Universidade Federal de Minas Gerais
Instituto de Ciências Biológicas
Departamento de Biologia Geral
Programa de Pós-Graduação em Genética

Tese de Doutorado

Hibridização e unidades evolutivas em peixes-bois (*Trichechus sp.*)

Autora: Camilla Savicius de Lima

Orientador: Fabrício Rodrigues dos Santos

Belo Horizonte - MG
2018

Camilla Savicius de Lima

Tese de Doutorado

Hibridização e unidades evolutivas em peixes-bois (*Trichechus spp*.)

Tese apresentada ao curso de Pós-graduação em Genética do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais como requisito parcial para obtenção de Título de Doutor em Genética.

Orientador: Fabrício Rodrigues dos Santos

Belo Horizonte - MG
2018

043 Lima, Camilla Savicius de.
Hibridização e unidades evolutivas em peixes-bois (*Trichechus sp*.) [manuscrito] / Camilla Savicius de Lima. – 2018.

99 f. : il. ; 29,5 cm.

Orientador: Fabrício Rodrigues dos Santos.
Tese (doutorado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas.

1. Genética. 2. Trichechus manatus. 3. Trichechus inunguis. 4. Peixe-boi. I. Santos, Fabrício Rodrigues dos. II. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. III. Título.

CDU: 575

Ficha catalográfica elaborada pela Biblioteca do Instituto de Ciências Biológicas da UFMG

Pós-Graduação em Genética
Departamento de Biologia Geral, ICB
Universidade Federal de Minas Gerais
Av. Antônio Carlos, 6627 - C.P. 486 - Pampulha - 31270-901 - Belo Horizonte - MG
e-mail: pg-gen@icb.ufmg.br FAX: (+31) - 3409-2570

"Hibridização e unidades evolutivas em peixes-bois (Trichech sp.)"

Camilla Savicius de Lima

Tese aprovada pela banca examinadora constituída pelos Professores:

Fabrício Rodrigues dos Santos - Orientador
UFMG

Mario Alberto Cozzuol
UFMG

Daniel Cardoso de Carvalho
PUC Minas

Mariana Freitas Nery
UNICAMP

Gustavo Campos e Silva Kuhn
UFMG

Belo Horizonte, 19 de setembro de 2018.

A minha avó Maria Savicius, *in memoriam*.

Aos meus pais, Ricardo e Verônica, e ao meu irmão, Ricardo Filho, que sempre acreditaram em mim e tornaram tudo isso possível. Eu não seria ninguém sem o apoio de vocês!

Aos meus verdadeiros amigos, que fiz ao longo de toda jornada e se mantiveram até hoje.

AGRADECIMENTOS

Agradeço à Universidade Federal de Minas Gerais (UFMG) e ao Programa de Pós-Graduação em Genética pelo ensino concedido.

À CAPES pela concessão da bolsa, à FAPEMIG, Fundação o Boticário e CNPq pelos auxílios financeiros dados ao Laboratório de Biodiversidade e Evolução Molecular (LBEM), tornando possível o desenvolvimento de todos os projetos.

Ao professor Fabrício Rodrigues dos Santos pela orientação dada, por todo conhecimento compartilhado, pela oportunidade de aprender cada vez mais e pela confiança ao me “presentear” com um dos seus projetos mais estimados.

Ao Instituto de Desenvolvimento Sustentável Mamirauá, ICMBio, AQUASIS e a ONG Kwata pelas amostras cedidas, essenciais para a realização do trabalho, e principalmente aos seus profissionais Dra Miriam Marmontel, Dra Fernanda Attademo e Dr Benoit de Thoisy, pela boa vontade e interesse em ajudar.

A todos os colegas e amigos do LBEM, com os quais dividi a maior parte dos meus dias nos últimos 4 anos, pelas conversas, risadas, trocas de experiências e cafezinhos. Especialmente ao Davidson Campos por sua amizade e paciência e ao Pedro Casemiro, pela disposição em ajudar e tornar as coisas mais fáceis pra mim.

Ao Rafael Felix por encarar o desafio das análises finais comigo, me ajudando, guiando e levando muito trabalho pra casa. Muito obrigada por tudo!

Aos ex-integrantes do laboratório, que se tornaram verdadeiros amigos: Carol Marinho, Marco Antônio Schettino e Samuel Bernardes. Poder contar com vocês no laboratório e na vida me deu forças para passar por tudo. Obrigada por estarem por perto em momentos tão importantes.

Àqueles que me apoiaram no dia-a-dia, ajudaram a me distrair e a manter o equilíbrio emocional nos momentos mais difíceis, principalmente ao meu namorado Ricardo Kloss.

A todos, enfim, que direta ou indiretamente contribuíram para o andamento e finalização deste trabalho, o meu mais sincero agradecimento.

“Every kid starts out as a natural-born scientist, and then we beat it out of them. A few trickle through the system with their wonder and enthusiasm for science intact.”

Carl Sagan

ÍNDICE

CAPÍTULO 1

CAPÍTULO 2

CAPÍTULO 3

LISTA DE FIGURAS

INTRODUÇÃO

CAPÍTULO 1

CAPÍTULO 2

CAPÍTULO 3

LISTA DE TABELAS

CAPÍTULO 1

CAPÍTULO 2

CAPÍTULO 3

LISTA DE ABREVIATURAS

°C: Graus Celsius
µg: Micrograma
µL: Microlitro
µM: Micromolar
AM: Amazônia
AP: Amapá
APOB: Apolipoprotein B
APP: Amyloid Precursor Protein
BLAST: Basic Local Alignment Search Tool
BMI1: Proto-Oncogene – BMI1
bp: Base Pair
CAPES: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CE: Ceará
CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico
COI: Cytochrome C Oxidase Subunity I
CR: Control Region
CREM: CAMP Responsive Element Modulator
ddRAD: Double Digest RADseq
DNA: Deoxyribonucleic Acid
dNTP: Deoxiribonucleotides
ESU: Evolutionary Significant Units
EUA: Estados Unidos da América
F1: Filial 1 hybrid
F2: Filial 2 hybrid
FAPEMIG: Fundação de Amparo à Pesquisa do Estado de Minas Gerais
FCA: Factorial Correspondence Analysis
FG: French Guiana
GMYC: Generalised Mixed Yule Coalescent
i.e.: *id est* (latim), isto é (português)
i.g.: *exempli gratia* (latim), por exemplo (português)
ICMBio: Instituto Chico Mendes de Conservação da Biodiversidade
IDSM: Instituto de Desenvolvimento Sustentável Mamirauá
INPA: Instituto Nacional de Pesquisas da Amazônia
IUCN: International Union for Conservation of Nature
K: Cluster

K2P: Kimura 2 parameter
Kg: Quilogramas
Km: Quilômetro
LBEM: Laboratório de Biodiversidade e Evolução Molecular
LR: Likelihood Ratio
m: Metros
Ma: Mega Annum (latim), Milhões De Anos (português)
MCC: Maximum Clade Credibility
MCMC: Markov Chain Monte Carlo
ML: Maximum Likelihood
mM: Milimolar
MSNC: Multispecies Network Coalescent
mtDNA: Mitochondrial DNA
MU: Management Units
MYA: Million Years Ago
NaCl: Sodium Chloride
NCBI: National Center for Biotechnology Information
NGO: Non-Governmental Organization
nuDNA: Nuclear DNA
ONG: Organização Não Governamental
PAN: Planos De Ação Nacional
PCR: Polymerase Chain Reaction
PEG: Polyethylene Glycol
PNPD: Programa Nacional de Pós Doutorado
PP: Posterior Probability
RAD: Restriction Site Associated DNA
RADseq: RAD-Sequencing
RN: Rio Grande do Norte
SISBIO: Sistema de Autorização e Informação em Biodiversidade
SNPs: Single Nucleotide Polymorphisms
TI: *Trichechus inunguis*
TM: *Trichechus manatus*
UFMG: Universidade Federal de Minas Gerais
UICN: União Internacional para Conservação da
USA: United States of America

RESUMO

Duas são as espécies de peixe-boi encontradas nas Américas: *Trichechus inunguis* (peixe-boi amazônico), endêmico da bacia do Rio Amazonas e habitando apenas regiões de água doce, e *Trichechus manatus* (peixe-boi marinho) com maior distribuição, encontrado em áreas costeiras e alguns rios e lagoas próximos ao mar, da Flórida (EUA) até Alagoas (Brasil). O cruzamento entre elas ocorre na área de simpatria, resultando em híbridos e foi identificado em estudos anteriores, através de DNA mitocondrial (mtDNA) e microssatélites. Apesar de algumas discordâncias a espécie de peixe-boi marinho é dividida em duas subespécies: *T. manatus latirostris* (Peixe-boi da Flórida) e *T. manatus manatus* (peixe-boi das Antilhas), sendo a primeira restrita a Flórida e a segunda ocupando o restante da extensão. Tendo em vista esses fatos, o objetivo geral desta tese foi auxiliar o entendimento genético das relações interespecíficas e intraespecíficas dos peixes-bois americanos (*T. inunguis* e *T. manatus*), sendo dividida em três partes (capítulos). A primeira visou o consenso entre a divisão taxonômica tradicional e as unidades evolutivas, fornecendo informações aplicáveis a conservação, manejo e desenvolvimento de planos de conservação através da delimitação de linhagens mitocondriais por Generalised Mixed Yule (GMYC). O resultado foi a obtenção de três linhagens evolutivas correspondentes aos Clusters já identificados, que coloca em questão o isolamento do peixe-boi da Flórida e sugere a revisão taxonômica do peixe-boi brasileiro. A segunda parte visou caracterizar a composição genômica dos peixes-bois da Guiana Francesa, utilizando SNPs identificados por ddRAD, quatro genes nucleares (nuDNA) e a região controle (CR) do mtDNA. Além de identificar híbridos de várias gerações e reportar, pela primeira vez, a ocorrência de um peixe-boi amazônico em um rio na Guiana Francesa, fora da bacia amazônica, o capítulo destaca a importância ecológica desta região. No último capítulo foram sequenciados seis fragmentos nucleares (2.768 bp) e a CR do mtDNA (410 bp) de *T. inunguis* (n = 44), *T. manatus* do Brasil (n = 43), Flórida (n = 23) e Porto Rico (n = 5) e peixes-bois da zona híbrida (n = 15) para análises filogenéticas estatísticas. Os resultados confirmaram a separação da população brasileira de peixe-boi marinho (0,26 MYA) e não encontrou distinção entre as populações da Flórida e Porto Rico. Além de identificar que a zona híbrida possui uma população exclusiva, estimamos a migração bidirecional entre as populações marinha e fluvial e reconstruímos a formação da zona híbrida, indicando uma maior e mais antiga contribuição de *T. inunguis* ($\gamma = 0,583$) do que as linhagens analisadas de *T. manatus* juntas ($\gamma = 0,296$). Nossos resultados destacam a importância da população do Brasil devido a suas características exclusivas e a dinâmica única da zona híbrida, que provavelmente foi formada como um "dreno" entre todas as populações vizinhas a ela, gerando uma mistura única e bem adaptada ao ambiente estuarino entre a bacia amazônica e o rio Orinoco, diretamente influenciado pela pluma amazônica.

ABSTRACT

There are two manatee species found in the America: *Trichechus inunguis* (Amazonian manatee) native to the basin of the Amazon River and inhabiting only freshwater regions, and, the *Trichechus manatus* (West Indian manatee) with a larger distribution, from Florida (USA) to Alagoas (Brazil), being found in coastal regions and in some rivers and lagoons close to the sea. Hybrids between these two species have been identified, in their sympatric region, through mitochondrial DNA (mtDNA) and microsatellites data. Although there is some disagreement, the West Indian manatee is divided in two subspecies: *T. manatus latirostris* (Florida manatee) and *T. manatus manatus (*Antilles manatee) with the first restricted to the Florida region and the other inhabiting the rest of the area. In light of these facts, the general aim of this thesis is to help the genetic understanding of the interspecific and intraspecific relationships of the American manatees (*T. inunguis* and *T. manatus*). This work is organized in three parts (chapters). The first part focused on the consensus between traditional taxonomic division and the evolutive units, which used the Generalised Mixed Yule method (GMYC) to define the mitochondrial lineage which supplied important data to the policy plan, management and conservation. This resulted in three evolutive lineages, corresponding to the already identified clusters, therefore, questioning the isolation of the Florida manatee and suggesting a taxonomic revision of the Brazilian manatee. The second part of this work regards the genomic composition of the manatees from the French Guiana which are outside the Amazon River basin, and which highlights the ecological importance of this region. In the last part of this work, we sequenced six nuclear fragments (2.768 bp) and region control of mtDNA (410 bp) to implement statistical phylogenetic analysis on them. The samples for this analysis were obtained from *T. inunguis* (n = 44), *T. manatus* from Brazil (n = 43), Florida (n = 23) and Puerto Rico (n=5) and also manatees from the hybrid zone (n=15). The results indicated the separation of the Brazilian manatee population (0.26 MYA) and no distinction between the populations of Florida and Puerto Rico. It also identified that the hybrid zone has its own population, with a bidirectional migration estimated between the marine and the fluvial populations and reconstructing the hybrid zone, indicating a major and ancient contribution of *T. inunguis* lineage (γ = 0,583) and a lesser of the all analyzed *T. manatus* lineage together (γ = 0,296). Finally, our results reinforce the importance of the Brazilian population due to their exclusive characteristics and the peculiar dynamics of the hybrid zone, that probably was formed as a “drain” for all its neighboring populations, forming an unique and well adapted admixture to the estuarine environment, between the Amazon Basin and the Orinoco River, directly influenced by the Amazon plume.

INTRODUÇÃO

Sirênios

A ordem Sirenia (termo originado da mitologia grega Sereias), pertencente à classe Mammalia, compreende duas famílias existentes: Trichechidae (peixes-bois) e Dugongidae (Dugongos). Os animais dessa ordem são os únicos mamíferos aquáticos herbívoros e encontrados em latitudes tropicais e subtropicais, habitando principalmente rios e águas costeiras mais quentes (Berta et al., 2015). Os representantes dessa ordem pesam entre 250 e 700 kg, apresentam corpo rotundo com pelos raros, esparsos e finos. Suas caudas e nadadeiras são achatadas. Possuem uma mama atrás de cada nadadeira peitoral, na região axilar. Os crânios são relativamente pequenos quando comparados a outros mamíferos, além de possuírem olhos pequenos e orelhas externas reduzidas a minúsculos orifícios nas laterais da cabeça. As duas narinas, localizadas dorsalmente no final do focinho, permitem que esses animais respirem com o restante do corpo submerso. A característica mais marcante na face dos sirênios é o grande disco oral carnudo, que facilita a localização e a manipulação dos alimentos, mesmo em ambientes mais escuros (Feldhamer, 2014).

Esses animais consomem aproximadamente 8% de sua massa corpórea por dia em plantas. Para isso o sistema digestório é monogástrico do tipo fermentativo, que permite que esses animais associem de 45 a 80% dos alimentos. A morfologia facial e a dentição refletem suas estratégias de alimentação e dieta. Os dentes são perdidos ao longo da vida, quando desgastados, sendo substituídos no sentido caudo cranial por 5 a 7 dentes no maxilar superior e inferior, chegando a 30 substituições por quadrante da mandíbula (Marsh et al., 2012).

Família Dugonguidae

Apenas a espécie *Dugong dugon* (Dugongo) representa atualmente a família Dugonguidae, sendo que a Vaca marinha de Steller (*Hydrodamalis gigas*) foi extinta no final do século XVIII. Ambas possuem a cauda bi-lobada e nadadeiras achatadas e sem unhas (Berta et al., 2015). Os dugongos habitam áreas costeiras entre o Japão, Austrália e do sudeste Asiático até o Mar Vermelho, além de parte da costa leste Africana. Toleram temperaturas de no mínimo 19°C. Possui a pré-maxila grande e flexionada em direção ventral. A dentição é composta por um par de incisivos superiores em cada mandíbula, incisivos anteriores vestigiais, segundo incisivo exposto nos machos mas ocluso na pré maxila das fêmeas e dentes molariformes colunares com coroas simples e planas. Sua dieta é estritamente bentônica e estima-se que esses animais consumam aproximadamente 28 a 40 kg de algas por dia. A profundidade máxima de mergulho para alimentação, é de 33 metros com duração média de 10 minutos (Marshall et al., 2000; Marsh et al., 2004). Essa espécie é classificada como Vulnerável pela IUCN (Deutsch et al., 2008).

A vaca marinha de Steller foi extinta pela exploração humana no final do século XVIII. Eram os maiores representantes da ordem, medindo até 10 metros e pesando até 11.000 kg. Diferente dos outros representantes da ordem, eram os únicos que não habitavam águas tropicais, sendo encontrados no mar de Bering subártico, e possuíam placas mastigatórias queratinizadas no lugar de dentes (Anderson, 1995).

Família Trichechidae

As três espécies pertencentes à família Trichechidae são compreendidas em um único gênero (i.e., *Trichechus*) e são classificadas como Vulneráveis pela IUCN (2016). As espécies dessa família possuem diferenças entre si que estão principalmente relacionadas à sua distribuição geográfica e hábitat de ocorrência (Figura 1). O tempo de geração dos peixes-bois é de 20 a 30 anos (Deutsch et al., 2008; Diagne, 2015; Marmontel et al., 2016), sendo que a maturidade sexual ocorre em média aos 3 anos de idade e a expectativa de vida é de no máximo 60 anos (Marmontel et al., 1992; Marsh et al., 2012). Os peixes-bois são K-estrategistas, pois possuem um tempo de vida longo e reprodução lenta, com baixa fertilidade e longos períodos de interparto e cuidado parental, o que os torna ainda mais susceptíveis à interferência humana (Meirelles, Marmontel & Mobley, 2016). O tempo de gestação é de aproximadamente 330 dias gerando, em média, apenas um filhote (Berta et al., 2015). A relação entre a mãe e o filhote é a mais forte em peixes-bois, já que estes animais geralmente são solitários (Meirelles, Marmontel & Mobley, 2016).

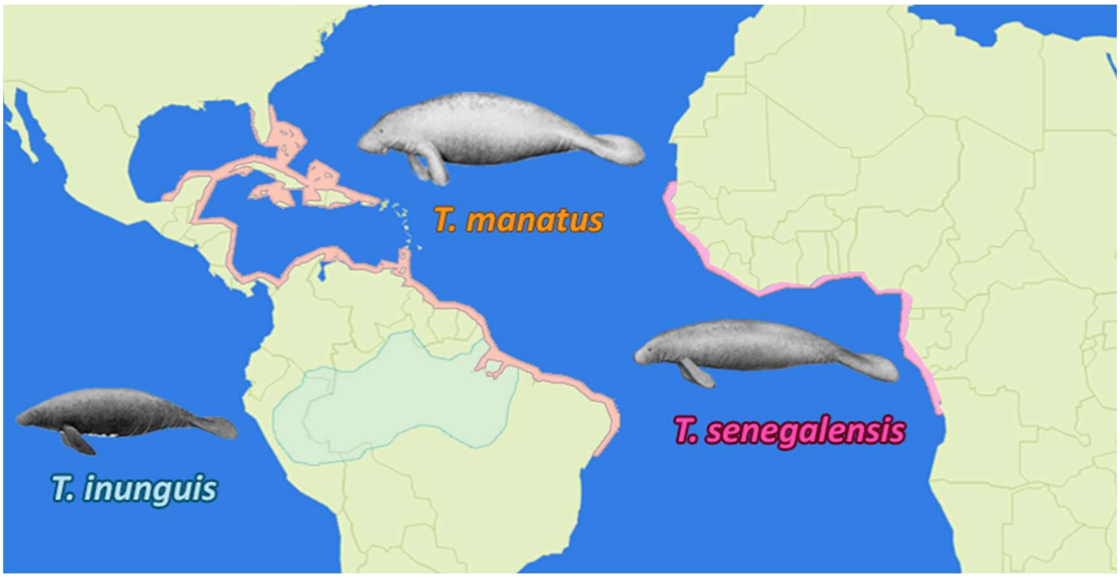


Figura 1. Distribuição das espécies de peixe-boi no mundo.

Trichechus senegalensis

O *T. senegalensis* (peixe-boi africano) é o menos estudado dos sirênios e pouco se sabe a respeito de sua biologia, sendo muitas vezes referido como o sirênio “esquecido” (Diagne, 2015). Esses animais apresentam morfologia externa semelhante ao peixe-boi marinho (*T. manatus*), porém seu corpo é menos robusto e seus olhos mais proeminentes (Figura 2). Também é encontrado em águas costeiras, rios, estuários e lagoas, distribuídos do Senegal ao norte da Angola, na África Ocidental, em águas com temperatura mínima de 18°C (Marsh et al., 2012; Parr et al., 2012) (Figura 1). Por serem muito negligenciados, apesar da caça de peixes-bois africanos ser proibida em toda sua área de ocorrência, sua exploração ainda é muito comum em países como Nigéria, Togo e Costa do Marfim para obtenção de pele, ossos e óleos usados na medicina tradicional e em rituais (Diagne, 2015).

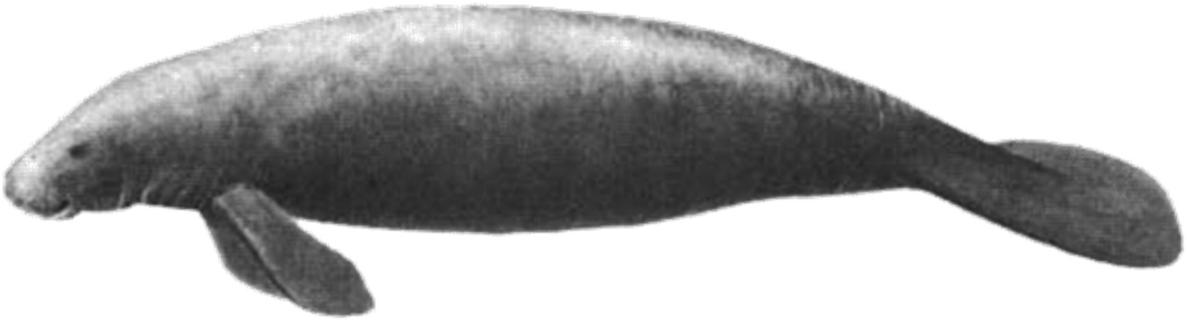


Figura 2. Peixe-boi Africano (Ilustração por P. Folkens de Berta et al., 2015).

Trichechus manatus

O *T. manatus* (peixe-boi marinho) pode medir de 3 a 5 m e pesar até 1620 kg. Apresenta coloração marrom-acinzentada, nadadeiras alongadas com unhas em suas extremidades e dentição composta de molares supranumerários substituíveis por toda a vida (Figura 3). Habita áreas costeiras e alguns rios e lagoas próximos ao mar, com temperatura mínima de 20°C, distribuindo-se da Flórida, Golfo do México, Mar do Caribe e Oceano Atlântico, das Antilhas até Alagoas no Brasil (Vianna et al., 2006; Parr et al., 2012) (Figura 1). Estudos com animais da Flórida reportam que essa espécie possui uma vascularização especial na córnea que provavelmente se trata de uma adaptação para movimentação entre os ambientes marinhos, estuarinos e manguezais, já que essa característica não é encontrada em nenhum outro grupo de mamíferos (Harper et al., 2005).

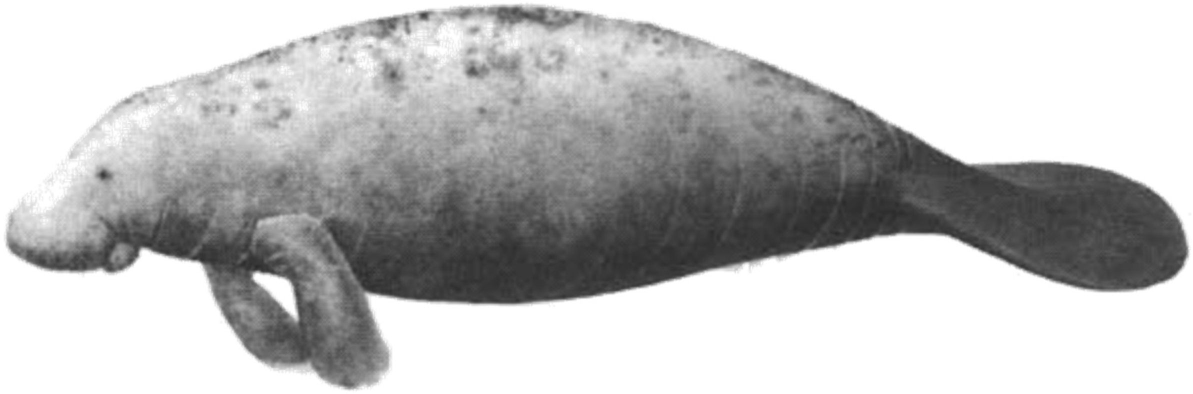


Figura 3. Peixe-boi marinho (Ilustração por P. Folkens de Berta et al., 2015).

Baseados em caracteres craniais quantitativos, Domning e Hayek (1986) dividiram a espécie do peixe-boi marinho em duas subespécies: *T. manatus latirostris* (Peixe-boi da Flórida) e *T. manatus manatus* (peixe-boi das Antilhas). Apesar da sua distribuição quase contínua nas Américas e da existência da subespécie restrita a Flórida, a população brasileira difere das demais por seu hábito predominantemente marinho, normalmente não habitando grandes extensões de água doce, adentrando a foz dos rios apenas para obtenção de água doce e alimentação, em algumas regiões, como mostrado na figura 4 (Meirelles, Marmontel & Mobley, 2016). Alguns autores, baseados em estudos genéticos e morfológicos, apontaram a necessidade de uma revisão taxonômica das subespécies (Vianna et al., 2006; Santos et al., 2016; Barros et al., 2017).

Até a década de 1990, as principais ameaças a essa espécie eram relacionadas à caça e captura acidental na pesca, em redes de emalhe e arpões, mas atualmente as causas são geralmente indiretas como encalhes, redução de habitat e ação antrópica (Deutsch et al., 2008). Apesar dos diversos programas de conservação, reintrodução e até mesmo translocação de peixes-bois que conseguiram aumentar o número de indivíduos em algumas localidades (Reynolds & Wetzel, 2008; UNEP et al., 2010; Service Fish and Wildlife, 2017), todas as populações, com exceção da Flórida, são tratadas como uma mesma unidade para conservação, sem levar em conta suas histórias evolutivas, diferenças genéticas e a existência de hibridização com a espécie amazônica (*T. inunguis*), fatores que podem causar depressão exogâmica nas populações, dificultando ainda mais sua conservação (Crandall et al., 2000; Vianna et al., 2006; Santos et al., 2016). No Brasil o peixe-boi marinho é assistido pelo Plano de Ação Nacional para a Conservação dos Sirênios (PAN Sirênios).

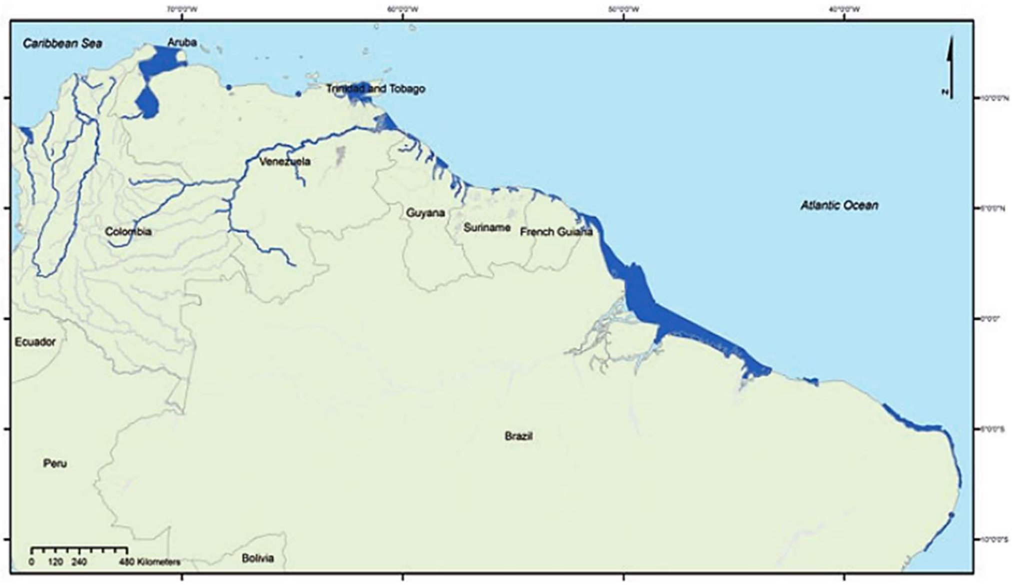


Figura 4. Distribuição do peixe-boi marinho (azul escuro) na América do Sul, incluindo rios, bacias e estuários. Os pontos azuis se referem a ocorrências isoladas de indivíduos da espécie (Meirelles, Marmontel & Mobley, 2016).

Trichechus inunguis

O *T. inunguis* (peixe-boi amazônico) é o menor representante da ordem Sirenia, com tamanho de 2,8 a 3,0 m e peso de até 450 kg. Possui coloração preta, podendo apresentar uma mancha branca na região peitoral. Suas nadadeiras não possuem unhas como os outros peixes-bois (daí seu epíteto específico) e seus dentes são menores, embora mais complexos e resistentes ao desgaste (Figura 5). É endêmico da bacia do Rio Amazonas e habita apenas o ambiente de água doce. Sua distribuição vai do Equador, Peru e Colômbia ao estuário no Brasil, preferindo temperaturas de 25°C a 30°C (Spiegelberger & Ganslosser, 2005) (Figura 1). A foz do Rio Amazonas ao redor da Ilha de Marajó é uma área de ocorrência comum entre as duas espécies americanas (*T. inunguis* e *T. manatus*) e, portanto, apresenta grande potencial de intercruzamento (Cantanhede et al., 2005; Vianna et al., 2006).

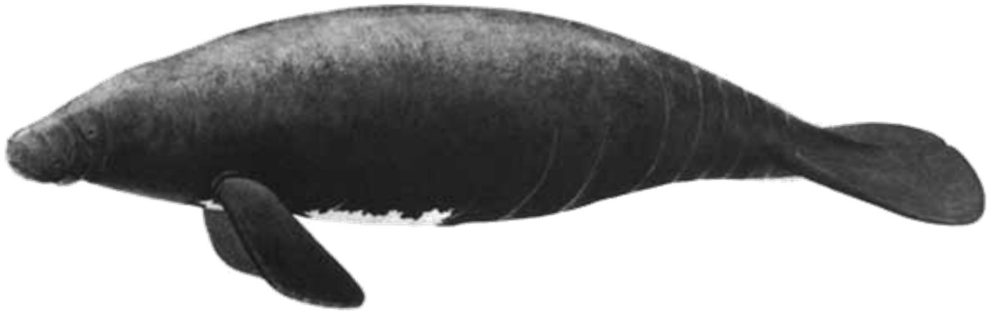


Figura 5. Peixe-boi amazônico (Ilustração por P. Folkens de Berta et al., 2015).

O peixe-boi amazônico é o mamífero aquático mais caçado do Brasil. O consumo da sua carne é tradição na Amazônia, sendo uma importante fonte de proteína animal do ribeirinho. Além disso, os impactos ambientais causados por mineradoras, garimpos e agropecuária também contribuem para a diminuição da população deste animal (Marmontel, Souza & Kendall, 2016). Iniciativas para conscientização ambiental e reabilitação de peixes-bois tem sido desenvolvida por instituições como o IDSM (Instituto de Desenvolvimento Sustentável Mamirauá), ICMBio (Instituto Chico Mendes de Conservação da Biodiversidade) e INPA (Instituto Nacional de Pesquisas da Amazônia), com o suporte do Plano de Ação Nacional para a Conservação dos Sirênios (Luna et al., 2011).

Evolução dos sirênios

A reconstrução da história evolutiva dos sirênios através de fósseis e dados morfológicos indica que os Protosirênios, primeiros representantes da ordem, chegaram ao litoral do continente americano no período Eoceno, sendo que a dispersão e o isolamento genético de suas populações deram origem a família Trichechidae (Gingerich & Cappetta, 2014). Os primeiros Trichechidae conhecidos, já exclusivamente aquáticos, foram divididos nas famílias Miorireninae (extinta) e Trichechinae. Todos os Trichechinae eram herbívoros e provavelmente os gêneros *Sirenotherium* (Brasil), *Patamosirenium* (Colômbia) e *Ribodon* (Argentina e Carolina do Norte, EUA) diversificaram-se entre o Mioceno e Plioceno. Provavelmente, até o final do Mioceno os Trichechidae ficaram restritos a América do Sul, habitando principalmente os rios costeiros e estuários, alimentando-se de plantas de água doce enquanto os Dungongidae habitavam as áreas do Atlântico Oeste e do Caribe, explorando as pradarias marinhas de plantas subaquáticas (Domning, 1982). O gênero *Trichechusteria* se originado entre o Plioceno e Pleistoceno.

Acredita-se que um grupo ancestral de *Trichechus* tenha adentrado a bacia Amazônica por uma passagem pelo Oceano Pacífico ou por alguma conexão do Caribe. O soerguimento dos Andes, entre o Mioceno e o Plioceno, mudou o curso da Bacia Amazônica e bloqueou a entrada para o mar acima da região amazônica (atual lago Maracaibo, na Venezuela formando temporariamente uma bacia fechada, fazendo com que grande quantidade de lodo e nutrientes escoasse para muitos rios sul-americanos, estimulando o crescimento de macrófitas aquáticas (principalmente gramíneas) (Hoorn et al., 2010). Os peixes-bois, isolados na bacia amazônica, provavelmente se adaptaram a essa fonte de alimentação e se diferenciaram mais tarde no atual *Trichechus inunguis* (Domning, 1982; Vianna et al., 2006). Já o *T. senegalensis* teria se originado pela dispersão de peixes-bois para a África durante o Plioceno ou Pleistoceno (Daryl P Domning, 1982; Vianna et al., 2006; Santos et al., 2016).

Estudos utilizando dados fósseis, genéticos e morfológicos indicam que o peixes-bois marinho e africano possuem um ancestral comum mais recente do que o peixe-boi amazônico (Domning, 1994; Springer et al., 2015). O primeiro estudo utilizando dados moleculares de DNA mitocondrial (mtDNA) de *T. inunguis* e de *T. manatus* estimou a divergência das duas espécies no Plioceno e demonstrou variações dentro da espécie de peixe-boi marinho, com a existência de três haplogrupos que não suportaram a existência das duas subespécies sugeridas em 1986 (Garcia-Rodriguez et al., 1998). Vianna e colaboradores (2006) através da região controle e Citocromo b do mtDNA suportaram o monofiletismo das espécies de *Trichechus* e dataram os nós ancestrais utilizando a divergência de nucleotídeos. Desta forma obtiveram datações de divergência dentro de cada espécie no Pleistoceno nos períodos de 621.000, 371.000 e 308.900 anos para *T. manatus*, *T. inunguis* e *T. senegalensis* respectivamente.

Estudos genéticos com peixes-bois

A composição cromossômica de*T. manatus* e *T. inunguis* foi estudada por alguns autores. Ambas as espécies diferem no número de cromossomos, sendo encontrados 56 cromossomos na espécie amazônica (Assis et al., 1988) e 48 na espécie marinha (White et al., 1976). Posteriormente, estudos genéticos e filogeográficos foram realizados utilizando mtDNA e microssatélites (Cantanhede et al., 2005; Vianna et al., 2006; Parr et al., 2012; Satizábal et al., 2012). Nestes estudos, as populações do peixe-boi amazônico demonstraram grande diversidade genética proveniente de um agrupamento de linhagens genéticas relacionadas, indicando uma origem recente e um sinal de expansão populacional nos últimos 120 mil anos (Vianna et al., 2006) que pode estar relacionada ao aparecimento de adaptações-chave que permitiram a sobrevivência desta espécie em ambiente amazônico (Cantanhede et al., 2005; Vianna et al., 2006).

Estudos feitos com peixes-bois marinhos (Garcia-Rodriguez et al., 1998, 2000; Vianna et al., 2006) abrangendo toda sua distribuição (Flórida, Caribe, Golfo do México e do Atlântico ao nordeste brasileiro) demonstram estruturação populacional por isolamento geográfico, formando duas unidades evolutivas significativas (ESU): uma composta por haplogrupos presentes da costa do Caribe e Golfo do México, da Venezuela à Flórida (EUA) e Antilhas e outra composta por apenas um haplogrupo restrito das Guianas ao Brasil (Figura 6) (Vianna et al., 2006; Parr et al., 2012). Essa separação de linhagens mitocondriais se deu, aparentemente, por processos vicariantes ocorridos durante as glaciações do Pleistoceno, com a diminuição do nível do mar e formação de barreiras pelas Antilhas, impedindo finalmente o contato entre as duas ESUs (Peltier & Fairbanks, 2006) há pelo menos 130 mil anos (Vianna et al., 2006). Posteriormente Barros et al. (2017) analisaram comparativamente peixes-bois marinhos do Brasil, Porto Rico, Flórida e outros indivíduos da América do Sul e Central, por bandeamento cromossômico CG e morfologia geométrica 3D dos crânios. Estes autores encontraram uma maior diferença dos peixes-bois marinhos entre Brasil e as demais populações. Como o peixe-boi da Flórida não apresentou diferenças morfológicas e citogenéticas em relação aos demais, através dessas análises a autora sugeriu a revisão taxonômica das subespécies de *T. manatus*, o que reforçou estudos preliminares com dados moleculares (Garcia-Rodriguez et al., 1998; Vianna et al., 2006).

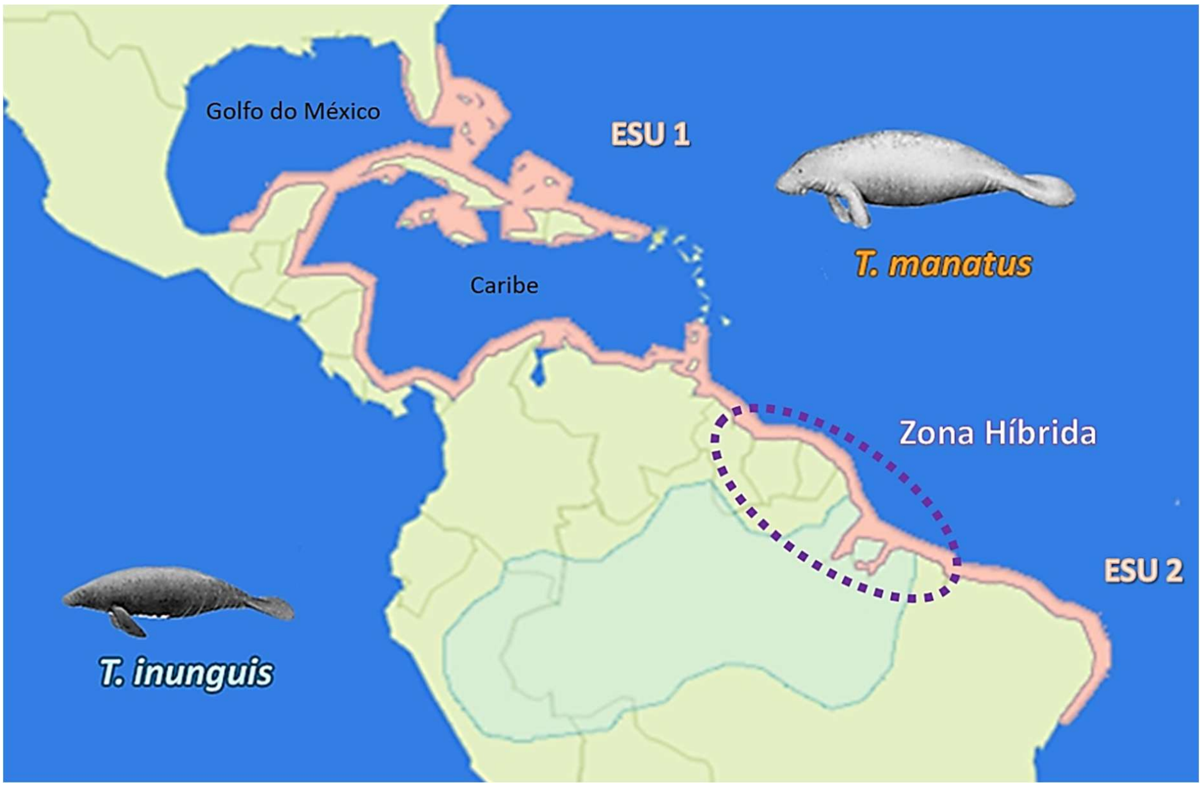


Figura 6. Distribuição das espécies americanas de peixe-boi e provável zona híbrida (elipse azul). Também são apresentadas as áreas de distribuição das duas unidades evolutivas significativas de *Trichechus manatus*: ESU-1 (Caribe e Golfo do México: Venezuela aos EUA e Antilhas) e ESU-2 (Atlântico: Brasil e Guianas).

