* e *T. senegalensis*, com estas espécies ficando em um mesmo clado, provavelmente derivadas do mesmo ancestral marinho. A explicação dos autores para esta divergência de topologia em relação aos dois marcadores utilizados foi que o *citocromo b* é uma região menos propensa à homoplasia do que a região controle. A conclusão gerada através da análise com o gene mitocondrial *citocromo b* está de acordo com a filogenia obtida através de caracteres cranianos e dentários, em um estudo que incluiu também fósseis de sirênios (Figura 4) (Domning, 1994).

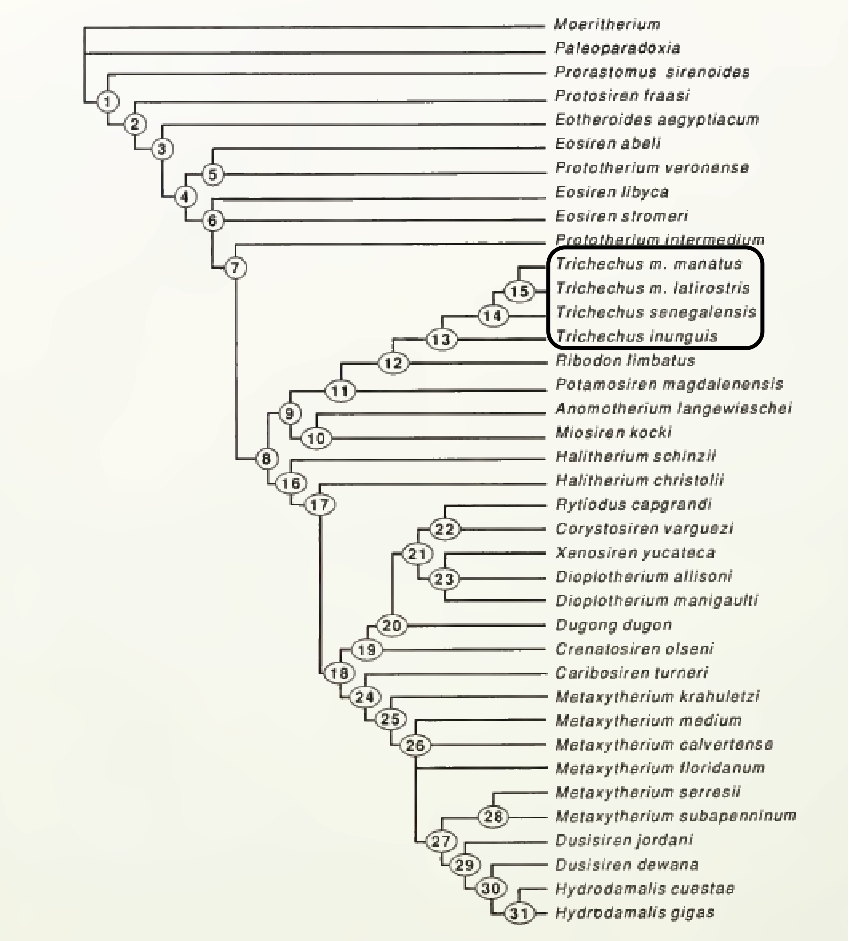
Adicionalmente, o trabalho de Vianna *et al*. (2006) apresentou o tempo de coalescência das linhagens do DNAmt atuais como uma aproximação grosseira dos eventos de especiação, indicando que o táxon mais recente é o peixe-boi amazônico, seguido pelo peixe-boi marinho e o peixe-boi africano. Os autores sugeriram que *T. inunguis* poderia ser a única espécie sobrevivente de uma linhagem ancestral adaptada à agua doce, para explicar o fato do peixe-boi amazônico ser cladisticamente mais basal e uma espécie recente comparada a *T. manatus* e *T. senegalensis*, estando sua origem relacionada à formação da bacia Amazônica.

Figura 3. Árvores de *neighbour-joining* (NJ) e de consenso de máxima parcimônia (MP) entre as cinco espécies modernas de sirênios (Ts: *Trichechus senegalensis*, Tm: *Trichechus manatus*, Ti: *Trichechus inunguis*, Hg: *Hydrodamalis gigas*, e Dd: *Dugong dugon*), baseadas nos aminoácidos correspondentes ao gene *citocromo b*.



Fonte: Vianna *et al*., 2006.

Figura 4. Árvore filogenética da ordem Sirenia baseada em caracteres morfológicos, destacando as relações entre as espécies e subespécies de peixe-boi.



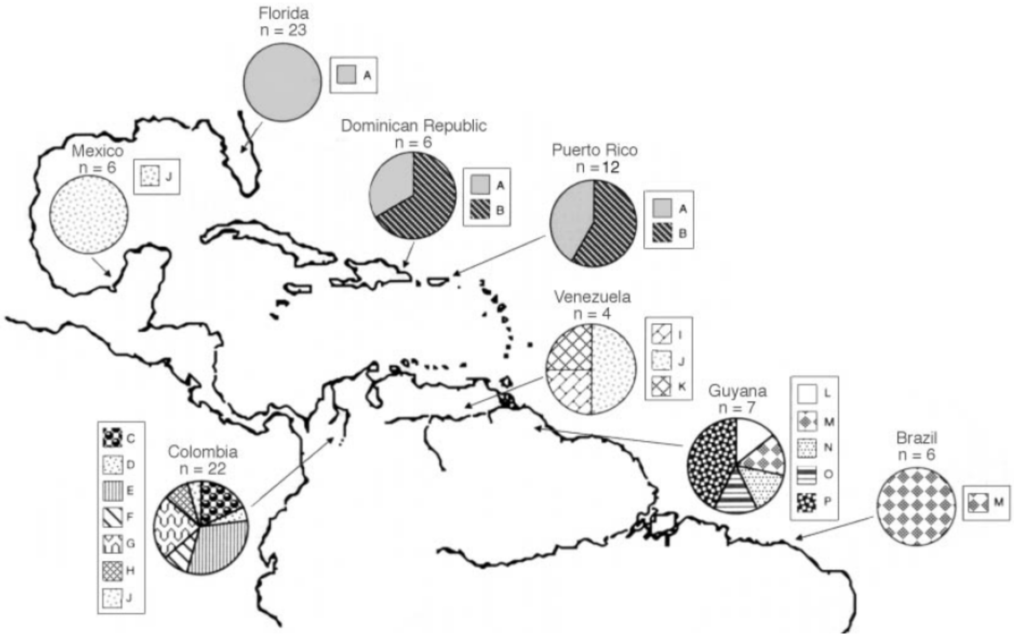
Fonte: Domning, 1994.

Em relação à designação subespecífica de *T. manatus* (*T. m. manatus* e *T. m. latirostris*), os resultados de estudos baseados em dados morfológicos e moleculares mostraram-se diferentes. Domning e Hayek (1986) confirmaram a distinção das subespécies de *T. manatus*, anteriormente proposta por Hatt (1934), por caracteres cranianos quantitativos, sendo seu uso como categorias taxonômicas justificável. Os primeiros autores verificaram que *T. m. latirostris* e *T. m. manatus* constituem populações distintas morfologicamente, e que barreiras física e climática no Estreito da Flórida e Golfo do México são efetivas para o fluxo gênico restrito entre os peixes-bois da Flórida e das Antilhas. As descontinuidades morfológicas encontradas neste estudo refletem a separação geográfica destes táxons, com *T.*

m. latirostris ocorrendo na Flórida e Golfo do México, e *T. m. manatus* sendo encontrado no México, Caribe e Américas Central e do Sul (Domning e Hayek, 1986).

Entretanto, o primeiro estudo filogeográfico realizado com *T. manatus*, utilizando dados de sequência da região controle do DNAmt, questionou sua divisão subespecífica, sendo propostas três linhagens distintas: cluster 1, amostras da Flórida, Porto Rico, República Dominicana e Colômbia; cluster 2, amostras do México, Colômbia e Venezuela; e cluster 3, amostras do Brasil e Guiana (Figura 5) (Garcia-Rodriguez *et al*., 1998).

Figura 5. Estruturação geográfica da diversidade de haplótipos do DNAmt nas populações de *T. manatus*.



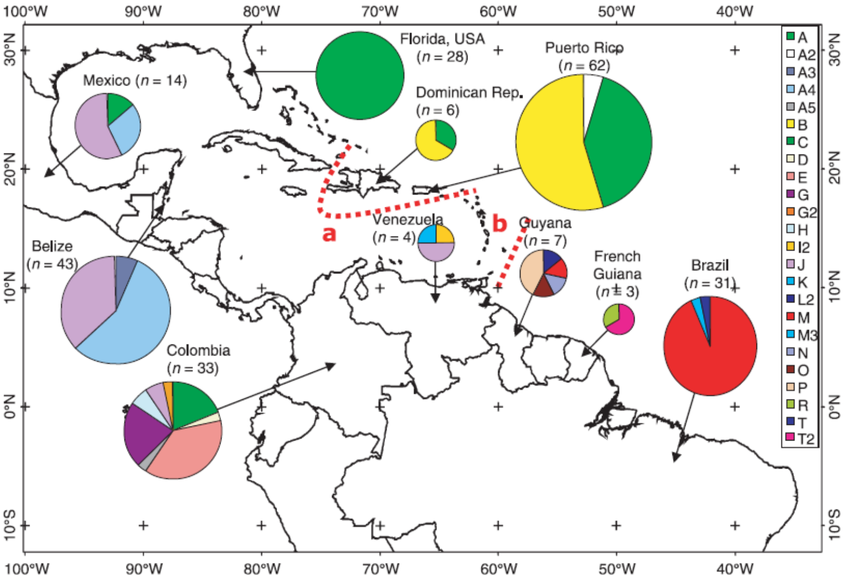
Fonte: Garcia-Rodriguez *et al*., 1998.

O trabalho de filogeografia realizado por Vianna *et al*. (2006) obteve um resultado similar ao de Garcia-Rodriguez *et al*. (1998), sendo utilizado o mesmo marcador molecular, mas abrangendo um maior número de populações de *T. manatus* de diferentes localidades. Foram evidenciados três grupos distintos de haplótipos, com uma distribuição geográfica heterogênea: (I) Flórida, México, Antilhas Maiores, América Central e costa caribenha da

América do Sul; (II) México, América Central e costa caribenha da América do Sul; e (III) costa nordeste da América do Sul (Brasil e Guianas). Enquanto os dois primeiros grupos apresentaram alguma sobreposição geográfica, o terceiro somente foi encontrado no Brasil e nas Guianas. Este estudo indicou a existência de uma barreira geográfica representada pela continuidade das Antilhas Menores até a ilha de Trinidad, próximo a boca do rio Orinoco na Venezuela, tendo restringido o fluxo gênico histórico em *T. manatus* (Figura 6). Os autores propuseram uma divisão das populações de *T. manatus* em duas unidades evolutivamente significativas (ESUs) distintas, através da barreira das Antilhas Menores, uma incluindo as populações do Brasil e das Guianas e outra com as populações das demais áreas de ocorrência.

Os resultados de ambos estudos moleculares (Garcia-Rodriguez *et al*., 1998; Vianna *et al*., 2006) não estão de acordo com a designação subespecífica para *T. manatus* proposta anteriormente por dados morfológicos (Hatt, 1934; Domning e Hayek, 1986), e evidenciaram que as populações do Brasil e das Guianas são diferentes das restantes.

Figura 6. Distribuição dos haplótipos da região controle do DNAmt encontrados nas populações de *T. manatus*.



Fonte: Vianna *et al*., 2006.

O trabalho de Vianna *et al*. (2006) também abordou padrões filogeográficos em *T. senegalensis* e *T. inunguis*, sendo encontrada, no peixe-boi africano, uma marcante estruturação populacional geográfica, com pelo menos dois grupos distintos de haplótipos: (I) Guiné-Bissau e (II) Gana, Chade e Níger. Entretanto, no peixe-boi amazônico foi observado um único grupo de expansão populacional, com uma alta diversidade de haplótipos relacionados, que está de acordo com os resultados obtidos por Cantanhede *et al*. (2005).

2.2. MORFOMETRIA GEOMÉTRICA

Os métodos de morfometria geométrica fornecem ferramentas poderosas para descrever e representar graficamente as mudanças de forma em estruturas biológicas. Desde sua origem, durante o final dos anos 80 (Corti *et al*., 2000), vários estudos têm sido realizados aplicando esta nova maneira de medir as diferenças de forma de uma determinada estrutura, entre grupos ou populações, comparando-os (Marcus *et al*., 2000; Astúa, 2009; Figueirido *et al*., 2012; Segura *et al*., 2013). Isto pode ser feito utilizando-se, por exemplo, marcos anatômicos previamente definidos que sejam homólogos entre os objetos de estudo, cujas coordenadas cartesianas, sejam em duas ou três dimensões, são as variáveis que descrevem a geometria das estruturas analisadas. A vantagem da utilização de coordenadas é a inclusão das informações sobre suas posições relativas, e assim, possibilitam a reconstrução gráfica da forma após a aplicação de estatísticas multivariadas, permitindo localizar e visualizar as variações dos marcos anatômicos entre uma configuração e outra (Rohlf e Marcus, 1993; Monteiro e Reis, 1999). Desta forma, variações morfológicas mínimas podem ser detectadas, gerando resultados estatísticos consistentes.

A técnica de morfometria geométrica permite analisar, separadamente, forma e tamanho (isométrico), cuja informação é relativa ao tamanho de centroide (centro geométrico da configuração dos marcos anatômicos). Este componente é um valor obtido através da raiz quadrada da soma das distâncias ao quadrado de cada marco ao centroide da configuração e constitui uma medida geral multivariada de tamanho, que é usado para redimensionar todas as configurações dos marcos anatômicos (Moraes, 2003).

Um método estatístico padrão e comumente usado na morfometria geométrica que permite separar os componentes de tamanho e forma é a Análise Generalizada de Procrustes (AGP) ou Sobreposição Generalizada de Procrustes (SGP). Após o tamanho de centroide de cada indivíduo ser calculado, estes são igualados, padronizando todas as configurações para esta mesma medida geral de tamanho. Além de remover o efeito do tamanho isométrico (dimensionamento), esta técnica estatística possibilita corrigir diferenças devido à posição (translação das configurações para um mesmo centroide) e orientação (rotação das configurações sobrepondo os marcos homólogos correspondentes à uma configuração de consenso), ocorridas durante a etapa de digitalização dos marcos anatômicos. Estes dois últimos passos são realizados baseando-se na distância mínima de Procrustes, que é a soma das distâncias ao quadrado entre os marcos correspondentes das configurações e a de consenso. Após estes procedimentos, as configurações dos marcos anatômicos mantêm a variação de forma não relacionada com o tamanho e variação de forma alométrica (contribuição da variação de tamanho às mudanças de forma) (Dujardin e Slice, 2007).

As análises de estatística multivariadas são essenciais em estudos de morfometria geométrica, sendo úteis na ordenação, agrupamento e redução da dimensionalidade de dados morfométricos, permitindo que parâmetros biológicos subjacentes às relações morfológicas entre espécimes ou espécies possam ser mais facilmente detectados e interpretados de maneira mais simples (Reis, 1988). Um destes métodos bastante utilizado para detectar as principais variações de forma e empregado para estudar questões em biologia evolutiva é a Análise de Componentes Principais (ACP). Esta análise leva em consideração as correlações entre as variáveis, sendo cada componente principal uma combinação linear dos caracteres morfométricos originais, porém a maior porcentagem da variação morfológica pode ser explicada pelos primeiros componentes principais, porque estes detêm a maior parte da informação estatística (variação estatística decrescente). A ACP não requer que os grupos sejam conhecidos *a priori*, pois é uma técnica de ordenação exploratória (Monteiro e Reis, 1999), assim, os agrupamentos revelados podem ser interpretados como tendo estruturas biológicas diferentes morfologicamente e as amostras podem ser tratadas como entidades biológicas heterogêneas.

A Análise de Função Discriminante (AFD) e a Análise de Variável Canônica (AVC) são métodos estatísticos multivariados usados para distinguir a variação de forma entre grupos determinados *a priori* (Reis, 1988). A diferença entre as duas técnicas é que somente dois grupos são considerados na AFD, sendo esta análise mais utilizada em comparações de grupos específicos, enquanto que a AVC pode ser usada em análises gerais da estrutura de um maior número de grupos em um conjunto de dados (Klingenberg, 2011).

Todas essas contribuições fazem da morfometria geométrica uma ferramenta mais robusta para estudar variações de forma do que abordagens morfométricas tradicionais, onde a mudança de forma é analisada por meio das distâncias entre pares de medidas lineares. A técnica de morfometria geométrica tem sido aplicada em vários grupos de organismos, em áreas como a biologia evolutiva, detectando variações intra e interespecíficas de tamanho e forma em caracteres quantitativos, sistemática e taxonomia (Swiderski *et al*., 2000; Monteiro-Filho *et al*., 2002; Bogdanowicz *et al*., 2005; Francoy *et al*., 2006; Porto *et al*., 2009; Viscosi e Cardini, 2011). Nas espécies de peixes-bois somente métodos de morfometria linear foram usados para avaliar variações morfológicas intra e interespecíficas (Domning e Hayek, 1986).

2.3. CITOGENÉTICA E EVOLUÇÃO

A descrição do cariótipo de um táxon representa um passo inicial para o estudo de comparações citogenéticas e, portanto, para o entendimento das mudanças cromossômicas que surgem durante a evolução dos organismos. As análises cromossômicas podem fornecer dados de diferenciação cariotípica entre táxons que são utilizados para o delineamento do *status* taxonômico (citotaxonomia), baseando-se no fato de que diferenças fixadas nos cromossomos podem indicar espécies distintas, pois estas diferenças influenciam na esterilidade de indivíduos heterozigotos (Lee *et al*., 1993; Xu e Arnason, 1996; Dobigny *et al*., 2002; Corti *et al*., 2005; Tagliarini *et al*., 2007; Frankham *et al*., 2008; Paiva *et al*., 2010).

As técnicas de citogenética clássica empregadas na caracterização cromossômica incluem a coloração convencional e diferencial (bandeamentos G, C e impregnação por nitrato de prata). O cariótipo convencional permite determinar o número diploide (2n), o número fundamental (NF), o sistema de determinação do sexo e variações intra e interespecíficas do número e morfologia cromossômicas. O padrão de bandeamento G fornece informações importantes no estudo comparativo da estrutura do cariótipo entre táxons proximamente relacionados, permitindo a identificação precisa dos cromossomos e podendo revelar rearranjos ocorridos durante a evolução cromossômica. Além disso, a detecção de rearranjos cromossômicos proporcionam dados que podem ser utilizados em análises filogenéticas (Mengden e Stock, 1980; Modi, 1987; Volleth *et al*., 2001; Santos *et al*., 2007; Barros *et al*., 2009). A heterocromatina constitutiva (HC) e as regiões organizadoras de nucléolos (RONs), obtidas através das técnicas de bandeamento C e impregnação por nitrato de prata ($AgNO_3$), respectivamente, constituem marcadores cromossômicos que podem variar entre ou dentro de espécies, sendo também usados em análises citogenéticas comparativas

(Dowler e Bickham, 1982; Odierna *et al*., 1985; Medeiros *et al*., 2003; Ipucha *et al*., 2008; Tagliarini *et al*., 2009; Lemos-Pinto *et al*., 2012).

O desenvolvimento de novas metodologias utilizando o DNA, mapeamento gênico e análise cromossômica permitiram um grande avanço na compreensão da organização, estrutura e evolução do complemento cromossômico. A técnica de hibridização *in situ* fluorescente (FISH) permitiu uma ligação entre os dados moleculares de sequências de DNA e a citogenética, podendo ser utilizada no mapeamento físico destas sequências e na identificação e caracterização de cromossomos ou segmentos cromossômicos (Schwarzacher e Heslop-Harrison, 2000).

A técnica de FISH tem permitido analisar a distribuição do DNA telomérico nos cromossomos, que consiste da sequência TTAGGG repetida em *tandem*. A sequência repetitiva (TTAGGG)n tem sido encontrada principalmente nos telômeros, mas sua detecção em sítios não-teloméricos (regiões intersticiais) também tem sido observada em uma variedade de espécies (Meyne *et al*., 1990; Nanda e Schmid, 1994; Metcalfe *et al*., 1998; Lear, 2001). A presença da sequência (TTAGGG)n em sítios não-teloméricos tem sido relacionada a resquícios de telômeros verdadeiros resultantes de rearranjos cromossômicos, tais como, inversões, fusões cêntricas ou em tandem, que ocorreram durante a evolução cariotípica (Finato *et al*., 2000; Metcalfe *et al*., 2002; Viera *et al*., 2004), como também tem sido reportada a ocorrência dessa sequência como um componente do DNA satélite, coincidindo com regiões de HC (Pagnozzi *et al*., 2000; Multani *et al*., 2001; Faria e Morielle-Versute, 2002). A ausência de sequências teloméricas intersticiais (ITs) não significa que eventos de rearranjos cromossômicos não ocorreram, mas que as ITs podem ter sido perdidas ou modificadas no curso da evolução, como também, podem apresentar sequências curtas que não são detectadas pela técnica de FISH (Meyne *et al*., 1990; Nanda *et al*., 1995).

Estudos citogenéticos desenvolvidos com peixes-bois ainda são incipientes. Através da coloração convencional, estabeleceu-se o número diploide de 2n=48 para o peixe-boi da Flórida, *T. m. latirostris* (White *et al*., 1976, 1977) e para o peixe-boi antilhano, *T. m. manatus*, do Brasil (Vianna *et al*., 2006), e de 2n=56 para a espécie amazônica, *T. inunguis* (Loughman *et al*., 1970). Adicionalmente, procedimentos de bandeamento cromossômico foram usados para a construção de cariótipos e idiogramas de *T. inunguis* (Assis *et al*., 1988), *T. m. latirostris* (Gray *et al*., 2002) e *T. m. manatus* de Porto Rico (Hunter *et al*., 2012).

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CAPÍTULO 3. MANUSCRITO 1*: VARIAÇÃO GEOGRÁFICA NO CRÂNIO DAS ESPÉCIES DE TRICHECHIDAE (MAMMALIA: SIRENIA) COM A DESCRIÇÃO DO CARIÓTIPO DO PEIXE-BOI ANTILHANO DO BRASIL

CHAPTER 3. MANUSCRIPT 1*: GEOGRAPHIC VARIATION IN THE SKULL OF TRICHECHIDAE SPECIES (MAMMALIA: SIRENIA) WITH A DESCRIPTION OF THE ANTILLEAN MANATEE KARYOTYPE IN BRAZIL

* Artigo padronizado de acordo com as normas do periódico *Zoological Journal of the Linnean Society*.

Geographic variation in the skull of Trichechidae species (Mammalia: Sirenia) with a description of the Antillean manatee karyotype in Brazil

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ABSTRACT

Here, we employed landmark-based 3D geometric morphometrics to assess cranial size and shape geographical variation between *Trichechus manatus manatus*, *Trichechus manatus latirostris*, *Trichechus inunguis* and *Trichechus senegalensis* and within their populations (except for the latter species). In addition, a detailed chromosome study using cytogenetic banding techniques was performed in *T. m. manatus* from Brazil. No sexual dimorphism was detected in size and shape in any of the species. *T. inunguis* was clearly separated from *T. senegalensis*, *T. m. manatus* and *T. m. latirostris* by skull shape changes, whereas some overlap was observed among the other taxa. *T. m. manatus* and *T. m. latirostris* had larger skull size than *T. inunguis*. Morphological discontinuity was noted only in *T. m. manatus* and *T. m. latirostris* populations, with the Brazilian *T. m. manatus* population being clearly separated from the *T. m. manatus* Caribbean and *T. m. latirostris* USA populations, while there was a partial overlap between the latter two. Considering that only individuals of northeastern Brazil were sampled and specimens from Suriname and Guyana had skull shape more similar to that one from the Caribbean population, we hypothesize that the Amazon River mouth may be acting as a barrier for *T. m. manatus* populations in Brazil. Moreover, Brazil Antillean manatee population exhibited morphological differences that correspond to the ones found between the Amazonian and West African species, besides structural chromosome differences were detected between that population and manatees in Puerto Rico and Florida, suggesting its reproductive isolation.

ADDITIONAL KEYWORDS: chromosome – landmarks – population differentiation – skull morphology – three-dimensional geometric morphometric – *Trichechus inunguis* – *Trichechus manatus manatus* – *Trichechus manatus latirostris* – *Trichechus senegalensis*.

INTRODUCTION

Currently, Trichechidae includes the 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, and discontinuously along the Caribbean coast of Central and South America, where its distribution reaches northeastern Brazil (Alagoas state) (Lefebvre *et al*., 2001; Luna *et al*., 2008b). 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 coastal regions, it is suggested that this species may sometimes also be found in salt water on the coast of Amapá state (Brazil) (Domning, 1981; Luna, 2013). The West African manatee (*T. senegalensis*) is distributed along the rivers, estuaries and coastal regions of western Africa from Senegal to Angola (Marsh, O'Shea & Reynolds III, 2012). 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).

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, *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 & Hayek (1986) confirmed the two subspecies of *T. manatus* based mostly on the analysis of linear cranial measurements and they 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, these authors proposed the name Antillean manatee for *T. m. manatus*.

However, molecular genetic studies of the West Indian manatee are not concordant with the current subspecies designation based on morphological characters. Mitochondrial DNA (mtDNA) control regions (Garcia-Rodriguez *et al*., 1998; Vianna *et al*., 2006) revealed three, rather than two, distinctive lineages for this species. These combined studies showed a strong geographical structuring of mtDNA diversity, revealing one cluster with 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 Guyanas and Brazil. There is some overlap between clusters one and two, but cluster three was found only in the Guyanas and Brazil, which supports the Lesser Antilles barrier hypothesis during the Pleistocene, isolating those populations, as suggested by Vianna *et al*. (2006). These authors also concluded that the Lesser Antilles divided *T. manatus* into two distinct evolutionary significant units (ESUs), one including the populations from the Guyanas and Brazil and another with the populations from the rest of the range.

In contrast to those phylogeographic studies, mtDNA and microsatellite data showed that the Florida manatee and the Antillean manatee from Puerto Rico were considered distinct populations and no admixture or recent migrants between those localities were observed (Hunter *et al*., 2012), supporting the traditionally recognized taxonomic classification at the subspecies level (Domning & Hayek, 1986).

Although manatees from Florida (*T. m. latirostris*) have been assigned to a separated population cluster from the manatees from Mexico (*T. m. manatus*) using microsatellites, migration events may occur from the growing manatee population in Florida to Mexico (Nourisson *et al*., 2011), indicating that there may be haplotypes sharing between those locations, as observed by Vianna *et al*. (2006).

Vianna *et al*. (2006) also proposed that a phylogeographic structure is likely present in *T. senegalensis* with at least two divergent clusters revealed by mtDNA. The first cluster is composed of Guinea-Bissau haplotypes and the second of Ghana, Chad and Niger haplotypes. However, for *T. inunguis* the authors reported the presence of a single compact cluster as observed by Cantanhede *et al*. (2005).

Up to now, the study assessing intra- and interspecific morphological variation in manatees was based on linear measurements (Domning & Hayek, 1986). Besides, no detailed chromosome study was performed in *T. m. manatus* from Brazil. In this sense, the current study employed three-dimensional geometric morphometric techniques to assess geographic variation in size and shape of the skull between *T. m. manatus*, *T. m. latirostris*, *T. inunguis* and *T. senegalensis* and within their populations (except for the latter species), in order to evaluate the presence of morphological discontinuities among geographical samples. Additionally, 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.

MATERIAL AND METHODS

MORPHOLOGICAL DATA

SAMPLES

We analyzed 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). These specimens were housed in the mammal collections of the following institutions: American Museum of Natural History (AMNH, New York); Associação de Pesquisa e Preservação de Ecossistemas Aquáticos (AQUASIS, Caucaia); Centro Nacional de Pesquisa e Conservação de Mamíferos Aquáticos, Instituto Chico Mendes de Conservação da Biodiversidade (CMA/ICMBio, Itamaracá); Instituto de Desenvolvimento Sustentável Mamirauá (IDSM, Tefé); Florida Museum of Natural History, University of Florida

(FLMNH, Gainesville); Field Museum of Natural History (FMNH, Chicago); Museu Paraense Emílio Goeldi (MPEG, Belém); National Museum of Natural History, Smithsonian Institution (NMNH, Washington DC); and U.S. Geological Survey (USGS, Gainesville).