O cruzamento entre peixe-boi amazônico e marinho, resultando em híbridos, foi identificado em um estudo realizado por Vianna e colaboradores (2006) com marcadores mitocondriais, microssatélites autossômicos e citogenética de 189 indivíduos de *T. manatus*, 93 de *T. inunguis* e seis de *T. senegalensis*. Foram identificados sete indivíduos híbridos de *T. manatus* e *T. inunguis* na zona costeira, sendo três na Guiana Francesa, três na Guiana e um no estado brasileiro do Amapá, todos morfologicamente identificados como *T. manatus*, porém possuidores de mtDNA de *T. inunguis*. Outro indivíduo híbrido foi identificado no Rio Amazonas, próximo a Belém (Pará), com morfologia de *T. inunguis* e mtDNA de *T. manatus*. Um dos indivíduos híbridos (da costa do Amapá) teve seu cariótipo estudado e apresentou 2n=50, número intermediário entre o cariótipo de *T. inunguis* (2n=56) e *T. manatus* (2n=48). Esses dados evidenciaram a ocorrência de, pelo menos, uma segunda geração de introgressão (F2), pois um híbrido com 2n = 50 pode ser filho de uma fêmea híbrida F1 (2n=52- *T. manatus* (macho) x *T. inunguis* (fêmea)) com outro macho *T. manatus*.

A hibridização na conservação

O naturalista britânico Charles Darwin (1876) em seu consagrado livro “A origem das espécies” já tentava entender os processos de hibridização e a presença de barreiras reprodutivas entre espécies. Desde então, a ocorrência de híbridos na natureza é uma questão amplamente discutida por diversos autores na área da biologia evolutiva, que buscam compreender as implicações ecológicas e taxonômicas das espécies envolvidas (Schwenk, 2008). A área de contato entre populações ou espécies distintas onde ocorre hibridização, independentemente da fertilidade da prole híbrida inicial (F1), é chamada “zona híbrida” (Allendorf et al., 2001). A incorporação de genes de uma espécie (ou população) através de um híbrido fértil que também hibridizou com uma das espécies (ou populações) parentais (≥ F2) é denominada “introgressão”. Por sua vez, uma população formada apenas por indivíduos de diversas gerações de introgressão, não dependendo de fluxo gênico com as parentais para sua manutenção, é chamada “hybrid swarm” (Mallet, 2005; Shurtliff, 2013).

Apesar de ser um processo que ocorre naturalmente em diversos grupos – como plantas, peixes (Schaefer et al., 2016), anfíbios (Fitzpatrick & Shaffer, 2007), aves (Ottenburghs et al., 2017) e mamíferos (Shurtliff, 2013) – a hibridização é um processo muito complexo e está associada tanto a impactos positivos quanto negativos para as espécies envolvidas (Allendorf et al., 2001). Quando é parte natural do processo evolutivo, a longo prazo, a introgressão contribui fortemente para a geração de diversidade e especiação (Kronforst et al., 2013), sendo muito frequente, principalmente em aves (Grant, 2015). Porém, quando a hibridização é influenciada por ações antropogênicas através de introdução intencional ou modificação do hábitat, pode resultar em processos deletérios como depressão exogâmica, competição (Bar-Zvi et al., 2017) e até extinção (Rhymer & Simberloff, 1996).

Os híbridos constituem uma das questões mais desafiadoras para a proteção e manejo das espécies, sendo ainda mais difícil quando há o envolvimento de espécies ameaçadas (Wayne & Shaffer, 2016). Para o desenvolvimento de estratégias e políticas de conservação, além de entender os processos de hibridização, é necessário compreender o contexto histórico e ecológico desses organismos, determinando se as causas foram naturais ou antropogênicas (Stronen & Paquet, 2013). Levando-se em conta a particularidade de cada caso e as controvérsias envolvidas, as políticas públicas devem ser flexíveis o suficiente para se adequarem a cada caso (Stronen & Paquet, 2013), fazendo com que alguns autores se preocupassem em desenvolver diretrizes (Allendorf et al., 2001) e fluxogramas para auxiliar nas tomadas de decisão (Jackiw et al., 2015). Ferramentas genéticas têm cada vez mais contribuído para identificar e interpretar esses eventos (Wayne & Shaffer, 2016; Martin & Jiggins, 2017).

A genética na conservação

Nas últimas décadas, ferramentas genéticas têm sido utilizadas no delineamento de ações para conservação de populações e espécies ameaçadas, com o uso de dados de variantes moleculares que podem ajudar a reconstruir tempos históricos e diversos eventos populacionais (Fraser & Bernatchez, 2001). Essas ferramentas vêm sendo comumente utilizadas para delimitações de planos e unidades de conservação, através da análise de diferentes marcadores genéticos que podem responder perguntas específicas e testar hipóteses (Funk et al., 2012). Estimativas de diversidade dentro e entre as populações, a quantificação de fluxo gênico ou isolamento, permitem por exemplo, inferir o padrão da estruturação das populações, identificando agrupamentos ou separações entre populações da mesma espécie, sugerindo em certos casos até a separação em subespécies ou sinonimização de espécies consideradas distintas (Allendorf et al., 2010).

O uso de marcadores mitocondriais têm sido aplicado para análises filogenéticas e estudos populacionais intraespecíficos a fim de descrever histórias relacionadas às linhagens maternas, devido ao fato que o mtDNA é virtualmente haplóide, herdado uniparentalmente (matrilinear) e estável, sendo composto de vários genes, sem sequências repetitivas, íntrons ou pseudogenes, porém com rápida evolução (Kocher et al., 1989). Marcadores microssatélites no DNA nuclear, têm sido usados para testes de paternidade, parentesco, estudos populacionais recentes, fluxo gênico interpopulacional, análises de *linkage* e mapeamento genômico, não sendo indicando processos adaptativos ou de médio e longo prazos (Tautz, 1989). Já o uso de sequências nucleares em genes autossômicos pode auxiliar na detecção de eventos mais lentos na evolução e processos adaptativos, incluindo divergências interespecíficas, permitindo também a análise detalhada de processos complexos como hibridização e especiação (Thomas et al., 2003).

Justificativa e objetivos

Devido à importância dos processos de hibridização e introgressão para a evolução e conservação das populações, tanto das espécies parentais quanto dos híbridos, a ocorrência desse evento se torna ainda mais relevante quando ocorre em espécies ameaçadas (Allendorf et al., 2001), como é o caso dos peixes-bois. Apesar da identificação desse fenômeno já ter sido relatada anteriormente (Garcia-Rodriguez et al., 1998; Vianna et al., 2006), a falta de estudos genéticos mais completos, que utilizem marcadores genéticos com histórias evolutivas distintas e ferramentas mais atuais, restringe muito a compreensão da dimensão real da hibridização e do seu impacto, o que demanda a realização de análises filogeográficas multilocus, englobando dados nucleares (genômicos) e mitocondriais das duas espécies e suas populações. Adicionalmente, esses mesmos dados podem subsidiar na resolução das controvérsias a respeito da existência das duas subespécies de *T. manatus* e da delimitação de suas populações, que são de extrema importância para a conservação e o manejo da espécie.

Desta maneira, o objetivo geral desta tese foi auxiliar o entendimento genético das relações interespecíficas e intraespecíficas dos peixes-bois americanos (*T. inunguis* e *T. manatus*), tendo como objetivos específicos:

- Selecionar regiões gênicas diagnósticas para identificação efetiva das espécies em questão.
- Identificar a composição genômica dos indivíduos da zona híbrida, através da identificação da origem específica dos fragmentos nucleares e mitocondriais, estimando suas proporções e identificando possíveis eventos de introgressão.
- Estimar as populações existentes nas duas espécies americanas, com inclusão da zona híbrida e de *T. manatus* do Brasil, Flórida e Porto Rico.
- Selecionar o modelo que melhor explique as relações entre as populações identificadas, estimando seus tempos de divergência, a existência e direção do fluxo gênico, diversidade genética e tamanho efetivo populacional.

Apresentação da tese

Esta tese está estruturada em três capítulos em forma de artigos científicos, estando os dois primeiros já submetidos a revistas científicas para análise por pares e publicação. Também são apresentados, em anexo, um capítulo de livro publicado e uma Short Communication submetida, ambos a convite de editores durante o doutorado.

No primeiro capítulo desta tese é realizada uma discussão das unidades de manejo dos peixes-bois marinhos e da existência de duas subespécies (*T. m. manatus* e *T. m. latirostris*) estabelecidas por Domning & Hayek (1986a) a fim de sugerir um consenso entre a divisão taxonômica tradicional e as unidades evolutivas para delimitar populações e fornecer informações mais precisas para a conservação, o manejo e o desenvolvimento de políticas efetivas. Para isso foi feita uma pequena revisão sobre os principais estudos com *T. manatus*, que obtiveram resultados relevantes a respeito da estruturação populacional, e sobre a importância da comunicação e entendimento entre ciência e políticas conservativas. Também foi realizada uma análise que se utiliza de árvores gênicas de mtDNA para delimitação de linhagens mitocondriais de maneira quantitativa (Generalised Mixed Yule - GMYC).

O segundo estudo teve como foco a população da zona híbrida entre *T. inunguis* e *T. manatus* identificada em estudos anteriores (Garcia-Rodriguez et al., 1998; Vianna et al., 2006). Feito em parceria com o Dr Benoit de Thoisy (ONG Kwata e Institut Pasteur de la Guyane, Guiana Francesa, França), Dra Camilla Mazzoni (Leibniz Institute for Zoo and Wildlife Research Berlin e Berlin Center for Genomics in Biodiversity Research, Alemanha) e Dra Sibelle Villaça (Trent University, Peterborough, Canadá), este estudo teve como objetivo caracterizar a composição genômica dos peixes-bois ao longo da costa da Guiana Francesa através de SNPs identificados por ddRAD (realizado na Universidade Livre de Berlin) e sequenciamento de genes nucleares e da região controle de mtDNA (obtidos no Laboratório de Biodiversidade e Evolução Molecular - UFMG). Com os dados obtidos, foram realizadas análises da composição genômica da população híbrida e simulações de hibridização que permitiram entender a magnitude da hibridização nessa área e discutir hipóteses ecológicas e evolutivas desse evento e sua implicação para conservação. Neste artigo também foi demonstrada pela primeira vez a ocorrência de peixe-boi amazônico (*T. inunguis*) em um rio na Guiana Francesa, fora da bacia amazônica.

Por fim, o terceiro capítulo traz um estudo mais abrangente das dinâmicas populacionais entre *T. inunguis* e *T. manatus*, incluindo a zona híbrida. O trabalho contou com a colaboração de pesquisadores como o Dr. Benoit de Thoisy (ONG Kwata e Institut Pasteur de la Guyane), Dra Miriam Marmontel (Instituto de Desenvolvimento Sustentável Mamirauá), Dra Fernanda Loffler Niemeyer Attademo (ICMBio) e Dra Ana Carolina Oliveira Meirelles (AQUASIS), sendo toda a parte genética e de filogeografia estatística desse estudo realizada no Laboratório de Biodiversidade e Evolução Molecular da UFMG. Para tal, as sequências de seis genes nucleares e da região controle do mtDNA foram analisadas a fim de caracterizar os híbridos, a estruturação populacional, fluxo gênico entre populações (e espécies), filogenia e tempo de divergência das espécies, considerando os eventos de introgressão e hibridização.

CAPÍTULO 1

Submitted manuscript to Marine Mammal Science

Looking for a consensus on intraspecific management units of West Indian manatees and its implication to conservation: a mini-review

CAMILLA S. LIMA, Laboratório de Biodiversidade e Evolução Molecular, Departamento de Biologia Geral, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil;

RAFAEL F. MAGALHÃES, Programa de Pós-Graduação em Zoologia, Departamento de Zoologia, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil;

FABRICIO R. SANTOS,[1] Laboratório de Biodiversidade e Evolução Molecular, Departamento de Biologia Geral, ICB, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, CP 486, 31270-010 Belo Horizonte, MG, Brazil.

[1] Corresponding author (e-mail: fsantos@icb.ufmg.br).

The Sirenia Order is composed by the Dugongidae and Trichechidae families. Trichechids are represented only by the *Trichechus* genus, with tree species: *T. manatus* (West Indian manatee), *T. inunguis* (Amazonian manatee) and *T. senegalensis* (African manatee), being all at risk of extinction. The West Indian manatee is the most widely distributed species (Deutsch et al., 2008), found along tropical coastal waters from Florida (USA) to Alagoas (Brazil), for which many efforts are made to preserve all of its geographical range (Normande et al., 2015; Ortega-Argueta & Castelblanco-Martínez, 2018). Two *T. manatus* subspecies are traditionally recognized: *T. m. latirostris*, restricted to the Florida peninsula, and *T. m. manatus*, found in the remaining areas where the species occurs (Domning & Hayek, 1986a). Previous studies allowed to characterize intraspecific variation within *T. manatus*, testing limits of subspecies and/or ESUs by evaluating morphological and genetic data (Table 1), but with no consensus.

The concept of subspecies is a matter of controversy (Guia & Saitoh, 2007), but many zoologists use this term to designate geographical varieties within species (Mayr, 1982). To Inger (1961) and Mayr (1982), this is not well applied, although it has some advantages to describe some particular taxa. With the advent of genetic techniques, some studies tried to define subspecies as differentiated populations in the process of speciation (quasi-species) or with different adaptations to particular environments (Ryder, 1986). To avoid the misuse of different subspecies concepts for conservation purposes, geneticists coined another intraspecific concept, called Evolutionary Significant Units (ESUs) (Ryder, 1986). As in the case of subspecies, this has also undergone modifications and is currently used to identify intraspecific populations that show limited gene flow between each other and relative evolutionary independency (Fraser & Bernatchez, 2001), mostly using phylogeographic methodologies (Moritz, 1994a; Palsbøll et al., 2006). However, in some cases where populations could not be classified as separate ESUs, but warrant conservation attention for any other reason, they could still be considered management units (MU) (Moritz, 1994a; Palsbøll et al., 2006). The identification of intraspecific units of management using subspecies, ESUs, and MUs criteria frequently results in controversy, sometimes involving political and economic considerations, more than biological reasons, where the intraspecific classification is definitive for the social appeal and financial investments (Gippoliti & Amori, 2007).

An important management practice for reducing the risk of extinction of threatened species, if well applied, can use individual translocations between areas (Pérez et al., 2012), where intraspecific lineages are taking into account by decision makers. This was important, for example, in the case of the population of Rocky Mountain bighorn sheep (*Ovis canadensis*) located in the National Bison Range (NBR; Montana, USA). Two separate translocations from sheep populations selected with genetics information reversed some local deleterious effects of bottleneck and inbreeding (Hogg et al., 2006; Miller et al., 2012). On the other hand, wrong management decisions can worse the conservation status of populations that are already endangered, as the initial reintroduction of the Arabian oryx (*Oryx leucoryx*) in the Oman Sanctuary (Pérez et al., 2012). The reintroduced individuals derived from captivity represented a small number of founding animals, resulting in inbreeding in many cases, but other individuals were originated from differentiated populations, whose intercross resulted in outbreeding depression with low fitness offspring (Marshall & Spalton, 2000). For these reasons, the identification of intraspecific divisions is so important, providing data for the diagnostics of interpopulation divergence and local adaptations, and the possible deleterious outcomes of outbreeding in future management.

The International Union for Conservation of Nature (IUCN) developed some management criteria considering information of geographic distribution, population data, species characteristics that may interfere with its response to environmental changes, associated threats, and existing conservation measures (IUCN, 2012). These criteria can be used by governments to define priority populations and species for conservation (MMA, 2014). However, many recent studies discuss the gap between conservation research and management practice, where genetic data are usually considered superficially in policy documents (Shafer et al., 2015; Cook & Sgrò, 2017; Taylor et al., 2017). Indeed, non-academics that make final decisions have difficulty to link fundamental research and real-world application (Hogg et al., 2017; Britt et al., 2018). Thus, it is undoubted the necessity to translate the scientific data into policy decisions, but another important point is reaching a consensus on the information provided in order to guide decisions more directly (Pressey et al., 2017).

The first studies describing *T. m. latirostris* as a subspecies restrict to Florida have taken into account only morphological data with a very limited sampling of data and individuals (Hatt, 1934; Domning & Hayek, 1986a, 1986b). Posteriorly, genetics data were generated for all West Indian manatee populations, obtaining contradictory results and interpretations (Table 1). The most complete phylogeographic studies, with a representative distribution of *T. manatus*, described tree mtDNA clusters (Garcia-Rodriguez et al., 1998; Vianna et al., 2006). Based on the mtDNA phylogeny and distribution, two ESUs were suggested: the first composed by the populations from Florida to Venezuela and West Indies (Caribbean ESU), and the second with eastern populations of Brazil and the Guianas coastline (Atlantic ESU), where the latter area revealed to be an interspecific hybrid zone with *T. inunguis* (Vianna et al., 2006; Santos et al., 2016). The West Indian manatee population structure was probably shaped by environmental instability mediated by Quaternary changes in ocean levels, making barriers to gene flow dynamic through time, which were likely reinforced by a more recent hybrid barrier (Vianna et al., 2006; Parr et al., 2012; Santos et al., 2016; Barros et al., 2017) (Vianna et al., 2006; Parr et al., 2012; Santos et al., 2016; Barros et al., 2017).

The appropriate management of ESUs should consider inbreeding and outbreeding depression impacts, taking the metastable balance between these consequences into account, to avoid the decreasing of fitness because of high homozygosis or rupture of population-specific adaptations, respectively (Joly, 2010; UNEP et al., 2010; Hedrick & Garcia-Dorado, 2016). For example, consider the initial plan of re-establishment of the manatee population in Guadeloupe Island (France), Lesser Antilles. In this Caribbean island, the species was extinct for many decades, and translocation of Brazilian manatees to there was initially considered (Reynolds & Wetzel, 2008; UNEP et al., 2010). Because Caribbean populations are historically isolated from Brazilian manatees (about 500 thousand years, according to Santos et al., 2016), the reintroduction of Brazilian individuals together other Caribbean manatees from captivity (Mexico, Singapore etc) in this area would disrupt a natural history of many thousands of years, which could lead to a likely outbreeding depression (Moritz, 1999). As observed by Edmands & Timmerman (2003) a wrong transfer of only one migrant per generation into a population of 100 individuals could cause the same or more damage (outbreeding depression) as a one-time 50:50 mixture. To make information clear and help the decision-makers, widely known and supported genetic data (such as mtDNA) can be used to better delimiting populations for conservation and management (Moritz, 1994b; Reid & Carstens, 2012).

To test more objectively the existence of significant intraspecific phylogenetic divisions, researchers began to use the Generalised Mixed Yule Coalescent (GMYC) (Mello et al., 2018), a tree-based method for delimitation of lineages. The GMYC model determines the point (or points) on a gene tree that represents the threshold between tokogenetic relationships and cladogenetic events (Pons et al., 2006; Fujisawa & Barraclough, 2013). For this reason, we used the GMYC to objectively define the intraspecific mtDNA lineages of *T. manatus*, providing clues for an interpretation of the ESU status of the populations from phylogenetic trees. As a practical consequence, our results will serve as support for the development of conservation policies considering the evolutionary history of the species. For this, we estimated a mtDNA gene tree using sequences from the control region (Garcia-Rodriguez et al., 1998; Vianna et al., 2006), including two new haplotypes, M5 found in Brazil and J2 found in French Guiana (unpublished data), and using *T. inunguis* as outgroup. The model of nucleotide substitution (*i.e.*, HKY+Γ) was selected through the corrected-Akaike Information Criterion implemented in jModeltest 2 (Darriba et al., 2012). Additionally, the null hypothesis of equal evolutionary rates through tree was rejected ($p < 0.001$) through a maximum likelihood (ML) test of molecular clock implemented in MEGA7 (Kumar et al., 2016). Therefore, we generated ultrametric gene trees of unique haplotypes, assuming the selected substitution model, the lognormal uncorrelated clock, and the Birth-Death tree prior, as implemented in BEAST2 software (Bouckaert et al., 2014), using 5 x 107 generations and thinning 1 x 104. The analysis was run twice and the stationarity of each replicate and the convergence between them was evaluated visually in Tracer v 1.6 (Rambaut et al., 2018). Both replicates were combined in LogCombiner (Rambaut & Drummond, 2010a) after a burn-in of 5%, with a total of 1 x 104 saved trees. Finally, the maximum clade credibility (MCC) tree was annotated using the TreeAnnotator software (Rambaut & Drummond, 2010b).

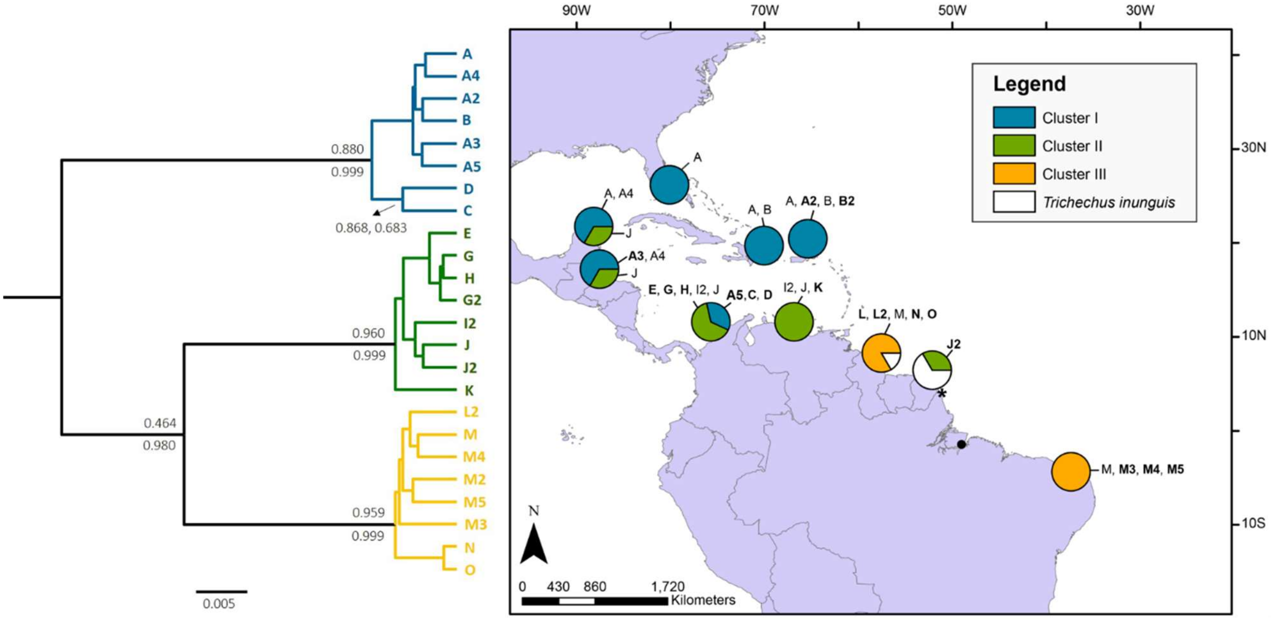


Figure 1. *Trichechus manatus* mtDNA lineages and distribution. Left, the maximum clade credibility (MCC) tree. The posterior probability (PP) of each clade is show below the nodes, and above them is represented the PP of each lineage estimated under the bGMYC model, which takes the phylogenetic uncertainty associated to the MCC into account. Right, the map of haplotype composition, with the percentage of each cluster in sampling countries. The haplotypes of each region are discriminated in pie charts, with the exclusive ones represented in bold. The haplotype L was reported by (Garcia-Rodriguez et al., 1998) in Guyana, but its sequence is not available or deposited in GenBank. To visualize the abundance of each of these haplotypes see references in table 1. The black asterisk and circle points represent two

isolated records of hybrids in Brazil: the first found close to the Oiapoque River presenting a *T. inunguis* haplotype, and another found in the Amazon River close to mouth, which has a *T. manatus* haplotype (M2).

The mtDNA lineage delimitation per se was made in two steps. In the first, we submitted the MCC tree to a ML GMYC version, using the single threshold algorithm implemented in the splits package of R software (Fujisawa & Barraclough, 2013; R Core Team, 2017). In this analysis, the null hypothesis of a single lineage on the tree was rejected (LR test = 17.23; $p < 0.001$). The optimal number of mtDNA lineages was three, with a confidence interval from three to six: Cluster I represented by the haplotypes A, A2, A3, A4, A5, B, C and D; Cluster II represented by E, G, G2, H, I2, J, J2 and K; and Cluster III represented by L2, M, M2, M3, M4, M5, N and O. In the second step, we submitted the remaining 9,999 trees to a Bayesian version of the GMYC model, implemented in the bGMYC package (Reid & Carstens, 2012; R Core Team, 2017), in order to account the phylogenetic uncertainty associated to topology differences and variance in branch lengths. The posterior probability (PP) of the delimited lineages was < 0.95 only for the Cluster I. This probably is due to the uncertainty of the positioning of the haplotypes D and C, that show PP = 68.3% in the MCC tree (Figure 1).

In short, our GMYC analysis confirms the three clusters previously reported (Table 1; Garcia-Rodriguez *et al.*, 1998a; Vianna *et al.*, 2006; Santos *et al.*, 2016) as independent mtDNA lineages with high probability. However, it is observed a sympatric occurrence of some mtDNA haplotypes and clusters (I and II), and currently a major vicariance is found between Caribbean (Florida to Venezuela and Antilles) and Atlantic (Brazil), where along the Guianas coastline appears an interspecific hybrid zone with the *T. inunguis* species. This result supports the interpretation of cyclical isolation-recontact dynamics during Pleistocene due to the variation on sea level, which formed the Antilles Arc, which likely isolated the Caribbean Sea and Mexico Gulf (until Florida) from northwestern Atlantic coast of South America (Peltier & Fairbanks, 2006; Vianna et al., 2006; Santos et al., 2016), and was further reinforced by a hybrid zone in the Guianas coastline.

Curiously, Florida manatees present only the A haplotype that displays a large distribution, occurring also in Mexico, Dominican Republic and Puerto Rico, which is part of mtDNA Cluster I that extends from Colombia to Florida (Figure 1). Besides, Cluster II mtDNA haplotypes are distributed from Mexico to Venezuela, and show overlay with Cluster I, making all manatees from Florida, Caribbean and Mexico Gulf highly connected between them, in accordance also with the karyotypic and morphological data (Barros et al., 2017). This shows that even though microsatellite data show a small but significant differentiation between Florida and Belize (Hunter et al., 2010), and Puerto Rico (Hunter et al., 2012), all belong to the same Caribbean ESU. Anyway, populations from Mexico, Belize and Colombia display some unique haplotypes, which may indicate local differentiation and make these areas also important management units to conservation. On the other hand, the Brazilian manatees present only Cluster III mtDNA haplotypes, and almost all haplotypes are exclusive (Figure 1), composing the Atlantic ESU. This, added to the distinctness in karyotype and shape of the skull in relation to all other populations from Florida, Mexico Gulf and the Caribbean (Barros et al., 2017), points to the need for reassessment of taxonomic status of the Brazilian population. Besides, when Barros et al. (2017) analysed the skulls of individuals from Suriname and Guyana, they observed that their skulls were more similar to the Caribbean populations (including Florida), even though they are geographically closer to the Brazilian ones. Additionally, it is important to highlight that Guyana and French Guiana manatees present introgression of *T. inunguis* genes (Garcia-Rodriguez et al., 1998; Vianna et al., 2006; Santos et al., 2016). This region between the Amazon River mouth and the Guianas coastline is a hybrid zone between the two American manatee species, which apparently reinforces the isolation and differentiation of Brazilian manatees in comparison to the other ones (Santos et al., 2016).

Currently, the southernmost limit of the species distribution is in Brazil, where the population show some peculiar characteristics, beyond the already discussed isolation, and morphological and genetic differentiation. Contrary to other West Indian manatee populations, the Brazilian manatees are predominantly marine (and they are called sea manatees in Portuguese), not entering large streams of fresh water in Brazil (Meirelles et al., 2018). Besides that, despite the wide distribution of the seagrass Halodule wrightii in all coastal America, only in Brazil this aquatic plant is most preferred in the manatees diet, especially in the states of Ceara and Alagoas, where the diet is mainly constituted of *H. wrightii* and algae (Ciotti, 2012; Meirelles et al., 2018). However, in the northern Brazilian states (north of Paraiba), the diet of manatees is mainly constituted by macroalgae and terrestrial plants from estuaries and mangroves, but not typical fresh water plants like other manatees feed (Meirelles et al., 2016, 2018). That distinct diet specialization, in addition to morphological and molecular differentiation, indicates that Brazilian manatees should be considered a top priority for conservation. Since the Brazilian government agencies usually disregard the use of ESUs for conservation management (ICMBio, 2012), we suggest considering this population at least in the same level as subspecies, and emphasize the need for a taxonomic review by the multidisciplinary specialists, which should have a direct impact on conservation policies regarding this population, and the whole distribution of West Indian manatees.

Based on the data presented here we raise some discussion about real cases of management. The genetic data with microsatellites of Florida manatees (Hunter et al., 2010, 2012) could be used to support the status of this population as MU, and considering their increasing population size (Service Fish and Wildlife, 2017), they could be considered in translocations for localities of Puerto Rico, Dominican Republic, and even Guadeloupe, as manatee source to increase genetic diversity (Tucker et al., 2012). Indeed, West Indian manatees were recently reclassified from Endangered to Threatened under the Endangered Species act of 1973, for presenting estimates of about 13,000 individuals throughout its range, particularly because of conservation efforts in Florida (USA), where population has increased to 6,350, and remaining animals are distributed in all other territories, and only 700 manatees along 4,000 km of coasts where it occurs in Brazil (Service Fish and Wildlife, 2017). Thus, the increase in the number of West Indian manatees was punctual and do not represent the reality of all populations, particularly in Brazil.

Along the extensive Brazilian coastline, manatees present no clear structuration, even though few individuals from Maranhão and Piauí coasts presented another single nucleotide variant of mtDNA Cluster III haplotypes (Luna et al., 2012). However, the mtDNA haplotype distribution along the Brazilian coast suggest a west to east gradient, with no reasonable point to be managed separately from the rest of the country as previously suggested (Luna et al., 2012), and they could be translocated to other parts in Brazil. On the other hand, the translocation of Brazilian manatees (and others from the hybrid zone) to Guadeloupe Island (Reynolds & Wetzel, 2008) is not recommended, because this could imply in extrinsic outbreeding depression caused by a likely rupture of local adaptations (Allendorf et al., 2001). Thus, there is an urgent need for consensual intraspecific science-based delimitation within *Trichechus manatus* (such as MU, ESU and/or subspecies), once remarkably different populations are still considered the same subspecies (Caribbean and Brazilian) and some closely related lineages are managed separately as different subspecies (Florida, Mexico Gulf and Caribbean). The review of these designations would provide greater understanding and clarity in conservation policies for the West Indian manatee as a whole.

Notwithstanding we recognize the necessity of genomic data to evaluate the historical and recent gene flow patterns between ESUs and MUs. Additionally, an essential research program should focus on assessing the local adaptation potential of each lineage and how the admixture between them can lead to outbreeding depression or alleviate the impacts of inbreeding. Nevertheless, the concordance between molecular and morphological data indicates that the most significant intraspecific division within *T. manatus* separates two ESUs, one from coasts in Brazil (Atlantic) and another (Caribbean) from other populations found in the Caribbean, Mexico Gulf and Florida (USA) coasts, making the unique and isolated Brazilian ESU a priority for taxonomic research and conservation policy.

ACKNOWLEDGEMENTS

CSL was supported by a CAPES (Brazil) fellowship, RFM by a PNPD (CAPES) fellowship, and FRS by a CNPq (Brazil) research fellowship. This project was performed under ICMBio/SISBIO permit 45028 and received funding support from FAPEMIG, CNPq and Fundaçãoo o Boticario from Brazil.

Table 1. Summary of the main studies that aimed to delimit West Indian manatee units for conservation.

Reference	Populations analyzed	Types of data analyzed	Main results	Authors' interpretation
Hatt, 1934	Puerto Rico, Honduras, Guatemala and Florida	Osteological characters of skull and bones	The adult manatees of the western Caribbean and Gulf of Mexico appear to be slightly longer snouted than corresponding specimens from Florida. The shape and size of scapulae are different between Florida and Puerto Rico manatees.	Suggestion that the geographic extremes of *T. manatus* are racially distinct, and one such from Florida, is recognized as subspecies, named *T. m. latirostris*.
Domning & Hayek, 1986	Florida, U.S. Gulf Coast west Florida, West Indies and (sic) Central or South America	Linear cranial measurements tested by discriminant analysis.	Differences between the measurements of rostrum, zygomatic arches and skull roof, mandible, occipital region and palate and dentition.	Confirmed the interpretation of Hatt (1934), diagnosing T.m.manatus and T.m.latirostris, which constitute morphologically distinct geographic populations
Garcia-Rodriguez et al., 1998	Florida, Mexico, Dominican Republic, Puerto Rico, Colombia, Venezuela, Guyana and Brazil	Control region of mtDNA	Identification of 15 haplotypes (mtDNA). Three distinctive mtDNA haplogroups were observed in intraspecific phylogeny, corresponding approximately to: (i) Florida and the West Indies; (ii) the Gulf of Mexico to the Caribbean rivers of South America; and (iii) the northeast Atlantic coast of South America. They provided the first report of putative hybrids between *T. inunguis* and *T. manatus*, in Guiana.	The three mtDNA haplogroups are discordant with the previous subspecies designation.
Vianna et al., 2006	Florida, Puerto Rico, Dominican Republic, Mexico, Belize, Colombia, Venezuela, Guyana, French Guiana and Brazil	Control region and cytochrome b of mtDNA, seven microsatellite loci and cytogenetic analysis	Identification of 20 haplotypes (mtDNA) and support to three distinctive mtDNA lineages previously found by García-Rodriguez (1998). Detection of a genetic distance from Guyana and Brazil, comparing with Colombia and Venezuela populations, showing the probably historic separation caused by the Lesser Antilles	Proposal of the existence of two evolutionary significant units (ESUs), east and west of the Lesser Antilles barrier, with the eastern ESU (Guyana and Brazil) resulting from an expansion following a bottleneck in the late Pleistocene.
Hunter et al., 2010	Belize and Florida	Control region of mtDNA and 16 microsatellite loci	The Belize population and Florida subspecies appear to be unrelated with microsatellite and mitochondrial FST values.	Subspecies designations are supported and SSR data, suggesting low vagility throughout the northern Caribbean habitat.
Hunter et al., 2012	Puerto Rico and Florida	Control region of mtDNA and 15 microsatellite loci	Highly divergent allele frequencies between Florida and Puerto Rico were identified in both microsatellite and mtDNA data. Bayesian cluster analyses from SSR detected two populations and no admixture or recent migrants between Florida and Puerto Rico.	The level of genetic differentiation between these populations supports Puerto Rico as a distinct evolutionary lineage, with independent management considerations.
Luna et al., 2012	Northeast region of Brazil	Control region of mtDNA	A new haplotype (M4) identified only in Maranhão and Piauí.	The need for caution in manatees management to or from this area, because of the potential to cause outbreeding depression.
Barros et al., 2017	Puerto Rico, Belize, Guatemala, Honduras, Nicaragua, Costa Rica, Guyana and Suriname, Brazil and Florida	Cranial morphometric (Landmark-based 3D geometric) and karyologic (chromosome G- and C-banding) techniques.	A morphological discontinuity was noted within *T. m. manatus*, with the southernmost Brazilian population discriminated from the Caribbean T. m. manatus and from *T. m. latirostris* (USA). The Brazilian population of west Indian manatee, when compared with the remaining ones, exhibited morphological divergence similar to those found between the Amazonian (*T. inunguis*) and African (*T. senegalensis*) species.	All results strongly indicate that the taxonomy of *T. manatus* needs to be revised, supporting preliminary evidence from molecular data and contrast with the currently accepted subspecies designation. Also shows that Brazilian manatees need to have their management and conservation strategies reviewed.

CAPÍTULO 2

Submitted manuscript to Estuarine, Coastal and Shelf Science journal (Revision requested)

Manatee genetics supports a special conservation area along the Guianas coastline under the influence of the Amazon River plume

Authors: Sibelle T. Vilaça[1,2,6#], Camilla S. Lima[3#], Camila J. Mazzoni[1,2], Fabricio R. Santos[3*], Benoit de Thoisy[4,5*]

[1]Leibniz Institute for Zoo and Wildlife Research, Department of Evolutionary Genetics, Berlin, Germany. [2]Berlin Center for Genomics in Biodiversity Research, Berlin, Germany. [3]Laboratório de Biodiversidade e Evolução Molecular, Departamento de Biologia Geral, ICB, Universidade Federal de Minas Gerais, Av. Antonio Carlos 6627, CP 486, 31270-010 Belo Horizonte, MG, Brazil. [4]Institut Pasteur de la Guyane, 97300, Cayenne, French Guiana, France. [5]Kwata NGO, 97300 Cayenne, French Guiana, France. 6 Current address: Trent University, Peterborough, ON, Canada.

#both authors contributed equally to this work.

*corresponding authors: benoit@kwata.net, fsantos@icb.ufmg.br

ABSTRACT

The West Indian manatee (Trichechus manatus) occurs along the Atlantic coastline and adjacent freshwater systems of South, Central and North America, from Alagoas (Brazil) to Florida (USA) and Greater Antilles. The Amazonian manatee (Trichechus inunguis) is the only sirenian adapted exclusively to freshwater and endemic to the Amazon River basin. Previous studies have reported hybrids between T. inunguis and T. manatus close to the mouth of the Amazon River, composing a likely extensive hybrid zone along the Guianas coast of South America under the influence of the Amazon River plume. We have used ddRAD SNP data, and sequences of nuclear and mtDNA loci to characterize the genomic composition of manatees along the French Guiana coastline. We found this population to be formed by introgressed or later generation interspecific hybrids, and also describe the first T. inunguis found outside the Amazon River basin. Our results indicate that T. inunguis can survive in the Amazon River plume and have colonized independent water streams of the Guianas coastline where they likely hybridize with T. manatus. This hypothesis offers a plausible explanation for the known extension of the hybrid zone between the two species along the Guianas coastline. It also reinforces the importance of the Amazon plume, which flows westwards to the Guianas coastline and favors the dispersion of the freshwater species. This habitat functions as a large estuary-like system that provides an ecological continuum from the Amazon River mouth to the disconnected waterflows of the Guianas, which deserves a status of a special conservation area.

Keywords: *Trichechus*, hybridization, hybrid zone, population genomics, ddRAD, Amazon River plume

INTRODUCTION

Manatees (*Trichechus spp.*) are charismatic and vulnerable aquatic species that can be found in the Americas (*T. manatus* and *T. inunguis*) and coastal regions of Africa (*T. senegalensis*) (Deutsch et al., 2008). The West Indian manatee, *T. manatus,* can live in both salt and freshwater environments. It is found in rivers and lake systems along its coastal distribution, with exception of the population inhabiting the northeastern coast of Brazil. This population prefers coastal and estuarine habitats and has never been reported in any of the large streams, like São Francisco and Parnaíba rivers (Meirelles et al., 2018). Previous observations have recorded *T. manatus* up to 1100 km upstream in the Orinoco River (Castelblanco-Martinez et al., 2003) and up to 650 km in the Magdalena River in Colombia (Montoya-Ospina et al., 2001). Conversely, the Amazonian manatee (*T. inunguis*) is highly specialized to freshwater environments and has only been reported in the Amazon River basin so far.