We pooled all specimens 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 and on the lack of geographic differentiation among the individuals in each region after performing preliminary morphometric analyses. *T. inunguis* population included individuals from Peru (PER, N = 2), Amazonas, Brazil (AM, N = 62) and Pará, Brazil (PA, N = 9). *T. 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. *T. 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). Brazilian *T. m. manatus* population included individuals from Ceará (N = 6), Paraíba (N = 2), Rio Grande do Norte (N = 1) and two specimens from Brazil of unknown origin. *T. m. latirostris* individuals were from United States of America (USA, N = 85) (Fig. 1).

Data on sex were available for 82 specimens of *T. m. latirostris* (44 females and 38 males), 16 individuals of *T. m. manatus* (seven females and nine 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.

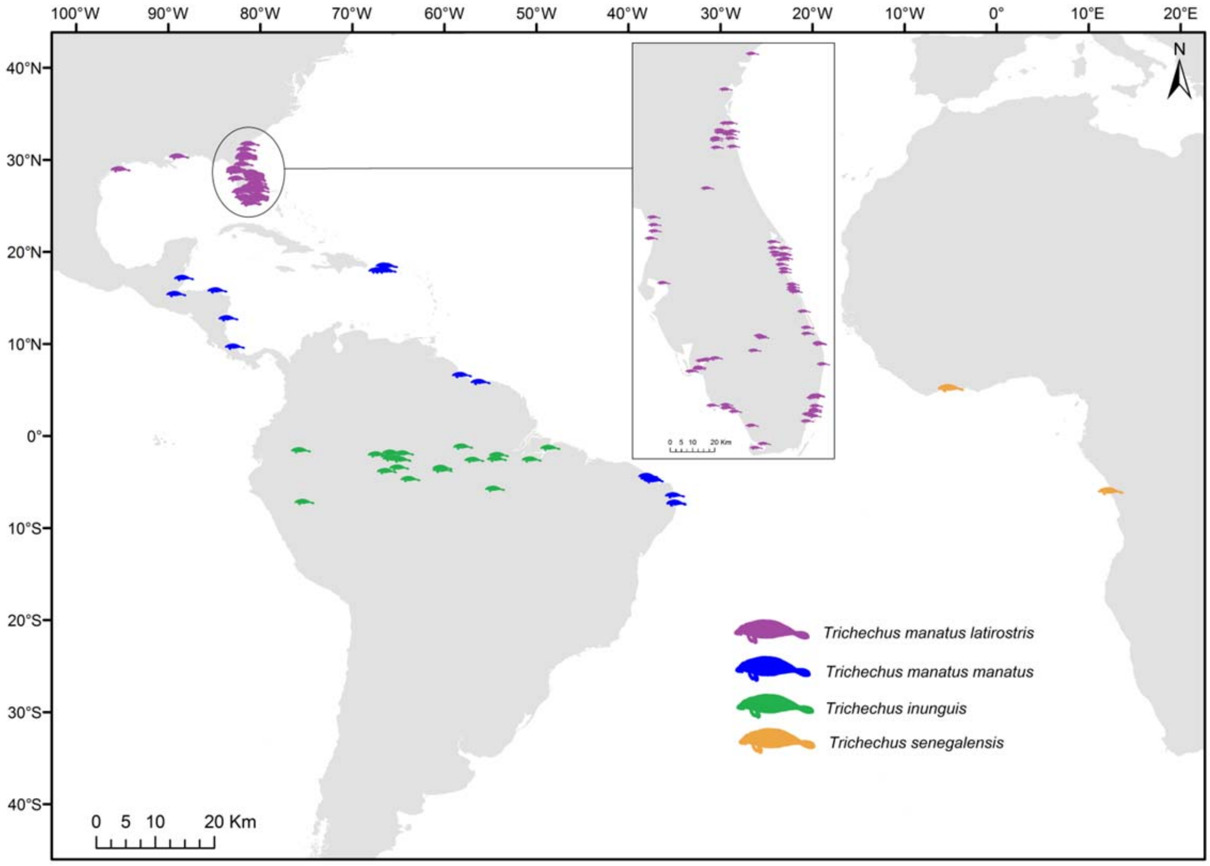

Figure 1. Collection localities of the skulls included in the present study. Localities are labelled according to current species and subspecies taxonomy, following Domning & Hayek (1986).

LANDMARKS

Geometric morphometrics was used to assess geographic variation in skulls of trichechid sirenians. This approach has showed to be efficient when compared to linear methods to study shape differences (Zelditch *et al*., 2004).

Three-dimensional coordinates were recorded with replicates for 60 landmarks in both dorsal and ventral sides of the skulls (Fig. 2; Table 1), using a MicroScribe 3DX digitizer. For 156 of 189 skulls, all landmarks were digitized. In this case, the dorsal and ventral views were combined into a single configuration of landmarks using FileConverter, and five landmarks were digitized from both views to fit them. Missing values were estimated for the 33 remaining specimens. For this, we used DVLR (Raaum, 2006) to fit the two views because it allows that missing landmarks are 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 Morpheus 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, 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 homologous along the sagittal plane.

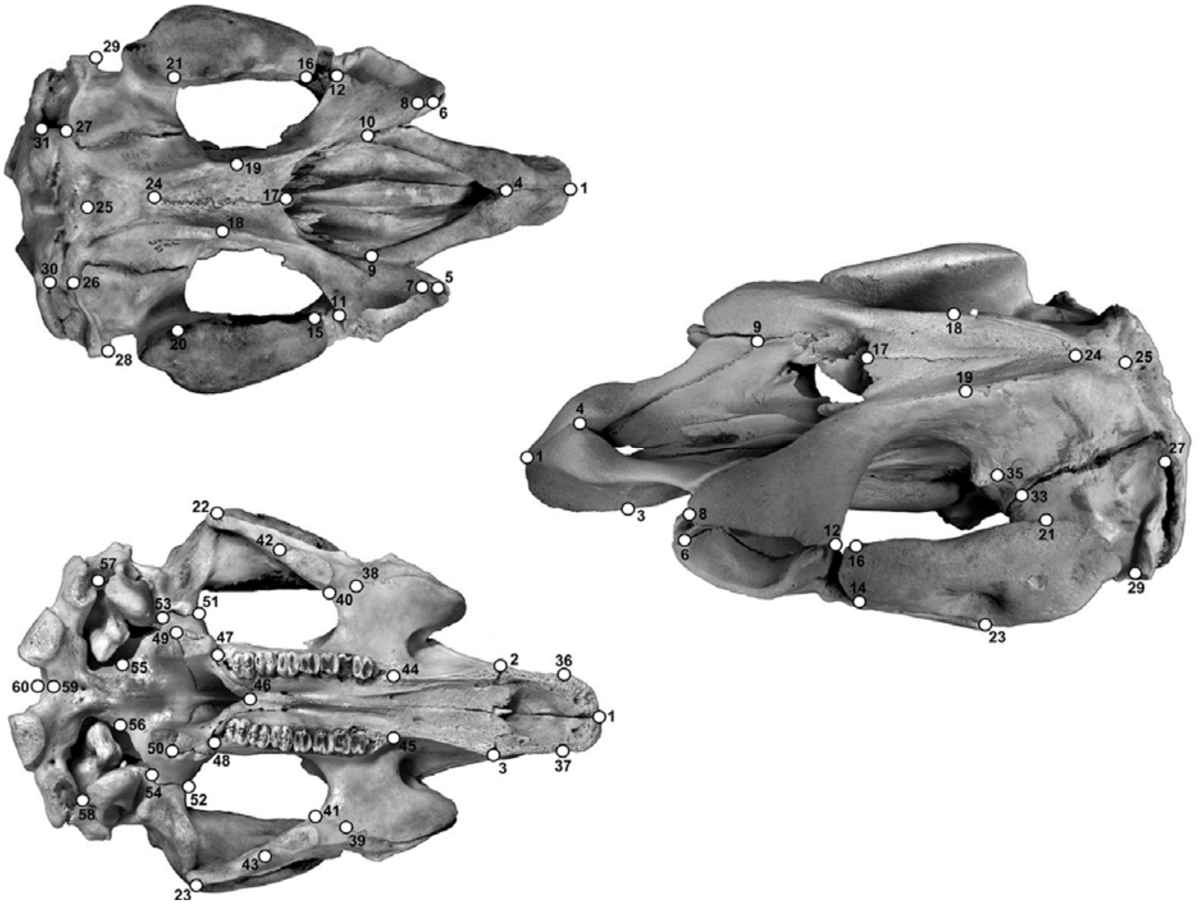

Figure 2. *Trichechus manatus manatus* specimen, illustrating locations of landmarks and their respective numbers in dorsal, ventral and lateral views of the skull. Anatomical positions of each landmark are defined in Table 1.

The raw data (coordinates) were imported into MorphoJ 1.05f (Klingenberg, 2011) where most analyses were performed. Data were then submitted to a Procrustes Fit, to remove effects of isometric size and orientation and a Procrustes ANOVA (Klingenberg & McIntyre, 1998), to lessen measurement error effects. This method allows quantifying shape variation between the same specimen measured twice (variation due to measurement error), but also variation among individuals and within-individual (biological variations), providing covariance matrices for each effect: error, individual variation and fluctuating asymmetry. Therefore, in subsequent shape analyses we used the individual values matrix generated after the Procrustes ANOVA because it is corrected for measurement error and fluctuating asymmetry.

Table 1. Definition and numbering of the landmarks used in both dorsal and ventral sides of the skulls.

Landmarks	
Number	Description
1	Anterior-most tip of premaxilla, midline
2 – 3	Most ventral maxilla-premaxilla suture, both sides
4	Superior tip of premaxilla as viewed dorsally, midline
5 – 6	Zygo-maxillary superior, both sides
7 – 8	Anterior-most tip of the supraorbital process of frontal, both sides
9 – 10	Posterior-most point of premaxilla as viewed dorsally, both sides
11 – 12	Posterior-most tip of the postorbital process of frontal, both sides
13 – 14	Inferior-most tip of the zygomatic process of squamosal, anterior position, both sides
15 – 16	Superior-most tip of the zygomatic process of squamosal, anterior position, both sides
17	Anterior-most point of suture between frontals, midline

18 – 19	Anterior-most 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, both sides
24	Bregma, frontal-parietal suture, midline
25	Most dorsal superior point of parietal, midline
26 – 27	Asterion, occipital-parietal-squamosal junction, both sides
28 – 29	Posterior-most tip of squamosal as viewed laterally, both sides
30 – 31	Posterior-most point of suture between supraoccipital-exoccipital, both sides
32 – 33	Squamosal-alisphenoid-parietal junction, both sides
34 – 35	Pterion, frontal-parietal-alisphenoid junction, both sides
36 – 37	Anterior-most point of premaxilla as viewed ventrally, both sides
38 – 39	Maxilla-jugal suture, most-anterior position, both sides
40 – 41	Zygo-maxillary inferior, both sides
42 – 43	Posterior-most 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	Posterior-most sphenoid-palatine suture on the pterygoid process, both sides
51 – 52	Squamosal-sphenoid junction, anterior-most position, both sides
53 – 54	Squamosal-sphenoid junction, posterior-most position, both sides
55 – 56	Basisphenoid-basioccipital suture at the limit with middle lacerate foramen, both sides
57 – 58	Extremity of jugular process, both sides
59	Basion, inferior point of foramen magnum, midline
60	Opisthion, superior point of foramen magnum, midline

DATA ANALYSES

Sexual shape dimorphism for each taxon was examined using a Discriminant Function Analysis (DFA). In order to assess the main shape variations a PCA was run. The PCA was first run with the complete dataset, and afterwards within each species, to evaluate intraspecific differences in shape. Because of its small sample size, *T. senegalensis* was used only in analyses among the taxa. Canonical Variates Analysis (CVA) between all the species and populations of *T. m. manatus* and *T. m. latirostris* was performed to confirm natural groupings previously suggested by the PCA. The Mahalanobis and Procrustes distances among the groups from the CVA were used to measure the degree of skull shape differentiation between the five groups studied (*T. inunguis*, *T. senegalensis*, *T. m. manatus* Brazil population, *T. m. manatus* Caribbean population and *T. m. latirostris* USA population), where a phenogram was generated from the neighbour-joining (NJ) method. To check for the presence of allometry for each taxon, the symmetric component of shape was regressed against centroid size (Drake & Klingenberg, 2008). DFA was also used to assess the separation between *T. m. manatus* and *T. m. latirostris* and among their populations based on skull shape, and to generate wireframes of shape differences between pairs of groups.

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). Sexual size dimorphism was determined using student's t-test for each taxon. We compared size among the taxa using one-way ANOVA, followed by a Tukey *post hoc* test. In addition, skull size among the populations of *T. m. manatus* from Brazil, Caribbean and *T. m. latirostris* from USA was assessed using the same analysis of variance. Similarly, size among *T. inunguis* populations (PER, AM and PA) was examined using one-way ANOVA.

Shape variation between species, subspecies and populations were evaluated by plotting first and second principal components (PC1 x PC2). We used the symmetric component average (exported from MorphoJ) of the species and subspecies and populations of *T. manatus* to generate polygons using the software Morphologika 2 v2.5 (O'Higgins & Jones, 2006).

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 under license from Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, SISBIO 21595-1) by the permanent veterinarian staff at the CMA, and at the same time to blood sampling to evaluate the health status of captive manatees, since this is a routine procedure at the CMA. 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).

Chromosome preparations were obtained by lymphocytes 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 & Black (1980), respectively. Metaphases were examined with an Olympus optical microscope and the images were captured using 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, Rasband & Eliceiri, 2012), because solid staining maintains chromosomal integrity. Each arm was measured from the midpoint of the primary constriction to the end of the arm. The relative total length and arm ratios (q/p) were calculated based on the lengths of the short (p) and long (q) arms. The bi-armed chromosomes were classified as metacentric (m), submetacentric (sm) and subtelocentric (st), and one-armed chromosomes as acrocentric (a), following Levan, Fredga & Sandberg (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 the sex chromosomes were depicted separately.

RESULTS

MORPHOLOGICAL DATA

Measurement error effect (differences between replicates) was non-significant when compared to the variation among individuals (F = 67.27), which means that measurement error is negligible (67.27 times smaller than the fluctuating asymmetry effect), and that the individual values generated after Procrustes ANOVA were suitable for use in all analyses performed here.