French Guiana (FG), an overseas department of France in South America, has a stable manatee population, allegedly belonging to the *T. manatus* species, estimated to have between 50 and 250 mature individuals (UICN France et al., 2017). The main threats to the FG manatee population are poaching, bycatches and degradation of coastal water quality (UICN France et al., 2017). Despite a significant manatee population decrease was reported during the last century, recolonization of some areas has been observed (de Thoisy et al., 2003). Due to small population sizes, few takes per year by local fishers are enough to suggest a regional threatened status (UICN France et al., 2017). Sighting records are widespread in FG, both in dense and well-preserved mangroves and on the lower parts of all river systems. Important seasonality in habitat preference has been noticed in relation to strong seasonal fluctuations in the relative contributions of marine and continental waters to the estuarine waters (Castelblanco-Martínez, dos Reis, & de Thoisy, 2017). However, FG manatees also make use of large freshwater streams, with records up to 100 km inland (UICN France et al., 2017).

Previous studies involving *T. manatus* populations (Garcia-Rodriguez et al., 1998; Vianna et al., 2006; Santos et al., 2016) have identified three deeply divergent mitochondrial DNA (mtDNA) clusters composing two Evolutionary Significant Units (ESUs) that occupy two major geographic regions: Caribbean coast of Antilles, South and Central America, Mexico Gulf coast and Florida (USA) (clusters I and II); and the northeastern Atlantic coast of South America (Brazil and the Guianas), where cluster III was exclusively found (Vianna et al., 2006). However, seven manatees found along the Guianas coastline (corresponding to Guyana, French Guiana and the state of Amapá, Brazil) and one manatee found in the Amazon River close to the mouth were reported to be hybrids with mixed morphology and unexpected mtDNA lineages for their habitat (Garcia-Rodriguez et al., 1998; Vianna et al., 2006). Among these hybrids, three manatees from FG were morphologically classified as T. manatus (presence of nails, absence of white patch on the chest), but presented mtDNA haplotypes typically found in T. inunguis (Vianna et al., 2006). Besides, microsatellite data confirmed the hybrid status of four of the eight manatees (two from FG and two from Brazil), and a single individual from Amapá (Brazil) presented a karyotype 2n = 50, an intermediary number between *T. inunguis* (2n = 56) and *T. manatus* (2n = 48). It indicated that this individual was a likely second-generation hybrid, a male offspring between a female F1 hybrid and a male *T. manatus* (Vianna et al., 2006). Due to the supposedly high prevalence of hybrids along the Guianas coast, this region located west of the Amazon River mouth was suggested to be a hybrid zone where *T. manatus* and *T. inunguis* individuals may contact and produce hybrids, further isolating the two *T. manatus* ESUs (Santos et al., 2016).

Hybridization has been recognized as a widespread phenomenon among animals, and it plays an important role in species evolution (Mallet, 2005). In endangered species, hybridization is often a consequence from declining population numbers due to anthropogenic activities (Fitzpatrick et al., 2015). When populations of closely related species – that have overlapping distribution and can still interbreed – decrease in size and consequently mating partners are rare, species hybridization is a likely outcome. Hybridization and introgression can have negative effects for a population (e.g. outbreeding depression), and the rise of hybrid individuals can further complicate the conservation status of a species, and policies to protect it. If hybrid frequency is shown to be increased due to anthropogenic population decline of parental species, other alternatives should be discussed to avoid further decline of rare parental species and populations. Otherwise, in populations where hybridization is a natural phenomenon and does not pose a threat to the non-hybrid populations, improved management strategies can be developed to ensure the protection of hybrids. However, hybrids are usually not taken into consideration in conservation regulation and plans (Stronen and Paquet, 2013), and therefore the individuals and their habitat may not be eligible to be protected (but see Allendorf et al., 2001). Therefore, understanding hybridization events (i.e. their extension in space and time, ecological factors, population size, survival rates, etc.) and their causes (i.e. anthropogenic *versus* natural) is fundamental so that hybrids can be considered for appropriate conservation efforts.

In this study, we used a genomic approach to (i) investigate a previously recognized hybrid zone between manatee species, in order to (ii) understand the extent of hybridization, and (iii) to discuss ecological and evolutionary hypothesis for their occurrence and conservation implications.

METHODS

Sampling

Tissue material from eight manatees from FG were collected from stranded animals, found dead on beaches and/or in mangroves along the coast. If the carcasses were not too degraded, phenotypical information was collected, such as presence of nails, skin color and extant of white patches on the chest and belly, expected to differentiate between West Indian and Amazonian manatees. Kidney and liver samples were obtained from a dead *T. manatus* individual from the Tierpark Berlin Zoo (here referred as "Tierpark"), a born in captivity and descendant of maternal grandparents from Georgetown, Guyana, and other unknown ancestors (Bell, 2001). Two *T. inunguis*, two *T. manatus* and a likely F2 hybrid from Amapá, Brazil (Vianna et al., 2006) were also included.

DNA extraction and Sanger sequencing

DNA extractions were performed using the Dneasy Blood and Tissue kit (Qiagen). The mtDNA D-loop region was amplified using the primers L15926 and H16498 (Kocher et al., 1989). The amplification cycle followed Vianna et al. (2006), with annealing temperature of 55°C. Nuclear genes (Apolipoprotein B - APOB, Amyloid precursor protein - APP, BMI1 proto-oncogene – BMI1, and CAMP Responsive element modulator – CREM), were amplified following Murphy et al. (2001) (Table S1). New primers for the APOB gene were designed using as reference the *T. manatus* GenBank sequence JN413954 (Meredith et al., 2011). For all loci, the PCR mix was prepared with final volume of 25 µL, containing 1X Taq reaction buffer; 1.5 mM of $MgCl_2$; 200 µM of dNTPs; 0.5 µM of each primer; 0.5 U of Platinum Taq DNA Polymerase (Thermo Fisher Scientific). PCR products were purified by the polyethylene glycol (PEG) method (20% PEG 8000, 2.5 M NaCl) (Sambrook et al., 1989) and sequenced on the ABI 3130xl Genetic Analysis (Applied Biosystems) using the BigDye Termination v3.1 Cycle Sequencing kit. The same primers used for amplification were used for DNA sequencing. Chromatograms were analyzed in *SeqScape 2.6* and consensus sequences were aligned using the *ClustalX* (Larkin et al., 2007) algorithm in the software *MEGA* 6 (Tamura et al., 2013). The relationships between haplotypes were inferred with the software *NETWORK* (Fluxusengineering) using the median-joining algorithm (Bandelt et al., 1999). Summary statistics were calculated in *DNAsp 5* (Rozas et al., 2003).

Double-digest RADseq

Double-digest RAD (ddRAD) libraries were prepared following a previous protocol (Peterson et al., 2012) with adapter modifications (Meyer and Kircher, 2010), including two *T. inunguis* from Brazil, five manatees from French Guiana, and a *T. manatus* from Tierpark Zoo (Germany). In brief, 1 µg of genomic DNA was digested with the enzymes *Mse*I and *Eco*RI at 37 °C for at least two hours in a reaction volume proportional of DNA. The ligation of adapters was performed immediately after the digestion. Inline barcodes of varying sizes (5 to 9 bp) were added to the P5 adapter. The adapters and overhangs matching the enzymes were added. A sixteen-cycle indexing PCR was then performed, in which one of the 50 barcodes described by Meyer and Kircher (2010) was added to the P7 adapter. Each product was cleaned with 1.5X AMPure XP magnetic beads and quantified in the Qubit 2.0 using the dsDNA HS assay (Life Technologies) and checked in the Agilent 2100 Bioanalyzer (Agilent Technologies). The indexed samples were then equimolarly pooled in two libraries and selected by size in the BluePippin using the 1.5% cassette with R2 marker (Sage Science). We selected the sequences in a range between 350-400 bp, including adapters, and checked each library with the Agilent 2100 Bioanalyzer High Sensitivity DNA chip. No PCR was performed after the samples were pooled to avoid chimera formation (DaCosta and Sorenson, 2014). The libraries were then characterized using the KAPA SYBR® FAST kit (Kapa Biosystems) and run on the in-house Illumina NextSeq 500 using a 300-cycle mid output v2 kit or on the in-house MiSeq with a 300-cycle kit.

Illumina reads were first demultiplexed according to P7 indexes. The software *ipyrad* 0.7.19 (Eaton, 2014) was used to demultiplex the P5 inline barcodes, trim the adapters and quality trimming. Since samples from FG were in an advanced state of decomposition, we first estimated the contamination level in each sample. A subsample of 20,000 merged sequences was randomly extracted and blasted against the NCBI nt database. BLAST results were visualized in *MEGAN6* (Huson et al., 2007) and levels of bacterial contamination were evaluated. Samples that remained in the analysis after evaluating the levels of contamination were submitted to a stepwise filtering process. First, all reads were blasted (BLASTN) against the Florida manatee genome (GenBank Accession Number GCA000243295) and to the African elephant genome (GCA000001905). Sequences with blast hits below an e-value threshold of 10e-50 were retained. Subsequently, reads with no hits to either genome were blasted against a *Clostridium noyi* genome (see Results for reasoning) with a similar threshold.

The remaining reads were finally blasted against a database of 8344 bacterial genomes and all reads presenting hits below the threshold were excluded from the clustering step. Clustering and SNP calling were performed in *ipyrad*. We used the following parameters: minimum coverage for a cluster: 8; clustering threshold: 0.90; data type: pairddrad; minimum samples in a final locus: 6. The remaining parameters were left as default.

Putative hybrid individuals used for the ddRAD analysis were originally from the Guianas region where the hybrid zone was reported (Vianna et al., 2006). As controls we used two Amazon manatees (*T. inunguis*) from Brazil (Table 1) and a West Indian manatee (*T. manatus*) represented by a Florida manatee (*T. manatus latirostris*) genome (Foote et al., 2015). The consensus sequences of each RAD locus were mapped to the Florida manatee genome using *bowtie2* (Langmead and Salzberg, 2012) in order to identify the homologous regions. In parallel, all raw reads of the Florida manatee genome available in GenBank were mapped back to the genome contig sequences, also using *bowtie2*. Resulting SAM files were analyzed using customized python scripts for the identification of alleles at the target loci. The Florida manatee genotypic information was then added to the ddRAD results for subsequent analyses. To generate a dataset of unlinked SNPs, we restricted the data analysis to only the first SNP per locus. Only bi-allelic SNPs were selected. All SNPs unique to the Florida manatee were excluded from the analysis.

The software *STRUCTURE 2.3.4* (Pritchard et al., 2000) was used to identify hybrids and possible different genetic groups. A total of twenty replicates were performed for each value of K from 1 to 5 (burn-in period: 20,000, 2 x 10^6 iterations) under the admixture model and the assumption of correlated allele frequencies among populations. The USEPOPINFO model was used to specify the two parental species (POPFLAG=1) by assigning the two *T. inunguis* samples from Brazil and the *T. manatus* from Florida, USA as learning samples, while ancestry was estimated for all FG samples. The software *CLUMPAK* (Kopelman et al., 2015) was used to summarize and visualize the results. The best value of K was estimated using the Evanno method (Evanno et al., 2005) implemented in the online tool Structure Harvester (Earl and voHoldt 2012). The observed heterozygosity (Hobs) was estimated using the R package *adegenet 2.1.0* (Jombart and Ahmed, 2011). The number of differences between samples were estimated in the software *Arlequin 3.5* (Excoffier and Lischer, 2010).

The proportion of *T. manatus* ancestry among hybrids was assessed using a maximum-likelihood estimator of hybrid index that calculates genome-wide admixture based on the proportion of alleles inherited from each parental species (Gompert and Buerkle 2009). The R package *INTROGRESS* (Gompert and Buerkle 2010) was used to estimate the hybrid index for all FG samples. The same samples assigned as learning samples in STRUCTURE were assigned as parentals.

Hybridization simulations using species-specific SNPs between the two-parental species were performed using the command *hybridize* in *adegenet* (Fig. S1). Only fixed SNPs (species-specific, n=359) between the two *T. inunguis* from Brazil and *T. m. latirostris* were used. Two mating scenarios for the FG population were simulated, backcrosses between hybrids and *T. manatus*, and crossings between hybrids. For the “Backcross” scenario, the first crossing (or generation) was between a simulated F1 hybrid and *T. manatus* (F1xTm), the second backcrossing was between the result of the first crossing and *T. manatus* ((F1xTm)xTm). The same rationale was repeated for seven generations. For the “Hybrid” scenario, the simulated F1 hybrids were crossed with other F1s (F1xF1), the resulting hybrids were crossed among them (F2xF2), until (F7xF7). The reasoning behind the two scenarios was to test if the FG population is composed by hybrids that mate predominantly with pure *T. manatus* (“Backcross”), or if the hybrid population tends to mate with another hybrids (“Hybrid”). Given the admixture results from STRUCTURE, no backcross with *T. inunguis* was simulated (see Results). In both tested scenarios, seven generations of crossings were simulated with five hybrid offspring per generation. Simulated hybrids were analyzed as described previously and results were compared with putative hybrid samples.

RESULTS

Sanger sequencing

A total of 410 bp of mtDNA D-loop and 1,919 bp of nuclear DNA was sequenced, although four samples (M348, M827, M2108 and M2772) were partially sequenced for some Sanger sequenced nuclear genes (Table 1). Alleles were considered as species-specific based on population studies using larger samples of both species (Vianna et al., 2006; Santos et al., 2016, and unpublished data). Summary statistics and diagnostic polymorphisms for all sequenced loci can be seen in Tables S2 and S3.

Sample	Sampling location	D-loop	Nuclear	SNPs	Overall classification
M113	Paranã do Castanho, AM, Brazil	Ti (T)	Ti (0/8)	Ti	Ti
M117	Tefé, AM, Brazil	Ti (Q2)	Ti (0/8)	Ti	Ti
M22 JAG	Awala, French Guiana	Tm (J4)	Introgressed (7/1)	Introgressed (0.75)	Introgressed
M297 JAG	Awala, French Guiana	Ti (T2)	Tm (8/0)	Introgressed (0.76)	Introgressed
M348 JAG	Awala, French Guiana	Ti (T2)	-	-	?
M423 JAG	Mana, French Guiana	Tm (J4)	Introgressed (7/1)	Introgressed (0.77)	Introgressed
M524 JAG	Kourou, French Guiana	Ti (T2)	Tm (8/0)	Introgressed (0.77)	Introgressed
M827 JAG	Riviere des Cascades, French Guiana	Ti (T2)	Ti (0/2)	Ti (0.02)	Ti
M2108 JAG	Marina de Dégrad des Cannes, French Guiana	Ti (T2)	-	-	?
M2772 JAG	Kaw River, French Guiana	Ti (T2)	Tm (6/0)	-	Introgressed
Tierpark	Tierpark zoo, Berlin, Germany	Tm (M)	Introgressed (7/1)	Introgressed (0.73)	Introgressed
M035	Oiapoque, AP, Brasil	Ti (T)	Introgressed (6/2)	-	Introgressed
M042	Baía Formosa, RN, Brazil	Tm (M)	Tm (8/0)	-	Tm
M047	Beberibe, CE, Brazil	Tm (M)	Tm (8/0)	-	Tm

Table 1. Sampling location and main results obtained for each sample. mtDNA D-loop haplotypes and Sanger sequenced nuclear haplotypes belonging to each species (*T. manatus*/*T. inunguis,* respectively) are shown in parenthesis. The column SNPs shows genomic proportion of *T. manatus* for each sample estimated from species-specific SNPs in *INTROGRESS*. Based on all molecular markers, the column "Overall classification" shows the classification of each sample. Abbreviations: Ti *T. inunguis*, Tm *T. manatus*.

Trichechus manatus and *T. inunguis s*amples from Brazil had both the mitochondrial and nuclear DNA consistent with their morphological classification and habitat, except for sample M035 (Table 1) from Amapá state (close to the frontier with French Guiana), which is a likely second-generation hybrid previously characterized by mtDNA, microsatellite and karyotype data (Vianna et al., 2006). Most of the samples from FG had mixed nuclear and mtDNA from both species (Table 1). Surprisingly, a new D-loop mtDNA haplotype (J04), related to the *T. manatus* cluster II (Fig. S1), was found in two FG samples (M22 and M423). All five FG samples with *T. inunguis* mtDNA had the haplotype T2, previously described in FG samples as hybrids (Vianna et al., 2006). Remarkably, a FG manatee (M827) found in a tributary (Riviere des Cascades, 40 km from the river mouth at sea) of the Cayenne River had both *T. inunguis* mtDNA and Sanger sequenced nuclear genes (Table 1), which suggests a non-hybrid individual. The analysis of Sanger sequenced nuclear genes, also indicates that the *T. manatus* from Tierpark Zoo, which is maternally descendant from a manatee from

Guyana (in the Guianas coastline), presents 1 out of 8 haplotypes derived from *T. inunguis*, thus a likely later generation hybrid born in captivity.

Most FG manatees were found dead in an advanced state of decomposition, but phenotypic information was obtained for two individuals. The individual M423 had a large white patch on its breast (Fig. S2) and no nails on its flippers, both characteristic of *T. inunguis*. The sample M524 had no nails but did not exhibit a white patch. Thus, both individuals presented phenotypes indicative of *T. inunguis* ancestry.

ddRAD

A total of 30,486,215 paired sequences was obtained for eleven *Trichechus* samples. Due to the elevated number of reads compared to other samples, a subsample of 2 million reads was used for sample M524. Eight samples presented a small number of reads with similarities to bacterial genomes (<1%), indicating a negligible contamination by opportunistic organisms. One sample (M827) had a high presence of bacterial reads, especially from the genus *Clostridium* (Fig. S3), and due to the large number of reads we were able to filter out bacterial sequences and include this sample in further analysis. On the other hand, three samples (M2772, M2108, M348) were mostly composed of bacterial sequences, primarily from the genus *Clostridium* and were excluded from further analysis. Within the *Clostridium* species, we identified the following potentially pathogenic species: *C. perfrigens*, *C. botulinum*, *C. novyi*, *C. tetani*. Even though previous studies have associated *C. perfrigens* with myotities and cutaneous abscesses in marine mammals (Buck et al., 1987; Hartmann 1997), clostridial bacteria are normal inhabitants of the manatee gut (Merson et al., 2014) and common in dead animals (Hyde et al., 2013). Another potentially pathogenic bacteria found in our samples include: *Klebsiella* sp., *Edwardsiella* sp., *Staphylococcus* sp., *Acinetobacter* sp., *Pseudomonas* sp., and *Vibrio* sp (Hartmann 1997; Higgins 2000). Despite the presence of putative pathogenic bacteria, we cannot confidently associate these bacteria as a cause of death in our samples.

After filtering for bacterial reads, samples had an average of 1,379,455 paired reads, although three samples had significant numbers of small fragment carry-over (DaCosta and Sorenson, 2014). All samples had overlapping fragments between 210-260 bp, and therefore all analyses were conducted within this range. Coverage depth varied greatly between clusters (Table S4), with many loci sequenced at low depth. Despite passing the paralog filter in *ipyrad*, five loci blasted in two separate regions of the *T. m. latirostris* genome and were excluded. The final dataset consisted of 947 clusters (or RAD loci) and 2287 SNPs. Genotypic data from the *T. m. latirostris* genome was obtained for 900 RAD loci and had a total of 118 unique SNPs.

A total of 720 bi-allelic unlinked SNPs was used in the *STRUCTURE* analysis. The preferred K was 2 (Fig. 1A, Fig. S4). The results are in accordance with the four sequenced autosomal loci, the FG sample M827 is a non-hybrid *T. inunguis*, the sample from Tierpark shows a small *T. inunguis* component, and all FG samples showed admixture between the two parental species (Fig. 1A and 1B). With K=3, all FG with *T. manatus* ancestry exhibit third group represented by the purple component. This substructure of FG manatees is in accordance with previous studies (Vianna et al., 2006) that showed differentiation between the Florida manatee and manatees from Brazil and the Guianas. When analyzing only the species-specific SNPs, the preferred K was 3 (Fig S4). The *STRUCTURE* results were largely concordant between the two datasets, with the exception for Tierpark that showed a larger *T. inunguis* component for the species-specific SNPs dataset.

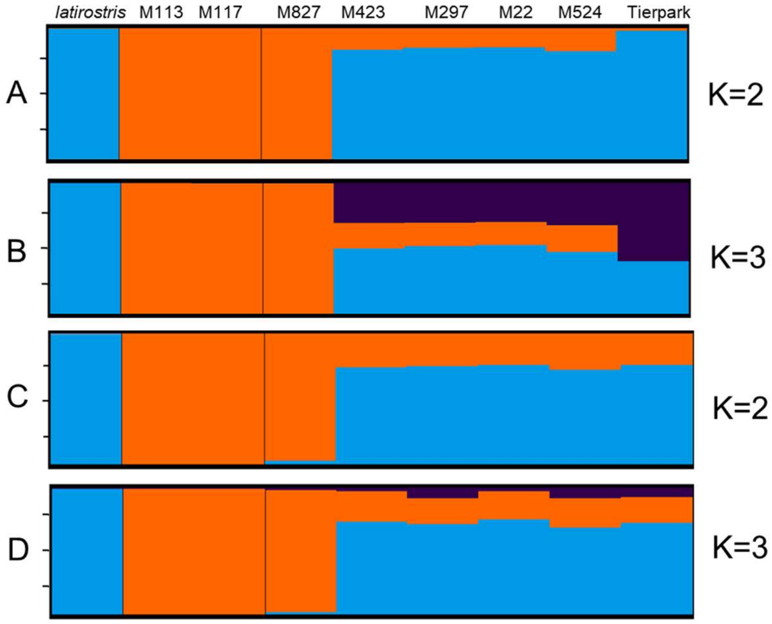


Figure 1. *STRUCTURE* results for unlinked SNPs. Results for 720 unlinked SNPs with K=2 (A) and K=3 (B). Results for 359 species-specific SNPs with K=2 (C) and K=3 (D). In all analysis, *T. m. latirostris*, and the *T. inunguis* samples M113 and M117 are used as “learning” samples.

The differences within samples (Table 2 and Table S5) showed a lower diversity in *T. inunguis* and *T. manatus* (Florida) when compared to *T. manatus* (Tierpark Zoo). Hybrid samples from FG were more similar to *T. manatus* from Tierpark (with maternal ancestors from Guyana) than from Florida (*T. m. latirostris*). The newly identified *T. inunguis* from FG (M827) was more closely related to *T. inunguis* from Brazil than to other FG samples, although it presents the typical mtDNA haplotype found in FG hybrids (Table 1).

	M827	M423	M297	M22	M524	*T. manatus* (Tierpark)	*T. inunguis*	*T. manatus* (Florida)
M827	24	73	77	74	83	86	0	134
M423	0.62	64	0	0	0	13	101	35
M297	0.67	0.00	52	0	1	17	102	36
M22	0.61	0.00	0.00	70	0	10	95	29
M524	0.61	0.00	0.01	0.00	84	8	109	37
T. manatus (Tierpark)	0.70	0.19	0.25	0.14	0.10	49	108	41
T. inunguis	0.00	0.77	0.79	0.75	0.76	0.80	21	157
T. manatus (Florida)	0.90	0.50	0.55	0.43	0.45	0.60	0.90	6

Table 2. Pairwise differences between manatee samples considering the 720 unlinked SNPs. Both *T. inunguis* samples from Brazil were considered as one group. Upper diagonal: Corrected pairwise differences between samples; Diagonal: Number of polymorphic sites within samples; Lower diagonal: Pairwise divergence between samples.

The simulations of hybridization scenarios (Fig. S4) and the *STRUCTURE* results showed a similar level of admixture between hybrids individuals (>F2). The admixture coefficients estimated with *INTROGRESS* agreed with *STRUCTURE* results. The sample M827 showed an almost exclusive *T. inunguis* component, while the other five FG samples had an 0.75 average proportion *T. manatus* alleles. The sample Tierpark had a slightly lower proportion of *T. manatus* alleles (0.72).

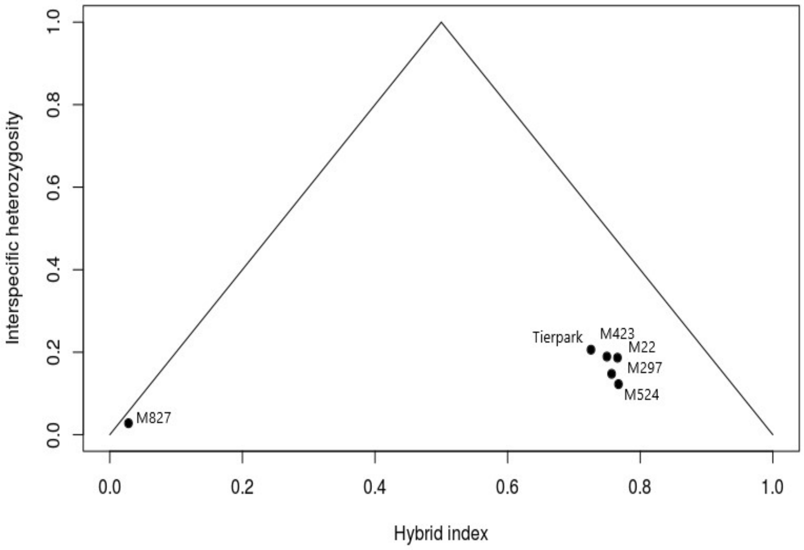


Figure 2. Maximum likelihood hybrid index estimates for FG manatee individuals. The hybrid index coefficient (X axis) is based on frequencies for 359 species-specific SNPs from two

parental populations (*T. inunguis* samples M113 and M117, and the Florida manatee *T. m. latirostris*), and gives the fraction of the genome shared with the two parental populations for each individual.

DISCUSSION

This paper explores the genetic diversity of manatees at the frontier region of the distribution areas of two American species along the Guianas coastline, with some characteristics of a large estuarine environment close to the Amazon River mouth. Here we (i) validated a pipeline to extract genotypic information from the raw reads of full genomes, overcoming samples limitations for ddRAD analysis, (ii) described an incongruence between mitochondrial and nuclear DNA in FG manatees, supporting the extent of the hybridization in this population, (iii) reported the first *T. inunguis* outside the Amazon Basin, in a French Guiana river at least 40 km away from the sea and (iii) discussed the implications for conservation and management of this hybrid population under influence of the Amazon River plume.

Low-quality DNA sample quality for population genomics

The combination of high-coverage sequencing and bioinformatic filtering allowed the use of low-quality samples from decomposing manatees found in FG rivers. The high number of bacterial reads was an indicative of the low sample quality. The presence of *Clostridium, Lactobacillus* and other Enterobacteriaceae sequences was previously associated with decomposing bodies (Hyde et al., 2013). The samples composed mostly of bacterial reads showed a higher bacterial diversity than the other samples, which could indicate a more advanced state of decomposition than the others. Nevertheless, ddRAD data can be used to assess sample quality, local bacterial diversity and bacterial composition of decaying samples. The use of decomposing animals, can be the only biological material usable to assess the genetic of small populations or that have difficulties in sampling. In this study, these samples have proven to be a valuable tool to identify SNPs to investigate the hybrid swarming phenomenon occurring along the Guianas coastline (Santos et al., 2016).

Manatees in French Guiana: a hybrid population

The presence of a *T. manatus* mtDNA clade II haplotype in FG, combined with the presence of *T. inunguis* individuals, is an indication that this population has a more complex dynamics than previously thought. Previous studies have identified that the distribution limit between *T. manatus* clades II and III was between Venezuela and Guyana (Vianna et al., 2006), where a possible historical barrier to gene flow was hypothesized. The presence of clade II in FG highlights the gene flow of western manatees into the hybrid zone, where they mix with eastern manatees (from Brazil), as shown by the presence of clade III mtDNA lineages (L, M, N, O) in Guyana (Garcia-Rodriguez et al., 1998) in the westernmost country of the Guianas coastline. Thus, this *T. manatus vs T. inunguis* hybrid zone is also a secondary contact zone between both *T. manatus* ESUs (Vianna et al., 2006; Santos et al., 2016).

The extent of the hybrid zone between *T. manatus* and *T. inunguis* as suggested previously (Vianna et al., 2006; Santos et al., 2016), and the unusual high rate of hybrids supports a possible ecological and biological advantage of hybrids in this peculiar environment. Since the generation time of American manatees is approximately 20-25 years, the identification of later generation hybrid manatees indicates that this region may have an old and slow process of hybridization, and hybrids may present higher fitness in the local ecological conditions. This remarkable finding of an extended distribution of a large aquatic mammal throughout riverine systems separated by hundreds of kilometers of sea implies the unique influence of the largest river basin in the world. The Amazon River discharge at the Atlantic Ocean shapes the distribution of many taxa of the marine biota (Spalding et al., 2007), sometimes promoting vicariance, as it can be seen for the disjoint distribution of West Indian manatees (Santos et al., 2016; Barros et al., 2017), with eastern and western ESUs separated by the hybrid zone acting as a barrier to gene flow. However, the presence of the Amazon River plume seems also to promote the connection of freshwater biota from separate river basins, like catfishes (Barthem et al., 2017), as we have also observed for the Amazonia manatee found in a river in French Guiana.

The Amazonian manatee outside the Amazon basin

The presence of *T. inunguis* reported here for the first time outside the Amazon basin indicates a possible connection between freshwater systems along the Guianas coastline and the Amazon River, in a region under influence of the Amazon plume that runs westwards because of the North Brazil Current (Muller-Karger et al., 1988). Indeed, the identification of a large Amazon River mammal in a French Guiana freshwater system supports the idea of a broad and unique low-salinity estuarine-like habitat along the Guianas coastline that connects freshwater biodiversity of further apart river basins under the influence of the Amazon River plume (Muller-Karger et al., 1988; Artigas et al., 2003; Anthony et al., 2013; Gensac et al., 2016; Barthem et al., 2017). From the Amazon River mouth to the west of the Guianas coast, the suitable habitats for *T. manatus* are narrow, due to the quite restricted alluvial coastal plain. The fluvial sediment supplies of the Amazon River shaped the coastal landscape during the Quaternary (Anthony et al., 2013; Gensac et al., 2016) and resulted in a continuous mangrovedominated ecosystem along the Guianas coastline from Amapá (Brazil) to Suriname, with rather close salinity and turbidity traits brought by the Amazon plume. This coastal geomorphology associated with the estuary-like characteristics may thus provide an ecological continuum (Artigas et al., 2003), from the currently accepted distribution of *T. inunguis* in the Amazon Basin towards the area of occurrence of *T. manatus* along the Guianas coastline. Furthermore, the North Brazil Current and the Amazon River plume (Froidefond et al., 2002; Fratantoni and Richardson, 2006) likely promote a westward movement of animals from the Amazon River mouth.

The newly reported *T. inunguis* in this study has the same mtDNA haplotype as the hybrids and has only been described in FG (Vianna et al., 2006), which implies this hybridization may be a local phenomenon, involving both species. The mtDNA haplotypes and the observed heterozygosity from simulated hybrids indicates that the hybridization phenomenon has been occurring for several generations and that later-generation hybrids in FG tend to mate with each other, thus initial hybrids (F1 and F2) are at least partially fertile, particularly the females, as occurs in other mammals (Allendorf et al., 2001; Stronen and Paquet, 2013; Fitzpatrick et al., 2015). Future sampling efforts at larger geographic extent could help understanding the ratio of hybrids to “pure” manatees in the Guianas coastline, and if hybrids from different generations (F1, F2, etc) are also present in the population.

Implications for conservation

The need for conservation of hybrids is a controversial and challenging issue. Within species, Conservation Units are independent population units that are used to help guide management and conservation initiative, at the more relevant spatial extent (Funk et al., 2012). Other commonly discussed conservation units correspond to ESUs and management units (MUs). An ESU can be defined as a population exhibiting high genetic and ecological distinctiveness (Crandall et al., 2000) as suggested for *T. manatus* (Vianna et al., 2006; Santos et al., 2016), while MUs are expected to be demographically independent (Palsbøll et al., 2007).

Distinctive genetic signatures of the manatees from the Guianas coastline and their likely adaptive characters to the ecological conditions of this peculiar coastline and drainages may allow considering them as a special Conservation Unit, as a distinct MU, if not even an ESU. In this case, this population would require a particular conservation effort. Even though global IUCN criteria do not consider hybrids as relevant taxa to be evaluated (IUCN Standards and Petitions Subcommittee, 2017), regional Red List assessment in FG (UICN France et al.,

2017) concluded that to be in a stable or decreasing trend, with a small population size due to restricted habitats, and ongoing threats such as incidental poaching, could be included in an "endangered" conservation status. Furthermore, there are increasing anthropogenic-related threats related to oil exploitation and coastal urbanization along the Guianas coastline (de Thoisy et al., 2003).

Other conservation implications should be considered for long-lived organisms like manatees, which display slow growth, delayed maturity, high survival, and resilience or resistance to major perturbations (Musick, 1999). However, those species may compensate for increases in mortality through increased survival, fecundity, changes in growth and maturity (Fordham et al., 2009) that may occur rapidly, and rapid and stochastic changes in demographic parameters (Spencer et al., 2010), such as survival and mortality rates that may favor hybridization.

Finally, our study provides evidence that most of manatees in FG (and other neighboring coastlines) are hybrids between *Trichechus manatus*, the coastal (and associated freshwater environment) species and *T. inunguis*, the freshwater adapted species from the Amazon River basin. Because genomic contribution to the local hybrids mostly comes from *T. manatus*, we conclude this hybridization is occurring over multiple generations with the likely input of *T. inunguis* individuals surviving along the Amazon River plume. The extent of the hybrid zone within the distribution area of *T. manatus* raises the question of the likely ecological advantages of this introgression of genes from a freshwater adapted genome. The understanding of associated genetic process cause could be investigated by searching for natural selection signs throughout the genome of both species and hybrids, which could also give clues for the putative dominance of characters and directionality in interbreeding.

If this particular aquatic ecosystem promoted by the Amazon River plume indeed influences the distribution and population dynamics of other species along this coastal area, a regional conservation program should be devised, with an emphasis to preserve this unique aquatic environment of the Guiana shield coastline

Acknowledgments

We thank Susan Mbedi (BeGenDiv) and Hannah Ebbinghaus for their technical support in next-generation sequencing and Max Driller for bioinformatic support. Claudia Szentkis (IZW) for providing the sample of the captive manatee. STV was supported by an Alexander von Humboldt Foundation fellowship, CSL by a CAPES (Brazil) fellowship, and FRS by a CNPq (Brazil) research fellowship. This project was performed under ICMBio/SISBIO permit 45028 and received funding support from FAPEMIG, CNPq and Fundação o Boticário from Brazil. Samples from Brazil are kept in the collection of the Laboratório de Biodiversidade e Evolução Molecular in UFMG, Belo Horizonte, with a collaboration with ICMBio and Instituto de Desenvolvimento Sustentável Mamirauá, as part of the National Action Plan for Conservation of Sirenians of Brazil. The use of genetic resources for this study is declared in the SisGen platform according to Brazilian law 13123/2015.

Samples from French Guiana are kept in the databank JAGUARS, at Cayenne, French Guiana. The JAGUARS tissue collection is supported by the INDIGEN project, funded by European Community (ERDF funds), the Collectivité Territoriale de Guyane, and the DEAL Guyane.

The use of the genetic resources was declared to the French Ministry of Environment under the reference TSP 48704, in compliance with the Access and Benefit Sharing procedure implemented by the Loi pour la Reconquête de la Biodiversité.

APPENDIX

Table S1. Primer sequences and annealing temperatures used for amplification and Sanger sequencing of nuclear and mitochondrial loci.

Genes	Primer sequences (5' - 3')	Annealing temperature	Reference
APOB	F: GGA GAA GCC ACT CTC CGA CGC R: GGG AAC GGA GGA GTC TTC TGG	58 °C	This study
APP	F: TCC AAG ATG CAG CAG AAC G R: CTA ATG TGT GCA CAT AAA ACA GG	48 °C	Murphy et al., 2001
BMI1	F: CAT TGG GCC ATA GTT TGT TAA TCT CAA R: CCA ATA TGG CAT TGT ACA ACA AGC	50 °C	Murphy et al., 2001
CREM	F: AGG AAC TCA AGG CCC TCA AA R: GGG AGG ACA AAT GTC TTT CAA	48 °C	Murphy et al., 2001
D-loop	L15926: TCA AAG CTT ACA CCA GTC TTG TAA ACC H16498: CCT GAA GTA GGA ACC AGA TG	55 °C	Kocher et al., 1989

Figure S1. Simulated Observed heterozygosity for two different mating schemes. Black Circles represent results from the "Backcross" scenario, while black triangles represent crosses from the "Hybrid" scenario. The green square represent first generation hybrids. The red circle represent the observed heterozygosity of FG hybrids.

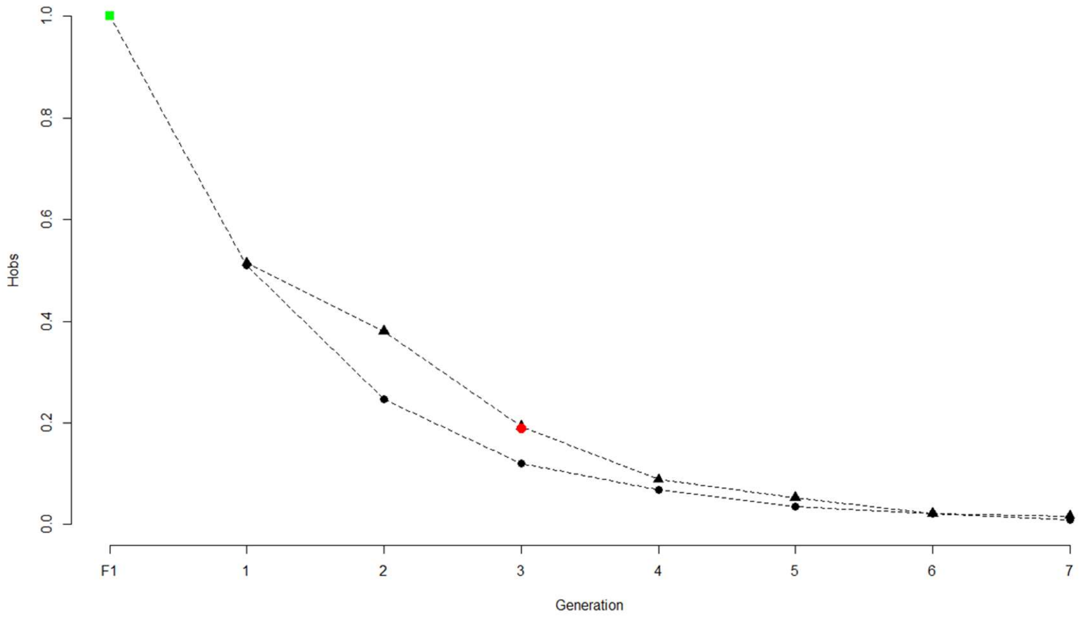


Table S2. Diagnostic sites for four nuclear Sanger sequenced loci. Polymorphisms restricted to *T. inunguis* are shown with an asterisk. Numbers correspond to polymorphic positions in each locus.

	Polymorphic sites							
Sample	APOB		APP			BMI1		CREM
	126	366	4	73	191	42	224	101
M113	G*	G*	T*	C*	T*	T*	G*	T*
M117	G*	G*	T*	C*	T*	T*	G*	T*
M22 JAG	A	A	T*/C	C*/T	T*/C	G	C	A
M297 JAG	A	A	C	T	C	G	C	A
M348 JAG	-			-		-		-
M423 JAG	G*/A	G*/A	C	T	C	G	C	A
M524 JAG	A	A	C	T	C	G	C	A
M827 JAG	G*	G*		-		-		-
M2108 JAG	-			-		-		-
M2772 JAG	A	A	C	T	C	G	C	-
Tiepark	A	A	C	T	C	G/T*	C/G*	A
M035	A	A	T*	C*	T*	G	C	A
M042	A	A	C	T	C	G	C	A
M047	A	A	C	T	C	G	C	A
T. m. latirostris	A	A	C	T	C	G	C	A

Table S3. Summary statistics for five Sanger sequenced loci. Only samples from French Guiana are included. Abbreviations: S, Number of variable sites; π, Nucleotide diversity; Hd, Haplotype diversity.

Loci	Length (bp)	N samples	N haplotypes	S	π	Hd
APOB	585	6	2	2	0.0014	0.409
APP	612	5	5	7	0.0039	0.822
BMI1	314	6	2	2	0.0011	0.167
CREM	396	5	1	0	0	0
D-loop	410	9	3	36	0.0351	0.556

Figure S2. Median joining network tree showing the relationships between *T. manatus* D-loop haplotypes. Haplotype names follow Vianna et al. (2006). Number of mutations are indicated by numbers on branches. The new haplotype (J2) is shown in gray.

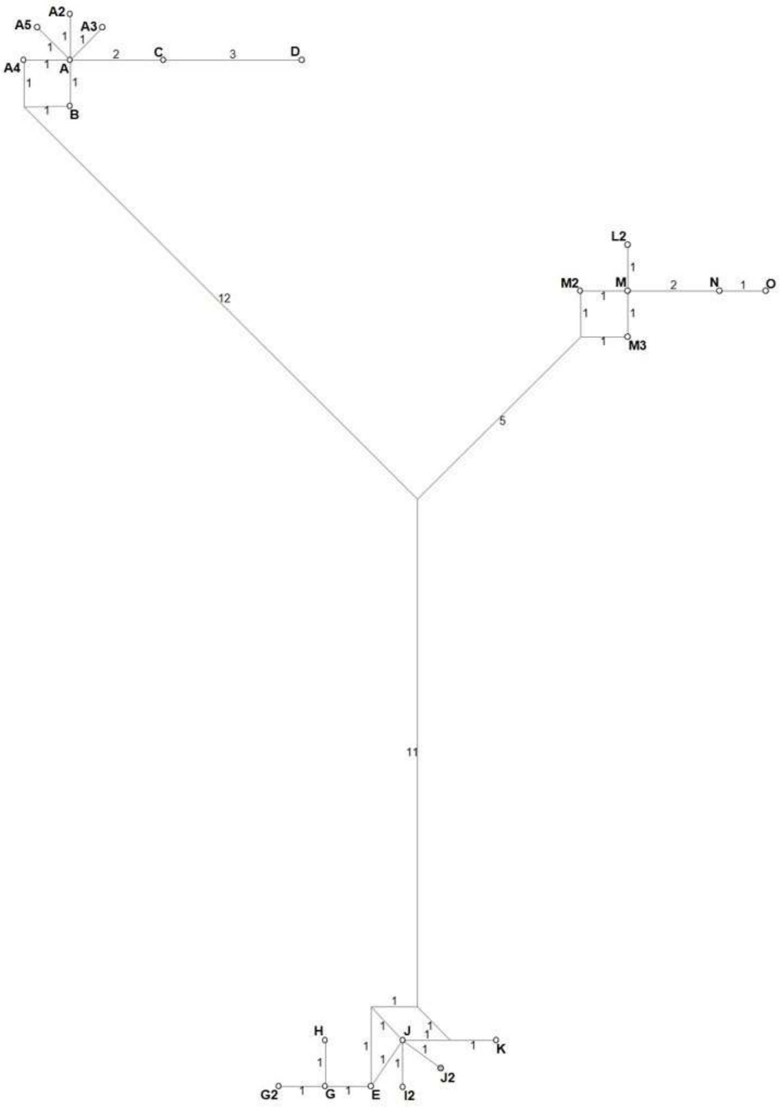


Figure S3. Photo of the individual M423, collected in French Guiana, with a large white patch on its breast and no nails on its flippers.

Figure S4. Taxon composition from a random subsample of 20,000 reads for each sequenced manatee sample analysed in this study. Colors represent Blast results. Results were ranked according to Class.

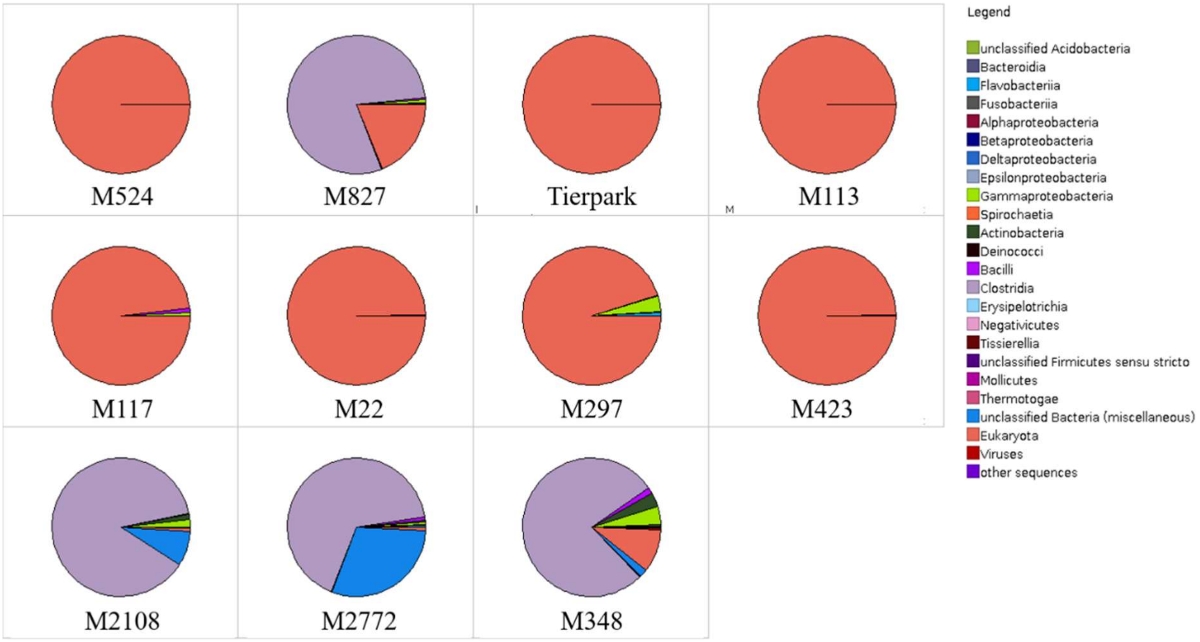


Table S4. Description of the ddRAD analysis. *N reads* represents the total number of reads obtained, *% bacterial reads* show the percentage of reads blasted against bacterial sequences out of a random subsample of 20,000 reads, *N reads used* show reads between 210-260 bp used for clustering after all filtering steps, N clusters is the total of clusters obtained per sample.

Sample	N reads	% bacterial reads	N reads used	Average cluster depth (total)	Average cluster depth (coverage >8)	N clusters
M113	3,878,104	0	2,072,092	5.41	17.88	950
M117	1,712,613	0.9	1,236,404	5	14.24	644
M22 JAG	1,969,364	0.1	1,352,150	3.53	12.85	905
M297 JAG	2,041,547	2.0	1,107,618	3.5	11.99	869
M348 JAG	935,443	7.8	-	-	-	-
M423 JAG	5,655,706	<0.1	880,626	5.32	15.43	854
M524 JAG	5,700,680	<0.1	1,996,670	4.97	16.92	946
M827 JAG	7,604,774	17.9	590,332	6.39	14.77	735
M2108 JAG	791,216	41.44	-	-	-	-
M2772 JAG	1,053,794	53.74	-	-	-	-
Tierpark	2,352,832	<0.1	1,799,752	7.16	15.73	917

Table S5. Pairwise divergence between samples for the species-specific SNP dataset. Samples M113 and M117 were lumped into *T. inunguis*.

	T.inunguis	*T.m.latirostris*	Tierpark	M827	M423	M297	M22
T.m.latirostris	1.00	-	-	-	-	-	-
Tierpark	0.94	0.72	-	-	-	-	-
M827	0.11	0.98	0.84	-	-	-	-
M423	0.92	0.58	0.17	0.78	-	-	-
M297	0.94	0.69	0.30	0.83	0	-	-
M22	0.91	0.51	0.15	0.76	0	0	-
M524	0.91	0.58	0.13	0.76	0	0.10	0

CAPÍTULO 3

Manuscript in preparation to Heredity or Mol.Phyl.Evolution.

Evolutionary dynamics of American manatee species on the northern coast of South America

Camilla Savicius de Lima[1], Rafael Felix de Magalhães[1,2], Benoit de Thoisy[3,4], Miriam Marmontel[5], Fernanda Loffler Niemeyer Attademo[6], Ana Carolina Oliveira Meirelles[7], Fabrício Rodrigues Santos[1,2].

1 Laboratório de Biodiversidade e Evolução Molecular, Departamento de Biologia Geral, ICB, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, CP 486, 31270-010 Belo Horizonte, MG, Brazil.
2 Programa de Pós-Graduação em Zoologia, Departamento de Zoologia, ICB, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, CP 486, 31270-010 Belo Horizonte, MG, Brazil.
3 Institut Pasteur de la Guyane, 97300, Cayenne, French Guiana, France.
4 Kwata NGO, 97300 Cayenne, French Guiana, France.
5 Instituto de Desenvolvimento Sustentável Mamirauá, Estrada do Bexiga, 2584, Fonte Boa, 69553-225 Tefé, Amazonas, Brazil.
6 Centro Nacional de Pesquisa e Conservação de Mamíferos Aquáticos, Instituto Chico Mendes de Conservação da Biodiversidade, Estrada do Forte Orange, s/n, Itamaracá, Pernambuco 53900-000, Brazil.
7 Associação de Pesquisa e Preservação de Ecossistemas Aquáticos (Aquasis), Av. José Alencar, 150, Sesc Iparaná, 61627-010 Caucaia, Ceará, Brazil.

*corresponding author: fsantos@icb.ufmg.br

Running title: Evolutionary dynamics of the American manatees’ hybrid zone

ABSTRACT

Hybridization has been historically neglected in research and conservation practice, but this process seems to be a common phenomenon in nature and several models were developed to characterize it genetically. Even though *T. inunguis* (Amazonian manatee) and *T. manatus* (West Indian manatee) present a deep divergence with large morphological, karyotipic and molecular differentiation, a parapatric hybrid zone was identified from the Amazon River mouth towards the Guianas coastline. Furthermore, this hybrid zone separates two major populations or ESUs within *T. manatus*, Caribbean and Atlantic, which may have promoted or reinforced isolation of both populations. Therefore, to elucidate the population dynamics of American manatees, we used nuclear and mtDNA sequences and statistical phylogeography tools to reconstruct their initial speciation, diversification through time and space, and secondary contact resulting in a hybrid zone, where the genome contribution of each parental species was also estimated, and different models to generate the current scenario were tested. All results confirm a long-time hybridization history, generating a stable and structured hybrid swarm. The hybrid zone is composed with individuals presenting usually a larger contribution of the West Indian manatee genomes. The selected model to explain the formation of the hybrid zone indicates that it is working as a genetic sink between neighboring populations and prevents gene flow between Caribbean and Atlantic populations, which further reinforces the isolation and differentiation of the Brazilian manatees.

Keywords: *Trichechus manatus*, *Trichechus inunguis*, Introgression, Hybrid zone, Model selection

INTRODUCTION

Most cases of speciation result of allopatric differentiation, where two populations evolve independently in separate locations and eventually become reproductively isolated from each other (Network, 2012). This allopatric process can also result in differentiated populations but without reproductive barriers, allowing a secondary contact of deeply divergent lineages that later merge into a single population (Abbott et al., 2013). Besides, many well-recognized species can also hybridize and generate at least partially viable descendants in a sympatric area (Bar-Zvi et al., 2017). Regardless of mode, hybridization seems to be a common phenomenon in nature that has been historically neglected in research and conservation practice, mainly because of the great importance is given to the biological species concept (Mayr, 1942), and its emphasis on reproductive isolation.

Although hybridization can be part of the natural evolutionary process, in many situations it can contribute to population extinction, linked directly and indirectly to anthropogenic causes (Allendorf *et al.*, 2001), such as translocation of animals and plants, habitat changes (Payseur, 2010), and recent population declines (Pinto et al., 2016). Furthermore, hybrid zones can reveal introgression, where F1 hybrids produce offspring with individuals of one or both parental species (Allendorf *et al.*, 2010). In many cases, introgressed alleles cause mosaicism across the genome (Elgvin et al., 2017), resulting in unpredictable fitness effects depending on the genetic background, varying from deleterious incompatibilities to local adaptation, or even associated to speciation (Martin & Jiggins, 2017). In some hybrid zones, parental forms can be fully replaced by introgressed individuals, resulting in a hybrid swarm, with a self-sustaining population, not requiring continued gene flow from parental sources to maintain novel genetic combinations (Shurtliff, 2013). Currently, several genetic models were developed to characterize hybridization and introgression events (Pinho and Hey, 2010; Yu *et al.*, 2014). These tools use diagnostic features from each parental species or populations to estimate the extent of admixture and time (generations) since hybridization occurred (Martin & Jiggins, 2017).

In this context, manatees can be investigated as a model for the study of hybridization between two deeply divergent mammal species. The divergence time between *T. inunguis* (Amazonian manatee) and *T. manatus* (West Indian manatee) has been dated to at least 3.34 Ma (Santos et al., 2016), and previous genetic studies (Garcia-Rodriguez et al., 1998; Vianna et al., 2006) reported the existence of some interspecific hybrids in the area around the Amazon River mouth, towards the Guianas coastline (Santos et al. 2016). Hybrids can present morphological characteristics of both species (i.e., a general morphology of *T. manatus*, associated with a white spot in abdomen and absence of some nails, as in *T. inunguis*).

Moreover, an individual from Amapá, Brazil (Vianna et al.2006) presented also a karyotype with an intermediate number of chromosomes (2n = 50) between the *T. inunguis* (2n = 56) and *T. manatus* (2n = 48). Besides, a recent study reported incongruence between mitochondrial and nuclear DNA in the population of French Guiana, suggesting a long-term admixture between individuals in this region (Villaça et al 2018). Additionally, although *T. inunguis* is considered an endemic freshwater mammal of the Amazon basin, Vilaça, at al. (2018) reported the presence of the Amazonian manatee in a French Guiana river (50 km away from the coast), showing the transit of at least one of the parental species in the hybrid zone, likely using the Amazon River plume.

The West Indian manatee is distributed in the coastal areas from Florida (USA) to Alagoas (Brazil) and, in 1986, Domning and Hayek used cranial analysis to divide it into two subspecies: *T. manatus latirostris* (restricted to Florida) and *T. m. manatus* (other locations). Many molecular studies evaluated the intraspecific divisions with *T. manatus* (Garcia-Rodriguez et al., 1998; Vianna et al., 2006; Hunter et al., 2010, 2012), but most of them presented divergent results with the current taxonomy. Indeed, *T. manatus* shows an intraspecific structure with two major populations with different traits (Santos et al. 2016, Barros et al. 2017), Caribbean (Caribbean, Mexico Gulf and Florida) and Atlantic (Brazilian), separated by an interspecific hybrid zone (Santos *et al.* 2016), which may have promoted or reinforced the differentiation observed between them (Abbott et al., 2013). Additionally, the first study that united cranial and chromosomal variation in manatees, showed that Brazilian manatees are clearly different from others, including that from Florida (Barros et al., 2017).

Despite independent data indicate a major intraspecific division of *T. manatus* that is composed by a hybrid zone, no study has focused on the population dynamics and origin of the manatee population in the hybrid zone from the Amazon River mouth towards the Guianas coastline. Mitochondrial data suggests that both, Caribbean and Atlantic (Brazilian) ESUs, are *T. manatus* sources for the admixed population with *T. inunguis* (Vilaça *et al*., 2018). Presumably, the relative isolation of Brazilian ESU was reinforced by the hybrid zone that forms a barrier between the two ESUs (by different karyotype number and accumulated adaptations), since this area present a large hybrid population in an immense estuary-like environment with distinct habitat characteristics when compared with the environments where *T. manatus* lives (Artigas et al., 2003; Castelblanco-Martínez, dos Reis, de Thoisy, et al., 2017; Meirelles et al., 2018). Then, it is important to understand the population dynamics and maintenance of the hybrid zone.

Nevertheless, the characterization of admixture events in a hybrid zone can be a challenge, since the cause of variation shared between related species may be also related to retention of ancestral polymorphisms due to incomplete lineage sorting (ILS) (Zhou et al., 2017). These processes difficult the reconstruction of admixture events, like interspecific hybridization and gene flow (Toews & Brelsford, 2012). Due to the discordance observed across the entire tree of life, several coalescent models were developed to help in the estimation of the history of related populations (Leaché et al., 2014). These models can be applied in statistical phylogeography, allowing to test between gene flow and ILS, besides estimating other demographic parameters (Knowles & Maddison, 2002). Although the available evidence points to a complex history of the hybrid zone between *T. manatus* and *T. inunguis*, there are still many open questions about its formation and maintenance (Santos *et al.*, 2016; Vilaça *et al.*, 2018). Therefore, it is important to elucidate the population dynamics of manatees, both in the hybrid zone and among populations of parental species, since this data could improve the management planning of these threatened species. Using diagnostic genes and statistical phylogeography tools, we tested the hypothesis that if there is a hybrid swarm in this area, which may be working as a genetic sink of diversity from parental species and divergent *T. manatus* populations, We aim to reconstruct the process of formation and maintenance of the hybrid zone in relation to its geographic position and ecological distinctness, seeking to understand the importance of this hybrid population for the evolution of both species, and for local conservation efforts.

MATERIALS AND METHODS

Sampling

Tissue samples (skin, muscle, and blood) were obtained from *Trichechus inunguis*, *Trichechus manatus* and putative hybrids between these two species. To confirm the identification of hybrids, we sampled pure individuals from parental species following Vilaça *et al.* (2018). *Trichechus inunguis* controls were from the interior Amazon River (n = 44) and *Trichechus manatus* were from the northeast coast of Brazil (n = 43), Florida, USA (n = 23) and Puerto Rico (n = 5). The putative hybrid individuals were sampled in the Amazon Delta and Amapa coast of Brazil (n = 3), and French Guiana (n = 12). The samples were preserved in 100% ethanol and DNA extraction was performed by standard phenol-chloroform protocol (Sambrook et al., 1989), and deposited in the Tissue and DNA collection of the Centro de Coleções Taxonômicas of Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil.

PCR and sequencing

We sequenced a control region (CR) fragment of mitochondrial DNA (mtDNA) (Vianna et al., 2006) and five nuclear (nuDNA) genes (APOB, APP, BMI1, CREM, and RAG1), both diagnostic of parental species. The PCR was performed following Villaça *et al.*, (2018) and

Lima *et al*., (2018), and the amplicons were purified by the polyethylene glycol (PEG) method (20% PEG 8000, 2.5M NaCl) with modifications (Santos Júnior *et al.*, 2015). The fragments were sequenced on the ABI 3130xl Genetic Analyzer (Applied Biosystems) using the BigDye Termination 3.1 Cycle Sequencing kit. The same amplification primers were used for DNA sequencing. Chromatograms were analyzed in SeqScape 2.6 and consensus sequences were aligned using the Clustal W algorithm (M. A. Larkin et al., 2007) implemented in the software MEGA7 (Kumar et al., 2016).

Haplotype inference and characterization of hybrids

The haplotypes were inferred in software DNAsp 5 using the algorithm Phase (Rozas et al., 2003), and parsimony relationships between them were estimated with the software POPART (Leigh & Bryant, 2015) using the median-joining algorithm (Bandelt et al., 1999). This analysis was made to identify diagnostic haplotypes of each manatee species and individuals were different species-specific alleles are found co-occurring. To characterize the relative genome contribution of both parental species to the hybrids through nuclear DNA haplotypes, we used the software STRUCTURE 2.3.4 (Pritchard et al., 2000) with K fixed in two and treating haplotypes as alleles. The analysis was made under the admixture model and the assumption of uncorrelated allele frequencies, with ten replicates a burn-in of 2.5×10^4, and 10×10^6 iterations each. The software CLUMPAK (Kopelman et al., 2015) was used to summarize the STRUCTURE results, and the DISTRUCT (Rosenberg, 2004) to visualize them. Additionally, we made a factorial correspondence analysis (FCA) implemented in GENETIX 4.02 (Belkhir et al., 2000) to co-validate the admixture results identified in the STRUCTURE, treating distinct haplotypes as alleles. To evaluate mtDNA and nuDNA sequences we estimated a multigenic nuclear distance matrix between the individuals in program POFAD (Joly & Bruneau, 2006), using the genpofad algorithm (Joly et al., 2015). Finally, this genetic dissimilarity matrix was visualized in SplitsTree4 software (Huson, 1998; Huson & Bryant, 2006), using the Neighbor-Net algorithm.

Population analyses

To estimate the optimal number of populations and their geographical boundaries, we implemented the spatial clustering approach of the Geneland 3.1.4 package (Guillot et al., 2005, 2012), available for the R software (R Core Team, 2017). This analysis was made with two datasets. In the first one, we joined all the individuals in the same analysis. In the second dataset, we searched an intraspecific structure within *T. manatus* outside the hybrid zone, to test the population division proposed in the literature (Vianna *et al*., 2006; Santos *et al.*, 2016; Lima *et al.*, 2018). As *T. manatus* occurs only on the continental shelf associated to coastal habitats, the spatial model implemented in Geneland (i.e., Voronoi polygons) may not represent the non-euclidean distances observed between populations of the species (Guillot et al., 2009). For this reason, two separate runs were made, considering either a spatial or non-spatial model. This allows us to evaluate the impact of geographic data on the clustering. These analyses were made with the uncorrelated allele model, and calculations performed through the Markov Chain Monte Carlo (MCMC) algorithm, using ten replicates of 2.5 x 10^7 iterations and 2.5 x 10^4 of thinning each (Guillot et al., 2012).

We used the isolation with migration model (Nielsen & Wakeley, 2001) to estimate demographic parameters and dynamics between fluvial (i.e., sampled in the Amazonas basin) and marine individuals, as implemented in IMa (Hey & Nielsen, 2007). To this analysis, we assumed the infinite sites mutation model to APOB gene and Hasegawa-Kishino-Yano (HKY) (Hasegawa et al., 1985) for the other loci. We made three replicates, where the runs had a burn-in period of 1 x 10^6 and a sampling period of 25 x 10^6 (thinning 2,500). The Metropolis-Hastings MCMC was implemented using 40 chains, with the geometric increment of term 1 and 2 being 0.8 and 0.9, respectively. The maximum values for integrated parameters were set according to previous exploratory tests (i.e., q1: 1.93, q2: 1.95, qa: 5.85, m1: 1.00, m2: 1.25 and t: 2.20). Since the full model was estimated, we used an information-theoretical approach through AIC to select the best between 16 reduced models estimated in IMa (Hey & Nielsen, 2007; Carstens et al., 2009). In this interim, we made two analyses, with distinct datasets. In the first one, we included all individuals (pure and putative hybrids), separated into two geographical groups, marine and fluvial. In the second one, we removed the putative hybrids from both groups. This sampling scheme was made to evaluate the impact of hybrids in gene flow estimates and, especially with the second analysis, to better estimate the effective population sizes of *T. manatus* and *T. inunguis*, since the presence of hybrids can inflate this.

Phylogenetic estimation and dating

The reconstruction of the calibrated species-tree of *Trichechus* was made in the BEAST 2.5 software (Bouckaert et al., 2014). For this, we included the three manatee species (*T. inunguis*, *T. senegalensis,* and *T. manatus*), considering *T. manatus* divided into two distinct lineages or demic populations (see Geneland results). Additionally, we also included two outgroups, the dugong (*Dugong dugon*) and the sea cow (*Hydrodamalis gigas*). The mtDNA an nuDNA sequences of *D. dugon*, *H. gigas*, and *T. senegalensis* were obtained from Genbank (accession numbers: JN413953, JN632735, JN633472, JN633590, KR827286, KR827273, KR827274, KR827287, KR827336, KR827337, KR827277, KR827278, KR827275, KR827276, KR827280, KR827288, KR827289, KR827323, KR827324, KR827335, KR827338, AY963894-98). These sequences were added in our data matrix, aligned and the best substitution model of each fragment was estimated using AICc, implemented in jModeltest2 (Darriba et al., 2012). The molecular clock model for each locus was tested using the maximum likelihood method (ML), under the best substitution model selected, in MEGA7 (Kumar et al., 2016). We used the calibrated Yule model (Steel & Mckenzie, 2001) combined with two fossil calibration intervals, one for the Sirenia ancestor (minimum 28.1 MYA, maximum 38.0 MYA) (Velez-Juarbe et al., 2012) and another for the Dugongidae one (minimum 41.3 MYA, maximum 59.2 MYA) (Vélez-Juarbe & Domning, 2014), as implemented by Springer et al. (2015). We made three distinct analyses with $5x10^8$ generations and thinning 5×10^4 each. The stationarity and convergence of parameters were checked in Tracer 1.7 (Rambaut et al., 2018). The tree files were combined in LogCombiner and summarized in TreeAnnotator, both available in the BEAST 2.5 package (Bouckaert et al., 2014).

With the substitution rates estimated for each fragment in the previous analyses, we reconstructed a species network under the multispecies network coalescent model (MSNC) (Yu et al., 2014), which considers reticulations as hybridization and introgression events. This analysis was implemented in the SpeciesNetwork package (Zhang et al., 2017) of BEAST 2.5. We applied the same site and clock models as the species-tree analysis. Two distinct runs with $2.5x10^8$ generations and thinning 2.5×10^4. The stationarity and convergence were checked as above, and programs IcyTree (Vaughan, 2017) and Dendroscope 3 (Huson & Scornavacca, 2012) were used to visualize the phylogenetic network.

RESULTS

We obtained a total of 2,768 bp high-quality sequences for nuDNA (APOB = 644 bp, APP = 642 bp, BMI1 = 401 bp, CREM = 767 bp and RAG1 = 767 bp) and 410 bp for mtDNA. All diagnostic haplotypes were exclusive of each parental species (Fig. S1, S2 and S3). On the other hand, all of these diagnostic haplotypes were found co-occurring in the individuals of the hybrid zone. Additionally, we discovered one new *T. manatus* mtDNA haplotype (M5) (Fig. S1) and eleven new *T. inunguis* haplotypes (Q5, Q6, Q7, Q7a, S4, S5, T16, U3, V2, V3, and W2) (Fig. S2). The localization of samples and proportion of haplotypes belonging to each species are represented in Figure 1.

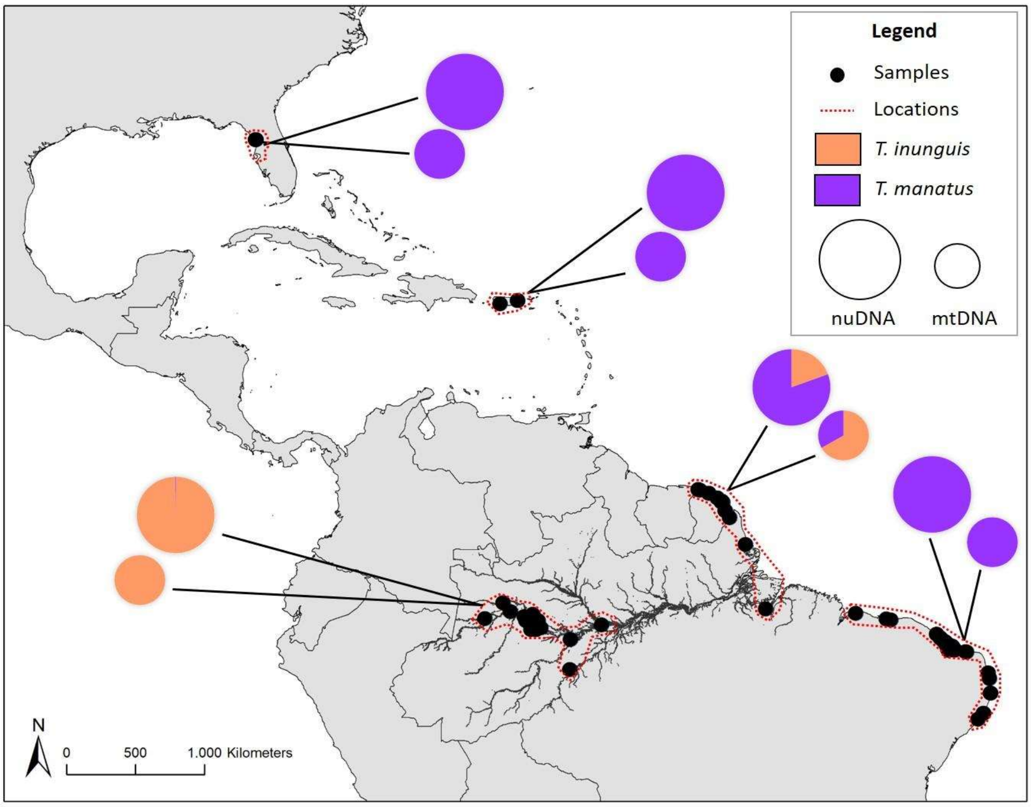


Figure 1. The proportion of diagnostic haplotypes of each species found in each location.

As seen in STRUCTURE, admixed individuals were found only in the hybrid zone (Fig. 2A), with the mean of proportion of individual admixture in this area being 20.3% and 79.7% of *T. inunguis* and *T. manatus,* respectively. The results of the factorial correspondence analysis (FCA) clearly show a separate clustering between *T. inunguis* and *T. manatus* populations. On the other hand, individuals from the hybrid zone are graphically dispersedly between the two species clusters (Fig. 2B), confirming the STRUCTURE results. Finally, the neighbor-net shows also a clear separation between *T. inunguis* and *T. manatus*, with most hybrids in the torso of the network. However, some hybrids are closely related to each one of the parental species. Two of the hybrids grouped together with *T. manatus*, while one of them grouped with *T. inunguis*. Besides, it is possible to observe the subdivision within *T. manatus*, forming two groups equivalent to the known ESUs (Fig. 2C), with the hybrids grouping with the Brazilian (Atlantic) one. This also indicates that most of the *T. manatus* genes found in the hybrids likely originated from the Brazilian population, as expected because of the sympatric area of both species in the Amazon River mouth, and the North Brazil current directing westwards the Amazon Plume.

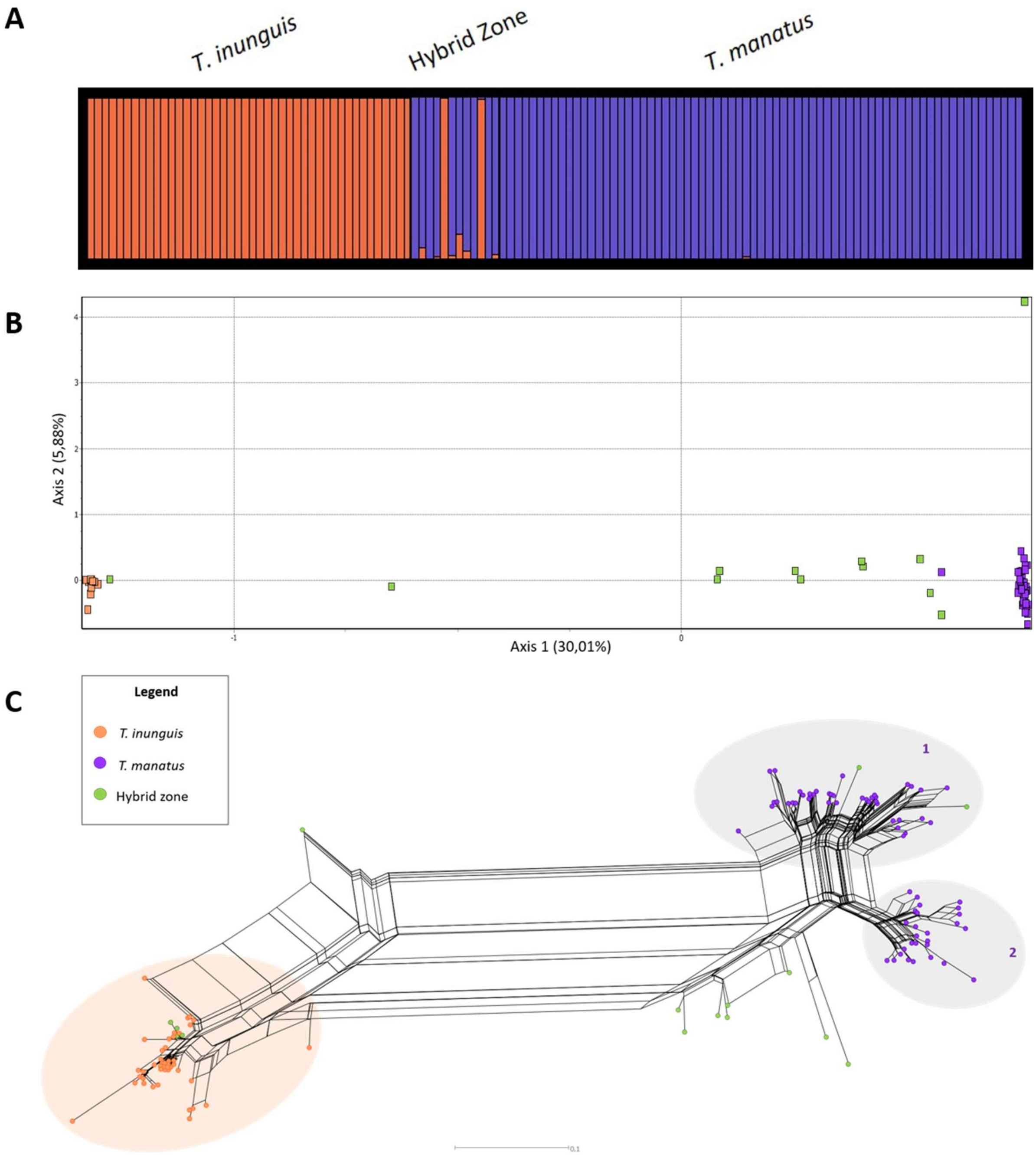


Figure 2. Inter-individual analyses of American manatees (A) Q-plot showing the genome composition (and admixture) of each individual from the hybrid zone and both parental species. (B) Factorial correspondence analysis (FCA) based on five nuDNA haplotypes, showing a clear clustering between *T. inunguis* (orange) and *T. manatus* (purple). Samples of the hybrid zone (green) occupied intermediate positions. (C) Neighbor-net based in genpofad distances. The blue ellipse 1 highlights the samples of *T. manatus* from Brazil, while the blue ellipse 2 samples from Florida and Puerto Rico. The orange ellipse highlights *T. inunguis* individuals. Observe some hybrid individuals grouping with pure ones.

The Geneland analysis including all individuals identified three populations, with a posterior probability (PP) of 60%. These clusters represented individuals from (I) *T. inunguis*, (II) *T. manatus,* and (III) hybrid zone. The spatial PP to each cluster, estimated for a K = 3, was 90% in mean (Fig. S4). For the spatialized analysis evaluating only "pure" *T. manatus* individuals, the obtained number of clusters was K = 2, with PP > 90%. These clusters grouped individuals from (I) Florida and Puerto Rico, and (II) Brazil. The non-spatialized analysis was congruent, recovering the same clusters (Fig. S5).

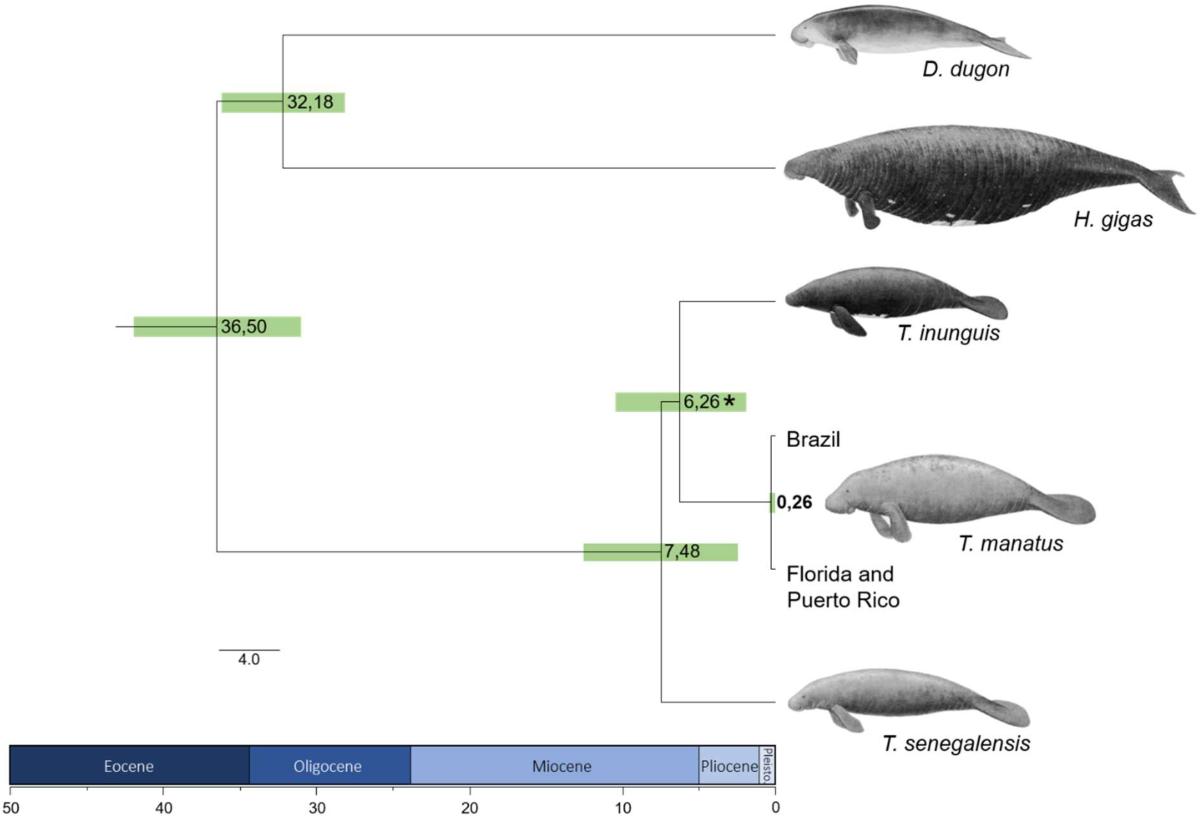


Figure 3. Bayesian phylogeny of species in the order Sirenia using calibration by fossils and mtDNA (CR) and five nuDNA (APOB, APP, BMI1, CREM, and RAG1). The node dates refer to millions of years (MA) and the green bar represents the 95% highest posterior density of the model. All nodes have posterior probability (PP) equal to 1, except the one signalized with an asterisk (0.4237).

The species tree (Fig. 3) recovers almost all nodes totally supported (PP = 1). Nevertheless, the relationship between the *Trichechus* branches was weakly supported (PP = 0.4237). The mean time of the most recent common ancestor (TMRCA) of *H. giga*s and *D. dugon* was 32.18 MYA, while the mean TMRCA of the sirenian ancestor was 36.50 MYA. Our results recovered the TMRCA of *T. inunguis* and *T. manatus* in the Messinian Age of Neogene (6.26 MYA). The time of the ancestor of all trichechids was estimated at 7.48 MYA, at the end of the Tortonian Age of Neogene. The intraspecific lineages of *T. manatus* share a recent ancestor dated around 0.26 MYA, in the Late Pleistocene. The substitution rate estimated for each fragment was (mean ± standard deviation): APOB ($1.766 \times 10^{-4} \pm 1.7473 \times 10^{-5}$), APP ($2.105 \times 10^{-4} \pm 5.3075 \times 10^{-5}$), BMI1 ($2.821 \times 10^{-4} \pm 7.9405 \times 10^{-5}$), CREM ($2.141 \times 10^{-4} \pm 5.8317 \times 10^{-5}$), CR ($1.064 \times 10^{-2} \pm 3.2226 \times 10^{-3}$), and RAG1 ($3.2548 \times 10^{-4} \pm 6.8261 \times 10^{-5}$).