SEXUAL DIMORPHISM

No sexual dimorphism was detected in size and shape of the skull in any of the taxa (Table 2). Therefore, sexes were pooled in subsequent analyses, including those specimens with unknown sex.

Table 2. Student's t-test and Discriminant Function Analysis (DFA) results from 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; SD: standard deviation; df: degrees of freedom; p: significance level for t-test and for Procrustes distance (DFA).

	Student's t-test									Procrustes distance
Taxon	N		Mean		SD					♂ - ♀
	♂	♀	CS ♂	CS ♀	♂	♀	t-value	df	p	p
TMM	9	7	809.66	854.90	69.87	83.30	-1.18	14	0.2567	0.2170
TML	38	44	824.72	842.81	87.37	82.58	-0.96	80	0.3383	0.2220
TI	34	25	732.41	748.29	82.12	51.85	0.85	57	0.3991	0.5700

INTERSPECIFIC SHAPE ANALYSES

The population of *T. inunguis* is clearly separated from the *T. senegalensis*, *T. m. manatus* and *T. m. latirostris* populations along the first two PCs from the PCA. *T. senegalensis* is separated from *T. m. latirostris*, but is fully included in the variation of *T. m. manatus*. In addition, there is some superimposition between the *T. m. manatus* and *T. m. latirostris* populations along the two axes (Fig. 3). *T. inunguis* can be separated from the other taxa by having a more elongated and narrower rostrum, less deflected snout, narrower and more retracted skull (mostly the face, basicranium and zygomatic arch regions), more flattened supraoccipital region, and beginning of tooth row with a more anterior position (Fig. 3).

The skull of *T. m. latirostris* exhibited greater morphological variation compared to the *T. inunguis* skull, since these taxa are located in the most extreme positions along the first PC. *T. m. latirostris* presents the following shape changes in relation to *T. inunguis*: shorter and wider rostrum, more deflected snout, enlargement of the skull as a whole, with frontal, maxilla, jugal and orbits expanded sideways and more frontally oriented, expanded zygomatic arches, more rounded supraoccipital region, and beginning of tooth row with a more posterior position. *T. senegalensis* and *T. m. manatus* have skull shape changes from *T. inunguis* to *T. m. latirostris*, with *T. senegalensis* presenting some single features, particularly: more slender rostrum, broader zygomatic arch, wider zygomatic process of the squamosal, and narrower superior region of the frontal (Fig. 3).

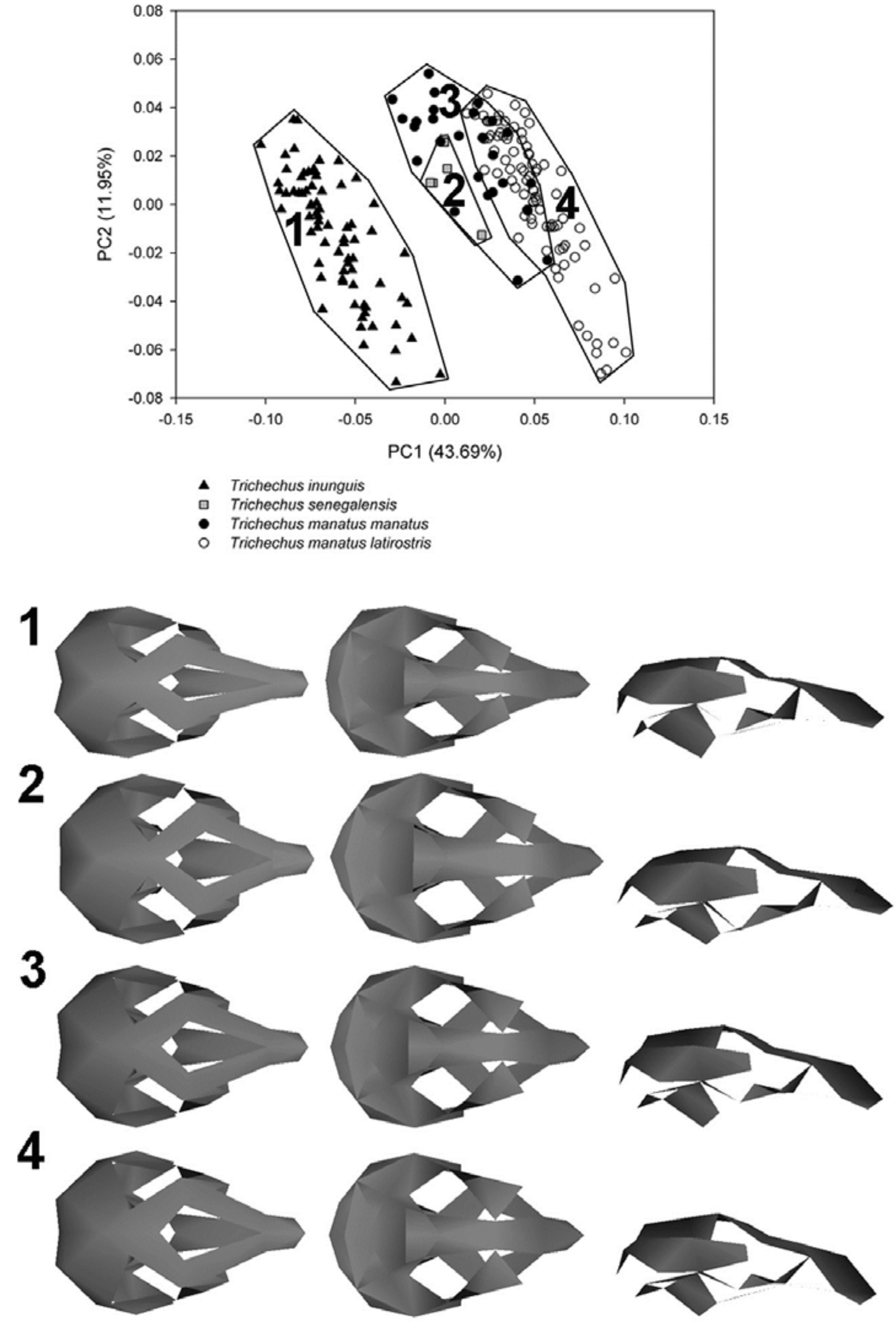

Figure 3. Scatterplot of PC1 vs. PC2 from the PCA among all species studied. Shape changes are visualized by the polygons constructed from the average of symmetric component of each taxon. From left to right: dorsal, ventral and lateral views, respectively.

The regressions between symmetric component and centroid size showed significant presence of allometry (Fig. 4A, B, C). In the individual analysis of *T. inunguis* (Fig. 4A), CS could predict 23.03% of total variance ($p < 0.0001$) which means that shape is influenced by size. Specimens with larger skull size present more elongated rostral and face regions, retracted zygomatic arch, superior tip of frontal with a more posterior position, larger tooth row, palatine with a more posterior position, narrower temporal fossa, and narrower and shorter neurocranial and basicranial regions. For *T. m. manatus* (Fig. 4B) and *T. m. latirostris* (Fig. 4C), allometric size explained, respectively, 18.31% and 30.11% of the shape variation ($p < 0.0001$), and like *T. inunguis* shape is also associated with size. The shape changes related with size are similar among these taxa.

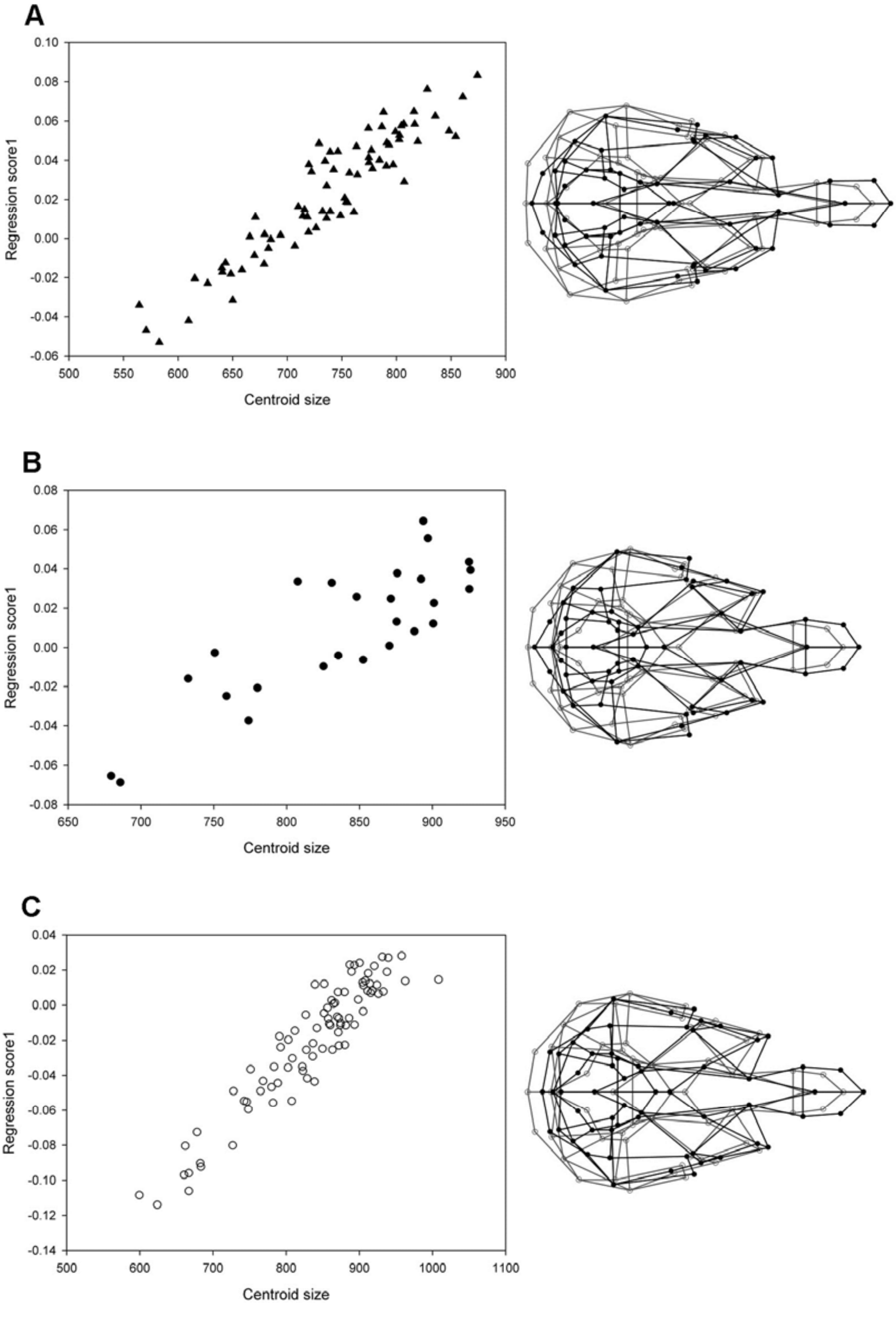

Figure 4. Scatterplot regression scores between symmetric component of shape and centroid size. (A) *Trichechus inunguis*; (B) *Trichechus manatus manatus*; (C) *Trichechus manatus latirostris*. Variations in landmarks positions related with size are showed (wireframe in gray represents smaller skull shape and wireframe in black represents larger skull shape).

The ANOVA showed that species differ significantly in size (F = 24.457, degrees of freedom = 3, p = 0.0000), and the a posteriori Tukey test revealed that *T. m manatus* and *T. m. latirostris* are different from *T. inunguis*, with the former species presenting larger skull size (CS mean = 838.56 and 835.79, respectively) than the latter one (CS mean = 736.99). *T. senegalensis* presents a skull CS mean of 816.91.

INTRASPECIFIC SHAPE ANALYSES

Trichechus manatus manatus and *Trichechus manatus latirostris*

The Brazilian *T. m. manatus* population is clearly separated from the *T. m. manatus* Caribbean and *T. m. latirostris* USA populations, which in turn overlap partially along the first two PCs extracted from the PCA (Fig. 5). The cranial features of the Brazilian *T. m. manatus* population are: superior tip of premaxilla with a more posterior position, more elongated and wider rostral region, end of premaxilla with a more anterior position, narrower superior region of the frontal and parietal, retracted face and zygomatic arch regions, wider zygomatic process of the squamosal, least deflected snout, wider medial region of the rostrum on the ventral side, superior point of foramen magnum with a more posterior position, and inferior point of foramen magnum with a more anterior position. *T. m. latirostris* USA population presents the most extreme scores along the two axes, exhibiting morphological variations such as: superior tip of the premaxilla with a more anterior position, shorter and narrower rostral region, end of premaxilla with a more posterior position, wider superior region of the frontal and parietal, enlargement of the face and zygomatic regions, with frontal, maxilla, jugal and orbits expanded sideways and more frontally oriented, narrower zygomatic process of the squamosal, most deflected snout, narrower medial region of the rostrum on the ventral side, superior point of foramen magnum with a more anterior position, and inferior point of foramen magnum with a more posterior position (Fig. 5).

Although the Caribbean and USA populations currently belong to separate subspecies, they showed some overlap along the first two PCs. *T. m. manatus* Caribbean population shows skull shape changes from the Brazilian *T. m. manatus* population to *T. m. latirostris* USA population (Fig. 5).

Procrustes distance from the DFA between both subspecies of *T. manatus* showed a significant discrimination ($p < 0.0001$), with 92.94% and 84.62% of the specimens of *T. m. latirostris* and *T. m. manatus*, respectively, being correctly classified from cross-validation. When *T. m. manatus* Caribbean and *T. m. latirostris* USA populations were pooled and a DFA was run comparing them with the Brazilian *T. m. manatus* population, a significant discrimination from the Procrustes distance was also found ($p < 0.0001$), but now with 100% of correct classification of all the specimens in both groups from cross-validation. The shape changes that allowed discriminating among the populations are similar to those ones that separated the Brazilian *T. m. manatus* population from the others by the PCA.

The ANOVA showed no skull size variation among the populations of *T. m. manatus* from Brazil, Caribbean and *T. m. latirostris* from USA ($F = 1.034$, degrees of freedom = 2, $p = 0.3592$).