In the IMa analysis including hybrids, the best model ranked by the AIC was the one with bidirectional migration and all θ equal (θA = θA = θA, m1, m2; AAADE). On the other hand, the second-best model only considers migration to marine population and independent θ values (θA, θ1, θ2, m2; ABC0D), as shown in Table S1. In the analysis without hybrids, the best model also includes unidirectional migration θA, θ1, θ2, m1 (ABCD0), but the second (ABA00) and third bests models (AAC00), both showing $\Delta i < 2.00$, indicate isolation between *T. inunguis* and *T. manatus* (Table S2). In short, the analysis to a better estimate of parameters kept a low migration to fluvial population, what may indicate a whilom introgression that currently cannot be identified ($2N_e m = 0.07$, Table S4), there is evidence of multiple hybridization events between the American manatees. Finally, the SpeciesNetwork shows that both, *T. inunguis* and the two lineages of *T. manatus*, contributed with the hybrid zone formation, being the inheritance probability from Amazonian manatee ($\gamma = 0.583$) bigger than from *T. manatus* ($\gamma = 0.296$), besides being older (Fig. 4). In addition, the MSNC indicates an ancient event of gene flow between the lineages of *T. manatus* ($\gamma = 0.512$).

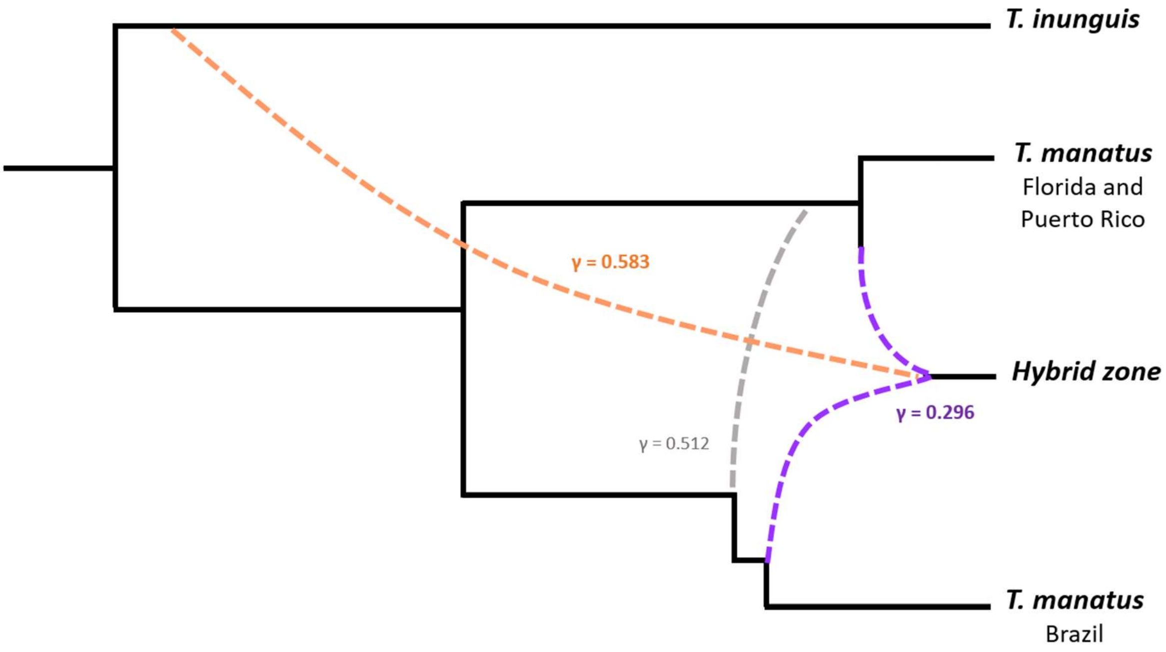


Figure 4. The best-supported species network (PP = 87.27%). The numbers in dotted reticulations indicate inheritance probabilities of the hybridization event.

DISCUSSION

Few algorithms were developed to identify hybrids of F1, F2 and further generations due to backcrossing (Anderson, 2003; Gompert & Buerkle, 2010), but those analyses do not consider additional genomic information. Because of this, there are limitations in cases of deep introgression and these are especially sensitive to missing data. As shown in our results, the use at least two integrative methods of identification as geography and DNA (mitochondrial and/or nuclear) analyses was very important to an efficient diagnostic of hybrids. For example, some hybrids with missing data were identified by show diagnostic haplotypes from one species typical environment from another. Furthermore, despite the increasing number of genomic tools and the application of high throughput sequencing to the identification of hybrids (Vilaça et al. 2018), the Sanger sequencing of six loci has shown to be reliable and less expensive, being applicable to both population and inter-individual analyses for diagnosis of genome composition of hybrids, population structure, and gene flow estimates that can be used in a conservation plan for both American manatees.

The genetic admixture found only in individuals from the hybrid zone is probably caused by the backcrossing events occurred between hybrids of this area and the West Indian manatee population. Additionally, the gene flow model demonstrates a more important number of effective migrants from fluvial to marine environment than the opposite flow (Tables S3 and S4). As confirmed by the spatial clustering analysis, the genetic mosaicism observed only in the hybrid zone individuals makes them a distinct population (or aggregation) from both parental species. The establishment of this supposedly locally-adapted population, likely reinforced the barrier between the ESUs Caribbean and Atlantic (Brazilian) of *T. manatus* (Vianna *et al.*, 2006; Santos *et al.*, 2016; Lima *et al.,* 2018), leading to isolation and differentiation of Brazilian manatees (Santos et al., 2016; Barros et al., 2017). In addition to the gene flow barrier in the hybrid zone, the Amazon River plume flow in the Atlantic coastline of the Guianas Shield may also be considered a biogeographic barrier to manatees, as it is to different marine species (Spalding et al., 2007; Luiz et al., 2012) because of changes in salinity and carried sediments, interfering in the incidence of sunlight and forming a physic obstacle by the westward water flow of the plume, guided by the North Brazil current (Geyer et al., 1996). Another relevant fact is that the maternal lineage (mtDNA) of individuals from the hybrid zone is mostly originating from female of Amazonian manatees which indices that in the F1 offspring the males are often sterile, as expected by Haldane's rule (Haldane, 1922).

The past population dynamics of manatees was likely influenced by climate changes and the sea level fluctuations (Edwards, 2013), and our multilocus analysis with fossil calibration provided a reliable dating in the Pleistocene. A low gene flow to *T. inunguis* population was identified, which indicates that the hybridization process may be cyclical and more ancient, difficult to identify currently. The current state of Amazon basin was reached about 2.5 MYA, and before that, the amount and the force of water were lower (Hoorn et al., 2010), which could facilitate the entrance of manatees in the Amazon River. In our species tree, only the node between *T. inunguis* and *T. manatus* was low supported, probably caused by the absence of nuclear sequences of *T. senegalensis*. This phylogenetic uncertainty is also found in other researches. For example, Santos *et al*. (2016) found a distinct topology using only the Cyt-*b* fragment, with *T. manatus* more related to *T. senegalensis* than to *T. inunguis*, a topology that was recovered with a good support using morphological data (Springer et al., 2015). So, we concluded that this phylogeny should be better investigated using more independent markers, especially for *T. senegalensis.* The dating was also quite different when comparing those works. Although Santos *et al*. (2016) showed a more recent divergence between the trichechid species, between trichechids and dugongids, and a more ancient divergence between lineages of *T. manatus* when compared with our results, they used only mtDNA and one fossil calibration point. Thus, we conclude that our dating using more markers and calibration points should be more precise. On the other side, Springer *et al*. (2015) showed more ancient divergence times than our dated phylogeny. This could be attributed to the distinct sampling made by these authors. They used many outgroups, including several Afrotheria, while we used only Sirenians. Rull (2011) demonstrates that the sampling design can affect the dating and modify the resolution. In this interim, deep lineages sampling (i.e., crown dating) results in more ancient nodes, while shallow sampling (i.e., species dating) results in more recent ones (Rull, 2011). The species dating is more adequate to investigate recent paleontological events and its effects on the diversification of the studied group (Rull, 2011), which proved to be adequate in our case. Nevertheless, it is important to note that our HPD intervals included the means obtained by Spriger *et al*. (2015).

Despite a long time of divergence between Amazon and West Indian manatees, dating to the Late Miocene and Early Pleistocene, all analysis suggests a high gene flow and introgression between them. The SpeciesNetwork (Fig. 4) evidence that the *T. inunguis* (γ = 0.583) contribution was larger and older than two lineages of *T. manatus* together (γ = 0.296), what is unexpected, once most of the hybrids were found in the Guianas coastline and usually they are morphologically identified as West Indian manatees (Vianna *et al*., 2018). Some points have to be considered to the interpretation of that result. Firstly, the absence of samples in Amazon River close to the mouth (as Pará and Amapá) can be forcing an ancient contribution of *T. manatus* to hybrid zone.This is because the unsampled region could be individuals with genomes more related . Moreover, the connections between the branches of Brazil and Florida + Puerto Rico may also represent a ghost lineage, probably of Caribbean region. Taking into consideration these facts, the results show that both species and all lineages contributed to the formation of the hybrid population.

Analyzing only the West Indian manatees, it was possible to distinguish two groups (Fig. 2C and S5), corresponding to geographic separation of two ESUs proposed by Vianna *et al.* (2006) and confirmed by Santos *et al.*, (2016) and Lima et al. (2018): The first ESU (Caribbean) is composed by populations from the Caribbean, Florida, and Gulf of Mexico, from Venezuela to Florida (USA) and the Antilles, and the second ESU (Atlantic) composed by only populations from Guianas to Brazil. In all our tests, the populations of Florida and Puerto Rico appear together, even though studies with microsatellite markers suggest a small differentiation between them (Hunter et al., 2012). Our results reinforce the importance of a special management strategy for the Brazilian manatee population, with suggestions to review its status to endemic subspecies because of morphological and karyotypic differences (Barros et al., 2017), and highly differentiated and exclusive mtDNA lineage, forming an independent ESU (Lima et al., 2018).

As *T. manatus* and *T. inunguis* are two threatened species with a reduced population, the N_e calculated for each one of them is an important parameter for conservation. Our analysis calculated a N_e similar to *T. manatus* and *T. inunguis* (43,962 and 44,196, respectively). The effective female population size was estimated to Amazonian manatees by Vianna *et al.* (2006) using mtDNA in three estimation methods, obtaining the numbers of 133,200, 479,558 and 510,823 females. As interpreted by the Vianna *et al.* (2006), our evaluation also seems to have been overestimated and may have suffered a bias caused by the type of fragment analyzed, which can be reflecting a most ancestral number, not representing the current populations.

The use of diagnostic markers, more conserved within species, allows reconstructing an older story of the dynamic intra- and interspecific (Meredith et al., 2011; Springer et al., 2015). Thus this study reconstructs the formation of the hybrid zone by secondary contact between two deeply divergent species of manatees, indicates that this hybrid zone deserves a special attention, once the conservation of hybrids is much discussed and few efforts are put into practice (Dong et al., 2003; Grobler et al., 2011; Fitzpatrick et al., 2015). The dynamics of that population revealed to be old and complex, serving as a genetic sink between neighboring populations. Since the populations of Brazil and Florida + Puerto Rico are separated by at least 0.25 MYA and form distinct populations, as suggested in previous studies (Vianna *et al.*, 2006; Barros *et al.*, 2017; Lima *et al.*, 2018), our reconstruction analyses corroborates the hypothesis of that hybrid zone also preclude the gene flow between the Caribbean and Atlantic populations, which further highlights the isolation and differentiation of the Brazilian manatees.

ACKNOWLEDGMENTS

CSL was supported by a CAPES (Brazil) Ph.D. fellowship, RFM by a CAPES-PNPD (Brazil) fellowship, and FRS by a CNPq (Brazil) research fellowship. This project was performed under ICMBio/SISBIO permit 45028 and received funding support from FAPEMIG, CNPq, PETROBRAS and Fundação o Boticário from Brazil. Samples from French Guiana are kept in the databank JAGUARS, at Cayenne, French Guiana. The JAGUARS tissue collection is supported by the INDIGEN project, funded by the European Community (ERDF funds), the Collectivité Territoriale de Guyane, and the DEAL Guyane. The use of the genetic resources was declared to the French Ministry of Environment under the reference TSP 48704, in compliance with the Access and Benefit Sharing procedure implemented by the Loi pour la Reconquête de la Biodiversité. The research with access to genetic resources of Brazil is registered under number ADDD66D in the SisGen platform of the Ministry of Environment.

SUPPLEMENTARY INFORMATION

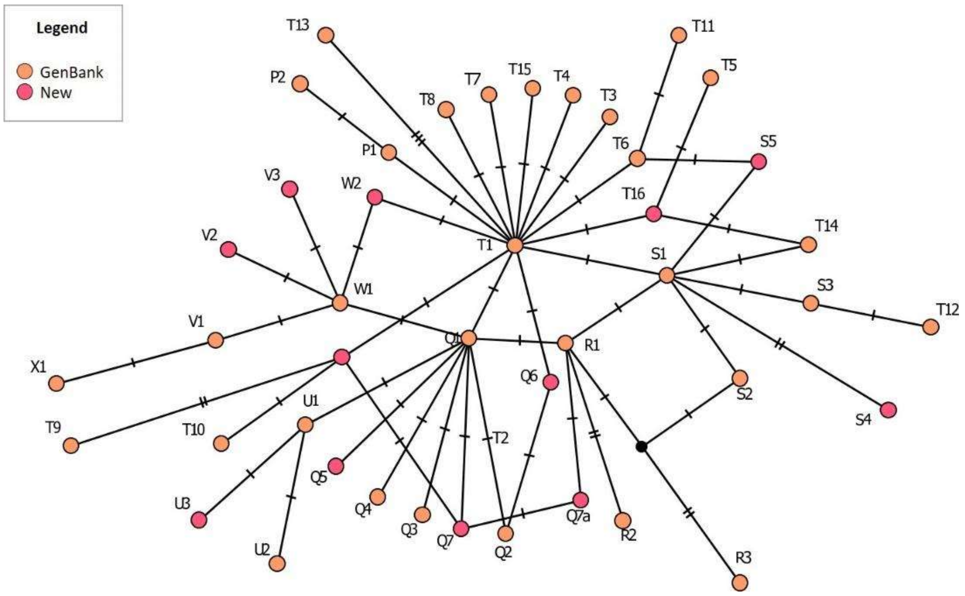


Figure S1. Median-joining networks of CR showing the relationships between new and previously published haplotypes of *T. inunguis*. The small traces in the branches indicate the mutations points.

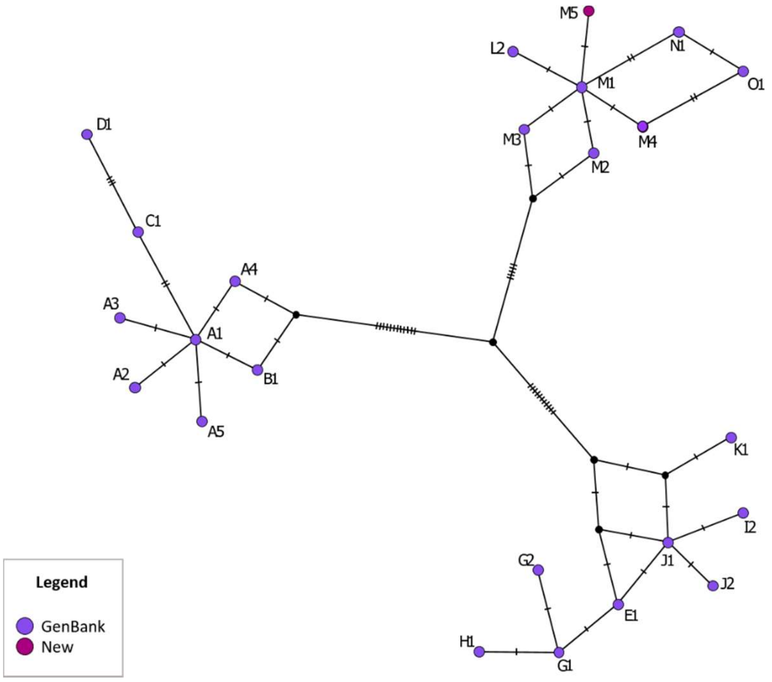


Figure S2. Median-joining networks of CR showing the relationships between new and previously published haplotypes of *T. manatus*. The small traces in the branches indicate the mutations points.

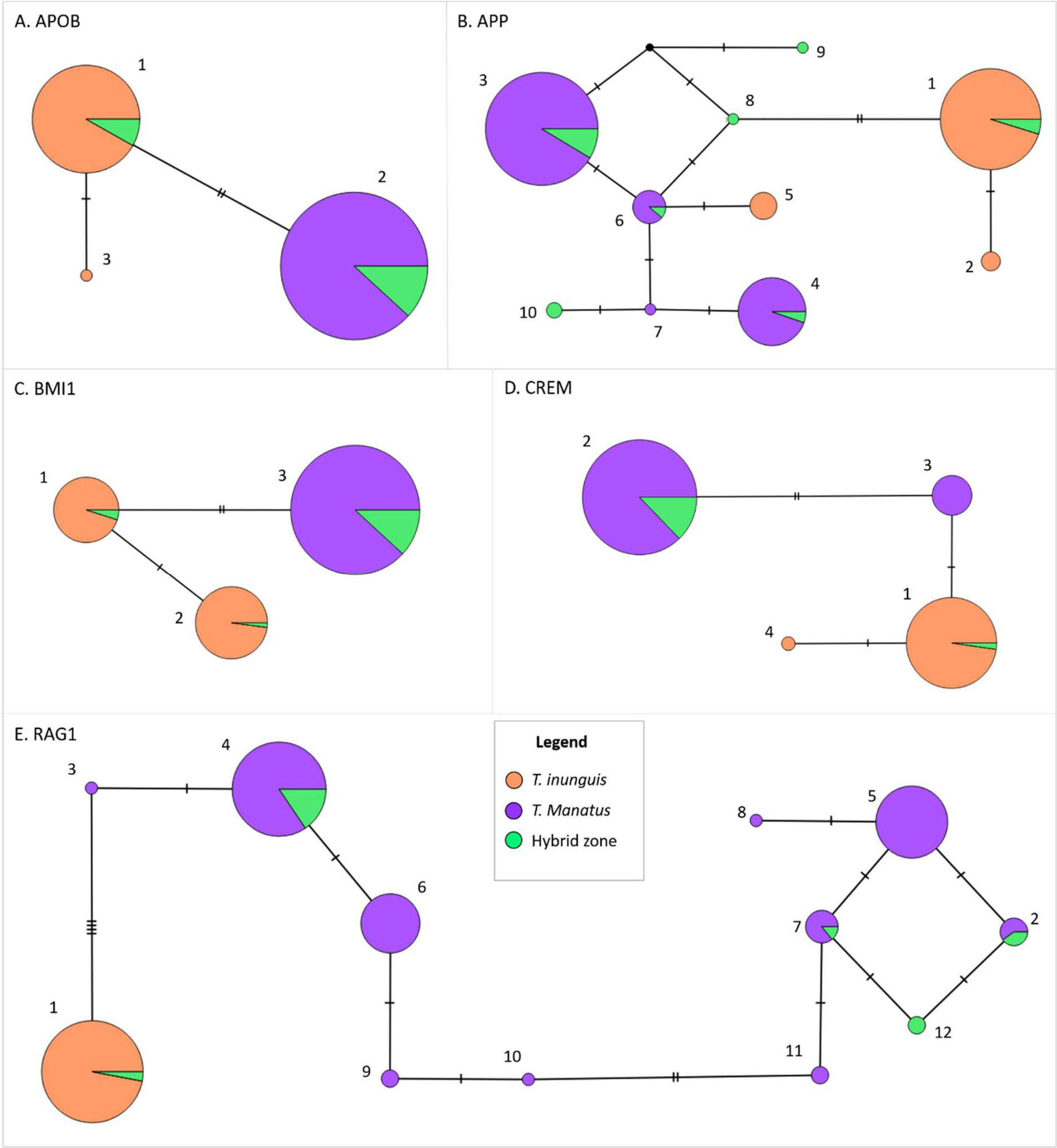


Figure S3. Median-joining networks showing the relationships between haplotypes to each gene. The diameter of the circles is proportional to the number of individuals. The small traces in the branches indicate the mutations points.

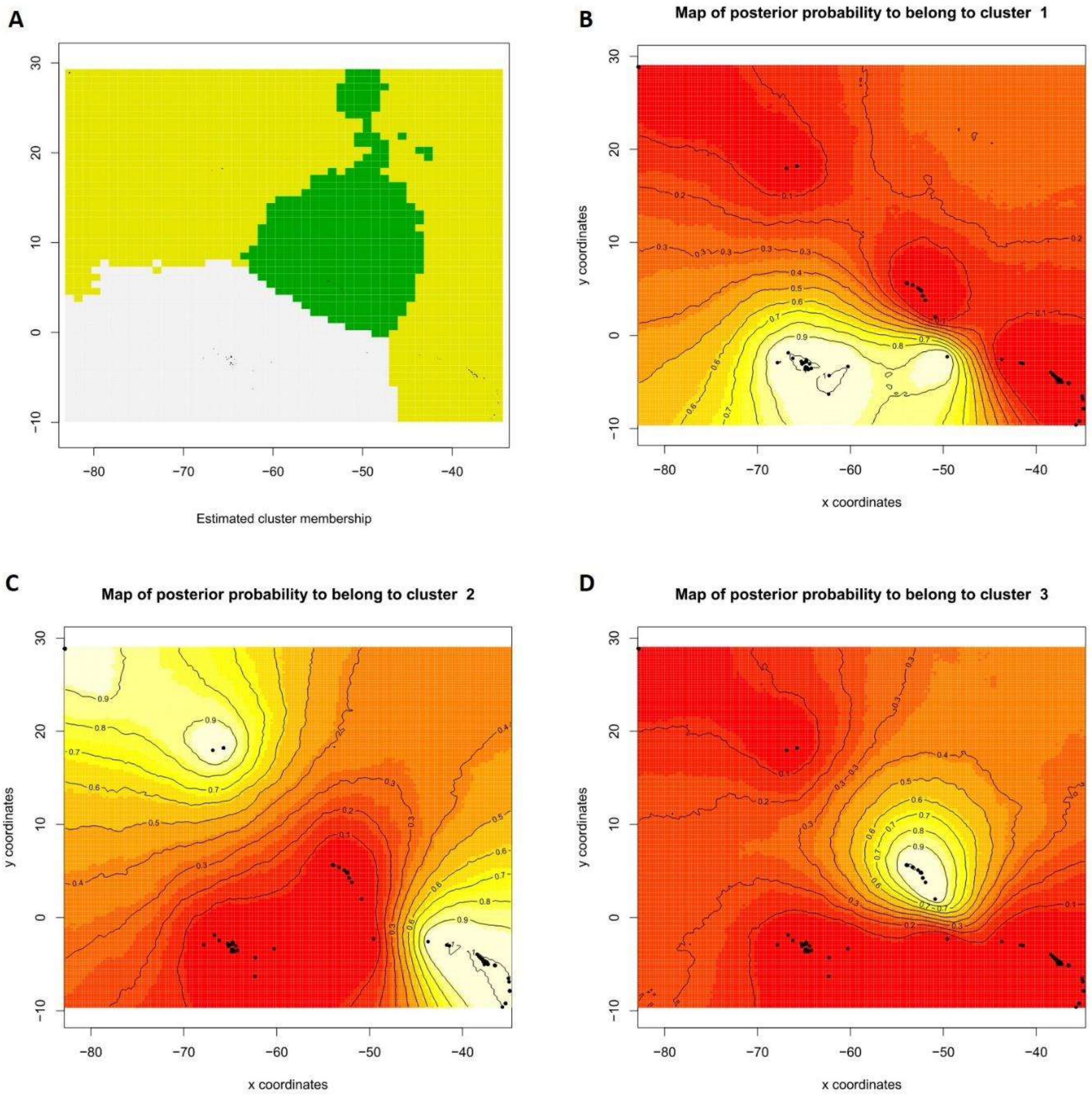


Figure S4. GENELAND' results for American *Trichechus* populations, with uncorrelated alleles and a spatial model. A is the result of the first analysis, to estimate the numbers of populations. B, C, and D are the results of three populations fixed.

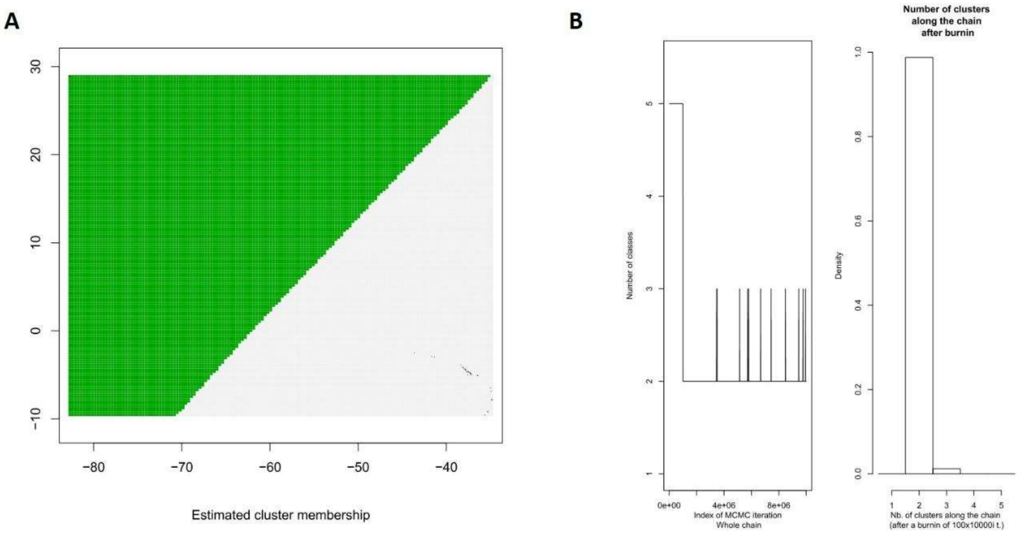


Figure S5. GENELAND' results for *T. manatus* populations, excluding the hybrid zone, with uncorrelated alleles and no spacial model. The A and B are the results of estimate the numbers of populations.

Table S1. All models considered by IMa to populations of analysis 1 (fluvial and marine manatees with hybrids), the number of parameters for each model, its AIC score, AIC differences (Δi), model likelihoods and model probabilities (*w*i) and the evidence ratio based in the best model. All values were calculated following Burnham & Anderson (2002).

	Model	k	log(P)	AIC	Δi	Model Likelihoods	wi	Evidence Ratio (best model)
$\theta_A\ \theta_A\ \theta_A\ m_1\ m_2$	**AAADE**	3	2.3352	1.3296	0	1	0.2590688	
$\theta_A\ \theta_1\ \theta_2\ m_2$	**ABC0D**	4	3.0539	1.8922	0.5626	0.754801861	0.1955456	1.324851
$\theta_A\ \theta_A\ \theta_2\ m_1\ m_2$	**AACDE**	4	2.891	2.218	0.8884	0.641337141	0.1661505	1.559242303
$\theta_A\ \theta/\theta_1\ m_1\ m_2$	**ABBDE**	4	2.6833	2.6334	1.3038	0.521054831	0.1349891	1.919183816
$\theta_A\ \theta/\theta_A\ m_1\ m_2$	**ABADE**	4	2.5879	2.8242	1.4946	0.473643666	0.1227063	2.111291826
$\theta_A\ \theta/\theta_2\ m_1\ m_2$	**FULL**	5	3.1468	3.7064	2.3768	0.304708408	0.0789404	3.281826083
$\theta_A\ \theta/\theta_A\ m_1\ m_1$	**ABADD**	3	-0.5505	7.101	5.7714	0.055815705	0.0144601	17.91610448
$\theta_A\ \theta/\theta_1\ m_1\ m_1$	**ABBDD**	3	-0.8437	7.6874	6.3578	0.041631425	0.0107854	24.02031667
$\theta_A\ \theta/\theta_2\ m_1\ m_1$	**ABCDD**	4	-0.255	8.51	7.1804	0.027592811	0.0071484	36.24132347
$\theta_A\ \theta_A\theta_A\ m_1\ m_1$	**AAADD**	2	-2.4547	8.9094	7.5798	0.022597862	0.0058544	44.25197486
$\theta_A\ \theta_A\theta_2\ m_1\ m_2$	**AACDD**	3	-1.7515	9.503	8.1734	0.016794564	0.0043509	59.54307499
$\theta_A\ \theta_1\ \theta_1$	**ABB00**	2	-54.5761	113.1522	111.8226	$5.22433.10^{-25}$	$1.353.10^{-25}$	$1.91412.10^{24}$
$\theta_A\ \theta_1\ \theta_2$	**ABC00**	3	-54.2516	114.5032	113.1736	$2.65867.10^{-25}$	$6.888.10^{-26}$	$3.76127.10^{24}$
$\theta_A\ \theta_1\ \theta_2\ m_1$	**ABCD0**	4	-54.2534	116.5068	115.1772	$9.76312.10^{-26}$	$2.529.10^{-26}$	$1.02426.10^{25}$
$\theta_A\ \theta_1\ \theta_A$	**ABA00**	2	-57.7494	119.4988	118.1692	$2.18718.10^{-26}$	$5.666.10^{-27}$	$4.5721.10^{25}$
$\theta_A\ \theta_A\ \theta_2$	**AAC00**	2	-66.9263	137.8526	136.523	$2.26156.10^{-30}$	$5.859.10^{-31}$	$4.42174.10^{29}$
$\theta_A\ \theta_A\theta_A$	**AAA00**	1	-73.0194	148.0388	146.7092	$1.38836.10^{-32}$	$3.597.10^{-33}$	$7.20274.10^{31}$

Σ Model Likelihoods = 3.859978273

Population 1: Fluvial manatees; Population 2: Marine manatees

Table S2. All models considered by IMa to populations from analysis 2, the number of parameters for each model, its AIC score, AIC differences (Δi), model likelihoods and model probabilities (*w*i) and the evidence ratio based in the best model. All values were calculated following Burnham & Anderson (2002).

	Model	k	log(P)	AIC	Δi	Model Likelihoods	wi	Evidence Ratio (best model)
$\theta_A\ \theta_1\ \theta_2\ m_1$	**ABCD0**	4	6.7292	-5.4584	0	1	0.209141031	
$\theta_A\ \theta_1\ \theta_A$	**ABA00**	2	4.1254	-4.2508	1.2076	0.546730109	0.114343699	1.829056024
$\theta_A\ \theta_A\ \theta_2$	**AAC00**	2	4.0037	-4.0074	1.451	0.484082467	0.101241506	2.065763724
$\theta_A\ \theta_A\theta_A\ m_1\ m_1$	**AAADD**	2	3.6682	-3.3364	2.122	0.346109528	0.072385704	2.889258804
$\theta_A\ \theta/\theta_2\ m_1\ m_1$	**ABCDD**	4	5.6182	-3.2364	2.222	0.329229567	0.068855411	3.037394271
$\theta_A\ \theta_1\ \theta_2\ m_2$	**ABC0D**	4	5.4228	-2.8456	2.6128	0.270793159	0.056633961	3.692855474
$\theta_A\ \theta_A\ \theta_A\ m_1\ m_2$	**AAADE**	3	4.4222	-2.8444	2.614	0.270630732	0.05659999	3.695071852
$\theta_A\ \theta/\theta_A\ m_1\ m_1$	**ABADD**	3	4.1926	-2.3852	3.0732	0.215111238	0.044988586	4.648757595
$\theta_A\ \theta_1\ \theta_2$	**ABC00**	3	4.1478	-2.2956	3.1628	0.205686935	0.043017578	4.861757509
$\theta_A\ \theta/\theta_1\ m_1\ m_1$	**ABBDD**	3	4.1233	-2.2466	3.2118	0.200708836	0.041976453	4.982341693
$\theta_A\ \theta_A\theta_2\ m_1\ m_2$	**AACDD**	3	4.1192	-2.2384	3.22	0.199887614	0.041804702	5.002811228
$\theta_A\ \theta/\theta_A\ m_1\ m_2$	**ABADE**	4	4.8732	-1.7464	3.712	0.156296568	0.032688025	6.398093146
$\theta_A\ \theta_A\ \theta_2\ m_1\ m_2$	**AACDE**	4	4.8509	-1.7018	3.7566	0.15284973	0.03196715	6.542373368
$\theta_A\ \theta/\theta_1\ m_1\ m_2$	**ABBDE**	4	4.8137	-1.6274	3.831	0.14726818	0.030799819	6.790333115
$\theta_A\ \theta_A\ \theta_A$	**AAA00**	1	1.6279	-1.2558	4.2026	0.122297338	0.025577391	8.176792837
$\theta_A\ \theta_1\ \theta_1$	**ABB00**	2	2.2776	-0.5552	4.9032	0.086155627	0.018018677	11.60690291
$\theta_A\ \theta/\theta_2\ m_1\ m_2$	**FULL**	5	4.6848	0.6304	6.0888	0.047624878	0.009960316	20.99742897

Σ Model Likelihoods = 4.781462506

Population 1: *T. inunguis*; Population 2: *T. manatus*

Table S3. Parameter estimates from the empirical data using IMa to populations of analysis 1 and 2. The high point, mean, and boundaries of the highest posterior density are given for each parameter, for two long runs.

	ϑ_1	ϑ_2	ϑ_A	m_1	m_2	t
Analysis 1						
HiPt	0.7444	0.9074	2.5042	0.0005	0.5193	2.1626
Mean	0.8101	1.0027	2.8874	0.0912	0.5992	1.4074
HPD$_{90Lo}$	0.4937	0.6767	0.5675	0.0005	0.2731	0.5808
HPD$_{90Hi}$	1.1167	1.3223	5.3390	0.2055	0.9219	2.1989
Analysis 2						
HiPt	0.7280	0.5855	2.3833	0.0385	0.0006	2.1747
Mean	0.7819	0.6222	2.5247	0.1127	0.0833	1.4686
HPD$_{90Lo}$	0.4786	0.3895	0.6827	0.0005	0.0006	0.4851
HPD$_{90Hi}$	1.0714	0.8495	4.7059	0.2335	0.1881	2.1989

Analysis 1 - 1: Fluvial manatees; 2: Marine manatees; Analysis 2 - 1: *T. inunguis*; 2: *T. manatus*

Table S4. Demographic parameter converted from model parameter estimates in analysis 1 and 2.

Demographic Parameter	Analysis 1		Analysis 2	
	Fluvial	Marine	*T. inunguis*	*T. manatus*
Mean of the mutation rates/year (U)	1.76 x 10^{-7}		1.77 x 10^{-07}	
Time since splitting (t) in units of years (U)	7,923,971		8,301,271.87	
Generation time (G)	25	20	25	20
Rate per gene per generation between populations (m)	0.09	0.60	0.11	0.08
Effective population size (N_e)	89,072.11	111,340.14	44,196.95	43,962.37
Time since splitting (t) in generations (G)	316,958.83	17,0261.28	332,050.87	415,063.59
Migration rate (m) per generation (G)	4.02 x 10^{-07}	2.12 x 10^{-06}	4.98 x 10^{-07}	2.95 x 10^{-07}
Migration rate (m) per year (U)	1.61 x 10^{-08}	1.06 x 10^{-07}	1.99 x 10^{-08}	1.47 x 10^{-08}
Effective migration rate ($2N_em$)	0.07	0.47	0.07	0.05

CONSIDERAÇÕES FINAIS

O presente estudo contribuiu para a compreensão da formação da zona híbrida entre *T. manatus* e *T. inunguis*, destacando a relevância dos 1500 km de área costeira das Guianas, entre os rios Orinoco e Amazonas. A ausência de híbridos de primeira geração, a grande prevalência de híbridos introgredidos e o relato do primeiro peixe-boi amazônico encontrado no oceano atlântico, indicam a existência de uma população distinta composta majoritariamente por híbridos que possuem vantagens adaptativas a esse habitat. Assim como em muitos casos de hibridização, é difícil avaliar a influência humana nesse processo, porém os indícios apontam para uma população bem adaptada a um ambiente peculiar, que deve ser considerada uma área relevante para o desenvolvimento de políticas conservativas.

Além da zona híbrida, a população brasileira de peixes-bois marinhos também merece ser considerada prioritária para políticas conservacionistas e revisão taxonômica. Apesar da espécie não ser mais considerada em risco eminente de extinção pelo Service Fish and Wildlife (2017) dos EUA por conta do aumento populacional na Flórida, essa estimativa não representa a realidade brasileira, já que enquanto a população da Flórida é composta por aproximadamente 6.350 animais, a do Brasil é composta por 700, distribuídos por 4.000 km de costa. Além disso, a população brasileira apresenta características biológicas únicas, é composta por uma ESU exclusiva e parece ter seu isolamento reforçado pela zona híbrida.

Por conta das diferenças encontradas entre as populações de *T. manatus*, é recomendado que as mesmas sejam manejadas separadamente (em especial a população brasileira) de modo a evitar uma depressão exogâmica. Esse cuidado deve ser expandido aos indivíduos da zona híbrida que, apesar de aparentemente ter se formado sem interferência humana, não deve ser estimulada nem extinguida, por conta de sua grande importância.

A ausência de amostras das áreas de transição da zona híbrida, tanto de *T. inunguis* quanto de *T. manatus* impossibilitou um estudo ainda mais acurado de toda a dinâmica genética que envolve essas populações. Esse fato reforça ainda mais a importância de ações conjuntas de todas as organizações (governamentais ou não) envolvidas na conservação dos peixes-bois no Brasil, para que o manejo desses animais possam ser feito de maneira segura e eficiente, levando-se em conta toda a história evolutiva e genética das populações envolvidas.

REFERÊNCIAS BIBLIOGRÁFICAS

ABBOTT, R., ALBACH, D., ANSELL, S., ARNTZEN, J.W., BAIRD, S.J.E., BIERNE, N., ET AL. (2013) Hybridization and speciation. *Journal of Evolutionary Biology*, 26, 229–246.

ALLENDORF, F.W., HOHENLOHE, P. A & LUIKART, G. (2010) Genomics and the future of conservation genetics. *Nature reviews. Genetics*, 11, 697–709. Nature Publishing Group.

ALLENDORF, F.W., LEARY, R.F., SPRUELL, P. & WENBURG, J.K. (2001) The problems with hybrids : setting conservation guidelines. *Trends in Ecology & Evolution*, 16, 613–622.

ANDERSON, E.C. (2003) User ' s Guide to the Program NewHybrids Version 1 . 1 beta. *Options*, 1–16.

ANDERSON, P.K. (1995) Competition, Predation, and the Evolution and Extinction of Steller's sea cow, *Hydrodamalis gigas*. *Marine Mammal Science*, 11, 391–394. Wiley/Blackwell (10.1111).

ANTHONY, E.J., GARDEL, A., PROISY, C., FROMARD, F., GENSAC, E., PERON, C., ET AL. (2013) The role of fluvial sediment supply and river-mouth hydrology in the dynamics of the muddy, Amazon-dominated Amapá-Guianas coast, South America: A three-point research agenda. *Journal of South American Earth Sciences*, 44, 18–24.

ARTIGAS, L.F., VENDEVILLE, P., LEOPOLD, M., GUIRAL, D. & TERNON, J.-F. (2003) Marine Biodiversity in French Guiana: Estuarine, Coastal, and Shelf Ecosystems Under the Influence of Amazonian Waters. *Gayana*, 67, 302–326.

ASSIS, M.F., BEST, R.C., BARROS, R.M. & YASSUDA, Y.Y. (1988) Cytogenetic study of Trichechus inunguis (Amazonian manatee). *Brazil journal of genetics*, 11, 41–50.

BANDELT, H.J., FORSTER, P. & RÖHL, A. (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular biology and evolution*, 16, 37–48.

BAR-ZVI, D., LUPO, O., LEVY, A.A. & BARKAI, N. (2017) Hybrid vigor: The best of both parents, or a genomic clash? *Current Opinion in Systems Biology*, 6, 22–27. Elsevier Ltd.

BARROS, H.M.D. D. R., MEIRELLES, A.C.O., LUNA, F.O., MARMONTEL, M., CORDEIRO-ESTRELA, P., SANTOS, N. & ASTÚA, D. (2017) Cranial and chromosomal geographic variation in manatees (Mammalia: Sirenia: Trichechidae) with the description of the Antillean manatee karyotype in Brazil. *Journal of Zoological Systematics and Evolutionary Research*, 55, 73–87.