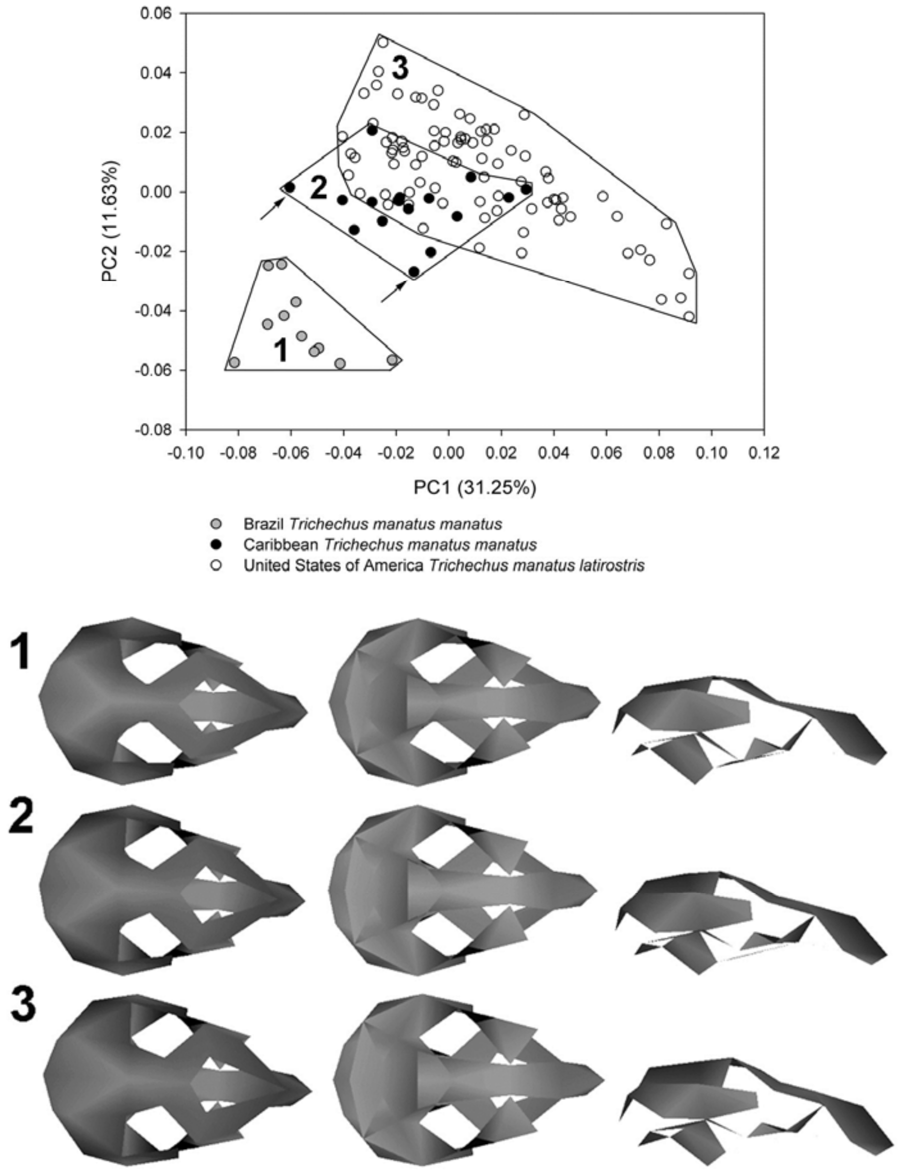

Figure 5. Scatterplot of PC1 vs. PC2 between the Brazilian, Caribbean *Trichechus manatus manatus* populations and United States of America *Trichechus manatus latirostris* population. Arrows represent individuals from Guyana (above) and Suriname (below). Shape changes are visualized by the polygons constructed from the average of symmetric component of each population. From left to right: dorsal, ventral and lateral views, respectively.

Trichechus inunguis

Distribution of PCs scores did not reveal any clear pattern for this species, with a wide superimposition of the three populations established based on geographic origin (Fig. 6). The ANOVA for size variation was non-significant among *T. inunguis* populations (PER, AM and PA; F = 0.682, degrees of freedom = 2, p = 0.5089).

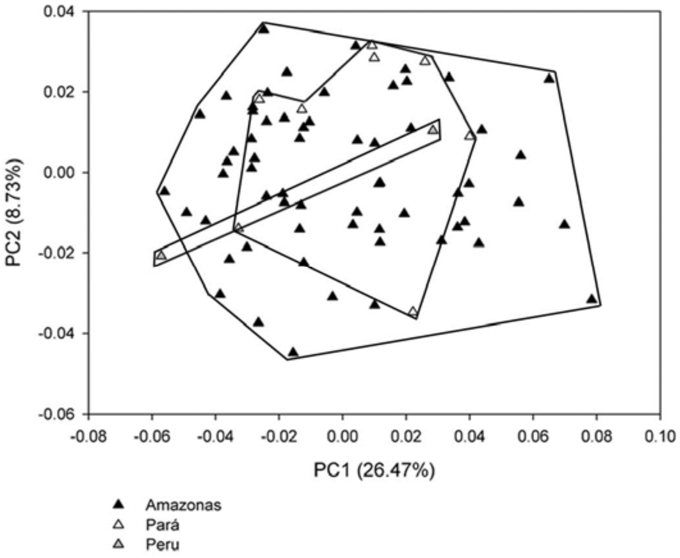

Figure 6. Scatterplot of PC1 vs. PC2 among *Trichechus inunguis* populations.

SHAPE ANALYSES BETWEEN SPECIES AND POPULATIONS OF *Trichechus manatus manatus* AND *Trichechus manatus latirostris*

The CVA of symmetric component of shape confirmed the existence of a priori groups suggested by the PCA due to skull shape differences. Mahalanobis and Procrustes distances were statistically significant among the five groups studied (Tables 3 and 4). The Caribbean *T. m. manatus* and *T. m. latirostris* USA populations are closer to one another than to the Brazilian *T. m. manatus* population, which in turn it is as differentiated as are the two well-recognized species of manatees (*T. inunguis* and *T. senegalensis*) (Fig. 7).

Table 3. Mahalanobis distances and p-values from the CVA 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).

Groups	TI	TML USA	TMM BRA	TMM CA	TS
TI	0	21.99 $p < 0.0001$	21.57 $p < 0.0001$	20.90 $p < 0.0001$	24.66 $p < 0.0001$
TML USA	21.99 $p < 0.0001$	0	18.17 $p < 0.0001$	7.76 $p < 0.0001$	19.66 $p < 0.0001$
TMM BRA	21.57 $p < 0.0001$	18.17 $p < 0.0001$	0	16.84 $p < 0.0001$	21.53 $p = 0.0001$
TMM CA	20.90 $p < 0.0001$	7.76 $p < 0.0001$	16.84 $p < 0.0001$	0	17.98 $p < 0.0001$
TS	24.66 $p < 0.0001$	19.66 $p < 0.0001$	21.53 $p < 0.0001$	17.98 $p < 0.0001$	0

Table 4. Procrustes distances and p-values from the CVA 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).

Groups	TI	TML USA	TMM BRA	TMM CA	TS
TI	0	0.11 $p < 0.0001$	0.09 $p < 0.0001$	0.10 $p < 0.0001$	0.10 $p < 0.0001$
TML USA	0.11 $p < 0.0001$	0	0.09 $p < 0.0001$	0.04 $p < 0.0001$	0.10 $p < 0.0001$
TMM BRA	0.09 $p < 0.0001$	0.09 $p < 0.0001$	0	0.07 $p < 0.0001$	0.09 $p = 0.0001$
TMM CA	0.10 $p < 0.0001$	0.04 $p < 0.0001$	0.07 $p < 0.0001$	0	0.08 $p < 0.0001$
TS	0.10 $p < 0.0001$	0.10 $p < 0.0001$	0.09 $p < 0.0001$	0.08 $p < 0.0001$	0

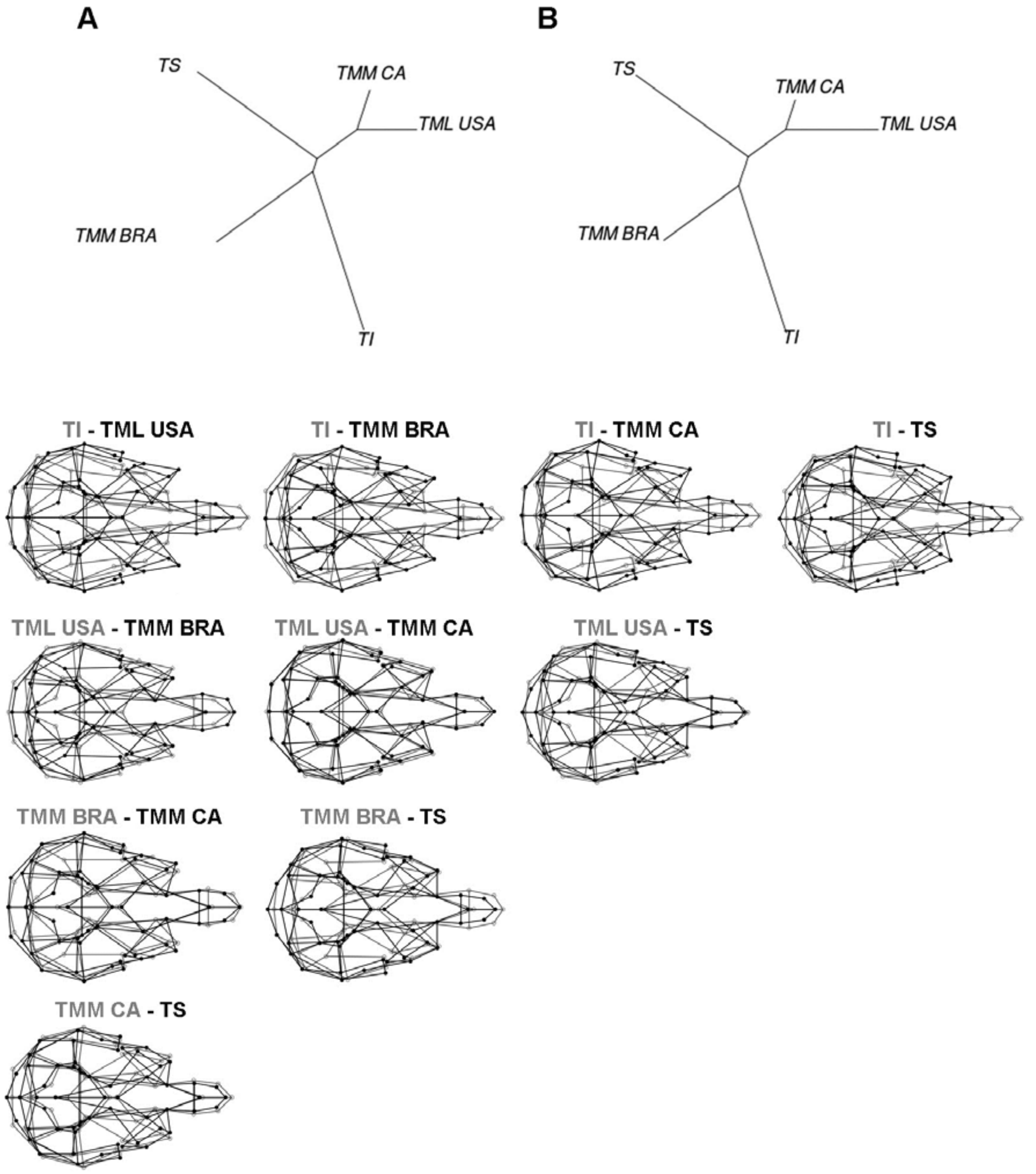

Figure 7. Neighbour-joining tree based on the Mahalanobis (A) and Procrustes (B) distances matrices from the CVA, expressing the degree of similarity among the five groups studied, *Trichechus inunguis* (TI), *Trichechus manatus latirostris* USA population (TML USA), *Trichechus manatus manatus* Brazil population (TMM BRA), *Trichechus manatus manatus* Caribbean population (TMM CA) and *Trichechus senegalensis* (TS). Superimposition of skull shape between each pair of groups is showed below (wireframe in gray represents starting shape and wireframe in black represents target shape).

KARYOLOGICAL DATA

The chromosomes of the Antillean manatee were arranged into seven groups (A-G) according to the similarity of the G-banding pattern between the two subspecies. The diploid chromosome number was the same (2n=48) of the previously established (Vianna *et al.*, 2006), 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. 8).

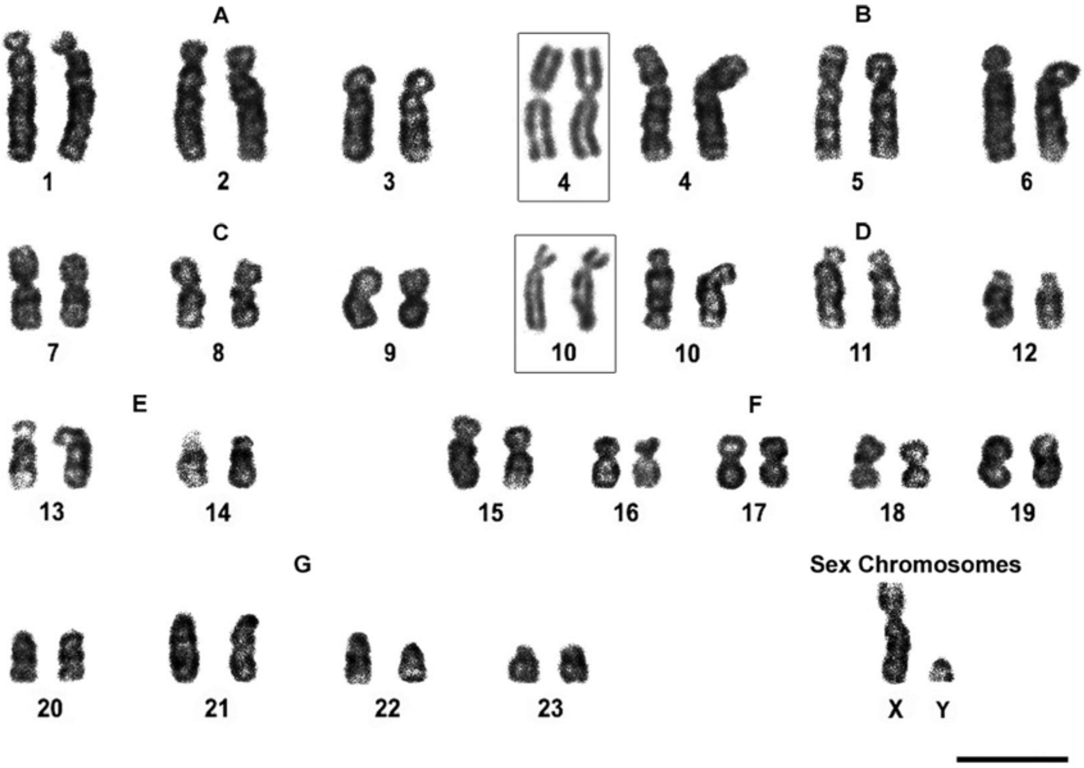

Figure 8. GTG-banded karyotype of *Trichechus manatus manatus* from Brazil (2n=48, XY) (inserts represent solid stained chromosome pairs 4m and 10sm allowing better visualization of chromosome morphology). Bar = 10 µm.