BARTHEM, R.B., GOULDING, M., LEITE, R.G., CAÑAS, C., FORSBERG, B., VENTICINQUE, E., ET AL.

(2017) Goliath catfish spawning in the far western Amazon confirmed by the distribution of mature adults, drifting larvae and migrating juveniles. *Scientific Reports*, 7, 1–13. Nature Publishing Group.

BELKHIR, K., BORSA, P., CHIKHI, L., RAUFASTE, N. & BONHOMME, F. (2000) GENETIX 4.02, Logiciel sous windows pour la génétique des populations. Laboratoire Génome, Populations, Interactions., Université de Montpellier II, France, Montpellier.

BELL, C.E. (2001) Encyclopedia of the World's Zoos. Taylor & Francis.

BERTA, A., SUMICH, J.L., KOVACS, K.M., FOLKENS, P.A. & ADAM, P.J. (2015) Sirenian and Other Marine Mammals: Evolution and Systematics. In *Marine Mammals: Evolutionary Biology* pp. 89–110, 3rd edition. Ed. Academic Press, Massachusetts.

BOUCKAERT, R., HELED, J., KÜHNERT, D., VAUGHAN, T., WU, C.-H., XIE, D., ET AL. (2014) BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. *PLoS Computational Biology*, 10, 1–6.

BRITT, M., HAWORTH, S.E., JOHNSON, J.B., MARTCHENKO, D. & SHAFER, A.B.A. (2018) The importance of non-academic coauthors in bridging the conservation genetics gap. *Biological Conservation*, 218, 118–123.

CANTANHEDE, A.M., DA SILVA, V.M.F., FARIAS, I.P., HRBEK, T., LAZZARINI, S.M. & ALVES-GOMES, J. (2005) Phylogeography and population genetics of the endangered Amazonian manatee, Trichechus inunguis Natterer, 1883 (Mammalia, Sirenia). *Molecular Ecology*, 14, 401–413.

CARSTENS, B.C., STOUTE, H.N. & REID, N.M. (2009) An information-theoretical approach to phylogeography. *Molecular Ecology*, 18, 4270–4282.

CASTELBLANCO-MARTÍNEZ, D.N., DOS REIS, V. & DE THOISY, B. (2017) How to detect an elusive aquatic mammal in complex environments? A study of the Endangered Antillean manatee Trichechus manatus manatus in French Guiana. *Oryx*, 1–11.

CASTELBLANCO-MARTÍNEZ, D.N., DOS REIS, V., DE THOISY, B., CASTELBLANCO-MARTÍNEZ, D.N., DOS REIS, V. & DE THOISY, B. (2017) How to detect an elusive aquatic mammal in complex environments? A study of the Endangered Antillean manatee Trichechus manatus manatus in French Guiana. *Oryx*, 52, 1–11.

CASTELBLANCO-MARTINEZ, D.N., ROSAS, F.C.W., BERMUDEZ, A. & TRUJILLO-GONZALES, T. (2003) Conservation status of the West Indian manatee, Trichechus manatus manatus, in the Middle Orinoco (Vichada, Colombia). In *Conference Proceedings, 15th Biennial Conference on the Biology of Marine Mammals, Greensboro, North Carolina* p. 30.

CIOTTI, L.L. (2012) Isótopos Estáveis de Carbono e Nitrogênio Aplicados ao Estudo da Ecologia Trófica do Peixe-boi Marinho (Trichechus manatus) no Brasil. Universidade Federal do Rio Grande.

COOK, C.N. & SGRÒ, C.M. (2017) Aligning science and policy to achieve evolutionarily enlightened conservation. *Conservation Biology*, 31, 501–512.

CRANDALL, K.A., BININDA-EMONDS, O.R.P., MACE, G.M. & WAYNE, R.K. (2000) Considering evolutionary processes in Conservation Biology. *Trends in Ecology and Evolution*, 15, 290–295.

DACOSTA, J.M. & SORENSON, M.D. (2014) Amplification Biases and Consistent Recovery of Loci in a Double-Digest RAD-seq Protocol. *PloS one*, 9, 1–14.

DARRIBA, D., TABOADA, G.L., DOALLO, R. & POSADA, D. (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, 9, 772–772.

DARWIN, C. (1876) The Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life, 6th edition. London.

DEUTSCH, C.., SELF-SULLIVAN, C. & MIGNUCCI-GIANNONI, A. (2008) *Trichechus manatus*. *The IUCN Red List of Threatened Species*, e.T22103A9.

DIAGNE, K. (2015) *Trichechus senegalensis*. *The IUCN Red List of Threatened Species*, e.T22104A9.

DOMNING, D.P. (1982) Fossil Sirenia from the Sahabi Formation. *Garyounis Scientific Bulletin*, 4, 29–32.

DOMNING, D.P. (1982) Evolution of manatees: A speculative history. *Journal of paleontology*.

DOMNING, D.P. (1982) Commercial exploitation of manatees Trichechus in Brazil c. 1785–1973. *Biological Conservation*, 22, 101–126.

DOMNING, D.P. (1994) A phylogenetic analysis of the Sirenia. *Proceedings of the San Diego Society of Natural History 29:177-189. 1994.*, 13.

DOMNING, D.P. & HAYEK, L.-A.C. (1986a) interspecific and intraspecific morphological variation in manatees (Sirenia:Trichechus). *Marine Mammal Science*, 2, 87–144.

DOMNING, D.P. & HAYEK, L.-A.C. (1986b) Morphological variation in manatees. *Marine Mammal Science*, 2, 87–144.

DONG, Z., XUE-DONG, L. & JIAN-ZHANG, M. (2003) Hybrid zone and its genetic analysis: implication for conservation. *Journal of Forestry Research*, 14, 167–170.

EATON, D.A.R. (2014) PyRAD: Assembly of de novo RADseq loci for phylogenetic analyses. *Bioinformatics*, 30, 1844–1849.

EDMANDS, S. & TIMMERMAN, C.C. (2003) Modeling Factors Affecting the Severity of Outbreeding Depression. *Conservation Biology*, 17, 883–892.

EDWARDS, H.H. (2013) Potential impacts of climate change on warmwater megafauna: The Florida manatee example (Trichechus manatus latirostris). *Climatic Change*, 121, 727–738.

ELGVIN, T.O., TRIER, C.N., TØRRESEN, O.K., HAGEN, I.J., LIEN, S., NEDERBRAGT, A.J., ET AL. (2017) The genomic mosaicism of hybrid speciation. *Science Advances*, 3, 1–16.

EXCOFFIER, L. & LISCHER, H.E.L. (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, 10, 564–567.

FELDHAMER, G.A. (2014) Mammalogy : adaptation, diversity, ecology4ª.

FITZPATRICK, B.M., RYAN, M.E., JOHNSON, J.R., CORUSH, J. & CARTER, E.T. (2015) Hybridization and the species problem in conservation. *Current Zoology*, 61, 206–216.

FITZPATRICK, B.M. & SHAFFER, H.B. (2007) Hybrid vigor between native and introduced salamanders raises new challenges for conservation. *Proceedings of the National Academy of Sciences*, 104, 15793–15798.

FOOTE, A.D., LIU, Y., THOMAS, G.W.C., VINAŘ, T., ALFÖLDI, J., DENG, J., ET AL. (2015) Convergent evolution of the genomes of marine mammals. *Nature Genetics*, 47, 272–275.

FRASER, D.J. & BERNATCHEZ, L. (2001) Adaptive evolutionary conservation: Towards a unified concept for defining conservation units. *Molecular Ecology*, 10, 2741–2752.

FRATANTONI, D.M. & RICHARDSON, P.L. (2006) The Evolution and Demise of North Brazil Current Rings*. *Journal of Physical Oceanography*, 36, 1241–1264.

FROIDEFOND, J.M., GARDEL, L., GUIRAL, D., PARRA, M. & TERNON, J.F. (2002) Spectral remote sensing reflectances of coastal waters in French Guiana under the Amazon influence. *Remote Sensing of Environment*, 80, 225–232.

FUJISAWA, T. & BARRACLOUGH, T.G. (2013) Delimiting Species Using Single-Locus Data and the Generalized Mixed Yule Coalescent Approach: A Revised Method and Evaluation on Simulated Data Sets. *Systematic Biology*, 62, 707–724.

FUNK, C.W., MCKAY, J.K., HOHENLOHE, P.A. & ALLENDORF, F.W. (2012) Harnessing genomics for delineating conservation units. *Trends Ecology and evolutin*, 27, 489–496.

GARCIA-RODRIGUEZ, A.I., BOWEN, B.W., DOMNING, D.P., MIGNUCCI-GIANNONI, A.A., MARMONTEL, M., MONTOYA-OSPINA, R.A., ET AL. (1998) Phylogeography of the West Indian manatee (Trichechus manatus): how many populations and how many taxa? *Molecular Ecology*, 7, 1137–1149.

GARCIA-RODRIGUEZ, A.I., MORAGA-AMADOR, D., FARMERIE, W., MCGUIRE, P.M. & KING, T.L. (2000) Isolation and characterization of microsatellite DNA markers in the Florida manatee(Trichechus manatus latirostris and their application in selected Sirenian species. *Molecular ecology*, 9, 2155–2234.

GENSAC, E., MARTINEZ, J.M., VANTREPOTTE, V. & ANTHONY, E.J. (2016) Seasonal and inter-annual dynamics of suspended sediment at the mouth of the Amazon river: The role of continental and oceanic forcing, and implications for coastal geomorphology and mud bank formation. *Continental Shelf Research*, 118, 49–62.

GEYER, W.R., BEARDSLEY, R.C., LENTZ, S.J., CANDELA, J., LIMEBURNER, R., JOHNS, W.E., ET AL. (1996) Physical oceanography of the Amazon shelf. *Continental Shelf Research*, 16, 575–616.

GINGERICH, P.D. & CAPPETTA, H. (2014) A new archaeocete and other marine mammals (cetacea and sirenia) from lower middle Eocene phosphate deposits of Togo. *Journal of Paleontology*, 88, 109–129.

GIPPOLITI, S. & AMORI, G. (2007) The problem of subspecies and biased taxonomy in conservation lists: the case of mammals. *Folia Zoologica*, 56, 113–117.

GOMPERT, Z. & BUERKLE, C.A. (2010) Introgress: A software package for mapping components of isolation in hybrids. *Molecular Ecology Resources*, 10, 378–384.

GRANT, W.S. (2015) Problems and cautions with sequence mismatch analysis and Bayesian skyline plots to infer historical demography. *Journal of Heredity*, 106, 333–346.

GROBLER, J.P., RUSHWORTH, I., BRINK, J.S., BLOOMER, P., KOTZE, A., REILLY, B., ET AL. (2011) Management of hybridization in an endemic species: decision making in the face of imperfect information in the case of the black wildebeest—Connochaetes gnou. *Eur J Wildl Res*, 57, 997–1006.

GUIA, A.P.O. DE & SAITOH, T. (2007) The gap between the concept and definitions in the Evolutionarily Significant Unit: the need to integrate neutral genetic variation and adaptive variation. *Ecological Research*, 22, 604–612.

GUILLOT, G., LEBLOIS, R., COULON, A. & FRANTZ, A.C. (2009) Statistical methods in spatial genetics. *Molecular Ecology*, 18, 4734–4756.

GUILLOT, G., MORTIER, F. & ESTOUP, A. (2005) GENELAND: A computer package for landscape genetics. *Molecular Ecology Notes*, 5, 712–715.

GUILLOT, G., RENAUD, S., LEDEVIN, R., MICHAUX, J. & CLAUDE, J. (2012) A unifying model for the analysis of phenotypic, genetic, and geographic data. *Systematic Biology*, 61, 897–911.

HALDANE, J.B.S. (1922) Sex ratio and unisexual sterility in hybrid animals. *Journal of Genetics*, 12, 101–109.

HARPER, J.Y., SAMUELSON, D.A. & REEP, R.L. (2005) Corneal vascularization in the Florida manatee (Trichechus manatus latirostris) and three-dimensional reconstruction of vessels. *Veterinary ophthalmology*, 8, 89–99.

HASEGAWA, M., KISHINO, H. & YANO, T. (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecuar Evolution*, 22, 160–174.

HATT, R.T. (1934) A manatee collected by the American Museum Congo Expedition, with observations on the recent manatees. *Bulletin American Museum of Natural History*, 66, 533–566.

HEDRICK, P.W. & GARCIA-DORADO, A. (2016) Understanding Inbreeding Depression, Purging, and Genetic Rescue. *Trends in Ecology & Evolution*, 31, 940–952.

HEY, J. & NIELSEN, R. (2007) Integration within the Felsenstein equation for improved Markov chain Monte Carlo methods in population genetics. *Proceedings of the National Academy of Sciences*, 104, 2785–2790.

HOGG, C.J., GRUEBER, C.E., PEMBERTON, D., FOX, S., LEE, A. V., IVY, J.A. & BELOV, K. (2017) “Devil Tools & Tech”: A Synergy of Conservation Research and Management Practice. *Conservation Letters*, 10, 133–138.

HOGG, J.T., FORBES, S.H., STEELE, B.M. & LUIKART, G. (2006) Genetic rescue of an insular population of large mammals. *Proceedings of the Royal Society B*, 273, 1491–1499.

HOORN, C., WESSELINGH, F.P., TER STEEGE, H., BERMUDEZ, M.A., MORA, A., SEVINK, J., ET AL. (2010) Amazonia Through Time : Andean. *Science*, 330, 927–931.

HUNTER, M.E., AUIL-GOMEZ, N.E., TUCKER, K.P., BONDE, R.K., POWELL, J. & MCGUIRE, P.M. (2010) Low genetic variation and evidence of limited dispersal in the regionally important Belize manatee. *Animal Conservation*, 13, 592–602.

HUNTER, M.E., MIGNUCCI-GIANNONI, A. A., TUCKER, K.P., KING, T.L., BONDE, R.K., GRAY, B. A. & MCGUIRE, P.M. (2012) Puerto Rico and Florida manatees represent genetically distinct groups. *Conservation Genetics*, 13, 1623–1635.

HUSON, D.H. (1998) SplitsTree: Analyzing and visualizing evolutionary data. *Bioinformatics*, 14, 68–73.

HUSON, D.H., AUCH, A.F., QI, J. & SCHUSTER, S.C. (2007) MEGAN analysis of metagenomic data. *Genome Research*, 17, 377–386.

HUSON, D.H. & BRYANT, D. (2006) Application of Phylogenetic Networks in Evolutionary Studies. *Molecular Biology and Evolution*, 23, 254–267. Oxford University Press.

HUSON, D.H. & SCORNAVACCA, C. (2012) Software for Systematics and Evolution Dendroscope 3: An Interactive Tool for Rooted Phylogenetic Trees and Networks. *Syst. Biol*, 61, 1061–1067.

HYDE, E.R., HAARMANN, D.P., LYNNE, A.M., BUCHELI, S.R. & PETROSINO, J.F. (2013) The Living Dead: Bacterial Community Structure of a Cadaver at the Onset and End of the Bloat Stage of Decomposition. *PLoS ONE*, 8, 1–10.

ICMBIO (2012) Procedimentos para a Elaboração, Aprovação, Publicação, Implementação, Monitoria, Avaliação e Revisão de Planos de Ação Nacionais para Conservação de Espécies Ameaçadas de Extinção ou do Patrimônio Espeleológico. In p. 64. Diário Oficial da União, Brasil.

INGER, R.F. (1961) Problems in the application of the subspecies concept in vertebrate taxonomy. In *Vertebrate Speciation* pp. 262–285.

IUCN (2012) IUCN Red List Categories and Criteria: Version 3.1. In *IUCN* p. Second. IUCN, Gland, Switzerland and Cambridge, UK.

JACKIW, R.N., MANDIL, G. & HAGER, H.A. (2015) A framework to guide the conservation of species hybrids based on ethical and ecological considerations. *Conservation Biology*, 29, 1040–1051.

JOLY, E. (2010) The existence of species rests on a metastable equilibrium between inbreeding and outbreeding. An essay on the close relationship between speciation, inbreeding and recessive mutations. *Biology Direct*, 6, 1–55.

JOLY, S. & BRUNEAU, A. (2006) Incorporating allelic variation for reconstructing the evolutionary history of organisms from multiple genes: An example from Rosa in North America. *Systematic biology*, 55, 623–636.

JOLY, S., BRYANT, D. & LOCKHART, P.J. (2015) Flexible methods for estimating genetic distances from single nucleotide polymorphisms. *Methods in Ecology and Evolution*, 6, 938–948.

JOMBART, T. & AHMED, I. (2011) adegenet 1.3-1: New tools for the analysis of genome-wide SNP data. *Bioinformatics*, 27, 3070–3071.

KNOWLES, L.L. & MADDISON, W.P. (2002) Statistical phylogeography. *Molecular Ecology*, 11, 2623–2635.

KOCHER, T.D., THOMAS, W.K., MEYER, A., EDWARDS, S. V., PAABO, S., VILLABLANCA, F.X. & WILSON, A.C. (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences*, 86, 6196–6200.

KOPELMAN, N.M., MAYZEL, J., JAKOBSSON, M., ROSENBERG, N.A. & MAYROSE, I. (2015) Clumpak: A program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources*, 15, 1179–1191.

KRONFORST, M.R., HANSEN, M.E.B., CRAWFORD, N.G., GALLANT, J.R., ZHANG, W., KULATHINAL, R.J., ET AL. (2013) Hybridization reveals the evolving genomic architecture of speciation. *Cell reports*, 5, 666–677. The Authors.

KUMAR, S., STECHER, G. & TAMURA, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, 33, 1870–1874.

LANGMEAD, B. & SALZBERG, S.L. (2012) Fast gapped-read alignment with Bowtie 2. *Nat Methods*, 9, 357–359.

LARKIN, M., BLACKSHIELDS, G., BROWN, N., CHENNA, R., MCGETTIGAN, P., MCWILLIAM, H., ET AL. (2007) ClustalW and ClustalX version 2. *Bioinformatics*, 23, 2947–2948.

LARKIN, M.A., BLACKSHIELDS, G., BROWN, N.P., CHENNA, R., MCGETTIGAN, P.A., MCWILLIAM, H., ET AL. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics*, 23, 2947–2948.

LEACHÉ, A.D., HARRIS, R.B., RANNALA, B. & YANG, Z. (2014) The Influence of Gene Flow on Species Tree Estimation: A Simulation Study. *Syst. Biol*, 63, 17–30.

LEIGH, J.W. & BRYANT, D. (2015) popart : full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, 6, 1110–1116. Wiley/Blackwell (10.1111).

LUIZ, O.J., MADIN, J.S., ROBERTSON, D.R., ROCHA, L.A., WIRTZ, P. & FLOETER, S.R. (2012)

Ecological traits influencing range expansion across large oceanic dispersal barriers: insights from tropical Atlantic reef fishes. *Proceedings of the Royal Society B: Biological Sciences*, 279, 1033–1040.

LUNA, F. DE O., SILVA, V.M.F. DA, ANDRADE, M.C.M. DE, MARQUES, C.C., NORMANDE, I.C., VELÔSO, T.M.G. & SEVERO, M.M. (2011) Plano de Ação Nacional para a Conservação dos Sirênios.

LUNA, F.O., BONDE, R.K., ATTADEMO, F.L.N., SAUNDERS, J.W., MEIGS-FRIEND, G., PASSAVANTE, J.Z.O. & HUNTER, M.E. (2012) Phylogeographic implications for release of critically endangered manatee calves rescued in Northeast Brazil. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 22, 665–672.

MALLET, J. (2005) Hybridization as an invasion of the genome. *Trends in ecology & evolution*, 20, 229–237.

MARMONTEL, M., SOUZA, D. & KENDALL, S. (2016) *Trichechus inunguis*. *The IUCN Red List of Threatened Species*, e.T22102A4.

MARMONTEL, M.M., ODELL, D.K. & REYNOLDS III, J.E. (1992) Reproductive Biology of South American Manatees. In *Reproductive Biology of South American Vertebrates* (ed W.C. Hamlett), p. 328. Springer-Verlag.

MARSH, H., LAWLER, I.R., KWAN, D., DELEAN, S., POLLOCK, K. & ALLDREDGE, M. (2004) Aerial surveys and the potential biological removal technique indicate that the Torres Strait dugong fishery is unsustainable. *Animal Conservation*, 7, 435–443.

MARSH, H., O'SHEA, T.J. & REYNOLDS III, J.E. (2012) Ecology and conservation of the sirenia: dugongs and manatees. In *Conservation Biology* p. .

MARSHALL, C.D., KUBILIS, P.S., HUTH, G.D., EDMONDS, V.M., HALIN, D.L. & REEP, R.L. (2000) Food-handling ability and feeding-cycle length of manatees feeding on several species of aquatic plants. *Journal of Mammalogy*.

MARSHALL, T.C. & SPALTON, J.A. (2000) Simultaneous inbreeding and outbreeding depression in reintroduced Arabian oryx. *Animal Conservation*, 3, 241–248.

MARTIN, S.H. & JIGGINS, C.D. (2017) Interpreting the genomic landscape of introgression. *Current Opinion in Genetics and Development*, 47, 69–74. Elsevier Ltd.

MAYR, E. (1982) Of what use are subspecies? *Auk*, 99, 593–595.

MEIRELLES, A.C.O. DE, CARVALHO, V.L. & MARMONTEL, M. (2018) West Indian Manatee Trichechus manatus in South America: Distribution, Ecology and Health Assessment. In

Advances in Marine Vertebrate Research in Latin America. Coastal Researche Library (eds M. Rossi-Santos & C. Finkl), pp. 263–291, 22nd edition. Springer, Cham.

MEIRELLES, A.C.O., MARMONTEL, M. & MOBLEY, R.S.S.L. (2016) Biology. In *West Indian Manatee: biology and conservation in Brazil / Peixe-boi marinho: biologia e conservação no Brasil* (eds A.C. Meirelles & V.L. Carvalho), pp. 29–50, 1st edition. Bambu Editora e Artes Gráfiacas, São Paulo.

MELLO, B., VILELA, J.F. & SCHRAGO, C.G. (2018) Conservation phylogenetics and computational species delimitation of Neotropical primates. *Biological Conservation*, 217, 397–406.

MEREDITH, R.W., JANEČKA, J.E., GATESY, J., RYDER, O. A, FISHER, C. A, TEELING, E.C., ET AL. (2011) Impacts of the Cretaceous Terrestrial Revolution and KPg extinction on mammal diversification. *Science*, 334, 521–524.

MEYER, M. & KIRCHER, M. (2010) Illumina sequencing library preparation for highly multiplexed target capture and sequencing. *Cold Spring Harbor Protocols*, 1–7.

MILLER, J.M., POISSANT, J., HOGG, J.T. & COLTMAN, D.W. (2012) Genomic consequences of genetic rescue in an insular population of bighorn sheep (*Ovis canadensis*). *Molecular Ecology*, 21, 1583–1596.

MMA (2014) Portaria n°445, de 17 de Dezembro de 2014. In *Diário Oficial da União - Seção 1* pp. 126–130. Brasil.

MONTOYA-OSPINA, R.A., CAICEDO-HERRERA, D., MILLA, S.L., MIGNUCCI-GIANNONI, A.A. & LEFEBVRE, L.W. (2001) Status and distribution of the West Indian manatee , Trichechus manatus manatus , in Colombia. *Biological Conservation*, 102, 117–129.

MORITZ, C. (1994a) Defining 'Evolutionarily Significant Units' for conservation. *Trends Ecology and evolutin*, 9, 373–375.

MORITZ, C. (1994b) Applications of mitochondrial DNA analysis in conservation: a critical review. *Molecular Ecology*, 3, 401–411.

MORITZ, C. (1999) Conservation units and traslocations: stategies for conserving evolutionary processes. *Hereditas*, 130, 217–228.

MULLER-KARGER, F.E., MCCLAIN, C.R. & RICHARDSON, P.L. (1988) The dispersal of the Amazon's water. *Nature*, 332, 56–59.

MURPHY, W.J., EIZIRIK, E., JOHNSON, W.E., ZHANG, Y.P., RYDER, O.A. & O'BRIEN, S.J. (2001) Molecular phylogenetics and the origins of placental mammals. *Nature*, 409, 614–618.

NETWORK, M.C.S. (2012) What do we need to know about speciation? *Trends in Ecology and Evolution*, 27, 27–39.

NIELSEN, R. & WAKELEY, J. (2001) Distinguishing Migration From Isolation: A Markov Chain Monte Carlo Approach. *Genetics*, 158, 885–896.

NORMANDE, I.C., DE OLIVIERA LUNA, F., MENDES MALHADO, A.C., GOMES BORGES, J.C., VIANA JUNIOR, P.C., NIEMEYER ATTADEMO, F.L. & LADLE, R.J. (2015) Eighteen years of Antillean manatee Trichechus manatus manatus releases in Brazil : lessons learnt. *Oryx*, 49, 338–344.

ORTEGA-ARGUETA, A. & CASTELBLANCO-MARTÍNEZ, D.N. (2018) Is captive breeding a priority for manatee conservation in Mexico? *Oryx*, 1–8.

OTTENBURGHS, J., MEGENS, H.-J., KRAUS, R.H.S., VAN HOOFT, P., VAN WIEREN, S.E., CROOIJMANS, R.P.M.A., ET AL. (2017) A history of hybrids? Genomic patterns of introgression in the True Geese. *BMC Evolutionary Biology*, 17, 201. BioMed Central.

PALSBØLL, P.J., BÉ RUBÉ, M. & ALLENDORF, F.W. (2006) Identification of management units using population genetic data. *Trends in Ecology and Evolution*, 22, 11–16.

PARR, L., SANTOS, F.R., WAYCOTT, M., VIANNA, J.A., MCDONALD, B., CABALLERO, S. & LOPES, M.J. DE S. (2012) Sirenian Genetics and Demography. In *Sirenian Conservation* (eds E.M. Hines, J.E. Reynolds III, L. V Aragones, A.A. Mignucci-Giannoni & M. Marmontel), pp. 168–179, 1st edition. University Press of Florida.

PELTIER, W.R. & FAIRBANKS, R.G. (2006) Global glacial ice volume and Last Glacial Maximum duration from an extended Barbados sea level record. *Quaternary Science Reviews*, 25, 3322–3337.

PÉREZ, I., ANADÓN, J.D., DÍAZ, M., NICOLA, G.G., TELLA, J.L. & GIMÉNEZ, A. (2012) What is wrong with current translocations? A review and a decision-making proposal. *Frontiers in Ecology and the Environment*, 10, 494–501.

PETERSON, B.K., WEBER, J.N., KAY, E.H., FISHER, H.S. & HOEKSTRA, H.E. (2012) Double digest RADseq: An inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PLoS ONE*, 7, 1–11.

PINTO, P.V., BEJA, P., FERRAND, N., GODINHO, R., PINTO, P.V., BEJA, P., ET AL. (2016) Hybridization following population collapse in a critically endangered antelope. *Scientific Reports*, 6, 1–9. Nature Publishing Group.

PONS, J., BARRACLOUGH, T.G., GOMEZ-ZURITA, J., CARDOSO, A., DURAN, D.P., HAZELL, S., ET

AL. (2006) Sequence-Based Species Delimitation for the DNA Taxonomy of Undescribed Insects. *Systematic Biology*, 55, 595–609.

PRESSEY, R.L., WEEKS, R. & GURNEY, G.G. (2017) From displacement activities to evidence-informed decisions in conservation. *Biological Conservation*, 212, 337–348.

PRITCHARD, J.K., STEPHENS, M. & DONNELLY, P. (2000) Inference of population structure using multilocus genotype data. *Genetics*, 155, 945–959. Genetics Soc America.

R CORE TEAM (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

RAMBAUT, A. & DRUMMOND, A.J. (2010a) LogCombiner version 1.6.1.

RAMBAUT, A. & DRUMMOND, A.J. (2010b) TreeAnnotator version 1.6.1.

RAMBAUT, A., DRUMMOND, A.J., XIE, D., BAELE, G. & SUCHARD, M.A. (2018) Posterior Summarization in Bayesian Phylogenetics Using Tracer 1.7. *Systematic Biology*, 1–5.

REID, N.M. & CARSTENS, B.C. (2012) Phylogenetic estimation error can decrease the accuracy of species delimitation: a Bayesian implementation of the general mixed Yule-coalescent model. *BMC Evolutionary Biology*, 12, 1–11.

REYNOLDS, J. & WETZEL, D. (2008) Reintroduction of Manatees Trichechus manatus into Guadeloupe , Lesser Antilles : Issues , Questions and Possible Answers. Sarasota, Florida.

RHYMER, J.M. & SIMBERLOFF, D. (1996) Extinction by hybridization and introgression. *Annu. Rev. Ecol. Syst*, 27, 83–109.

ROSENBERG, N.A. (2004) DISTRUCT: A program for the graphical display of population structure. *Molecular Ecology Notes*, 4, 137–138.

ROZAS, J., SÁNCHEZ-DELBARRIO, J.C., MESSEGUER, X. & ROZAS, R. (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, 19, 2496–2497. Oxford Univ Press.

RYDER, O.A. (1986) Species conservation and systematics: the dilemma of subspecies. *Trends Ecology and evolutin*, 1, 9–10.

SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. (1989) Molecular cloning. *Society*, 68, 1232–1239.

SANTOS, F.R., BARROS, H.M., SCHETINO, M.A.A. & LIMA, C.S. DE (2016) Genetics. In *West Indian Manatee: biology and conservation in Brazil / Peixe-boi marinho: biologia e* *conservação no Brasil* (eds A.C. Meirelles & V.L. Carvalho), pp. 63–75, 1st edition. Bambu Editora e Artes Gráfiacas, São Paulo.

SANTOS JÚNIOR, J.E., SANTOS, F.R. & SILVEIRA, F.A. (2015) Hitting an Unintended Target: Phylogeography of Bombus brasiliensis Lepeletier, 1836 and the First New Brazilian Bumblebee Species in a Century (Hymenoptera: Apidae). *PLoS ONE*.

SATIZÁBAL, P., MIGNUCCI-GIANNONI, A. A, DUCHÊNE, S., CAICEDO-HERRERA, D., PEREA-SICCHAR, C.M., GARCÍA-DÁVILA, C.R., ET AL. (2012) Phylogeography and sex-biased dispersal across riverine manatee populations (Trichechus inunguis and Trichechus manatus) in South America. *PloS one*, 7, e52468.

SCHAEFER, J., DUVERNELL, D. & CAMPBELL, D.C. (2016) Hybridization and introgression in two ecologically dissimilar Fundulus hybrid zones. *Evolution*, 70, 1051–1063.

SCHWENK, K. (2008) Introduction. Extent, processes and evolutionary impact of interspecific hybridization in animals. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 363, 2805–2811.

SERVICE FISH AND WILDLIFE (2017) Endangered and Threatened Wildlife and Plants; Reclassification of the West Indian Manatee From Endangered to Threatened. In p. 145. Federal register, United States.

SHAFER, A.B., WOLF, J.B., ALVES, P.C., BERGSTRÖ, L., BRUFORD, M.W., BRÄ NNSTRÖ, I., ET AL. (2015) Genomics and the challenging translation into conservation practice. *Trends in Ecology & Evolution*, 30, 78–87.

SHURTLIFF, Q.R. (2013) Mammalian hybrid zones: A review. *Mammal Review*, 43, 1–21.

SPALDING, M.D., FOX, H.E., ALLEN, G.R., DAVIDSON, N., FERDAÑA, Z.A., FINLAYSON, M., ET AL. (2007) Marine Ecoregions of the World: A Bioregionalization of Coastal and Shelf Areas. *BioScience*, 57, 573–583.

SPIEGELBERGER, T. & GANSLOSSER, U. (2005) Habitat analysis and exclusive bank feeding of the Antillean manatee (*Trichechus manatus manatus* L. 1758) in the Coswine Swamps of French Guiana, South America. *Tropical Zoology*, 18, 1–12.

SPRINGER, M.S., SIGNORE, A. V., PAIJMANS, J.L. A. A., VÉLEZ-JUARBE, J., DOMNING, D.P., BAUER, C.E., ET AL. (2015) Interordinal gene capture, the phylogenetic position of Steller's sea cow based on molecular and morphological data, and the macroevolutionary history of Sirenia. *Molecular phylogenetics and evolution*, 91, 178–193. Elsevier Inc.

STEEL, M. & MCKENZIE, A. (2001) Properties of phylogenetic trees generated by Yule-type speciation models. *Mathematical Biosciences*, 170, 91–112.

STRONEN, A. V. & PAQUET, P.C. (2013) Perspectives on the conservation of wild hybrids. *Biological Conservation*, 167, 390–395. Elsevier Ltd.

TAMURA, K., STECHER, G., PETERSON, D., FILIPSKI, A. & KUMAR, S. (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, 30, 2725–2729.

TAUTZ, D. (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic acids research*, 17, 6463–6471.

TAYLOR, H.R., DUSSEX, N. & VAN HEEZIK, Y. (2017) Bridging the conservation genetics gap by identifying barriers to implementation for conservation practitioners. *Global Ecology and Conservation*, 10, 231–242.

DE THOISY, B., SPIEGELBERGER, T., ROUSSEAU, S., TALVY, G., VOGEL, I. & VIÉ, J.-C. (2003) Distribution, habitat, and conservation status of the West Indian manatee Trichechus manatus in French Guiana. *Oryx*, 37, 431–436.

THOMAS, J.W., TOUCHMAN, J.W., BLAKESLEY, R.W., BOUFFARD, G.G., BECKSTROM-STERNBERG, S.M., MARGULIES, E.H., ET AL. (2003) Comparative analyses of multi-species sequences from targeted genomic regions. *Nature*, 424, 788–793. Nature Publishing Group.

TOEWS, D.P.L. & BRELSFORD, A. (2012) The biogeography of mitochondrial and nuclear discordance in animals. *Molecular Ecology*.

TUCKER, K.P., HUNTER, M.E., BONDE, R.K., CLARK, A.M., BECK, C. A., MCGUIRE, P.M., ET AL. (2012) Low genetic diversity and minimal population substructure in the endangered Florida manatee: implications for conservation. *Journal of Mammalogy*, 93, 1504–1511.

UICN FRANCE, MNHN, GEPOG, KWATA, BIOTOPE, HYDRECO & OSL (2017) La Liste rouge des espèces menacées en France - Chapitres de la Faune vertébrée de Guyane. Paris, France.

UNEP, QUINTANA-RIZZO, E. & REYNOLDS III, J. (2010) Regional Management Plan for the West Indian Manatee (*Trichechus manatus*). CEP Technical Report No. 48. Kingston, Jamaica.

VAUGHAN, T.G. (2017) IcyTree: Rapid browser-based visualization for phylogenetic trees and networks. *Bioinformatics*, 33, 2392–2394.

VÉLEZ-JUARBE, J. & DOMNING, D.P. (2014) Fossil Sirenia of the West Atlantic and Caribbean region. Ix. *Metaxytherium albifontanum* , sp. nov. *Journal of Vertebrate Paleontology*, 34, 444–464. Taylor & Francis Group .

VELEZ-JUARBE, J., DOMNING, D.P. & PYENSON, N.D. (2012) Iterative evolution of sympatric seacow (Dugongidae, Sirenia) assemblages during the past ~26 million years. *PLoS ONE*, 7, e31294. Public Library of Science.

VIANNA, J.A., BONDE, R.K., CABALLERO, S., GIRALDO, J.P., LIMA, R.P., CLARK, A., ET AL. (2006) Phylogeography, phylogeny and hybridization in trichechid sirenians: implications for manatee conservation. *Molecular ecology*, 15, 433–447.

WAYNE, R.K. & SHAFFER, H.B. (2016) Hybridization and endangered species protection in the molecular era. *Molecular Ecology*, 25, 2680–2689. Wiley/Blackwell (10.1111).

WHITE, J.R., HARKNESS, D.R., ISAACKS, R.E. & DUFFIELD, D.A. (1976) Some studies on blood of the Florida manatee, Trichechus manatus latirostris. *Comp Biochem Physiol*, 55.

YU, Y., DONG, J., LIU, K.J. & NAKHLEH, L. (2014) Maximum likelihood inference of reticulate evolutionary histories. *Proceedings of the National Academy of Sciences of the United States of America*, 111, 16448–16453.

ZHANG, C., OGILVIE, H.A., DRUMMOND, A.J. & STADLER, T. (2017) Bayesian Inference of Species Networks from Multilocus Sequence Data. *Molecular Biology and Evolution*, 35, 504–517.

ZHOU, Y., DUVAUX, L., REN, G., ZHANG, L., SAVOLAINEN, O. & LIU, J. (2017) Importance of incomplete lineage sorting and introgression in the origin of shared genetic variation between two closely related pines with overlapping distributions. *Heredity*, 118, 211–220.

ANEXO 1

Submitted manuscript to Annals of the Brazilian Academy of Sciences

A hybrid swarm of manatees along the Guianas coastline, a peculiar environment under the influence of the Amazon River plume

Camilla S. Lima, Rafael F. Magalhães, Miriam Marmontel, Ana Carolina Meirelles, Vitor Luz Carvalho, Anne Lavergne, Benoit de Thoisy, Fabrício R. Santos

Camilla S. Lima: Laboratório de Biodiversidade e Evolução Molecular, Departamento de Genética, Ecologia e Evolução, ICB, Universidade Federal de Minas Gerais, Av. Antonio Carlos 6627, CP 486, 31270-010 Belo Horizonte, MG, Brazil.

Rafael F. Magalhães: Programa de Pós-Graduação em Zoologia, Departamento de Zoologia, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil.

Miriam Marmontel: Instituto de Desenvolvimento Sustentável Mamirauá, Estrada do Bexiga, 2584, Fonte Boa, 69553-225 Tefé, Amazonas, Brazil.

Ana Carolina O. Meirelles: Associação de Pesquisa e Preservação de Ecossistemas Aquáticos (Aquasis), Av. José Alencar, 150, Sesc Iparana, 61627-010 Caucaia, Ceará, Brazil

Vitor Luz Carvalho: Associação de Pesquisa e Preservação de Ecossistemas Aquáticos (Aquasis), Av. José Alencar, 150, Sesc Iparana, 61627-010 Caucaia, Ceará, Brazil

Anne Lavergne, Institut Pasteur de Guyane, 23 Avenue Pasteur, BP 6010, Cayenne Cedex 97306, French Guiana, France

Benoit de Thoisy: Kwata NGO and Institut Pasteur de Guyane, 23 Avenue Pasteur, BP 6010, Cayenne Cedex 97306, French Guiana, France

Fabrício R. Santos (Corresponding author): Laboratório de Biodiversidade e Evolução Molecular, Departamento de Genética, Ecologia e Evolução, ICB, Universidade Federal de Minas Gerais, Av. Antonio Carlos 6627, CP 486, 31270-010 Belo Horizonte, MG, Brazil. E-mail fsantos@icb.ufmg.br

Keywords: Conservation Genetics, Hybrid zone, Introgression, Amazon River plume, Guianas coastline, Trichechus.

Running title: A hybrid swarm of manatees on the Guianas coastline
Academy Section Biological Sciences

ABSTRACT

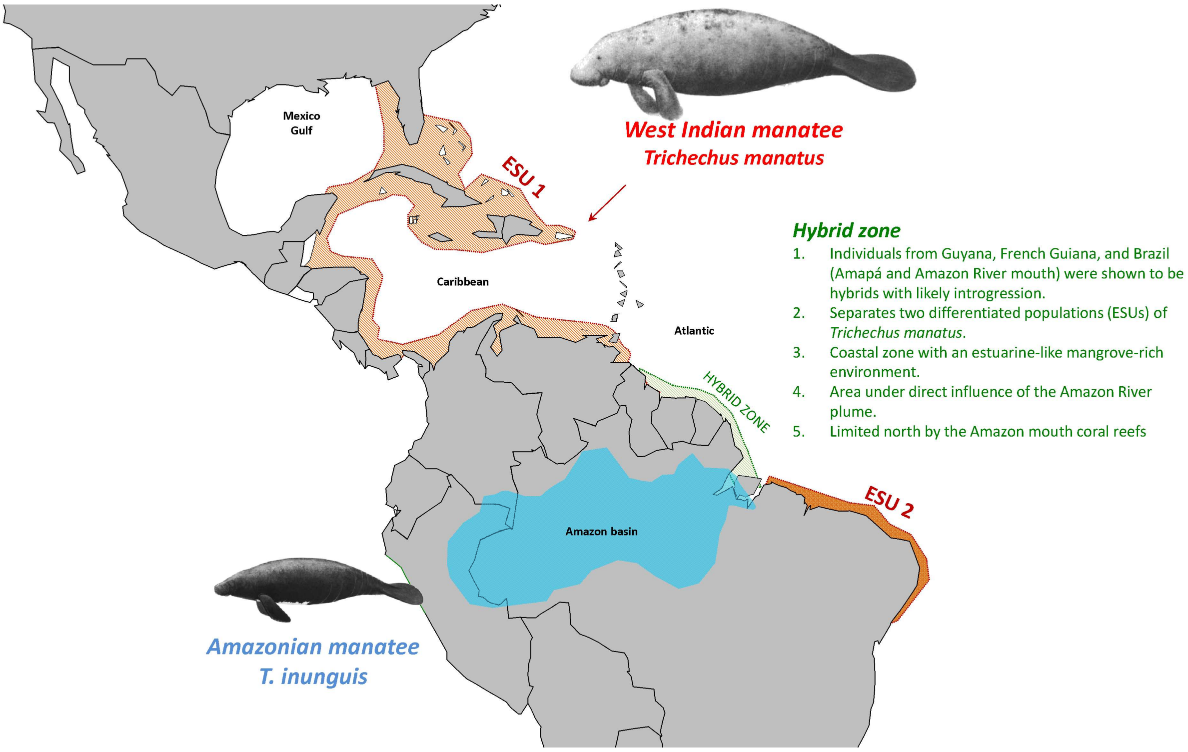
The West Indian (*Trichechus manatus*) and Amazonian (*Trichechus inunguis*) manatees have a sympatric occurrence at the mouth of the Amazon River. A result of this interspecific encounter is the occurrence of hybrids, which are frequently found along the coasts of Amapá state in Brazil, French Guiana and Guyana. Here we present new genetic evidence indicating the occurrence of a hybrid swarm along the Guianas Shield coastline, which is an interspecific hybrid zone that also separates *T. manatus* populations located east (Brazil) and west (Caribbean, Gulf of Mexico, Florida and Antilles). Besides, we suggest that this hybrid population occupies a peculiar mangrove-rich environment under strong influence of the Amazon River plume, which requires an independent management and should be considered a special conservation area.

SHORT COMMUNICATION

Conservation genetics provides important evidence for management of threatened species in nature and captivity (Frankel, 1974; Caughley, 1994). Currently, genetic data are used to understand the evolutionary dynamics of natural populations, which can be applied in conservation strategies to mitigate anthropogenic effects, allowing the persistence of species and their ecosystems in the medium and long term (Allendorf et al., 2010; Arif et al., 2011; Stronen and Paquet, 2013). However, the conservation of interspecific hybrid populations is a matter of controversy, with the origin and context of hybridization (e.g., natural or anthropogenic) having to be considered in conservation policies (Jackiw et al., 2015). This conservation controversy is mostly due to association of hybrids with outbreeding depression (OD) and possible deleterious effects on both parental species (Allendorf et al., 2001), especially in cases of direct or indirect anthropogenic influence, causing human-mediated population decline of endangered species under sympatry due to intrinsic OD (e.g., Pinto et al., 2016).

In some cases, hybrids are at least partially fertile and may cross with one of the parent species, resulting in introgression (Turner and Harr, 2014; Balcova et al., 2016). These hybrids can compete for resources and reproductive priority, which may reduce the adaptive value of local populations of parental species and eventually lead to local or global extinction (Brumfield, 2010; Wayne and Shaffer, 2016). However, some hybrid populations may be more well adapted for survival in peculiar environments as compared to their non-hybrid parents (Allendorf et al., 2001; Fitzpatrick and Shaffer, 2007), and may deserve particular protection (Jackiw et al., 2015). This is the case of hybrid zones with intermediate environments between the parental ones, where the hybrids possess ideal combinations of genes to live and thrive (Dong et al., 2003).

A case of interspecific hybridization in animals is found in South American manatees, aquatic mammals represented by three species of the *Trichechus* genus (Mammalia, Sirenia, Trichechidae). In the Americas there are two species, the West Indian manatee (*Trichechus manatus*) inhabiting the tropical Caribbean and Atlantic coastlines, estuaries and adjacent riverine and lake systems; and the Amazonian manatee (*T. inunguis*), restricted to the Amazon River basin. Previous genetic studies have identified the occurrence of interspecific hybrids between these two manatee species close to the Amazon River mouth and along the Guianas Shield coastline, from Amapá state in Brazil to Guyana (Garcia-Rodriguez et al., 1998; Vianna et al., 2006; Santos et al., 2016). This area is found separating two largely differentiated populations (or ESUs) of *T. manatus*, one along the Brazilian coast, and the other distributed from Venezuela to Florida (EUA) and the Antilles (Figure 1).



Hybrid zone

1. Individuals from Guyana, French Guiana, and Brazil (Amapá and Amazon River mouth) were shown to be hybrids with likely introgression.
2. Separates two differentiated populations (ESUs) of *Trichechus manatus*.
3. Coastal zone with an estuarine-like mangrove-rich environment.
4. Area under direct influence of the Amazon River plume.
5. Limited north by the Amazon mouth coral reefs

Figure 1. Map representing the distribution of *Trichechus inunguis* and *T. manatus* populations and the interspecific hybrid zone along the Guianas Shield coastline. The west and eastern distribution of both *T. manatus* evolutionary significant units (ESUs) is represented, according to genetics, cytogenetics and skull morphology evidence (Vianna et al., 2006; Santos et al., 2016; Barros et al., 2017).

To illustrate this hybridization event, we sequenced one nuclear (Recombination activating gene 1; RAG1) and one mitochondrial DNA (control region; CR) gene fragments of *T. inunguis* (Amazonas, Brazil) and *T. manatus* (Rio Grande do Norte, Brazil) as references, plus one hybrid previously identified by Vianna et al. (2006) from Amapá (Brazil), and five samples from Iracoubo, Awala, Mana, Kourou and an unknown locality (French Guiana). We amplified the gene fragments using primers previously reported (Murphy et al., 2001). For each locus, the PCR mix was prepared with final volume of 25 µL, containing: 20 ng of genomic DNA; 1× buffer; 1.5 mM MgCl2; 0.5 µM each primer, 200 µM dNTPs, and 0.5 U Platinum™ Taq DNA polymerase (Thermo Fisher Scientific). The cycles of amplification followed the conditions described by Vianna et al. (2006), with the annealing temperature of 58°C to RAG1. PCR products were purified by the polyethylene glycol (PEG) method (20% PEG 8000, 2.5M NaCl) (Santos-Júnior et al., 2015) and sequenced on the ABI 3130xl Genetic Analysis (Applied Biosystems) using the BigDye Termination v3.1 Cycle Sequencing kit, with the same primers used for amplification. Chromatograms were analyzed in SeqScape v2.6 and consensus sequences were aligned using the Clustal W algorithm (Larkin et al., 2007) in the software MEGA7 (Tamura et al, 2013). A total of 767 bp and 410 bp were sequenced for RAG1 and CR, respectively.

We made a phylogenetic analysis to show the relationship of the individuals from the hybrid zone and the parental species, using the CR and RAG-1 concatenated. We selected the best partition scheme and nucleotide substitution models using model selection implemented in PartitionFinder v.2.1.1 (Lanfear et al., 2016), taking the codon positions of RAG-1 into account. For this, we used the linked model of branch lengths and an exact search. The model selection was made using a corrected-Akaike Information Criterion. We reconstructed the phylogeny in MrBayes v3.2 (Ronquist et al., 2012), setting the best partition scheme and its optimal models (i.e., HKY+I for CR, HKY for 1st and 2nd codon positions of RAG1, and K80+I for the 3rd position). We made this analysis with two replicates, 1 x 106 generations, thinning 5000 and a burn-in of 20% of first generations. The stationarity and convergence were checked using the Tracer v1.6 (Rambaut et al., 2018).

The topology of the consensus tree reveals a polytomy between the individuals, with a clade constituted by all *T. manatus*, other clade formed by *T. inunguis* and individuals from the hybrid zone, and one individual from the hybrid zone alone (Figure 2). The absence of monophyly of *T. inunguis* in relation to individuals from the hybrid zone is an expected pattern observed in many studies that include introgression or backcross hybrids (Haines et al., 2014; Halas and Simons, 2014; Lecaudey et al., 2018). Although our data suggest that introgressed individuals are more related to *T. inunguis* than to *T. manatus*, this may be an artefact from our limited data, especially because the concatenation results generally are biased towards the fragment with more variation (Liu et al., 2015), in our case the mtDNA CR. For mtDNA, the greatest relatedness between hybrids and *T. inunguis* had already been reported for French

Guiana populations (Vianna et al., 2006). For this reason, this phylogenetic positioning should be better investigated using more independent markers.

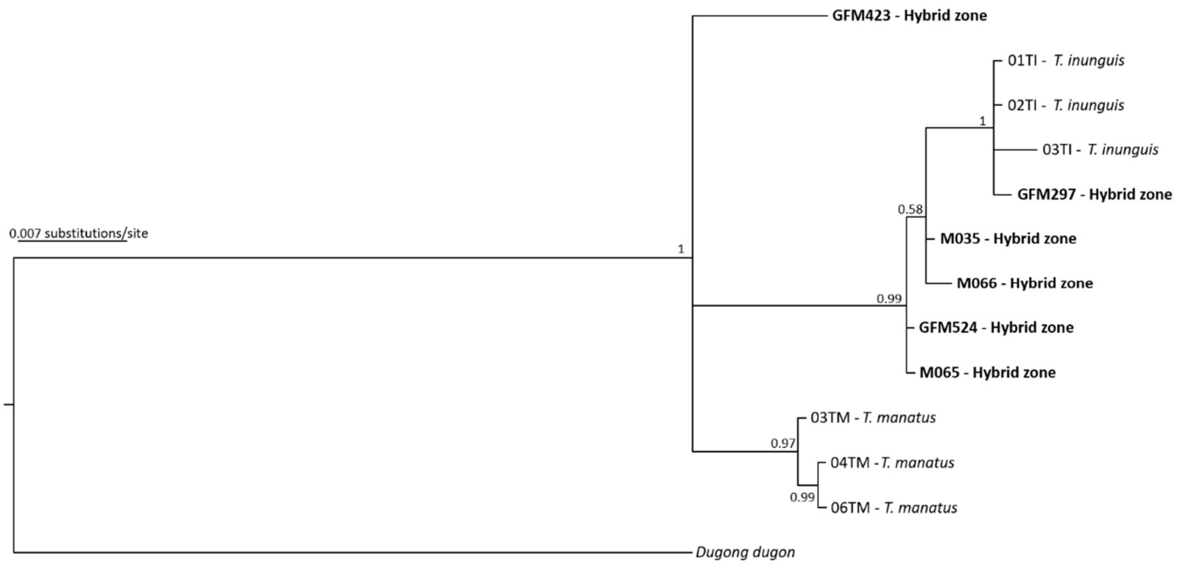


Figure 2. Phylogenetic consensus tree of *T. manatus*, *T. inunguis* and hybrid zone's sample (bold) to CR and RAG-1 loci, using *Dugong dugon* as the outgroup. On the node labels are the posterior probability (PP) of each clade.

The biogeographic patterns of the western Atlantic Ocean have a direct influence on distribution and fitness of local marine biota, showing the Guianas coastline as a barrier between many species and populations (Spalding et al., 2007). The presence of hybrid manatees in this area under Amazon plume influence seems to be associated to the isolation of the western and eastern West Indian manatees (Figure 1) and the sympatric area between Amazonian and West Indian manatees (Vianna et al., 2006; Santos et al., 2016; Barros et al., 2017). The region between the mouths of the Amazon and Orinoco rivers (extension of 1500 km of coastal South America) is known by peculiar characteristics caused by the Amazon River plume along this oceanic coast (Anthony et al., 2013). The unique coastal dynamics is largely influenced by the massive suspended-sediment discharge, causing a mega-turbidity on the shelf, a growth of a subaqueous delta and an intermediary salinity (Anthony et al., 2013; Anthony et al., 2014). All these factors have a direct influence on oceanic biodiversity, including commercial marine species, coral reef endemics and threatened species completely dependent on ecosystems such as estuaries, mudflats, sandy beaches and, particularly, littoral mangroves (Artigas et al., 2003).

The wild population of manatees on the Guianas coastline is composed by an introgressed population derived from interspecific crossings between *T. manatus* and *T. inunguis* (Vianna et al., 2006; Santos et al., 2016), likely happened on the sympatric area at the Amazon River mouth. Even though the recent collapse of the population of both parental species may be associated with some hybridization level, the hybrid zone appears to have existed for many generations in this area, with an intermediate coastal habitat under influence of the Amazon River plume. Indeed, because of this peculiar estuarine-like habitat in a 1500 km of coastal area between the mouths of the Orinoco and Amazon rivers, hybrids may have some advantages over the parental species, probably due to a singular combination of adapted genes to these intermediate conditions. The large predominance of hybrids of a large aquatic mammal in an area shown to be a vicariant zone for many marine species, and a distinctive habitat for many estuary-associated animals, emphasizes the independent management of the Guianas coastline as a special protection area with a peculiar environment and biota.

ACKNOWLEDGEMENTS

CSL was supported by a CAPES (Brazil) PhD fellowship, RFM by a CAPES-PNPD (Brazil) fellowship, and FRS by a CNPq (Brazil) research fellowship. This project was performed under ICMBio/SISBIO permit 45028 and received funding support from FAPEMIG, CNPq, PETROBRAS and Fundação o Boticário from Brazil.

REFERENCES

ALLENDORF FW, HOHENLOHE PA AND LUIKART G. 2010. Genomics and the future of conservation genetics. Nat Rev Genet 11: 697–709.

ALLENDORF FW, LEARY RF, SPRUELL P AND WENBURG JK. 2001. The problems with hybrids: setting conservation guidelines 16: 613–622.

ANTHONY EJ, GARDEL A AND GRATIOT N. 2014. Fluvial sediment supply, mud banks, cheniers and the morphodynamics of the coast of South America between the Amazon and Orinoco river mouths. Geol Soc London, Spec Publ 388: 533–560.

ANTHONY EJ, GARDEL A, PROISY C, FROMARD F, GENSAC E, PERON C, WALCKER R AND LESOURD S. 2013. The role of fluvial sediment supply and river-mouth hydrology in the dynamics of the muddy, Amazon-dominated Amapá-Guianas coast, South America: A three-point research agenda. J South Am Earth Sci 44: 18–24.

ARIF IA, KHAN HA, BAHKALI AH, AL HOMAIDAN AA, AL FARHAN AH, AL SADOON M AND SHOBRAK M. 2011. DNA marker technology for wildlife conservation. Saudi J Biol Sci 18: 219–25.

ARTIGAS LF, VENDEVILLE P, LEOPOLD M, GUIRAL D AND TERNON J-F. 2003. Marine biodiversity in French Guiana: Estuarine, coastal, and shelf ecosystems under de influence of Amazonian waters. Gayana 67: 302–326.

BALCOVA M, FALTUSOVA B, GERGELITS V, BHATTACHARYYA T, MIHOLA O, TRACHTULEC Z, KNOPF C, FOTOPULOSOVA V, CHVATALOVA I, GREGOROVA S AND FOREJT J. 2016. Hybrid Sterility Locus on Chromosome X Controls Meiotic Recombination Rate in Mouse. PLoS Genet 12: 1–16.

BARROS HMD, MEIRELLES ACO, LUNA FO, MARMONTEL M, CORDEIRO-ESTRELA P, SANTOS N AND ASTÚA D. 2017. Cranial and chromosomal geographic variation in manatees (Mammalia: Sirenia: Trichechidae) with the description of the Antillean manatee karyotype in Brazil. J Zool Syst Evol Res 55: 73–87.

BRUMFIELD RT. 2010. Speciation genetics of biological invasions with hybridization. Mol Ecol 19:5079–5083.

CAUGHLEY G. 1994. Directions in conservation biology. J Anim Ecol 63: 215–244.

DONG Z, XUE-DONG L AND JIAN-ZHANG M. 2003. Hybrid zone and its genetic analysis: implication for conservation. J For Res 14: 167–170.

FITZPATRICK BM AND SHAFFER HB. 2007. Hybrid vigor between native and introduced salamanders raises new challenges for conservation. Proc Natl Acad Sci 104: 15793–15798.

FRANKEL OH. 1974. Genetic conservation: our evolutionary responsibility. Genetics 78: 53–65.

GARCIA-RODRIGUEZ AI, BOWEN BW, DOMNING D, MIGNUCCI-GIANNONI AA, MARMONTEL M, MONTOYA-OSPINA RA, MORALES-VELA B, RUDIN M, BONDE RK AND MCGUIRE PM. 1998. Phylogeography of the West Indian manatee (Trichechus manatus): how many populations and how many taxa? Mol Ecol 7: 1137–1149.

HAINES ML, MOUSSALLI A, STUART-FOX D, CLEMANN N AND MELVILLE J. 2014. Phylogenetic evidence of historic mitochondrial introgression and cryptic diversity in the genus Pseudemoia (Squamata: Scincidae). Mol Phylogenet Evol 81: 86–95.

HALAS D AND SIMONS AM. 2014. Cryptic speciation reversal in the Etheostoma zonale (Teleostei: Percidae) species group, with an examination of the effect of recombination and introgression on species tree inference. Mol Phylogenet Evol 70: 13–28.

JACKIW RN, MANDIL G AND HAGER HA. 2015. A framework to guide the conservation of species hybrids based on ethical and ecological considerations. Conserv Biol 29: 1040–1051.

LANFEAR R, FRANDSEN PB, WRIGHT AM, SENFELD T AND CALCOTT B. 2016. PartitionFinder 2: New Methods for Selecting Partitioned Models of Evolution for Molecular and Morphological Phylogenetic Analyses. Mol Biol Evol 34: 772–773.

LARKIN M, BLACKSHIELDS G, BROWN N, CHENNA R, MCGETTIGAN P,

LECAUDEY LA, SCHLIEWEN UK, OSINOV AG, TAYLOR EB, BERNATCHEZ L AND WEISS SJ. 2018. Inferring phylogenetic structure, hybridization and divergence times within Salmoninae (Teleostei: Salmonidae) using RAD-sequencing. Mol Phylogenet Evol 124: 82–99.

LIU L, XI Z, WU S, DAVIS CC AND EDWARDS SV. 2015. Estimating phylogenetic trees from genome-scale data. Ann NY Acad Sci 1360: 36–53.

MCWILLIAM H, VALENTIN F, WALLACE I, WILM A, LOPEZ R, THOMPSON J, GIBSON T AND HIGGINS D. 2007. ClustalW and ClustalX version 2. Bioinformatics 23: 2947–2948.

MURPHY WJ, EIZIRIK E, JOHNSON WE, ZHANG YP, RYDER OA AND O'BRIEN SJ. 2001. Molecular phylogenetics and the origins of placental mammals. Nature 409: 614–618.

PINTO PV, BEJA P, FERRAND N AND GODINHO R. 2016. Hybridization following population collapse in a critically endangered antelope. Sci Rep 6: 1-9.

RAMBAUT A, DRUMMOND AJ, XIE D, BAELE G AND SUCHARD MA. 2018. Posterior Summarization in Bayesian Phylogenetics Using Tracer 1.7. Syst Biol 1–5.

RONQUIST F, TESLENKO M, VAN DER MARK P, AYRES DL, DARLING A, OHNA S, LARGET B, LIU L, SUCHARD MA AND HUELSENBECK JP. 2012. Software for Systematics and Evolution MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. Syst Biol 61: 539–542.

SANTOS-JÚNIOR JE, SANTOS FR AND SILVEIRA FA. 2015. Hitting an Unintended Target: Phylogeography of Bombus brasiliensis Lepeletier, 1836 and the First New Brazilian Bumblebee Species in a Century (Hymenoptera: Apidae). PLOS One. 10:e0125847.

SANTOS FR, BARROS HM, SCHETINO MA AND LIMA CS. 2016. Genetics. In MEIRELLES AC AND CARVALHO VL (Eds.), West Indian Manatee Biol. Conserv. Brazil / Peixe-boi Mar. Biol. e Conserv. no Bras. 1st ed., pp. 63–75. São Paulo, SP: Bambu Editora e Artes Gráficas.

SPALDING MD, FOX HE, ALLEN GR, DAVIDSON N, FERDAÑA ZA, FINLAYSON

M, HALPERN BS, JORGE MA, LOMBANA A, LOURIE SA, MARTIN KD, MCMANUS E, MOLNAR J, RECCHIA CA AND ROBERTSON J. 2007. Marine Ecoregions of the World: A Bioregionalization of Coastal and Shelf Areas. Bioscience 57: 573–583.

STRONEN AV AND PAQUET PC. 2013. Perspectives on the conservation of wild hybrids. Biol. Conserv 167: 390–395.

TAMURA K, STECHER G, PETERSON D, FILIPSKI A AND KUMAR S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30: 2725–2729.

TURNER LM AND HARR B. 2014. Genome-wide mapping in a house mouse hybrid zone reveals hybrid sterility loci and Dobzhansky-Muller interactions. Elife 3: 1–25.

VIANNA JA, BONDE RK, CABALLERO S, GIRALDO JP, LIMA RP, CLARK A, MARMONTEL M, MORALES-VELA B, DE SOUZA MJ, PARR L, RODRÍGUEZ-LOPEZ MA, MIGNUCCI-GIANNONI AA, POWELL JA AND SANTOS FR. 2006. Phylogeography, phylogeny and hybridization in trichechid sirenians: implications for manatee conservation. Mol Ecol 15: 433–447.

WAYNE RK AND SHAFFER HB. 2016. Hybridization and endangered species protection in the molecular era. Mol Ecol 25: 2680–2689.

ANEXO 2

Capítulo escrito no livro **West Indian Manatee: biology and conservation in Brazil / Peixe-boi marinho: biologia e conservação no Brasil** (eds A.C. Meirelles & V.L. Carvalho), pp. 63–75, 1° edição. Bambu Editora e Artes Gráfiacas, São Paulo, 2016.

Genética
Genetics

Fabricio R. Santos
Programa de Pós-Graduação em Genética, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
Postgraduate Program in Genetics, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil

Helen M.D.R. Barros
Universidade Federal de Pernambuco, Recife, PE, Brasil
Federal University of Pernambuco, Recife, PE, Brazil

Marco A.A. Schetino & Camilla S. Lima
Programa de Pós-Graduação em Genética, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
Postgraduate Program in Genetics, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil

Genética da conservação e marcadores moleculares
Conservation genetics and molecular markers

A genética da conservação é uma disciplina científica que incorpora o estudo de variantes hereditárias, genotípicas ou fenotípicas à pesquisa da biologia da conservação, com a finalidade de gerar conhecimento sobre a diversidade populacional para a elaboração de estratégias que visem a conservação de espécies e ecossistemas.

Atualmente, estudos de história natural de espécies e suas populações nativas fornecem alguns dos principais subsídios que são utilizados para determinar prioridades e estratégias de conservação da biodiversidade. Os dados genéticos de espécies ameaçadas permitem compreender a dinâmica evolutiva de suas populações naturais, a qual deve ser mantida ou restaurada para mitigar os efeitos antropogênicos e garantir a persistência destas populações na natureza em médio e longo prazo. Por exemplo, dados sobre parentesco, diversidade genética remanescente, tamanho efetivo e níveis de endogamia são parâmetros importantes para estimar a viabilidade das populações na natureza e minimizar problemas devido à depressão endogâmica. Outros dados interpopulacionais, como fluxo gênico, estrutura genética ao longo da distribuição espacial da espécie, padrões filogeográficos e adaptações gênicas locais permitem planejar estratégias apropriadas de translocação de indivíduos de acordo com a dinâmica evolutiva das populações, as quais permitem aumentar a diversidade genética local e minimizar outros efeitos negativos como a depressão exogâmica.

Nos estudos de genética da conservação da biodiversidade, o DNA mitocondrial (DNAmt) tem sido muito utilizado desde o final da década de 1980. Por ser uma molécula circular simples, portadora de vários genes em um arranjo estável, sem DNA repetitivo, íntrons ou pseudogenes, de rápida evolução, herdado uniparentalmente (via materna), sem recombinação e virtualmente haploide, o DNAmt oferece diversas vantagens no estudo da história natural das espécies e de suas populações (AVISE *et al.*, 1987; FRANKHAM; BALLOU; BRISCOE *et al.*, 2008). Além disto, diferentes regiões codificadoras e não codificadoras podem ser escolhidas, adequando a taxa evolutiva requerida à profundidade temporal a ser investigada. Geralmente, a região controladora ou HVSI do DNAmt é de evolução rápida, portanto, útil para investigar eventos recentes (intraespecíficos), enquanto genes codificadores de proteínas, como o Citocromo c Oxidase Subunidade I (COI), são úteis em estudos interespecíficos. Por essas características, o DNAmt tem sido também muito utilizado em estudos de biogeografia histórica por meio de análises filogeográficas e de genética populacional. Os resultados filogeográficos permitem compreender a dinâmica evolutiva de linhagens genéticas (ou haplótipos), no tempo e no espaço, a qual está associada aos eventos de divergência populacional, às flutuações demográficas e ao padrão de fluxo gênico interpopulacional que moldaram a distribuição da diversidade genética atual de cada espécie.

Atualmente, várias abordagens alternativas em genética são possíveis com o aumento do conhecimento dos genomas. Isto permite o desenvolvimento de novos marcadores moleculares que complementam e refinam as análises feitas com DNAmt nas diferentes escalas temporais. Entre estes marcadores, os mais

Conservation genetics is a scientific discipline that incorporates the study of hereditary variants, genotypic or phenotypic, to conservation biology research, with the purpose of generating knowledge about population diversity for the elaboration of strategies aimed at the conservation of species and ecosystems.

Currently, studies of the natural history of species and their native populations provide major subsidies to determine priorities and strategies for biodiversity conservation. Genetic data from threatened species bring an understanding about the evolutionary dynamics of their natural populations, which must be maintained or restored to mitigate anthropogenic effects and guarantee the persistence of these populations in nature in the medium and long terms. For example, data on kinship, remaining genetic diversity, effective size and inbreeding levels are important parameters to estimate the viability of populations in nature and minimize problems due to inbreeding depression. Other inter-population data, such as gene flow, genetic structure along the species' spatial distribution, phylogeographic patterns and local genic adaptations, permit the planning of appropriate strategies for the translocation of individuals according to the evolutionary dynamics of populations, which result in increased local genetic diversity and minimize other negative effects, such as outbreeding depression.

In studies of genetics for biodiversity conservation, the mitochondrial DNA (mtDNA) has been widely used since the end of the eighties. For being a simple circular molecule, carrier of several genes in a stable arrangement, without repetitive DNA, introns or pseudogenes, of rapid evolution, inherited uniparentally (maternally), without recombination and virtually haploid, the mtDNA offers several advantages in the study of the natural history of species and their populations (AVISE et al., 1987; FRANKHAM; BALLOU; BRISCOE et al., 2008). In addition, different coding and non-coding regions can be chosen, adjusting the evolutionary rate required to the temporal depth to be investigated. Usually, the mtDNA control region, or the HVSI, is fast evolving, therefore, useful to investigate recent events (intraspecific), while protein coding genes, such as the cytochrome c oxidase subunit I (COI), are useful in interspecific studies. For these characteristics, the mtDNA has also been widely used in historical biogeography studies through phylogeographic and population genetics analysis. The phylogeographic results reveal the evolutionary dynamics of genetic lineages (or haplotypes), in time and space, which is associated to population divergence events, demographic fluctuations and the inter-population gene flow pattern that shaped each species' current genetic diversity distribution.

Currently, several alternative approaches in genetics are possible with the increased knowledge of the genomes. This allows the development of new molecular markers that complement and refine the analysis of mtDNA in different time-scales. Among these markers, microsatellites are the most popular. These are biparental nuclear repetitive segments (inherited from both mother and father), which are very useful in recent scale analysis (last decades and centuries), as in studies of paternity, kinship, interpopulation gene flow and inbreeding estimates. In longer time scales (thousands to millions of years), the association of various markers representative of the entire genome allows one to generate more robust intra and interspecific phylogenetic trees that can clarify doubts in the systematics and natural history of some taxonomic groups (FRANKHAM et al., 2008). Therefore, with the growing availability of genomic data for several species, including the manatee (http://www.ncbi.nlm.nih.gov/bioproject/68243), new nuclear markers based on sequencing or genotyping of point mutations (SNPs) can be developed and used to complement genetic data obtained with mtDNA and microsatellites.

populares são os microssatélites, segmentos repetitivos nucleares biparentais (herdados do pai e da mãe) que se mostram muito úteis nas análises em escalas recentes (últimas décadas e séculos), tal como nos estudos de paternidade, parentesco, fluxo gênico interpopulacional e estimativas de endogamia. Nas escalas temporais antigas (milhares a milhões de anos), a associação de vários marcadores representativos de todo o genoma permite gerar filogenias intra e interespecíficas mais robustas, que podem esclarecer dúvidas em sistemática e história natural de alguns grupos taxonômicos (FRANKHAM *et al.*, 2008). Portanto, com a disponibilização crescente de dados genômicos de vários organismos, inclusive do peixe-boi-marinho (http://www.ncbi.nlm.nih.gov/bioproject/68243), novos marcadores nucleares baseados em sequenciamento ou genotipagem de mutações de ponto (SNPs) podem ser desenvolvidos e utilizados para complementar os dados genéticos obtidos com DNAmt e microssatélites.

Fundamentos genéticos para a prática da conservação
Genetic fundamentals for conservation practice

No nível intraespecífico, a genética lida com toda a variação hereditária genotípica e fenotípica que é necessária para a continuidade evolutiva das populações ameaçadas. Quando esta variação é quantificada dentro de populações, ela permite entender aspectos relacionados à biologia reprodutiva (poliginia, poliandria, paternidade extra-par, nível de parentesco, etc) e à diversidade genética que ainda existe (FRANKHAM *et al.*, 2008). Normalmente, esta diversidade é quantificada com marcadores neutros (não influenciados pela Seleção Natural), mas com os novos dados genômicos e a identificação de genes relacionados a processos adaptativos ou reprodutivos, a quantificação da variação diretamente ligada à adaptação populacional poderá ser futuramente utilizada. Com este tipo de evidência genética intrapopulacional é possível, por exemplo, sugerir manejos para o aumento da diversidade genética e diminuição da homozigosidade (nos casos de endogamia) das populações naturais, ou nos casos de cativeiro, planejar acasalamentos que evitem, por exemplo, a endogamia e a manifestação de desordens recessivas (FRANKHAM *et al.*, 2008, Figura 1).

Outras análises estimam também a diversidade que existe entre populações, as quais podem ser mais ou menos diferenciadas, dependendo do grau de isolamento de cada uma delas, ou seja, o nível de fluxo gênico entre elas. Estes resultados permitem inferir o padrão de estruturação populacional, que na maior parte dos casos está relacionado com a distribuição geográfica das populações. Estas medidas de estruturação populacional permitem revelar agrupamentos de indivíduos ou populações muito divergentes que, em certos casos, podem ser consideradas subespécies ou *quasi*-espécies (espécies incipientes). Na prática da conservação, estas análises de estruturação populacional e filogeografia permitem identificar unidades evolutivas significativas (ESUs), que são populações com relativa independência evolutiva e são geralmente tratadas como unidades diferentes de manejo ou populações prioritárias para conservação. Isto permite a implantação de métodos de manejo que visem perpetuar a dinâmica evolutiva das populações naturais, minimizando impactos antrópicos recentes e futuros problemas advindos do manejo inadequado (FRANKHAM *et al.*, 2008).

A estruturação populacional, principalmente aquela de longo prazo (vários milhares de anos), deve ser seriamente considerada nos casos de manejo envolvendo a translocação de indivíduos entre populações divergentes. Embora o aumento da diversidade genética seja um dos objetivos do manejo na natureza e um critério importante para reverter efeitos negativos da endogamia, o cruzamento entre indivíduos de populações que possuem grandes diferenças genéticas, seja por adaptações exclusivas para cada localidade ou por combinações adaptadas de genes (epistasia), pode resultar na menor viabilidade ou problemas reprodutivos na prole (FRANKHAM *et al.*, 2008). Este efeito deletério é conhecido como depressão exogâmica, uma diminuição do valor adaptativo populacional devido à inviabilidade relativa da prole destes cruzamentos que pode levar à extinção local de populações com número reduzido (Figura 1). Isto se dá porque os indivíduos oriundos destes cruzamentos não são tão adaptados para a sobrevivência quanto os seus pais, nos seus respectivos ambientes originais (FRANKHAM *et al.*, 2008).

Os efeitos da depressão exogâmica são ainda mais drásticos quando há hibridização entre espécies diferentes, podendo ocorrer uma acentuada diminuição da viabilidade populacional (ALLENDORF *et al.*, 2001). Em raros casos, alguns híbridos são férteis e podem cruzar com uma das espécies parentais, resultando em introgressão. Estes híbridos podem competir por recursos e prioridade reprodutiva, diminuindo o valor adaptativo das populações locais das espécies parentais e, eventualmente, levar à extinção local ou global. A depressão exogâmica intra e interespecífica (Figura 1) aparece, normalmente, por consequência de dois tipos de efeitos antropogênicos: indiretos, quando o declínio populacional resulta em aumento da hibridização (principalmente entre espécies); ou diretos, quando o manejo inadequado das translocações põe em contato indivíduos de populações ou espécies muito divergentes geneticamente.

At the intraspecific level, genetics deals with the entire genotypic and phenotypic hereditary variation that is required for the evolutionary continuity of threatened populations. When this variation is quantified within populations, it clarifies aspects related to reproductive biology (polygymy, polyandry, extra-pair paternity, level of kinship, etc.) and to remaining genetic diversity (FRANKHAM et al., 2008). Normally, this diversity is quantified with neutral markers (not influenced by natural selection), but with new genomic data and the identification of genes related to adaptive or reproductive processes, the quantification of variation directly linked to the population adaptation may be used in the future. With this type of intra-population genetic evidence it is possible, for example, to suggest management measures to increase genetic diversity and decrease homozygosis (in cases of inbreeding) of natural populations, or, in captivity cases, to plan matings which avoid, for example, inbreeding and the manifestation of recessive disorders (FRANKHAM et al., 2008, Figure 1).

Other analyses also estimate the diversity between populations, which can be more or less differentiated, depending on the degree of isolation of each one of them, i.e. the level of gene flow between them. These results allow us to infer the population-structuring pattern, which in most cases relates to the geographic distribution of the populations. These measures of population structuring reveal very divergent groupings of individuals or populations, which may, in certain cases, be considered subspecies or quasi-species (incipient species). In conservation practice, these analyses of population structuring and phylogeography allow the identification of Evolutionary Significant Units (ESUs), which are populations with relative evolutionary independence that are generally treated as different management units or populations of conservation priority. This enables the development of management methods aimed at perpetuating the evolutionary dynamics of natural populations, minimizing recent anthropic impacts and future problems evolving from inadequate management (FRANKHAM et al., 2008).

The population structuring, especially in the long term (several thousands of years), should be seriously considered in cases of management involving the translocation of individuals between divergent populations. Although increasing genetic diversity is one of the management objectives in nature and an important criterion to revert the negative effects of inbreeding, the mating between individuals from genetically different populations, because of either adaptations unique to each locality or adapted combinations of genes (epistasy), may result in lower viability or reproductive problems in the offspring (FRANKHAM et al., 2008). This deleterious effect is known as outbreeding depression, a decrease in the population adaptive value due to the relative unviability of the offspring from these matings that can lead to the local extinction of small populations (Figure 1). This is because the individuals born from these matings are not as adapted as their parents for the survival in their respective original environments (FRANKHAM et al., 2008).

The effects of outbreeding depression are even more drastic when there is hybridization between different species, possibly leading to a marked reduction of the population viability (ALLENDORF et al., 2001). In rare cases, some hybrids are fertile and can mate with one of the parental species, resulting in introgression. These hybrids can compete for resources and reproductive priority, decreasing the adaptive value of local populations of the parental species and possibly lead to local or global extinction. The intra and interspecific outbreeding depression (Figure 1) appears, normally, as a result of two types of anthropogenic effects: indirect, when the population decline results in increased hybridization (mainly between species); or direct, when the inadequate management of translocations brings into contact individuals from genetically divergent populations or species.

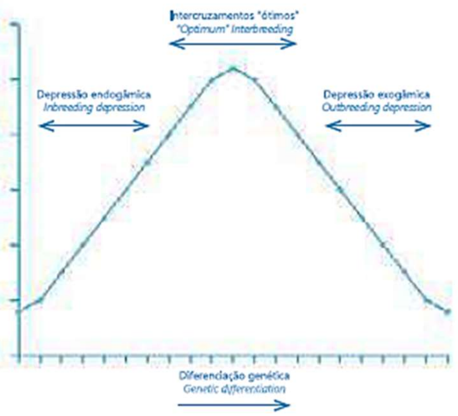


Figura 1. Gráfico que mostra a associação entre viabilidade e/ou sucesso reprodutivo da prole e a diferenciação genética entre os pais. A parte interna da curva revela a "zona ótima" de intercruzamentos e nas extremidades aparecem as zonas com efeitos negativos (depressão) dos intercruzamentos extremos endogâmicos e exogâmicos.
Figure 1. Graphic that shows the association between the reproductive viability and/or success of the offspring and the genetic differentiation between the parents. The centre of the curve reveals the "optimal" breeding zone and in the extremities appear the areas with negative effects (depression) from extreme inbreeding and outbreeding.

Estudos genéticos com peixes-bois
Genetic studies with manatees

Os primeiros estudos de citogenética indicaram composições cromossômicas diferentes entre a espécie marinha (*Trichechus manatus*) e a espécie amazônica (*T. inunguis*) (ASSIS *et al.*, 1988; GRAY *et al.*, 2002; WHITE *et al.*, 1976). O peixe–boi–marinho apresenta número diploide (2n) de 48 cromossomos, com cariótipo constituído principalmente por cromossomos de dois braços (metacêntricos, submetacêntricos e subtelocêntricos), enquanto que o peixe-boi-amazônico possui 56 cromossomos, sendo encontrados vários pares cromossômicos acrocêntricos (um braço). A espécie do ocidente africano (*T. senegalensis*) não tem dados cariotípicos e o dugongo (*Dugong dugon*), sirênio do oriente africano, sul da Ásia e Oceania, possui 50 cromossomos (SHORT, 1984).

Recentemente, uma análise citogenética comparativa (BARROS, 2014) entre *T. manatus* e *T. inunguis* com a técnica de hibridização *in situ* fluorescente (FISH) e sondas teloméricas revelou sinais de hibridização apenas nas regiões teloméricas, mas não foram observados remanescentes de telômeros intersticiais nos seus cromossomos, os quais seriam indicativos de inversões e fusões cromossômicas durante a diferenciação cariotípica. Este resultado está de acordo com a proposta de Gray *et al.* (2002), que indica que a evolução das diferenças cariotípicas observadas entre estas duas espécies poderia ser melhor explicada pela ocorrência de uma variedade de mecanismos de rearranjos intra e intercromossômicos e não somente por rearranjos do tipo fusão ou fissão cromossômica, como sugerido por Assis *et al.* (1988). Análises cariotípicas detalhadas (BARROS, 2014) também revelaram algumas diferenças no nível intraespecífico entre a população de *T. manatus* do Brasil e as populações de *T. manatus* que habitam Porto Rico e Flórida (EUA). Os pares cromossômicos 4 e 10 se mostraram metacêntrico e submetacêntrico, respectivamente, na população brasileira, enquanto nas populações do Caribe e da Flórida, estes pares são submetacêntrico e subtelocêntrico, respectivamente (GRAY *et al.*, 2002; HUNTER *et al.*, 2012). Assim, embora os peixes-bois que ocorrem em Porto Rico sejam tradicionalmente classificados como parte da subespécie *Trichechus manatus manatus*, estes apresentaram um cariótipo similar ao observado em *T. m. latirostris* (HUNTER *et al.*, 2012) e diferente da população brasileira que também é classificada como *T. m. manatus*.