All homologs were identified using G-banding (Fig. 8). C-banding detected centromeric heterochromatin in all chromosomes (Fig. 9), and nucleolar organizer regions were observed on the short arms of chromosome pair 20st (Fig. 10).

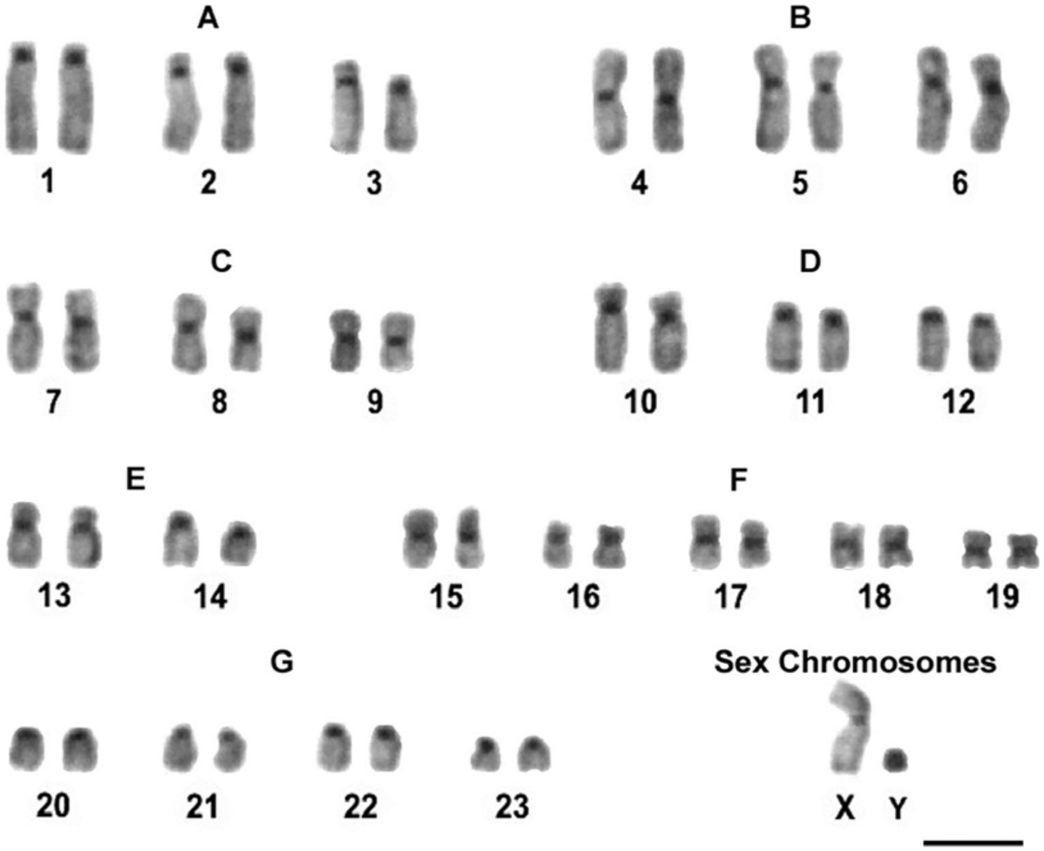

Figure 9. CBG-banded karyotype of Brazil Antillean manatee. Bar = 10 μm.

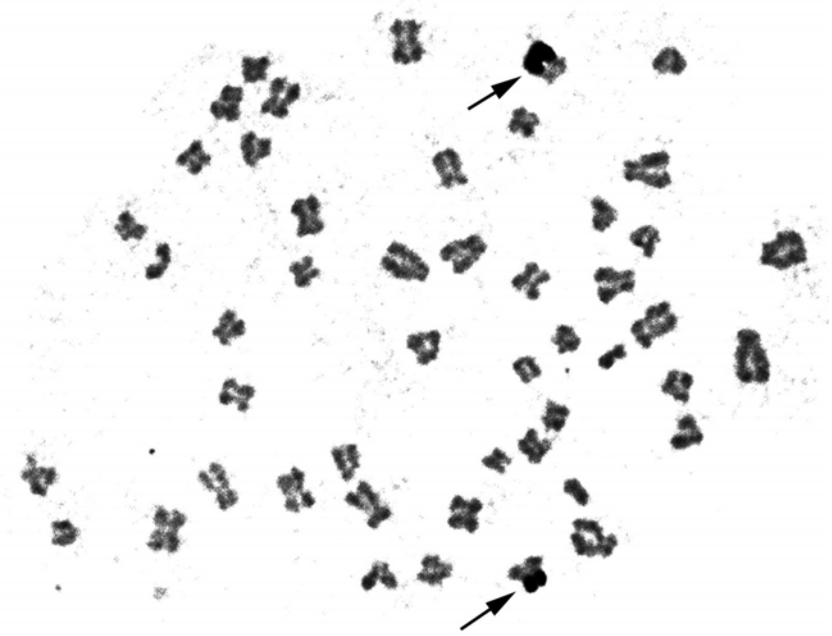

Figure 10. AgNOR staining of Brazilian *Trichechus manatus manatus* (arrows indicate positive NOR regions).

DISCUSSION

MORPHOLOGICAL DATA

SEXUAL DIMORPHISM

Our results showed no sexual dimorphism in size and shape in skulls of *T. m. manatus*, *T. m. latirostris* and *T. inunguis*. The previous study by traditional morphometric performed with manatee species also detected no significant sexual dimorphism in skulls of *T. manatus* and *T. inunguis* (Domning & Hayek, 1986). This likely reflects the manatee mating system, where there is 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 appears to exist (Marsh *et al*., 2012) and physical confrontations during the breeding behavior are rare (Reynolds III & Powell, 2002), thus robustness in male and females skulls seems to be not required.

INTERSPECIFIC SHAPE ANALYSES

The separation of *T. inunguis* from *T. senegalensis*, *T. m. manatus* and *T. m. latirostris* is supported by shape differences spread throughout the skull. Domning & Hayek (1986) gathered a suite of characters, including cranial data, from where their conclusions were mostly based, to study morphological variation in manatees. They evidenced that *T. inunguis* was the most divergent species compared to the other manatees, presenting apomorphic characters 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 studies on sirenians chromosomal evolution are not available so far in the literature). The probable origin of this species is allopatric speciation by the Andean uplift, which isolated the manatees that reached the Amazon Basin during the Pliocene. As a result, *T. inunguis* evolved more rapidly in response to the new environment and new food resource than coastal manatees (Domning, 1982). Our data, which show that *T. inunguis* exhibited differences in skull size and shape, agree with the previous study carried out by Domning & Hayek (1986), reflecting its adaptation to the Amazon Basin, a habitat that differs from the ones of the other manatee species.

Our results showed overlap along the first two PCs between *T. senegalensis* and *T. m. manatus* (mainly the Brazilian population), and between *T. m. manatus* (mainly the Caribbean population) and *T. m. latirostris*, which means that there are greater morphological similarities within each group, as would be expected according to phylogeny and speciation events (Domning, 1994; Domning & Hayek, 1986; Vianna *et al*., 2006). *T. senegalensis* is considered sister group of *T. m. latirostris* and *T. m. manatus*, sharing with each other some synapomorphies, and all of which are likely derived from the same marine ancestor. Those species appear to show little changes from their ancestor until the present. In addition, *T. senegalensis* likely evolved from South America coastal manatees through a transatlantic dispersal during the Pliocene or Pleistocene, and afterwards the populations of coastal manatees expanded their distribution through the Caribbean into coastal North America (speciation of *T. manatus*) (Domning & Hayek, 1986; Marsh *et al*., 2012).

Some of the cranial features observed in this study were also reported by Domning & Hayek (1986), and some of them are considered likely autapomorphies, including a long and narrow rostrum, and supraoccipital more flattened and swollen, allowing the skull almost always stand upright in this region (for *T. inunguis*); broader zygomatic arches (for *T. senegalensis*); and more deflected snout (for *T. m. manatus* and *T. m. latirostris*, particularly the Florida subspecies).

The least deflected snouts of the Amazonian and West African manatees reflect their likely adaptation for feeding on emergent and floating vegetation, which are often available in their habitats (Marsh *et al*., 2012). On the other hand, the Antillean and Florida manatees, especially the latter one, have the most deflected snout, reflecting a more bottom feeding habit. Indeed, when submerged vegetation is available, the Florida manatee prefers it to eat, although this subspecies, as well as the other one, have a wide feeding niche (freshwater, estuarine and marine environments) (Domning, 1982; Domning & Hayek, 1986; Marsh *et al*., 2012).

Our results indicated the presence of allometry and the allometric patterns of variation in skull morphology were similar in *T. inunguis*, *T. m. manatus* and *T. m. latirostris*. A probable explanation for this is because the separation between *T. inunguis* from *T. senegalensis* and *T. manatus* did not take place much earlier in time than that one between the two latter species (3 Myr and 1-2 Myr, respectively) (Domning, 1982; Domning & Hayek, 1986). As in the case of the manatees, shape variation associated with size has also been observed in other mammalian groups (Marroig & Cheverud, 2004, 2005; Marroig *et al*., 2009).

Despite the similarity of allometric patterns between *T. inunguis*, *T. m. manatus* and *T. m. latirostris*, difference in skull size was noted among these taxa. *T. m. manatus* and *T. m. latirostris* have significantly larger skull than *T. inunguis*, but no size difference was found between *T. senegalensis*, *T. m. manatus* and *T. m. latirostris*, and between *T. senegalensis* and *T. inunguis*. According to the body length and weight, the Amazonian manatee is the smallest and lightest of manatees and the Antillean and Florida manatees are larger and heavier than the West African manatee (Marsh *et al.*, 2012).

INTRASPECIFIC SHAPE ANALYSES

Trichechus manatus manatus and *Trichechus manatus latirostris*

There was a clear morphological discontinuity between the Brazilian *T. m. manatus* population and the *T. m. manatus* Caribbean and *T. m. latirostris* USA populations by PCA, DFA and CVA. Although Suriname and Guyana are countries geographically closer to Brazil, individuals from those localities presented skull shape more similar to that one from the Caribbean population (Fig. 5). Our results indicate that the Amazon River mouth could be acting as a barrier, isolating the Brazilian *T. m. manatus* population from the other ones, considering that only samples from northeastern Brazil were collected.

The Amazon continental shelf is extremely energetic and highly dynamic, reflecting a combination of different sources, including tides and tidal currents, waves, the North Brazil current, large amount of freshwater (which causes 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, supply and sediment reworking, physico-chemical of waters and biota organization (Lima, Tourinho & Costa, 2001). As a result, the Amazon River outflow has been suggested to act as a major barrier between the Brazilian and Caribbean biogeographical provinces, mainly separating the shallow water faunas (Floeter & Gasparini, 2000; McCartney, Keller & Lessios, 2000; Lessios, Kane & Robertson, 2003; Rocha, 2003; Robertson *et al*., 2006; Floeter *et al*., 2008; Rocha *et al*., 2008; Nunes, Norris & Knowlton, 2011). In addition, because of its width at the mouth (~ 300 km) the Amazon River is considered a greater barrier to dispersal for passerine birds (Hayes & Sewlal, 2004). Those factors may also be adverse for the Antillean manatee, influencing its occurrence at the Amazon River mouth, but this region offers more favorable environmental conditions to the occurrence of the Amazonian manatee (Luna *et al*., 2008a).

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 have been isolated in Brazil by the Amazon barrier, suggesting a speciation event by vicariance in the Amazon River mouth, which may lead to skull shape geographical variation, observed in the Brazilian population.

The previous morphological study using quantitative cranial characters 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 are effective barriers to gene flow between Florida and Antillean manatees (Domning & Hayek, 1986). However, phylogeographic analyses of *T. manatus* based on mtDNA data 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 the clusters one and two (individuals from Florida, Mexico, Central and South America), and the cluster three was found only in the Guyanas and Brazil, Vianna *et al*. (2006) proposed that a geographic barrier represented by the continuity of the Lesser Antilles to Trinidad Island appears to have restricted the gene flow historically in *T. manatus*. As a consequence the populations from the Guyanas and Brazil seem to have been separated by this barrier and they presented haplotypes that were identified only in these locations. In addition, these authors suggest that further analysis will indicate a subspecies status for both ESUs.

The skull shape variation presented here, which allowed the separation of the Brazilian *T. m. manatus* population from *T. m. manatus* Caribbean and *T. m. latirostris* USA populations, with some overlap between these taxa, supports partly the conclusions from molecular studies. Samples from Guyana and also from Suriname were grouped with *T. m. manatus* Caribbean population, suggesting that the Amazon barrier may have had a greater impact on the evolutionary history of *T. manatus* than previously thought (Haemig, 2011). Unfortunately, only one individual from Guyana and another from Suriname were available for use in the present work. Probably both barriers, Amazon River mouth and Lesser Antilles, may be involved in the process of morphometric and genetic differentiation of the South American Antillean manatee populations.

The phylogeographic division within the northeast Brazilian manatee populations previously described (Luna *et al*., 2012), was not detected by the morphological data from the current study, and the populations of *T. m. manatus* from Brazil were not different from each other in relation to the skull size and shape. Those authors observed that three haplotypes were found in the most northern states of the region (Maranhão and Piauí), and only a single haplotype was identified in the southern portion (Ceará, Rio Grande do Norte, Paraíba, Pernambuco and Alagoas states), suggesting the presence of two genetic groups with a central mixing zone.