O primeiro estudo de variação molecular com o peixe-boi-marinho (GARCIA-RODRIGUEZ *et al.*, 1998) identificou baixa diversidade nos dois extremos da distribuição da espécie, nas populações da Flórida (EUA) e do Brasil, assim como algumas descontinuidades na distribuição, que influenciam o padrão de fluxo gênico interpopulacional. Este estudo inicial também mostrou a existência de três haplogrupos ou linhagens mitocondriais (Figura 2) que não eram coerentes com a divisão de subespécies sugerida por análises morfológicas com um número limitado de amostras (DOMNING; HAYEK, 1986). Essas primeiras evidências genéticas foram corroboradas por um estudo posterior com marcadores mitocondriais e nucleares em uma grande amostragem (VIANNA *et al.*, 2006a,b), de 189 indivíduos de *T. manatus*, 93 de *T. inunguis* e seis de *T. senegalensis*. Este primeiro estudo de genética comparada entre as três espécies de peixe–boi

The first cytogenetic studies indicated different chromosomal compositions between the marine species (Trichechus manatus) and the Amazonian species (T. Inunguis) (ASSIS et al., 1988; GRAY et al., 2002; WHITE et al., 1976). The West Indian manatee has a diploid number (2n) of 48 chromosomes, with a karyotype consisting primarily of chromosomes with two arms (metacentric, submetacentric and subtelocentric), while the Amazonian manatee has 56 chromosomes and several pairs of acrocentric (one arm) chromosomes can be found. The African species (T. senegalensis) has no karyotypic data and the Dugong (Dugong dugon), the East African, South Asian and Oceania sirenian, has 50 chromosomes (SHORT, 1984).

Recently, a comparative cytogenetic analysis (BARROS, 2014) between T. manatus and T. inunguis, with the technique of fluorescence in situ hybridization (FISH) and telomeric probes, showed signs of hybridization in the telomeric regions only, but no interstitial telomere remnants were observed in their chromosomes, which would be indicative of chromosomal inversions and fusions during the karyotypic differentiation. This result is in agreement with Gray et al. (2002), which proposed that the evolution of the karyotype differences observed between these two species could be better explained by the occurrence of a variety of intra and inter-chromosomic rearrangement mechanisms and not only by chromosomal fusion or fission-type rearrangements, as suggested by Assis et al. (1988). Detailed karyotypic analyses (BARROS, 2014) also revealed some differences at the intraspecific level between the population of T. manatus from Brazil and the populations of T. manatus that inhabit Puerto Rico and Florida (USA). The chromosomal pairs 4 and 10 are metacentric and submetacentric, respectively, in the Brazilian population, while in the populations from the Caribbean and Florida these pairs are submetacentric and subtelocentric, respectively (GRAY et al., 2002; Hunter et al., 2012). Thus, while the manatees that occur in Puerto Rico are traditionally classified as part of the Trichechus manatus manatus subspecies, these presented a karyotype similar to that observed in T. m. latirostris (HUNTER et al., 2012) and different from the Brazilian population that is also classified as T. m. manatus.

The first molecular variance study with the West Indian manatee (GARCIA-RODRIGUEZ et al., 1998) identified low diversity at the two extremes of the species distribution, in populations of Florida (USA) and Brazil, as well as some discontinuities in the distribution, which influence the pattern of interpopulation gene flow. This initial study also showed the existence of three haplogroups, or mitochondrial lineages (Figure 2), that were not consistent with the subspecies division suggested by morphological analysis with a limited number of samples (DOMNING; HAYEK, 1986). These first genetic evidences were corroborated by a later study with nuclear and mitochondrial markers in a large sample size (VIANNA et al. 2006a,b) of 189 individuals of T. manatus, 93 of T. inunguis and six of T. senegalensis. This first comparative genetics study between the three species of manatees showed different evolutionary dynamics between them, as can be seen in the compared phylogeography study between the American species (Figure 2). The African manatee (T. senegalensis) presented divergent lineages associated with different river basins in west Africa, while the Amazonian manatee (T. inunguis) configured as a species of more recent origin than T. manatus (Figure 2), whose population began to expand a little more than 100 thousand years ago, probably from ancestors with unique adaptations to the Amazon river environment.

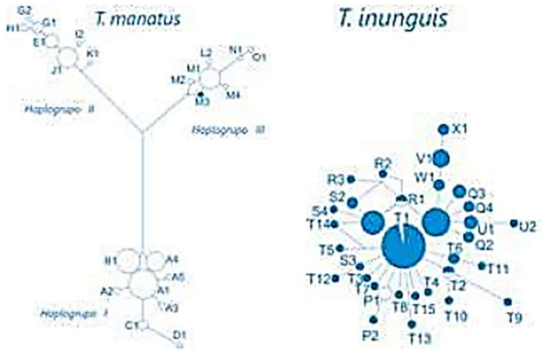


Figura 2. Filogeografia comparada das espécies americanas de peixe-boi com dados da região controle do DNAmt (VIANNA *et al.*, 2006a). Cada círculo representa uma sequência nucleotídica diferente de DNAmt, chamada de haplótipo, cuja área é proporcional ao número de indivíduos. O comprimento das linhas (ramos) é proporcional às diferenças nucleotídicas (mutações) entre os haplótipos. A cor dos círculos indica origem materna do DNAmt: branca (*T. manatus*) e azul (*T. inunguis*). Os haplótipos M3, R1, T1, T2 e P1 representam alguns indivíduos híbridos encontrados neste estudo e o haplótipo M4 foi descrito recentemente na população do Brasil (LUNA *et al.*, 2012). Para a espécie *T. manatus* foram detectados três agrupamentos (haplogrupos I, II e III) de haplótipos que são filogeneticamente muito divergentes entre si.

Figure 2. Compared phylogeography of the American manatee species with data from the mtDNA control region (VIANNA et al., 2006a). Each circle represents a different mtDNA nucleotide sequence, called haplotype, the area of which is proportional to the number of individuals. The length of the lines (branches) is proportional to the nucleotide differences (mutations) among the haplotypes. The color of the circles indicates maternal origin of the mtDNA: white (T. manatus) and blue (T. inunguis). The haplotypes M3, R1, T1, T2 and P1 represent some hybrid individuals found in this study and the haplotype M4 was recently described in the population from Brazil (LUNA et al., 2012). For the species T. manatus three groupings were detected (Haplogroups I, II and III) of haplotypes that are phylogenetically very divergent.

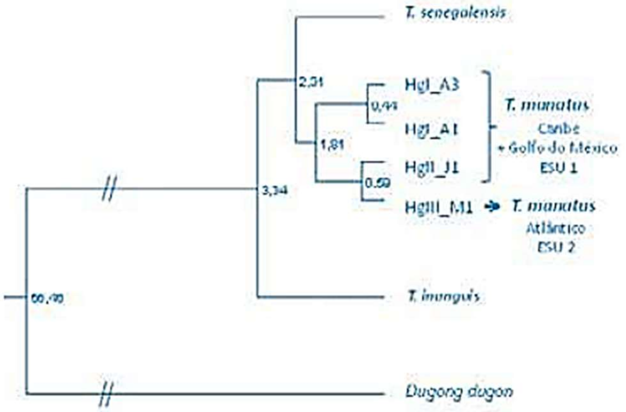


Figura 3. Filogenia bayesiana das linhagens e espécies de peixes–bois utilizando datações calibradas por fósseis (software Beast v2.2, BOUCKAERT *et al.*, 2014) a partir de dados de Citocromo B (GARCIA-RODRIGUEZ *et al.*, 1998, VIANNA *et al.*, 2006a). Os fósseis de sirênios utilizados definem datas para a divergência entre *Trichechus* e *Dugong* entre 47,6 e 66 milhões de anos (DOMNING *et al.*, 2010). Foi utilizado relógio molecular estrito ou relaxado e diferentes grupos externos compostos por Dugongo ou por outros Afrotheria como elefantes e hirax. Todas as filogenias apresentaram topologia e datações muito similares. As datas das separações dos ramos se referem a milhões de anos (MA). HgI, HgII e HgIII se referem aos haplogrupos de *T. manatus* previamente identificados (Figura 2).

Figure 3. Bayesian phylogeny of manatee lineages and species using dating calibrated by fossils (Beast software v2.2, BOUCKAERT et al., 2014) from cytochrome b data (GARCIA-RODRIGUEZ et al., 1998; VIANNA et al., 2006a). The sirenian fossils used define dates for the divergence between Trichechus and Dugong between 47.6 and 66 million years (DOMNING et al., 2010). Relaxed or strict molecular clocks and different external groups composed by Dugong or by other Afrotheria, such as elephants and hyrax, were used. All phylogenetic trees presented very similar topologies and datings. The branch separation dates refer to millions of years (MA). HgI, HgII and HgIII relate to the previously identified T. manatus haplogroups (Figure 2).

demonstrou diferentes dinâmicas evolutivas entre elas, como pode ser visto no estudo de filogeografia comparada entre as espécies americanas (Figura 2). O peixe-boi-africano (*T. senegalensis*) apresentou linhagens divergentes associadas a diferentes bacias fluviais no oeste da África, enquanto o peixe-boi-amazônico (*T. inunguis*) se configurou como uma espécie de origem mais recente que *T. manatus* (Figura 2), cuja população começou a se expandir há pouco mais de 100 mil anos, provavelmente a partir de ancestrais com adaptações únicas para o ambiente fluvial amazônico.

Foram feitos estudos com uma grande amostragem do peixe-boi-marinho (*T. manatus*) ao longo de sua distribuição nos litorais americanos do Caribe, Golfo do México e do Atlântico, da Flórida (EUA) ao nordeste do Brasil (GARCIA-RODRIGUEZ *et al.*, 1998; VIANNA *et al.*, 2006a). A espécie marinha apresentou uma separação significativa entre populações e áreas geográficas (Figura 2), representadas por uma ESU (unidade evolutiva significativa) com haplogrupos I e II e que habita as costas do Caribe e Golfo do México, da Colômbia à Flórida (EUA) e Antilhas, e outra ESU com indivíduos do haplogrupo III e que habita o nordeste do Atlântico da América do Sul, das Guianas ao Brasil (VIANNA *et al.*, 2006a,b; PARR *et al.*, 2012). As primeiras análises filogenéticas com métodos de datação baseados em "árvores linearizadas" implementadas no programa Mega v3 (KUMAR *et al.*, 2004) indicavam uma separação de pelo menos 130 mil anos (VIANNA *et al.*, 2006a) entre as duas unidades evolutivas significativas (ESUs), as populações do Atlântico (Guianas e Brasil) e do Caribe+Golfo do México. As duas ESUs surgiram, aparentemente, por processos vicariantes ocorridos durante as glaciações do Pleistoceno, quando o nível do mar estava até 120 m abaixo do nível atual e as Antilhas formaram uma barreira (Arco das Antilhas), tornando o mar do Caribe (e Golfo do México) relativamente isolado do nordeste Atlântico (PELTIER; FAIRBANKS, 2006). Estes eventos de vicariância marinha são a provável causa da grande diferenciação genética das linhagens mitocondriais (haplogrupos I, II e II) e do longo tempo de separação entre as populações do Atlântico e aquelas do Caribe e Golfo do México, que é estimada em 590 mil anos, data obtida com filogenia bayesiana de DNAmt e calibração cronológica com fósseis de sirênios (Figura 3).

Os primeiros estudos genéticos com populações de peixe-boi-amazônico foram publicados por dois grupos independentes com amostragens diferentes (CANTANHEDE *et al.*, 2005; VIANNA *et al.*, 2006a). Os resultados das análises filogeográficas e de genética populacional de ambos os estudos indicaram que esta espécie possui uma grande diversidade genética remanescente, mas que apresenta um agrupamento compacto de linhagens genéticas relacionadas (Figura 2). Isto indicou uma origem mais recente para esta espécie, que apresentou uma expansão populacional nos últimos 120 mil anos (VIANNA *et al.*, 2006a), podendo estar relacionada ao aparecimento recente de novas adaptações para sobrevivência no ambiente amazônico, permitindo a esta espécie colonizar vários afluentes com diferentes características. O peixe-boi-amazônico não apresentou uma diferenciação e estruturação geográfica tão grande quanto o peixe-boi-marinho, mas esta divergência interpopulacional variou de 10 a 22 % (*Fst*), dependendo de qual tipo de agrupamento geográfico era utilizado (VIANNA *et al.*, 2006a).

Vários outros estudos genéticos foram feitos recentemente com *T. manatus*, utilizando marcadores baseados em microssatélites e DNAmt. Tucker *et al.* (2012) utilizaram 18 microssatélites e compararam indivíduos de quatro unidades de manejo da Flórida (EUA), das costas leste dos EUA e do Golfo. Os dados obtidos demonstraram baixa diversidade genética nos peixes-bois-marinhos da Flórida e pouca diferenciação entre elas. Hunter *et al.* (2010) utilizaram microssatélites e sequências de DNAmt e demonstraram a baixa diversidade genética na população de Belize, com diferenciação significativa entre grupos localizados em City Cayes e Southern Lagoon, que são habitats diferentes. Com os mesmos marcadores, Hunter *et al.* (2012) mostraram diferenças significativas entre as populações da Flórida (EUA) e de Porto Rico, sugerindo que não há mistura recente (últimos séculos) entre as duas localidades.

Nourisson *et al.* (2011) utilizaram DNAmt e microssatélites para estudar a distribuição das populações do México que habitam os sistemas costeiros e zonas húmidas do Golfo do México e da costa do Caribe. Os dados revelaram um agrupamento diferenciado no Golfo do México e outro na Baía de Quetumal (Caribe), com uma zona de mistura entre elas na Baía de Ascención. Foi também identificado um fluxo gênico no sentido do Golfo do México para a costa do Caribe, e demonstrado que vários haplótipos de DNAmt encontrados no Caribe estão também presentes no Golfo do México, na Flórida (EUA) e em Belize. Estes dados de Nourisson *et al.* (2011) demonstram claramente a conexão destas populações do Caribe, do Golfo do México e Flórida, ressaltando a importância de se manter esta rota natural de migração, garantindo uma maior diversidade genética e a dinâmica evolutiva destas populações.

Estudos genéticos com os peixes-bois-marinhos e amazônicos nos rios da Colômbia (SATIZÁBAL *et al.*, 2012) também revelaram estruturas populacionais significativas e uma tendência de dispersão sexo-específica restrita a machos. Os dados de DNAmt e de microssatélites indicaram a existência de duas populações diferenciadas de peixe-boi-amazônico, separando a Amazônia colombiana e peruana. Já o peixe-boi-marinho apresentou diferenciação populacional significativa entre os rios colombianos com o DNAmt, mas não com os dados de microssatélites, indicando fluxo gênico promovido por machos, provavelmente através de migração pelo litoral.

No Brasil, as populações de peixe-boi-marinho da costa nordeste (Alagoas, Pernambuco, Paraíba, Rio Grande do Norte, Ceará, Piauí e Maranhão) foram analisadas com DNAmt (LUNA *et al.*, 2012) e foi encontrado um novo haplótipo (M04) em dois indivíduos do Maranhão e um do Piauí, além dos haplótipos previamente descritos (VIANNA *et al.*, 2006a). Luna *et al.* (2012) sugeriram que por terem um haplótipo diferente de DNAmt, indivíduos capturados ou encalhados nesta área não poderiam ser liberados em outros lugares como Alagoas, por exemplo. No entanto, os autores não apresentaram a análise filogenética. Uma reanálise dos dados realizada pelos autores deste capítulo (Figura 2) demonstra que M04 se diferencia do principal haplótipo encontrado na população brasileira (M01) por apenas uma mutação, que pode acontecer espontaneamente em uma geração. Então, consideramos que o argumento de possível depressão exogâmica levantado por Luna *et al.* (2012) para não liberação em áreas diferentes, não procede.

Studies were conducted with a large sample size of the West Indian manatee (T. manatus) along their distribution in the American coasts of the Caribbean, Gulf of Mexico and the Atlantic, from Florida (USA) to northeastern Brazil (GARCIA-RODRIGUEZ et al., 1998, VIANNA et al., 2006a). The marine species presented a significant separation between populations and geographical areas (Figure 2), represented by an Evolutionarily Significant Unit (ESU) with haplogroups I and II, which inhabit the coast of the Caribbean and Gulf of Mexico, from Colombia to Florida (USA) and the Antilles, and another ESU with individuals from haplogroup III, that live in the Northeast Atlantic of South America, from Guyana to Brazil (VIANNA et al., 2006a,b; PARR et al., 2012). The first phylogenetic analyses with dating methods based on " linearised" trees implemented in the program Mega v3 (KUMAR et al., 2004) indicated a separation of at least 130 thousand years (VIANNA et al., 2006a) between the two ESUs, the populations of the Atlantic (Guyanas and Brazil) and the Caribbean + Gulf of Mexico. The two ESUs arose, apparently, by vicariant processes occurring during the Pleistocene glaciers, when the sea level was up to 120 m below the current level and the Antilles formed a barrier (Antilles Arc), making the Caribbean Sea (and Gulf of Mexico) relatively isolated from the North East Atlantic (PELTIER; FAIRBANKS, 2006). These vicariant marine events are the likely cause of the great genetic differentiation between the mitochondrial lineages (haplogroups I, II and II) and the long period of separation between the populations of the Atlantic and the Caribbean and Gulf of Mexico, which is estimated at 590 thousand years, obtained with mtDNA Bayesian phylogeny and chronological calibration with sirenian fossils (Figure 3).

The first genetic studies with populations of the Amazonian manatee were published by two independent groups with different samplings (CANTANHEDE et al., 2005; VIANNA et al., 2006a). The results of the phylogeographic and population genetics analyses from both studies indicated that this species has a large remaining genetic diversity, but that it has a compact grouping of related genetic lineages (Figure 2). This indicated a more recent origin for this species, which underwent a population expansion in the last 120 thousand years (VIANNA et al. 2006a), and may be related to the recent emergence of new adaptations for survival in the Amazonian environment, allowing this species to colonize several tributaries with different characteristics. The Amazonian manatee did not show a differentiation and geographic structuring as large as the West Indian manatee, but this inter-population divergence ranged from 10 to 22 % (FST), depending on which type of geographic grouping was used (VIANNA et al. 2006a).

Several other genetic studies were recently performed with T. manatus, using markers based on microsatellites and mtDNA. Tucker et al. (2012) used 18 microsatellites and compared individuals from four management units in Florida (USA), from the east coast and the Gulf. The data obtained showed low genetic diversity in manatees from Florida and little differentiation between them. Hunter et al. (2010) used microsatellites and mtDNA sequences and demonstrated the low genetic diversity in the population of Belize, with significant differentiation between groups located in City Cayes and Southern Lagoon, which are different habitats. With the same markers, Hunter et al. (2012) showed significant differences between the populations of Florida (USA) and Puerto Rico, suggesting that there has been no recent mixing (past few centuries) between the two locations.

Nourisson et al. (2011) used mtDNA and microsatellites to study the distribution of the populations that inhabit the coastal systems and wetlands of the Gulf of Mexico and the Caribbean coast. The data revealed a differentiated grouping in the Gulf of Mexico and another in the Bay of Chetumal (Caribbean), with a mixing zone between them in the Bay of Ascención. A gene flow was also identified in the direction from the Gulf of Mexico to the Caribbean coast, and it was demonstrated that several mtDNA haplotypes found in the Caribbean are also present in the Gulf of Mexico, in Florida (USA) and in Belize. The data from Nourisson et al. (2011) clearly show the connection between these Caribbean populations, the Gulf of Mexico and Florida, emphasizing the importance of maintaining this natural migration route, ensuring a greater genetic diversity and the evolutionary dynamics of these populations.

Genetic studies with West Indian and Amazonian manatees in the rivers of Colombia (SATIZÁBAL et al., 2012) also revealed significant population structures and a sex-specific dispersion tendency restricted to males. The mtDNA and microsatellite data indicated the existence of two differentiated Amazonian manatee populations, separating the Colombian Amazon and Peru. The West Indian manatee, on the other hand, presented significant population differentiation between the Colombian rivers with mtDNA, but not with the microsatellite data, indicating gene flow promoted by males, probably through coastal migration.

In Brazil, the manatee populations from the northeast coast (Alagoas, Pernambuco, Paraíba, Rio Grande do Norte, Ceará, Piauí and Maranhão) were analyzed with mtDNA (LUNA et al. 2012) and a new haplotype (M04) was found in two individuals from Maranhão and Piauí, in addition to the previously described haplotypes (VIANNA et al. 2006a). Luna et al. (2012) suggested that because this was a different mtDNA haplotype, individuals captured or stranded in this area should not be released in other places such as Alagoas, for example. However, the authors did not present the phylogenetic analysis. A review of the data held by the authors of this chapter (Figure 2) shows that M04 differentiates from the main haplotype found in the Brazilian population (M01) by only one mutation, which can occur spontaneously in one generation. Thus, we believe that the argument of possible outbreeding depression raised by Luna et al. (2012) not to release in different areas, is unfounded.

Hibridização interespecífica entre Sirênios da América do Sul
Interspecific hybridization amongst South American Sirenians

A formação de híbridos entre as espécies de peixe–boi–amazônico e marinho foi demonstrada pela análise de diferentes marcadores mitocondriais, nucleares e dados citogenéticos por Vianna *et al.* (2006a,b) e Parr *et al.* (2012). Foram identificados sete indivíduos híbridos entre *T. manatus* e *T. inunguis* na zona costeira do Atlântico norte da América do Sul, sendo três na Guiana Francesa, três na Guiana e um no estado brasileiro do Amapá. Estes híbridos costeiros foram reconhecidos morfologicamente como peixe-boi-marinho, mas possuíam DNAmt de *T. inunguis*, indicando uma origem materna da espécie amazônica. Também foi identificado um híbrido no Rio Amazonas, próximo a Belém (Pará, Brasil), identificado inicialmente como peixe–boi–amazônico, mas com DNAmt de peixe-boi-marinho. Um híbrido macho que se encontra em cativeiro e de nome Poque, oriundo da região costeira do Amapá (próximo ao Rio Oiapoque), foi estudado também por citogenética e apresentou 50 cromossomos, um número intermediário entre a espécie marinha (2n=48) e a espécie amazônica (2n=56) (VIANNA *et al.*, 2006a; PARR *et al.*, 2012). Esta configuração cromossômica e a composição de alelos de microssatélites indicam que este indivíduo deve ser um híbrido de segunda geração (F2), filho de um pai da espécie marinha com uma mãe híbrida F1 (que teria provavelmente 52 cromossomos e DNAmt da espécie amazônica).

As evidências genéticas identificaram uma zona de hibridização ao redor da foz do Rio Amazonas (Figura 4), com ocorrência de híbridos principalmente no litoral oeste da foz, na região das Guianas (incluindo o Amapá), que perfazem mais de 40% da população analisada (VIANNA *et al.*, 2006a e dados não publicados).

The hybridization between Amazonian and West Indian manatees was demonstrated by analysis of different mitochondrial and nuclear markers and cytogenetic data by Vianna et al. (2006a,b) and Parr et al. (2012). Seven hybrid individuals were identified between T. manatus and T. inunguis in the coastal zone of the north Atlantic of South America, three in French Guiana, three in Guyana and one in the Brazilian state of Amapá. These hybrids were recognized morphologically as West Indian manatees but had T. inunguis mtDNA, indicating that the maternal lineage came from the Amazonian manatee. A hybrid was also found in the Amazon River, close to Belém (Pará, Brazil), initially identified as Amazonian manatee, but with West Indian mtDNA. A hybrid male called Poque which is in captivity and came from the coastal region of Amapá (near the Oiapoque River) was also studied by cytogenetics and presented 50 chromosomes, an intermediary number between the marine species (2n=48) and the Amazonian species (2n=56) (VIANNA et al., 2006th; PARR et al., 2012). This chromosomal configuration and microsatellite allele composition indicates this individual is probably a second-generation (F2) hybrid, the son of a West Indian father with an F1 hybrid mother (which would probably have 52 chromosomes and Amazonian mtDNA).

Genetic evidence identified a hybridization zone around the mouth of the Amazon River (Figure 4), with the occurrence of hybrids mainly on the west coast of the estuary, in the Guyana region (including Amapá), which make up more than 40% of the studied population (VIANNA et al., 2006a and unpublished data).

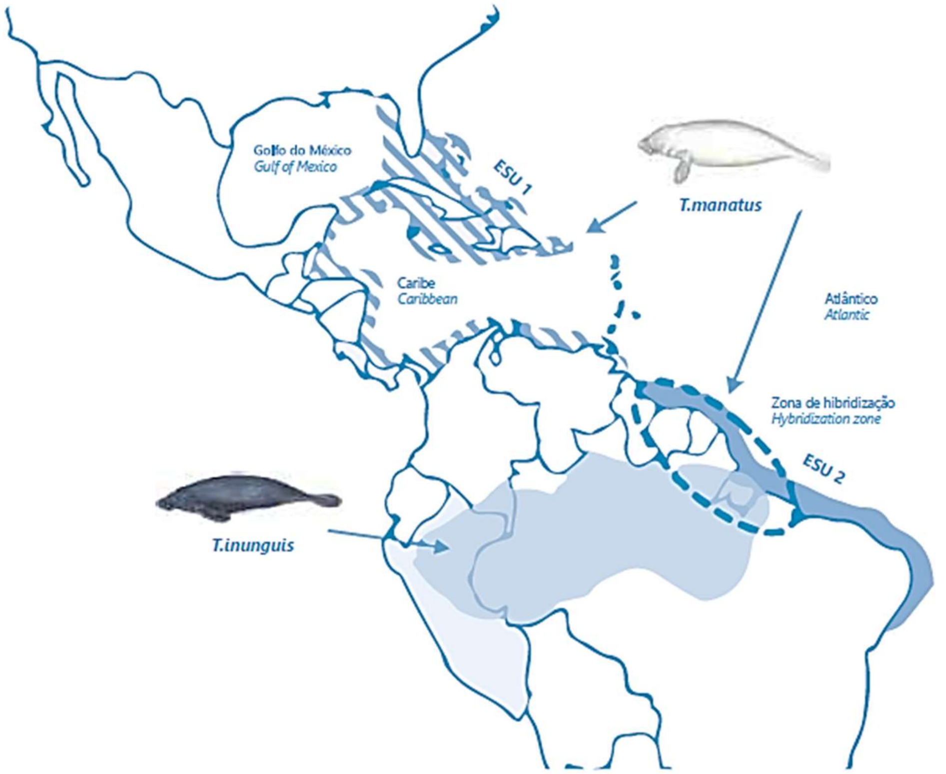


Figura 4. Distribuição das espécies americanas de peixe-boi e a zona de hibridização interespecífica (elipse azul). Também são apresentadas as áreas de distribuição das duas unidades evolutivas significativas de *Trichechus manatus*: ESU-1 (Caribe e Golfo do México: Venezuela aos EUA e Antilhas) e ESU-2 (Atlântico: Brasil e Guianas).
Figure 4. American manatee species distribution and interspecific hybridization zone (blue ellipse). The distribution areas of the two evolutionary significant units of Trichechus manatus are also shown: ESU-1 (Caribbean and Gulf of Mexico: Venezuela to the USA and the Antilles) and ESU-2 (Atlantic: Brazil and the Guyanas).

Implicações dos dados genéticos para a conservação dos peixes-bois

Implications of genetic data for manatee conservation

O único estudo genético feito com a espécie de peixe-boi-africano (VIANNA *et al.*, 2006a) indica que há estruturação populacional de acordo com as bacias fluviais da África ocidental. No entanto, esta espécie carece urgentemente de estudos genéticos e ecológicos, sendo a menos conhecida e está atualmente muito ameaçada, não apenas pela caça, mas também pela construção de várias barragens nos rios do oeste da África, que estão isolando artificialmente as populações que antes eram conectadas.

Embora as populações de peixe-boi-amazônico não tenham apresentado uma estruturação populacional marcada como visto para a espécie marinha, a diferenciação interpopulacional moderada (Fst~0.20) indica que pode haver populações amazônicas com um relativo isolamento das demais (VIANNA *et al.*, 2006a; SATIZÁBAL *et al.*, 2012). No entanto, ainda falta um estudo detalhado das populações de várias sub-bacias para desvendar a estrutura e dinâmica evolutiva populacional desta espécie, com finalidade de sugerir translocação de indivíduos entre as diversas áreas na Amazônia. Com os dados existentes, o ideal é manter os eventos de translocação de indivíduos, caso se façam necessários, restritos a uma mesma sub-bacia (SATIZÁBAL *et al.*, 2012).

O peixe-boi-marinho é a espécie de sirênio mais estudada cientificamente, principalmente a população da Flórida, nos Estados Unidos. Também é a espécie com mais dados genéticos gerados a partir de uma amostragem representativa de quase toda a sua distribuição atual. Por isto, *T. manatus* é a espécie de sirênio com mais evidências genéticas que já podem ser utilizadas na prática da conservação e elaboração de estratégias de manejo *ex-situ* e *in-situ*. Como evidenciado em vários estudos genéticos com DNAmt e microssatélites, muitas populações têm baixa diversidade e são endogâmicas, por exemplo, as populações do extremo da distribuição, na Flórida (EUA) e Brasil. A liberação de indivíduos nestas áreas pode ser promovida com a intenção de aumentar a diversidade genética local ou até mesmo recolonizar uma área onde houve extinção local da espécie. Para isto, temos de utilizar evidências da dinâmica evolutiva nesta espécie, tal como a identificação de duas ESUs (Figura 4), os dois grupos populacionais que foram separados por tempo suficiente para o acúmulo de diferenças adaptativas. Estas diferenças moleculares entre as ESUs são corroboradas por análises citogenéticas e análises de morfometria geométrica dos crânios (BARROS, 2014) demonstrando que a população brasileira de *T. manatus* possui características diferentes das populações do Caribe e da Flórida (EUA). Portanto, as evidências moleculares, citogenéticas e morfológicas (VIANNA *et al.*, 2006a,b; NOURISSON *et al.*, 2011; PARR *et al.*, 2012; BARROS, 2014) não estão de acordo com a classificação subespecífica reconhecida para *T. manatus* (DOMNING e HAYEK 1986), sugerindo que deve ser feita uma revisão taxonômica neste táxon.

No manejo de *T. manatus*, os processos de translocação e reintrodução de peixes-bois devem considerar as duas ESUs como unidades totalmente independentes de manejo, isto é, as liberações de indivíduos ao ambiente natural não devem ocorrer entre diferentes ESUs, separadas há vários milhares de anos (Figura 3). Outras considerações podem ser feitas no nível microgeográfico para tentar manter o padrão de fluxo gênico de curto prazo, isto é, a preferência de acasalamento filopátrico das populações de peixe-boi-marinho como previamente identificado por alguns estudos (HUNTER *et al.*, 2010, 2012; NOURISSON *et al.*, 2011; TUCKER *et al.*, 2012; SATIZÁBAL *et al.*, 2012). No entanto, este tipo de manejo só é aconselhável se as populações forem relativamente grandes ou em expansão, e o aumento da diversidade genética local ou recolonização de áreas não forem metas prioritárias. Na população brasileira de *T. manatus*, mesmo que existam diferenças genéticas e alguma estruturação geográfica ao longo do litoral, todos os indivíduos pertencem à mesma ESU (Atlântico) e cruzamentos entre quaisquer indivíduos desta ESU, inclusive das Guianas, não devem resultar em depressão exogâmica, exceto se houver híbridos interespecíficos envolvidos (ver abaixo). Portanto, como foi demonstrada uma baixa diversidade genética na população brasileira (VIANNA *et al.*, 2006a; LUNA *et al.*, 2012), a translocação de indivíduos entre áreas diferentes do litoral brasileiro pode ser uma estratégia viável para diminuir a perda de diversidade por deriva e o aumento da endogamia local.

Entretanto, outra grande preocupação de conservação apareceu na América do Sul com a detecção de hibridização interespecífica entre os peixes-bois-marinhos e amazônico (VIANNA *et al.*, 2006a). Sabe-se que o processo de hibridização interespecífica ocorre naturalmente na evolução de vários grupos taxonômicos (ALLENDORF *et al.*, 2001), porém este fenômeno pode ser disparado por ações antropogênicas que promovem o declínio populacional das espécies ameaçadas, fazendo com que os indivíduos de sexos e espécies diferentes possam cruzar mais frequentemente, já que se tornam raros os encontros de indivíduos de mesma espécie (VILAÇA *et al.*, 2012). A hibridização pode gerar indivíduos com menor sobrevivência e/ou viabilidade reprodutiva e aumentar a chance de extinção das espécies, principalmente aquelas que já estão com uma população muito diminuída (ALLENDORF *et al.*, 2001). Este resultado negativo da hibridização é conhecido como depressão exogâmica, que também pode ser visto em alguns cruzamentos intraespecíficos, quando este se dá entre indivíduos de populações divergentes e com adaptações locais diferentes, como entre as duas ESUs de peixe-boi-marinho (Figura 4).

Por causa das várias diferenças citogenéticas, genômicas, morfológicas e ecológicas (adaptações) entre as duas espécies sul-americanas de peixe-boi, estes híbridos devem possuir baixa viabilidade e fertilidade na natureza. No entanto, visto que foi encontrado pelo menos um híbrido de segunda geração (VIANNA *et al.*, 2006a; PARR *et al.*, 2012), parece não haver infertilidade completa entre as fêmeas híbridas F1, possibilitando que os genes de uma espécie possam ser transferidos para a outra, resultando no fenômeno da introgressão. Assim, alguns indivíduos desta área de hibridização no litoral ao redor da foz do Amazonas (Figura 4) podem ser animais introgredidos de geração F2 ou mais, que podem ocupar um nicho parecido ao das espécies parentais. De qualquer forma, a presença de híbridos de sobrevivência reduzida, estéreis ou com baixa fertilidade nesta zona de hibridização deve reduzir bastante o valor adaptativo populacional, que consequentemente, pode levar à extinção local.

Devido à ocorrência de híbridos ao redor da foz do Amazonas e nas áreas costeiras das Guianas (e Amapá), além do Pará e Maranhão, assim como nos trechos fluviais próximos à foz do Rio Amazonas (Figura 4), qualquer translocação ou reintrodução envolvendo indivíduos oriundos desta área deve ser monitorada geneticamente. Isto deve ser feito para evitar a depressão exogâmica nas áreas onde estes animais forem liberados, tanto para as populações de peixe-boi-marinho quanto amazônico. Portanto, caso os indivíduos candidatos à reintrodução sejam identificados como híbridos, eles não deveriam ser reintroduzidos na natureza.

The only genetic study carried out with the African manatee (VIANNA et al., 2006a) indicates population structuring according to the West African river basins. However, this species is in urgent need of genetic and ecological studies, being the lesser known manatee species and currently highly threatened not only by poaching, but also by the construction of several dams in the rivers of West Africa that are artificially isolating the populations that were previously connected.

Although the Amazonian manatee populations have not exhibited a marked population structuring as seen in the marine species, the moderate inter-population differentiation (Fst~0.20) indicates there may be some Amazonian populations relatively isolated from others (VIANNA et al., 2006a; SATIZÁBAL et al., 2012). However, we still lack a detailed study of the populations from several sub-basins to unveil the structure and evolutionary dynamics of this species, with the purpose of suggesting the translocation of individuals among the various areas in the Amazon. With the existing data, the ideal is to maintain the translocation of individuals, whenever required, restricted to a same sub-basin (SATIZÁBAL et al. 2012).

The West Indian manatee is the most scientifically studied sirenian species, mainly the population from Florida in the United States. It is also the species with most genetic data generated from a representative sample of almost all its current distribution. For this reason, T. manatus is the sirenian species with most genetic evidence that can already be used in conservation practice and preparation of ex-situ and in-situ management strategies. As evidenced in several genetic studies with mtDNA and microsatellites, many populations have low diversity and are endogamous, for example, the populations of the extreme of the distribution, in Florida (USA) and Brazil. The release of individuals in these areas can be promoted with the intention of increasing the local genetic diversity or even recolonizing an area where the species was locally extinct. For this, evidences of evolutionary dynamics in this species must be taken into account, such as the identification of two ESUs (Figure 4), the two population groups that were separated long enough for the accumulation of adaptive differences. These molecular differences between the ESUs are corroborated by cytogenetic examination and analyses of skull geometric morphometrics (BARROS, 2014), demonstrating that the Brazilian T. manatus population has different characteristics from the populations in the Caribbean and Florida (USA). Therefore, the molecular, cytogenetic and morphological evidence (VIANNA et al., 2006a,b; NOURISSON et al., 2011; PARR et al., 2012; BARROS, 2014) are not in accordance with the sub-specific classification recognized for T. manatus (DOMNING and HAYEK, 1986), suggesting the need for a taxonomic revision of this taxon.

In T. manatus management, the processes of translocation and reintroduction should consider the two ESUs as fully independent management units, i.e. the release of individuals to the natural environment should not occur between different ESUs, which have been separated for several thousand years (Figure 3). Other considerations can be made at the microgeographic level to attempt the maintenance of the short term gene flow pattern, that is, the preference for philopatric mating of manatee populations as previously identified by some studies (HUNTER et al., 2010; 2012; NOURISSON et al., 2011; TUCKER et al., 2012; SATIZÁBAL et al., 2012). However, this type of management is only advisable if the populations are relatively large or growing, and the increase of local genetic diversity or the recolonization of areas are not priority goals. In the Brazilian population of T. manatus, even if there are genetic differences and some geographic structure along the coast, all individuals belong to the same ESU (Atlantic) and breeding between any individuals of this ESU, including the Guyanas, should not result in outbreeding depression, except if interspecific hybrids are involved (see below). Therefore, given that a low genetic diversity was demonstrated in the Brazilian population (VIANNA et al., 2006a; LUNA et al., 2012), the translocation of individuals between different areas of the Brazilian coast may be a viable strategy to reduce the loss of diversity from drifting and increased local inbreeding.

However, another significant conservation concern arose in South America with the detection of interspecific hybridization between the Amazonian and West Indian manatees (VIANNA et al., 2006a). It is a known fact that the process of interspecific hybridization occurs naturally in the evolution of various taxonomic groups (ALLENDORF et al., 2001). This phenomenon, however, may be triggered by anthropogenic actions that promote the decline of endangered species populations, causing individuals of different sexes and species to come across more often, since the meetings of individuals of the same species become rarer (VILAÇA et al., 2012). Hybridization can generate individuals with lower survival and/or reproductive viability and increase the chance of species extinction, mainly for those populations that are already reduced (ALLENDORF et al., 2001). This negative result of hybridization is known as outbreeding depression, and can also be observed in some intraspecific breeding, when this occurs between individuals of differing populations with different local adaptations, such as between the two West Indian manatee ESUs (Figure 4).

Because of the multiple cytogenetic, genomic, morphological and ecological (adaptations) differences between the two South American manatee species, these hybrids should have low viability and fertility in nature. However, since at least one second generation hybrid has been found (VIANNA et al. 2006a; PARR et al., 2012), it seems there is no complete infertility among the F1 hybrid females, enabling the genes of a species to be transferred to another, resulting in the phenomenon of introgression. Thus, some individuals of this hybridization area on the coast around the mouth of the Amazon (Figure 4) can be introgressed animals of F2 generation or more, which may occupy a niche similar to that of the parental species. In any case, the presence of hybrids of reduced survival, sterile or with low fertility in this hybridisation zone should greatly reduce the population's adaptive value, which, consequently, can lead to local extinction.

Due to the occurrence of hybrids around the mouth of the Amazon and in the coastal areas of the Guyanas (and Amapá), besides Pará and Maranhão, as well as in the fluvial stretches near the mouth of the Amazon River (Figure 4), any reintroduction or translocation involving individuals originating from this area must be genetically monitored. This must be done to avoid exogamous depression in areas where these animals are released, for both the West Indian and the Amazonian manatee populations. Therefore, individuals identified as hybrids should not be reintroduced to nature.