Trichechus inunguis

The pattern observed for this species with no geographic differentiation in skull shape among the populations studied agrees with the results obtained by mtDNA data, which reported no geographical structure, with this species most likely behaving as a panmictic population, constituting a single expanding population cluster (Cantanhede *et al.*, 2005; Vianna *et al.*, 2006). Chromosome data also supported a probable recent overall population expansion, because of the occurrence of the same chromosomal variants in different geographical areas and distinct ones in the same region of the Amazonian manatee distribution range, besides the existence of heterozygous karyotypic variants, indicating a recent chromosomal evolution (Barros *et al.*, unpublished data).

Unfortunately, more precise information on specimens' collection localities was unavailable for testing additional hypothesis, such as differences between populations from different rivers.

SHAPE ANALYSES BETWEEN SPECIES AND POPULATIONS OF *Trichechus manatus manatus* AND *Trichechus manatus latirostris*

The phenogram between the two species of manatees and three populations of *T. m. manatus* and *T. m. latirostris* presented in this study showed surprising results. Differences in skull shape exhibited by the Brazilian *T. m. manatus* population are considerable, and they are in magnitude as large as are those ones found between *T. inunguis* and *T. senegalensis*. This strongly suggests reproductive isolation of that population. On the other hand, the Caribbean *T. m. manatus* and *T. m. latirostris* USA populations are closer to each other, reflecting the overlapping among some individuals observed by PCA.

This scenario supports conclusions from molecular studies that suggest the existence of three distinct lineages and two ESUs within *T. manatus*, with populations of *T. m. manatus* from Guyana and Brazil being very different from other ones (Garcia-Rodriguez *et al*., 1998; Vianna *et al*., 2006).

KARYOLOGICAL DATA

Divergences in karyotype were detected between the Brazil Antillean manatee and Puerto Rico Antillean and Florida manatees. The chromosome pairs 4 and 10 were (m) and (sm), respectively (Fig. 8) in the first taxon, however, these pairs were (sm) and (st), respectively in the latter two taxa (Gray *et al*., 2002; Hunter *et al*., 2012). Unfortunately, the G-banding alone was not sufficient to state with certainty the types of rearrangements, probably balanced ones, which led to structural chromosome differentiation observed between these three populations.

Gray *et al*. (2002) suggested that the evolution of the morphological karyotypic differences observed between *T. m. latirostris* and *T. inunguis* may have occurred by a variety of both interchromosomal and intrachromosomal rearrangement mechanisms. Therefore, the chromosomal rearrangements that occurred among trichechid sirenians appear to be subtle and they will be best revealed by comparative chromosome painting, allowing propose a scenario of Trichechidae chromosome evolution.

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

CONCLUSIONS

The current study explored levels of morphometric differentiation between species and populations within Trichechidae, providing clear differences among the taxa studied based on skull shape. We also presented a detailed chromosome study for *T. m. manatus* from Brazil, finding divergences in karyotype between this population and other ones.

The scores of *T. inunguis* are clearly separated along the first two PCs from the PCA, as a result of the difference in its skull shape. *T. senegalensis*, *T. m. manatus* and *T. m. latirostris* skulls are more similar to each other in shape, since there is some overlap among them by the PCA. It is observed that cranial shape variation is influenced by size (presence of allometry), *T. m. manatus* and *T. m. latirostris* have larger skull size than *T. inunguis* and there is no sexual dimorphism in skull size and shape in any of the species.

The skull shape of the Brazilian *T. m. manatus* population is distinguished by PCA, DFA and CVA, while there is some overlap between *T. m. manatus* Caribbean and *T. m. latirostris* USA populations, although they are traditionally considered different subspecies. In addition, individuals from Suriname and Guyana have skull shape more similar to that one from the Caribbean population, although these countries are geographically closer to Brazil. Our results indicate that the Amazon barrier may have interrupted or it may be interrupting gene flow in *T. m. manatus* populations from Brazil, consistent with models of allopatric speciation. The degree of morphological differentiation observed in the Brazil Antillean manatee population corresponds to that one found between the Amazonian and West African species, suggesting reproductive isolation of this population. The karyological differences detected between *T. m. manatus* from Brazil and Puerto Rico and Florida manatees reinforce the conclusions from the geometric morphometric technique.

Although it is necessary to record specimens of *T. m. manatus* from other states of Brazil, mainly of Amapá, and more individuals from the Caribbean region, which may not be readily available, we recommend a special attention to the current taxonomy of *T. manatus*, since our results support preliminary evidence from molecular data and these combined conclusions do not coincide with the accepted subspecies designation based on skull linear measurements.

In contrast, there is no geographic differentiation in skull shape among *T. inunguis* populations and it is necessary to record a greater number of *T. senegalensis* specimens to investigate differences in skull shape among their geographical populations.

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APPENDIX

SPECIMENS RECORDED IN THIS STUDY

Trichechus inunguis. AMNH: 73594; 76813; 93124; 94167; 98691. CMA: 01S0121/293. IDSM: 00/01; 00/04; 00/06; 00/07; 00/09; 01/01_01/46; 01/13; 01/14; 01/34; 01/39; 01/43; 01/44; 01/47; 02/03; 03/06; 03/07; 03/11; 05/27_05/28; 05/55_05/64; 05/67; 05/68; 05/69_06/25; 06/52; 06/53_06/58; 06/67; 07/03_07/04; 07/09_07/10; 11/02; 592; 600; 93/01; 94/04; 94/06; 95/03; 95/04; 95/14; 95/17; 96/01; 96/02; 96/07; 97/01; 97/02; 97/03; 97/07; 97/08; 97/10; 97/12; 98/01; 98/02; 98/09; 98/10; 99/02; 99/03; 9999/01; 99/04; 99/05; 99/07; 99/08; 99/10; SN. MPEG: 1518; 4635; 4637; 4638; 4639; 4640; 4641.

Trichechus manatus latirostris. AMNH: 100152; 100323; 24295; 70364; 90178; 91096. FLMNH: 13877; 15111; 15112; 15114; 15115; 15120; 15121; 15159; 15162; 15169; 15174; 15177; 15186; 15187; 15190; 15191; 15193; 15195; 15200; 15202; 15203; 15206; 15207; 19134; 19135; 20594; 20595; 20598; 20600; 20601; 20602; 20608; 20609; 24954; 24955; 24958; 24959; 24960; 24961; 24965; 24966; 24967; 24969; 24971; 24972; 24980; 24993; 24996; 25001; 25003; 25005; 25008; 25009; 25010; 25014; 25017; 25018; 25019; 25021; 25025; 25026; 25028; 25030; 25031; 25032; 25033; 25035; 25036; 25037; 25038; 25040. NMNH: 530313; 551656; 551665; 551671; 551672; 551681; 554180; A1375.

Trichechus manatus manatus. AMNH: 35566. AQUASIS: 2; 10; 24; 33; 36; 38. CMA: 01S0111/13; 01S0111/14; 01S0110/57; 01S0112/135; 01S0111/152. FMNH: 13888; 13890. NMNH: 257674; 257675; 258298; 550416; 550417; 550418; 550419; 550420; 554181; A13009; A20948. USGS: SB-Belize_I.

Trichechus senegalensis. AMNH: 53939. FMNH: 81513; 81514. NMNH: 571420; 571421.

CAPÍTULO 4. MANUSCRITO 2*: HETEROMORFISMO E POLIMORFISMO CROMOSSÔMICOS DO PEIXE-BOI AMAZÔNICO DO BRASIL

CHAPTER 4. MANUSCRIPT 2*: CHROMOSOME HETEROMORPHISM AND POLYMORPHISM OF THE AMAZONIAN MANATEE IN BRAZIL

* Artigo padronizado de acordo com as normas do periódico *Caryologia*.

Chromosome heteromorphism and polymorphism of the Amazonian manatee in Brazil

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Abstract

Chromosome heteromorphism and polymorphism in *Trichechus inunguis* Brazilian population have been described. All the specimens had 2n = 56 chromosomes, but one chromosome pair was heteromorphic and polymorphic regarding to acro- and submetacentric chromosomes. Additionally, fluorescence *in situ* hybridization technique was employed in *Trichechus inunguis* (2n = 56) and *Trichechus manatus manatus* (2n = 48) to investigate the occurrence of interstitial telomeric sequences (ITs) in metaphase cells of both species, but none of the taxa studied presented ITs. Our results showed that the chromosomal variants appear to be spread throughout the Amazonian manatee distribution range and they have not yet become fixed in the population, because we observed the same karyotype in different geographical areas and distinct karyotypes in the same region, although the acrocentric homozygous pair is the most common among the individuals studied in relation to the submetacentric homozygous pair and submetacentric and acrocentric heterozygous pair. The existence of heterozygous karyotypic variants may be an indication of a recent chromosomal evolution undergone by this species.

Keywords: *Trichechus inunguis*; cytogenetics; heteromorfism; polymorphism; chromosomal evolution

Introduction

The Amazonian manatee, *Trichechus inunguis* (Natterer, 1883) (Order Sirenia, i.e. manatees and dugongs) is a member of the family Trichechidae, which includes three other manatee taxa, *Trichechus manatus manatus* Linnaeus, 1758, *Trichechus manatus latirostris* (Harlan, 1824) and *Trichechus senegalensis* Link, 1795. *Trichechus inunguis* is endemic of the Amazon River basin, occurring from Marajó Island (Brazil) to rivers in Colombia, Peru and Ecuador (Domning 1981; Best 1984; Shoshani 2005). Morphologically, this species differs by its smaller size, having a dark and smooth skin, no nails on its flippers and the presence of a clear patch in the ventral region.

Trichechus inunguis is considered vulnerable in the Red List of the International Union for Conservation of Nature (IUCN 2013) and Red Book of the Brazilian Threatened Species List (MMA 2003). Today, the main threat to this species is illegal hunting (Hines et al. 2012).

Previous cytogenetic on the Amazonian manatee reported a diploid number of 2n = 56 (Loughman et al. 1970) and provided data on GTG- and CBG-banding and AgNOR staining (Assis et al. 1988), but chromosome variations were not noted. Analyses based on sequences of mitochondrial DNA (mtDNA) have shown that *T. inunguis* maintained a relatively high level of genetic diversity, despite the historic intensive hunting. In addition, it presents no geographical structure, constituting a single expanding population cluster (Cantanhede et al. 2005; Vianna et al. 2006).

Here, we report a case of heteromorphism and polymorphism involving one chromosome pair in *T. inunguis* from Brazil. Additionally, fluorescence *in situ* hybridization technique has been employed in *T. inunguis* (2n = 56) and *T. m. manatus* (2n = 48) to investigate the occurrence of interstitial telomeric sequences as a consequence of fusion rearrangements.

Material and methods

We analyzed 120 metaphase cells from six captive Amazonian manatee male specimens at the Centro de Preservação e Pesquisa de Mamíferos e Quelônios Aquáticos (CPPMQA), Balbina, Amazonas state, Brazil, from different geographic origins (Table 1).

Samples were collected under license from Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, SISBIO 21595-1) by the permanent veterinarian staff at the CPPMQA, and at the same time to blood sampling to evaluate the health status of captive manatees, since this is a routine procedure at the CPPMQA. 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).

Chromosome preparations were obtained by lymphocytes 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 were performed according to Seabright (1971) and Sumner (1972), respectively. Metaphases were examined with an Olympus optical microscope and the images were captured using Leica IM50 imaging system.

Fluorescence *in situ* hybridization technique (FISH) was carried out in both taxa *Trichechus inunguis* and *Trichechus manatus manatus* (Antillean manatee subspecies) by amplification by polymerase chain reaction (PCR) of telomeric sequence, and this was obtained in the absence of template DNA (Ijdo et al. 1991). It is in a total volume of 14 µl reaction mixture containing: 1.5 µl buffer 10X; 0.15 of *Taq DNA Polymerase* (5 U/ul); 3.0 µl dNTP (1 mM); 0.6 µl of each primer (5 pmol) and 8.15 µl H_2O Milli-Q. The PCR reaction utilized the following primers and amplification conditions using F $(5' \text{ TTAGGG-3'})_5$ and R $(5' \text{ CCCTAA-3'})_5$ (Ijdo et al. 1991), 10 cycles of 1 min at 94 ºC, followed by 30 sec at 55 ºC, 1 min at 72 ºC; 30 cycles of 1 min at 94 ºC, 30 sec at 60 ºC, 1 min and 30 sec at 72 ºC with 5 min at 72 ºC. PCR products were labeled with biotin-14-dATP for nick translation (BioNickTM Labeling System Invitrogen). FISH was performed under high stringency conditions (77%) (Pinkel et al. 1986). The hybridized metaphase chromosomes were observed using an Olympus BX51 epifluorescence microscope, and images were captured using Image-PRO MC 6.3 software.

The bi-armed chromosomes were classified as metacentric (M) and submetacentric (SM), and one-armed chromosomes as acrocentric (A) (Levan et al. 1964). GTG- and CBG-banded karyotypes were constructed by pairing chromosomes and grouping them into each morphological group, where the chromosomes were arranged in order of decreasing size. The sex chromosomes were depicted separately.

Table 1. Captive Amazonian manatee specimens at the Centro de Preservação e Pesquisa de Mamíferos e Quelônios Aquáticos (CPPMQA), whose blood samples were collected. A: acrocentric; SM: submetacentric.

Specimen	City/State	River	Chromosome heteromorphic and polymorphic pair (No. of cells)
Tintin	Urucará/Amazonas	Amazonas	A/A (20)
Preto	Rio Preto da Eva/Amazonas	Branco	A/A (17), SM/SM (2) and A/SM (1)
Marcelo	Barcelos/Amazonas	Negro	A/A (18) and SM/SM (2)
Capitari	Iranduba/Amazonas	Negro	A/A (19) and A/SM (1)
Itati	Itaituba/Pará	Tapajós	A/A (19) and SM/SM (1)
Manai	Santarém/Pará	Amazonas	A/A (18), SM/SM (1) and A/SM (1)

Results

We found the same diploid chromosome number for the Amazonian manatee (2n=56) as that previously established (Loughman et al. 1970; Assis et al. 1988). The fundamental number (FN) varied from 86 to 88. The chromosome set was composed of five metacentric (pairs 1-5), 11 or 12 submetacentric (pairs 6-16 or 6-17) and 10 or 11 acrocentric (pairs 18-27 or 17-27) autosome pairs. The X chromosome was submetacentric and Y was acrocentric (Figure 1a, b).

One autosome pair was heteromorphic and polymorphic in respect to (A) and (SM) chromosomes (Figure 1c; Table 1). Because of these heteromorphism and polymorphism, the FN and chromosome morphology were different.

All homologs were identified using G-banding (Figure 1a) and C-banding detected centromeric heterochromatin in all chromosomes (Figure 1b). FISH analysis of the telomere sequence revealed the presence of brightly fluorescent signals at the end of all the chromosomes and interstitial telomeric sequences (ITs) were not observed in either species (Figure 2a, b).

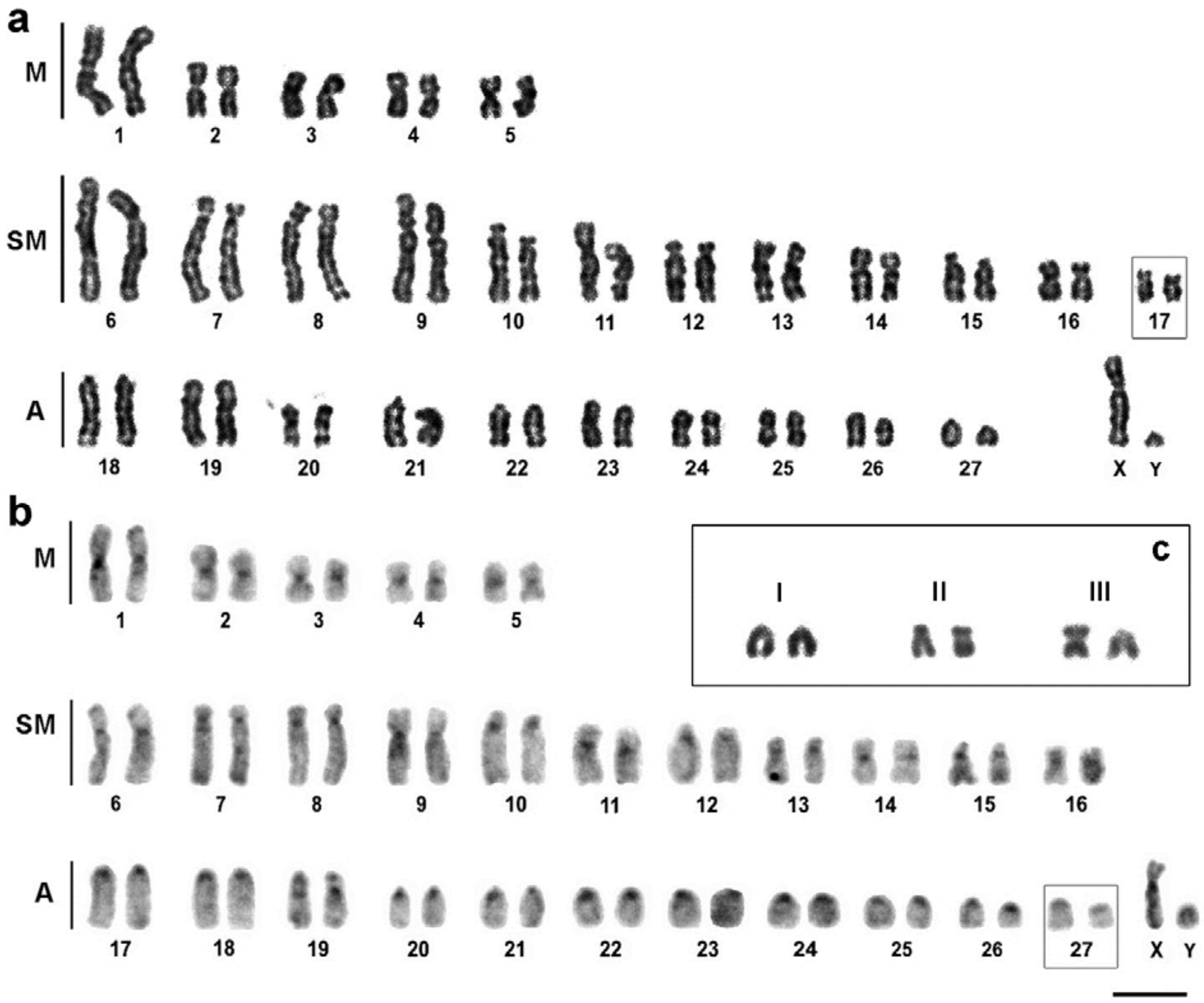

Figure 1. Chromosomal composition of *Trichechus inunguis* from Brazil (2n=56, XY). (a) GTG-banded karyotype, (b) CBG-banded karyotype, (c) chromosomal variants (I: acrocentric homozygous pair; II: submetacentric homozygous pair; III: submetacentric and acrocentric heterozygous pair). Smaller inserts represent chromosome heteromorphic and polymorphic pair regarding to submeta- and acrocentric chromosomes. Bar = 10 µm.

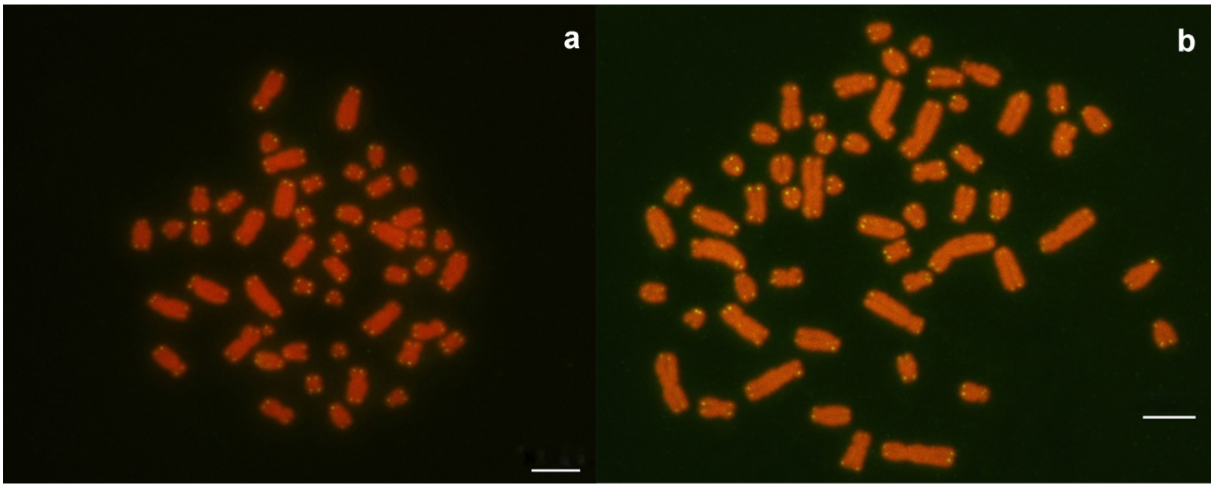

Figure 2. FISH to detect telomere repeat sequences signals on mitotic metaphase chromosome with the telomeric probe $(TTAGGG)_n$. (a) *Trichechus manatus manatus*, (b) *Trichechus inunguis*. Bar = 10 µm.

Discussion

The morphological variations observed in the present study must have arisen by pericentric inversions in *T. inunguis* acrocentric and submetacentric chromosomes leading to a submetacentric and acrocentric state, respectively. Balanced rearrangements of the translocation type may also have occurred because differences in the size of chromosome arms have been noted.

These results indicate that the chromosomal variants appear to be spread throughout the Amazonian manatee distribution range and they have not yet become fixed in the population, because we observed the same karyotype in different geographical areas and distinct karyotypes in the same region, although the acrocentric homozygous pair is the most common among the individuals studied in relation to the submetacentric homozygous pair and submetacentric and acrocentric heterozygous pair (Table 1). The existence of heterozygous karyotypic variants in *T. inunguis* from Brazil may be an indication of a recent chromosomal evolution and it is likely that there is some adaptive advantage for heterozygous form, as it has remained in the population. The adaptive advantage generated by rearrangements could replace the effects caused by inefficient meiotic pairing.

Molecular data provide information that is consistent with our chromosomal findings. Cantanhede et al. (2005), using mtDNA control region sequences, observed relatively high genetic diversity and effective population sizes in the Amazonian manatee, and near absence of geographical structuring (this species most likely behaves as a panmictic population). Additionally, a significant excess of recent mutations in the mtDNA of *T. inunguis* were observed, most likely explained by demographic expansion resulting from cessation of historic intensive hunting, species protection from 1967 and enforcement of conservation measures. This species is undergoing a possible recovery in the last 30-40 years (Cantanhede et al. 2005) after a long time of intensive exploitation (1780-1954) (Domning 1982).

These data are important for understanding the mechanisms that contributed to the rapid spread of chromosomal variants, suggesting that the migration rate of the Amazonian manatee may enable rearrangements spread through the population. In fact, the Amazon River basin is constituted by várzea (flooded periodically by white water rivers) and igapó (flooded periodically by black water rivers), and these floodplains form highly interconnected aquatic ecosystems. As *T. inunguis* has seasonal movements according to the flood regime of the Amazon River basin, it has many opportunities for extensive movement and migration, increasing the possibility of gene flow (Cantanhede et al. 2005).

We identified 16 or 17 bi-armed and 10 or 11 one-armed pairs (FN = 86-88) because of the heteromorphism and polymorphism. The previous detailed chromosome study for *T. inunguis* (eight animals from Brazil, Amazonas state, near the city of Manaus; and one animal from Colombia, near the city of Leticia), demonstrated 14 (M) and (SM) pairs and 13 (A) pairs of autosomes (FN = 82) (Assis et al. 1988). The animals sampled by Assis et al. (1988) were captive at the Instituto Nacional de Pesquisas da Amazônia (INPA), and probably these specimens were rehabilitated in the 1970s, when the Amazonian manatee *ex situ* conservation program began (1974). Moreover, this period coincides with the species hunting cessation. As mentioned above, *T. inunguis* probably has a recent overall population expansion and chromosome mutations are probably recent as well. It is thus likely that the karyotypes of animals sampled by us are different from those ones sampled by Assis et al. (1988), as it is observed by increase of bi-armed and decrease of one-armed chromosomes resulting from balanced rearrangements.

Assis et al. (1988) proposed fusion or fission rearrangements involving four bi-armed autosome pairs in *T. m. latirostris* (Florida manatee subspecies) or eight pairs of acrocentric autosomes in *T. inunguis,* when compared with the karyotype of *T. m. latirostris* (White et al. 1976, 1977), as the mechanism responsible for the karyotypic divergence between the Amazonian manatee and the Florida manatee. However, Gray et al. (2002) suggested that fusion or fission would not be the sole mechanism by which karyotypic divergence of these two species occurred. They suggested that the evolution of the morphological karyotypic differences observed between these two species could be best explained by the occurrence of a variety of both interchromosomal and intrachromosomal rearrangement mechanisms, such as inversions, whole-arm translocations, tandem fusions, and others. Our FISH results revealing no ITs and the occurrence of rearrangements of the pericentric inversion and translocation type in the *T. inunguis* karyotype support the Gray et al. (2002) proposal.

The karyotype instability observed in the Brazilian Amazonian manatee reflects an admixture between individuals with high genetic variability and a long-distance dispersal within the Amazon basin, although a greater number of individuals from other geographical areas must be sampled to confirm these patterns.

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CAPÍTULO 5. CONCLUSÕES GERAIS

1) Não houve dimorfismo sexual de tamanho e forma do crânio em *T. m. manatus*, *T. m. latirostris* e *T. inunguis*. Também não foi observada variação dentro das populações com relação ao tamanho do crânio;

2) *Trichechus inunguis* apresentou forma de crânio diferente, quando comparada à de *T. senegalensis*, *T. m. manatus* e *T. m. latirostris,* os quais se sobrepuseram parcialmente. O crânio de *T. inunguis* é mais estreito e retraído, o rostro é mais alongado, fino e com deflexão menos acentuada;

3) As mudanças de forma craniana foram influenciadas pelo tamanho (presença de alometria);

4) Os crânios de *T. m. manatus* e *T. m. latirostris* foram maiores que o de *T. inunguis*;

5) Variação geográfica na forma do crânio foi observada nas populações de *T. m. manatus* e *T. m. latirostris*. A população do Brasil de *T. m. manatus* foi claramente separada das populações de *T. m. manatus* do Caribe e *T. m. latirostris* dos Estados Unidos, havendo sobreposição entre estas;

6) Não houve uma clara separação entre as populações geográficas de *T. inunguis*, e não foi possível realizar uma análise da variabilidade intraespecífica na forma do crânio em *T. senegalensis* devido ao pequeno tamanho amostral obtido;

7) As variações na forma do crânio da população brasileira de *T. m. manatus* são em magnitude tão grandes quanto são as encontradas entre as duas espécies bem reconhecidas de peixes-bois (*T. inunguis* e *T. senegalensis*), sendo as populações de *T.*

m. manatus do Caribe e *T. m. latirostris* dos Estados Unidos mais similares entre si morfologicamente;

8) A descrição do cariótipo de *T. m. manatus* (2n = 48) do Brasil revelou diferenciação cromossômica estrutural entre este táxon e *T. m. manatus* de Porto Rico e *T. m. latirostris*;

9) A população do Brasil de *T. inunguis* (2n = 56) apresentou heteromorfismo e polimorfismo envolvendo um par cromossômico, com relação à morfologia acrocêntrica e submetacêntrica, mostrando um padrão que pode ser indicativo de evolução cromossômica recente, devido à existência de variantes cariotípicas heterozigotas;

10) A técnica de hibridização *in situ* fluorescente não revelou a ocorrência de sequências teloméricas intersticiais nos cromossomos de *T. inunguis* e *T. m. manatus*;

11) A morfometria geométrica indicou que a foz do rio Amazonas pode ter interrompido ou estar interrompendo o fluxo gênico nas populações de *T. m. manatus* do Brasil, condizente com modelos de especiação alopátrica;

12) As diferenças morfológicas e cariotípicas apresentadas neste estudo para a população brasileira do peixe-boi antilhano sugerem seu isolamento reprodutivo e suportam a evidência preliminar dos dados moleculares, não estando de acordo com a classificação subespecífica atualmente reconhecida para *T. manatus*.